NEURAL CIRCUITRY AND AFFERENT CHARACTERISTICS OF UROGENITAL REFLEXES

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To my mom, Linda Bartels Ferrero
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LIST OF ABBREVIATIONS

BSM        Bulbospongiosus muscle
C-LTMR     C fiber low threshold mechanoreceptor
DAB        Diaminobenzidine
Dil        1,1'-dilinoleyl-3, 3', 3' tetramethylindocarbocyanine percholate
DNP        Dorsal nerve of the penis
DNPg       Glans DNP afferents
DNPi       Ischiorectal DNP
DREZ       Dorsal root entry zone
DRG        Dorsal root ganglia
DRP        Dorsal root potential
EAS        External anal sphincter
EUS        External urethral sphincter
Gi         Gigantocellularis
GiA        Gigantocellularis pars alpha
GLANS NON-PAS Glans afferents that are not transsynaptically connected to the BSM
GPAs       Glans pudendal circuit afferents
IB4        Isolectin I-B4
IR         Ischiorectal
LPGi       Lateral paragigantocellularis
MRF        Medullary reticular formation
NFM        Neurofilament-M
NK-1       Neurokinin-1
NON-GLANS PAS Afferents that are non-glans in origin which are transsynaptically connected to the BSM
NRGi       Nucleus reticularis gigantocellularis
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<td>PAD</td>
<td>Primary afferent depolarization</td>
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<td>PMC</td>
<td>Pontine micturition center</td>
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<td>PMRD</td>
<td>Pudendal motoneuron reflex discharges</td>
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<td>PN</td>
<td>Pelvic nerve</td>
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<td>PRV</td>
<td>Pseudorabies virus</td>
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<td>SCI</td>
<td>Spinal cord injury</td>
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<td>SP</td>
<td>Substance P</td>
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NEURAL CIRCUITRY AND AFFERENT CHARACTERISTICS OF UROGENITAL REFLEXES

By

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Functional recovery of urogenital reflexes is of top priority for the spinal cord-injured (SCI) human population, yet there is little known about the neural circuitry in normal or spinal-injured humans and animals. Pudendal motoneurons innervating perineal muscles such as the bulbospongiousus muscle (BSM) are involved in ejaculation and micturition. In males, the dorsal nerve of the penis (DNP) innervates regions such as the glans and distal urethra; the pelvic nerve (PN) innervates regions such as the bladder and proximal urethra. Stimulation of either the DNP or PN bilaterally activates these motoneurons in the pudendal reflex. In the present study using a rodent model, transsynaptic anatomical tracing experiments of the BSM circuitry with pseudorabies virus demonstrated the bilateral nature and L6 dorsal root ganglion (DRG) location of glans afferent somas. Electrophysiological experiments revealed that the lateral paragigantocellularis (LGPi) modulated the pudendal reflex via primary afferent depolarization (PAD) of DNP afferents (but not PN afferents), resulting in bilateral presynaptic inhibition. DNP reflex afferents also were bilaterally presynaptically inhibited segmentally by other DNP afferents, particularly by myelinated fibers. These inhibitory mechanisms may play a role in the coordination/timing of
perineal muscle contractions. Behavioral experiments on bladder function following incomplete spinal injury showed that the recovery of micturition reflexes was correlated with sparing of bulbospinal pathways in the central portions of the lateral funiculus at T8, corroborating the electrophysiological data. Immunohistochemical tracing/labeling experiments on DRG neurons showed that, compared to putative reflex-connected urethral afferents, reflex-connected glans afferents had significantly more substance P (SP) immunoreactivity. SP-immunoreactive glans afferents not connected to the BSM circuitry, compared to those that were, tended to be the smallest/unmyelinated population. Neurofilament-immunoreactive glans afferents not connected to the BSM circuitry, compared to those that were, tended to be the largest/myelinated population. In addition, putative unmyelinated urethral afferents that are synaptically connected to the BSM circuitry were larger compared to those of glans afferents. Findings may provide targets for post-SCI therapies; for example, promoting the sparing of at least one side of the cord, or harnessing the sprouting ability of Aδ or C fiber afferents involved in the pudendal reflex.
Neural Control of Urogenital Circuitry

Compared to the extensive literature available that focuses on locomotion and sensation in the limbs, the neural circuitry of urogenital functions remains a relatively little-studied topic. One reason for this may be the complexity of such circuits; urogenital functions involve coordination of somatic and autonomic (both sympathetic and parasympathetic) pathways. These functions also involve two spinal centers at different levels of the cord which are interconnected with one another. These segmental components of urogenital reflexes are in turn interconnected with regions of the brainstem, cerebral cortex, and many other brain areas (Andersson, 2001; de Groat et al., 1981; de Groat and Booth, 1993; McKenna, 2000a, 2000b; Giuliano and Clement, 2005; Giuliano and Rampin, 2004; Johnson, 2006; Carro-Juárez and Rodríguez-Manzo, 2003; Steers, 2000; Sugaya et al., 2003). Also the complexity and interrelation of indirect neural pathways such as between special senses (i.e., visual, olfactory) and the spinal reflexes (Steers, 2000) is epitomized by a study observing that, in an animal model, males tend to transfer larger ejaculates to higher quality females (Kelly and Jennions, 2011). Even when solely considering the reflexive aspect, the eliminative processes of micturition, defecation and ejaculation are all very well-orchestrated. They first involve retention and then expulsion of some material, whether be it urine, feces or semen. Each process involves coordinated contraction of specific perineal muscles and also coordination of these striated muscles with smooth muscle activity.

Micturition and ejaculation share several characteristics. For example, afferents in the pelvic nerve (PN) are involved in both processes (Johnson, 2006). Dorsal nerve of
the penis (DNP, or pudendal) afferents, or of the clitoris in females, are known primarily for their role in sexual reflexes (Giuliano and Rampin, 2004; McKenna and Nadelhaft, 1989; Pacheco et al., 1997), but both DNP and PN afferents are transsynaptically connected to the bulbospongiosus muscle (BSM) (McKenna and Nadelhaft, 1989). The BSM surrounds the base of the penis and, with the appropriate timing and sequence of contractions, aids in both the expulsive phase of ejaculation (Giuliano and Rampin, 2004; Holmes et al., 1991; McKenna, 2000a) as well as micturition (Cruz and Downie, 2005; Yasuhiro et al., 2004). Both the processes of ejaculation (Johnson and Hubscher, 1998) and micturition (Sugaya et al., 2003) are under control from supraspinal sites as well as segmentally. Both processes involve a spino-bulbo-spinal loop. One of the supraspinal sites controlling ejaculation is the lateral paragigantocellularis (LPGi), located in the medullary reticular formation (MRF) (Hubscher and Johnson, 1999b). Our past lesion studies with male rats have shown that the central lateral funiculus of the spinal cord is the location where the descending tract from the LPGi runs to the segmental ejaculatory circuitry, becoming bilateral somewhere below the T8 level (Johnson et al., 2011). The lesion studies discussed in Chapter 4 investigate whether the descending pathway controlling micturition runs in a similar location in the cord with similar laterality. The most widely known supraspinal site which is part of the loop controlling micturition is the pontine micturition center or PMC as well as a “continence” center in the lateral pons (Holstege, 2005b). There also is evidence of a contributing pathway from the MRF (Sugaya et al., 2003) as is the case for ejaculation (Johnson and Hubscher, 1998).
Urogenital Dysfunction in Spinal Cord Injury

While being relatively unexplored, the area of urogenital neuroscience has proven to be important from not only a research but also clinical perspective. According to a survey administered to the spinal cord-injured population by Anderson in 2004, regaining sexual function is of highest priority for paraplegics. In addition, the survey shows that regaining bladder and bowel control is of shared importance to both quadriplegic and paraplegic groups. Infertility is an important concern of the spinal cord injured population (Sarkarati et al., 1987), which is mostly composed of young men who have not yet had children. Loss of eliminative functions such as bladder control can lead to several health complications as well as physical and psychosocial discomfort (Anderson, 2004). While spinal cord injury is associated with a variety of serious problems, disruption of sexual function in particular has been largely ignored, possibly because of the stigma or perceived unimportance. Such views are changing, however. Procreation is definitely a valid concern because although there exist therapies such as vibratory and electroejaculation (Sarkarati et al., 1987), various issues nevertheless persist such as poor sperm motility. In addition, inability to be intimate with one’s partner often results in reduced quality of life (Anderson, 2004).

The level of the lesion resulting from spinal cord injury (SCI) often dictates the severity of sexual dysfunction. For the purpose of this discussion and the applicability of this research, we will consider only injuries cranial to T10 (Johnson, 2006; Anderson et al., 2007a). Even when supraspinal input has been severed, as with patients with clinically complete spinal cord injuries cranial to T10, the spinal reflex arcs for erection and ejaculation remain intact. Most patients with such injuries display reflexogenic erections in response to very slight penile stimulation (ver Voort, 1987; Sarkarati et al.,
While these erections are easily initiated, they often are not sustained (Bodner et al., 1987) which may result from a change in sensory perception from the penis (Goldstein, 1988). However, despite an intact spinal reflex arc allowing easily initiated erection, ejaculation is either severely impaired or impossible for nearly all patients with spinal injuries cranial to T10 (Ver Voort, 1987; Seftel et al., 1991). This suggests that the ejaculatory circuitry is more dependent on supraspinal facilitation or disinhibition than the erection circuitry. The decrease in penile sensation also may be related to ejaculatory dysfunction. For spinal cord-injured patients who do not respond to normal tactile penile stimulation, more intense vibratory stimulation to the ventral midline of the penis may be sufficient to elicit ejaculation (Sonksen and Ohl, 2002). This suggests that recruitment of both low and high threshold penile mechanoreceptors provides sufficient input to the spinal circuitry for ejaculation to occur.

The fact that the terms for the conditions of anejaculation and anorgasmia cannot be used interchangeably demonstrates the distinction between processes required for the emission versus the expulsion phases of ejaculation (Wolters and Hellstrom, 2006). While several definitions of the term orgasm exist, physiologically it is closely associated with contraction of the BSM and/or other perineal muscles (known as the pudendal, or bulbocavernosus reflex). Patients with anorgasmia often have an absent bulbocavernosus reflex (Brindley and Gillan, 1982). In these patients, emission is still possible as evidenced by release of semen using electroejaculation (Brindley and Gillan, 1982). Hence, there may be ejaculation without orgasm. There also may be orgasm (i.e., BSM contraction), without ejaculation, termed anejaculation (Wolters and Hellstrom, 2006).
Many patients with chronic spinal cord injuries lose the synergy between the bladder and its outlet that is necessary for normal voiding of its contents. This micturition dysfunction, known as detrusor-urethral sphincter dyssynergia, results from inappropriate contraction or failure of relaxation of the striated external urethral sphincter (EUS) coincident with detrusor (bladder) muscle contraction (de Groat, 1995, Gerridzen et al., 1992, Rudy et al., 1988; Tai et al., 2006; Yalla et al., 1977). Many patients with chronic spinal cord injuries also lose conscious control of defecation. Some patients are able to defecate reflexively via anorectal stimulation (MacDonagh et al., 1992). Some patients suffer from chronic constipation and fecal incontinence (Glick et al., 1984; Longo et al., 1989; MacDonagh et al., 1992). Both urinary bladder dysfunction and severe constipation are causes of significant morbidity and mortality in this population (Glick et al., 1984), although renal failure is the most common (Hackler, 1977).

**Segmental Circuitry**

**Primary Afferents from the Male Urogenital Tract and Colon**

The lumbosacral segments (L5-S1 in the rat) contain a center for erection and for the propulsive phase of ejaculation (Coolen et al., 2004; Giuliano and Rampin, 2004). These functions are mediated by preganglionic parasympathetic motor neurons which run in the pelvic nerve, and somatic motor neurons running in the motor branch of the pudendal nerve supplying the striated perineal muscles of the pelvic floor, respectively (Coolen et al., 2004; Giuliano and Rampin, 2004). The peripheral sensory limb of the segmental reflexes for erection and ejaculation is carried primarily by afferents in the DNP (Johnson, 2006; Yang and Bradley, 1998), which is part of the predominantly sensory branch of the pudendal nerve (Steers, 2000). DNP stimulation results in
bilateral pudendal motoneuron reflex discharges (McKenna and Nadelhaft, 1989) and BSM contraction, termed the pudendal reflex. Desensitization of the penis which is produced by local anesthesia or dorsal nerve section causes impairment of reflexogenic erection, intromission, and ejaculation (reviewed by Hart and Leedy, 1985). Experiments by Johnson and co-workers have characterized the primary afferent population in the DNP (Kitchell et al., 1982; Johnson, 1988; Johnson and Halata, 1991; Johnson and Murray, 1992); the DNP includes mucocutaneous afferents innervating the penis (Giuliano and Rampin, 2004), prepuce, external urethral orifice, corpus cavernosum/spongiosum as well as visceral afferents innervating the distal urethra (Yang and Bradley, 1999).

The neural pathways from the gastrointestinal and lower urinary tracts also have been the subject of intense research (see review by Sengupta and Gebhart, 1995). Briefly, afferents innervating these organs (including the colon) travel to the spinal cord via the pelvic (otherwise primarily parasympathetic) and hypogastric and lumbar colonic (otherwise primarily sympathetic) nerves. Rectal pain in humans, for example, is believed to be the result of noxious information conveyed centrally to the thoracolumbar cord, whereas conscious perception of normal rectal sensations such as fullness and urgency is thought to be mediated by sacral (i.e., pelvic) afferents (see Bernstein et al., 1996 for review). Afferents in the pelvic nerve innervate the midline perineal region (Martinez-Gomez et al., 1998; Cruz et al., 2004) and pelvic viscera (Martinez-Gomez et al., 1998) such as the proximal urethra. The afferents terminate in the spinal cord at approximately the same level as pudendal afferents (Coolen et al., 2004). As with DNP stimulation, PN (Johnson and Hubscher, 1998) or urethral stimulation triggers bilateral
pudendal motoneuron reflex discharges, demonstrating these afferents are part of the ejaculatory segmental circuitry (Carro-Juárez and Rodríguez-Manzo, 2000; Johnson, 2006). Stimulation of proximal or distal urethral afferents triggers the pudendal reflex and these different regions of the urethra have been shown to be innervated by different peripheral afferents, namely PN and DNP afferents (Delcambre et al., 2011; Sarica and Karacan, 2005).

Studies of fibers innervating various viscera suggest a functional difference between fibers with low and high thresholds for response, a distinction that recent work in our lab has supported. Whereas low threshold fibers respond to peristaltic contractions, high threshold fibers do not, suggesting that low threshold fibers innervating the viscera are involved in regulatory functions under normal physiological conditions, whereas high threshold fibers are not (Sengupta and Gebhart, 1995).

The immunohistochemical signatures of the afferents that are specifically involved in the pudendal reflex are not well known. Cellular components such as neurofilament, glycoprotein or substance P (SP) content have been studied for other populations of afferents such as nociceptors (Fang et al., 2006; Matsuka et al., 2007; Petruska et al., 2000a, b). Neurofilament-M (NFM) is commonly used as a marker for larger, myelinated afferents. NFM immunoreactivity has been correlated with soma size, axon size, and conduction velocity (Lawson and Waddell, 1991; Lee et al., 1986). Lectin I-B4 (IB4) is a protein that binds to terminal α-galactose and thus is used as a label for a population of small and medium sized neurons that contain certain glycoproteins and glycolipids (Benton et al., 2008; Fullmer et al., 2004). SP is well known for its role in nociception including bladder pain (Rudick et al., 2009) and it also has been proposed
to play a role in sexual reflexes, since the receptor it often binds to (neurokinin-1 receptor) has been found in most of the lumbar spinothalamic neurons proposed to be part of a spinal ejaculation generator (Xu et al., 2006). Chapter 3 is committed to elucidating the histochemical signatures and functions of the population of afferents specifically involved in the pudendal reflex.

**Pudendal Motoneurons Innervating Perineal Musculature**

Pudendal motoneurons innervate striated perineal muscles and sphincters (Chambille and Rampin, 2002), namely the levator ani, bulbospongiosus muscle (BSM), ischiocavernosus muscle (IC) (Figure 1-1), external urethral sphincter (EUS), and external anal sphincter (EAS) (Coolen et al., 2004; Holstege, 2005a; McKenna and Nadelhaft, 1989; Pacheco et al., 1997). These motoneurons primarily innervate the sphincters in the female (McKenna and Nadelhaft, 1989) although thin bands of striated muscle analogous to the male bulbospongiosus and ischiocavernosus do surround the vagina (Thor and de Groat, 2010). Pudendal afferent stimulation results in activation of all four main perineal muscles (BSM, IC, EUS and EAS) (Holstege, 2005a; McKenna and Nadelhaft, 1989), although their patterns of contraction are not identical; for example, the EUS must first relax to allow semen through this proximal part of the tract, then undergo contraction to prevent retrograde ejaculation (Lipshultz et al., 1981) while other striated muscles undergo rhythmic contractions for successful expulsion of semen (Wolters and Hellstrom, 2006). The pudendal motor neurons are unique compared to other somatic motor neurons and have several characteristics similar to autonomic effector neurons (Holstege, 2005a). They are trophically dependent on testosterone (Breedlove and Arnold, 1980; Hamson et al., 2009), and they are unaffected by amyotrophic lateral sclerosis (Carvalho et al., 1995) and paradoxical sleep-triggered
inhibition (Mann et al., 2003). They also lack a monosynaptic reflex arc from their homonymous muscle spindle receptors (Bowens et al., 1984; McKenna and Nadelhaft, 1989; Collins et al., 1991). Also, pudendal motoneurons are susceptible to autonomic motor neuron disorders (Sung et al., 1979; Dubrovsky and Filipini, 1990). In the rat, their cell bodies are located in the L5-L6 ventral horn of the spinal cord (Chambille and Rampin, 2002; Holstege, 2005a; McKenna and Nadelhaft, 1986), and are organized into two groups (Coolen et al., 2004; McKenna and Nadelhaft, 1986; Collins et al., 1991); the dorsomedial group (or nucleus) contains a dispersed mixture of motoneurons to the BSM and EAS, and the dorsolateral group contains separate regions for IC motor neurons and EUS motoneurons (McKenna and Nadelhaft, 1986). In dogs, cats, monkeys, and humans, the dorsomedial and dorsolateral groups are combined in a more central location and termed Onuf’s nucleus (Coolen et al., 2004; Schroder, 1985; Beattie et al., 1993; Zempoalteca et al., 2008). The pudendal motor neurons are bilaterally organized to allow for simultaneous contraction, and their dendrites cross the midline to communicate with their contralateral counterparts electrically via gap junctions (Collins et al., 1991; Coleman and Sengelaub, 2002; McKenna and Nadelhaft, 1986). In addition, the pudendal motor nucleus is densely innervated by 5-HT and norepinephrine terminals, and regulation by these neurotransmitters also is implicated in micturition (Jost and Marsalek, 2004). The sustained contractions of the ischiocavernosus and bulbospongiosus contribute to rigidity during penile erection (Giuliano and Rampin, 2004; Giuliano et al., 1995; Holmes et al., 1991; McKenna, 2000a; Rampin, 2004; Tang et al., 1998; Zempoalteca et al., 2008), in part by producing venous outflow restriction (Zempoalteca et al., 2008). During the expulsion phase of
ejaculation, the perineal muscles including the BSM and IC undergo synchronous
rhythmic contractions which result in expulsion of semen from the urethra (Bernabe et
al., 2007; Coolen et al., 2004; Giuliani and Rampin, 2004; Johnson, 2006; McKenna,
2000a; Xu et al., 2005). In humans these contractions are 600 ms apart at the start of
ejaculation and increase by 100 ms with each subsequent contraction (Bohlen et al.,
1982; Coolen et al., 2004). The bulbospongiosus also has roles in micturition. In
humans, adequate inhibition of the BSM is required for normal voiding (Yasuhiro et al.,
2004). During micturition in rats, the BSM undergoes rhythmic contractions
(approximately 50 ms apart) resulting in expulsion of urine (Cruz and Downie, 2005).

Retroviral tracers have been of excellent use for elucidating the connections
between pudendal motoneurons and other neurons in the circuitry involved in sexual,
bladder and bowel functions (Marson and Carson, 1999; Vera and Nadelhaft, 2000).
The pseudorabies virus (PRV) has been shown to have a strong affinity for
motoneurons in rats when injected into either muscle or nerve (Card et al., 1990). The
virus is transported to the motoneuron somata in the spinal cord and replicates. It then
infects only those neurons synaptically connected to the primarily infected motoneurons
(Card et al., 1993; Card et al., 1990; Rinaman et al., 1993). The virus is live and
continues to replicate and infect more neurons. With longer survival periods, tertiary
and quaternary infections become evident. These even include the primary afferent
neurons involved in reflexes of specific pathways, with either Becker PRV or an
attenuated PRV strain (Bartha PRV) (Jasmin et al., 1997). PRV tracing by Johnson et
al. (2011) have confirmed the bilateral nature of pudendal motoneuron projections.
Spinal Interneurons

The pudendal reflex is polysynaptic; there are no monosynaptic connections between DNP or pelvic nerve afferents and pudendal motoneurons; all connections are regulated by interneurons (McKenna and Nadelhaft, 1989). Electrophysiological (Johnson, 2006) and immunohistochemical (Wiedey et al., 2008) techniques have been used to investigate single spinal cord interneurons in the dorsal horn and intermediate zone, primarily of the L6-S1 segments which receive input from DNP afferents. Johnson (2006) found that all penile-responsive interneurons exhibit receptive fields on the penis that are significantly larger than the receptive fields for single primary afferent neurons, thereby demonstrating a central convergence of penile sensory input. Almost all these penile-responsive interneurons have receptive fields on both sides of the body, and their electrical characteristics suggest a monosynaptic input from both ipsilateral and contralateral DNP fibers. There also is an extensive representation from the distal glans region in these spinal cord interneurons. DNP afferents produce bilateral (crossed and uncrossed) reflex firing of pudendal motoneurons located in L5-L6 (Johnson, 2006).

The location of interneurons involved in this reflex arc has been confirmed in transneuronal tracing studies (Collins et al., 1991; Marson and McKenna, 1996). These interneurons are located bilaterally in the medial gray, around the central canal, ranging from the upper lumbar to sacral spinal levels (Coolen et al., 2004; Johnson, 2006). This is consistent with the location of the spinothalamic cells proposed to act as a central pattern generator for ejaculation- specifically, lamina X of the L3-L4 cord (Xu et al., 2005; Xu et al., 2006; Clement et al., 2006; Truitt and Coolen, 2002).
Electrophysiological techniques also have been used to investigate single spinal cord interneurons in the dorsal horn and intermediate zone of lumbosacral spinal cord regions that receive input from the bladder, distal colon, and rectum in the rat. Many neurons responsive to visceral distention have been characterized, along with the sources of their cutaneous convergent inputs (Ness and Gebhart, 1987; Cadden and Morrison, 1991; Berkley et al., 1993). In addition, Ness and Gebhart (1987) revealed many L6-S1 colorectal distention-responsive neurons under tonic descending inhibition.

The interneurons identified as being involved in sexual, bladder and bowel regulatory circuitry can be excitatory or inhibitory. Some of the inhibitory interneurons are GABA-ergic and may play a role in presynaptically inhibiting DNP afferents, a topic that will be discussed later.

**Brainstem Circuitry**

**Sensory Information Conveyed from Pelvic/Visceral Territories to the Medulla**

Studies of female rats demonstrate that neurons located in the medullary reticular formation (MRF) and specifically the nucleus reticularis gigantocellularis (NRGi) and its surrounding regions respond to probing the vaginal canal (Hornby and Rose, 1976) and are involved in the female circuitry responsible for lordosis behavior (Modianos and Pfaff, 1979). In addition, neurons in the NRGi have been shown to receive ascending sensory information from other somatic and visceral sources (Chan, 1984; Peschanski and Besson, 1984). Studies have found pathways linking penile afferents to the MRF (Cothron et al., 2008; Hubscher and Johnson, 1999b). In our initial studies in male rats, we demonstrated that neurons receiving inputs from the male urogenital tract could be found throughout the NRGi nuclear complex. Using bilateral electrical stimulation of the DNP as our search stimulus, we found DNP-responsive neurons in all of the NRGi
subdivisions in intact male rats (Hubscher and Johnson, 1996). Single neurons responsive to bilateral electrical stimulation of the DNP were isolated and characterized throughout the NRGi. The majority of these neurons also exhibited an excitatory response to mechanical stimulation of receptive fields on either side of the penis (especially the distal glans region). All of the neurons sampled responded to both ipsilateral and contralateral DNP stimulation and had no background activity. Many of these neurons required wind-up with bilateral electrical stimulation of the DNP in order to respond. Many DNP-responsive neurons were additionally responsive to bilateral electrical stimulation of the PN. We also have shown that these neurons respond to selective A or C fiber afferent electrical stimulation (Petruska et al., 1998).

Transsynaptic tracing techniques using PRV have been used to identify brainstem neurons involved in sexual and bladder functions. Using the attenuated PRV strain (Bartha PRV), many labeled MRF neurons have been identified following injections of the striated perineal muscles (ischiocavernosus, bulbospongiosus) (Marson and McKenna, 1996; Johnson, 2006), prostate (Orr and Marson, 1998), urinary tract (Nadelhaft and Vera, 1995, 1996; Vizzard et al., 1995; Marson, 1997), penis (Marson et al., 1993) and clitoris (Marson, 1995). De Groat and co-workers (1996) used PRV injections of the colon in normal and T8 transected animals to determine spinal nerve versus vagal sources of supraspinal pathways. The MRF (and NRGi) is one of many regions of the brainstem that have been shown to receive inputs from pelvic visceral territories (Al-Chaer et al., 1996; Ding et al., 1997; Ness et al., 1998). Many of these other regions are likely interconnected with the MRF (Jones, 1995).
Motor Output from NRGi to Lumbosacral Circuitry Mediating Pelvic/Visceral Functions

The brainstem contains many regions with descending projections to circuitries controlling pelvic/visceral functions (Hubscher and Johnson, 2000; Johnson and Hubscher, 1998; Inatomi et al., 1998; Loewy et al., 1979; Murphy et al., 1996; Sasa and Yoshimura, 1994; Standish et al., 1992). Stimulating the NRGi produces electrophysiologically recorded field potentials in the lumbosacral spinal cord in close proximity to pudendal motor nuclei (Tanaka and Arnold, 1993), and anatomical tracing studies have shown that this same region of the spinal cord is neuroanatomically connected to the NRGi (Marson and McKenna, 1990; Monaghan and Breedlove, 1991; Shen et al., 1990; Hermann et al., 2003). The gigantocellularis (Gi) region appears to have a tonic inhibitory effect on erection (Marson et al., 1992) and ejaculation (Peeters and Giuliano, 2008). Large lesions that included the ventral Gi, Gi pars alpha and lateral paragigantocellularis (LPGi) nuclei reduced ejaculatory bursts in perineal muscles (Marson and McKenna, 1990). Smaller lesions of the nucleus raphe obscurus or LPGi had significant effects on EAS and perineal muscle reflexes, respectively (Holmes et al., 2002). This region of the MRF also contains a modulatory pathway for micturition (Sugaya et al., 2003). Based on a recent anatomical tracing study which looked at the connections between the MRF and perineal motoneurons (Hermann et al., 2003), the LPGi region contains neurons which appear to modulate the reflex activity of the perineal muscles involved in defecation, micturition, and ejaculation.

In earlier studies, our lab electrically microstimulated areas in the MRF where DNP-responsive neurons were found (Johnson and Hubscher, 1998; Hubscher and Johnson, 2000). In anesthetized male rats, microstimulation of the LPGi produced a
decrease in amplitude and increase in latency of the short latency pudendal motoneuron reflex discharges (PMRD) elicited by stimulation of the DNP. No effects on PN-elicited PMRD were found. The most robust reflex depression was observed when the LPGi was microstimulated bilaterally. Nearly 40% of the bilateral electrode tracks within the microstimulation matrix contained pudendal motoneuron modulation sites. The reflex depression consisted of an increase in the reflex latent period followed by a decrease in the size of the PMRD. The PN-elicited PMRD, however, were not appreciably modulated at any location within the MRF microstimulation matrix. A significant finding was that bilateral MRF conditioning was always more effective than unilateral microstimulation for motoneuron pools on either side of the cord. The MRF microstimulation (conditioning stimulus alone) never produced a direct firing of myelinated pudendal motoneurons. The latency of effect strongly suggested the spinal mechanism involves presynaptic inhibition of DNP afferents. This may involve descending serotonergic LPGi fibers interacting with GABA-ergic interneurons which in turn act on GABA receptors on DNP afferents. These likely would be GABA-A receptors, based on the relatively short latency of effect compared to that of GABA-B receptors. GABA-receptor activation on DNP afferent terminals allows chloride to flow out of the terminal due to the local gradient, thus depolarizing the cell. Presynaptic inhibition of pudendal and urethral afferents has been shown in the spinal micturition circuit in cats (reviewed by Shefchyk, 2002). The possibility of presynaptic inhibition on DNP afferents from LPGi microstimulation was investigated and is discussed in Chapter 2.
Presynaptic Inhibition

Based on our previous studies, the interneurons to which the LPGi projects may presynaptically inhibit the pudendal afferent terminals in the lumbosacral cord and produce rapid and transient primary afferent depolarization (PAD) which, as will be discussed, has an inhibitory effect on incoming action potentials. Moreover, we have shown that the LPGi descending circuitry does not appear to cause postsynaptic inhibition of pudendal motoneurons since the pelvic nerve elicited reflex is not affected (internal control) at the same LPGi site. Interestingly, the pelvic nerve afferents do not show evidence of LPGi-elicited presynaptic inhibition and are not as sensitive to rate depression in the segmental circuitry.

Periodic presynaptic inhibition may be responsible for the depression of DNP-elicited pudendal motoneuron reflex discharges, which may play a role in the coordination of ejaculation; each incidence of DNP afferent inhibition lasts 50-75 ms, which corresponds to the period of quiescence between bulbospongiosus contractions during ejaculation in the rat (Meisel and Sachs, 1994). A similar brainstem loop is known to exist for the process of micturition; the pontine micturition center in the brainstem projects to sacral GABA-ergic interneurons which inhibit the EUS (Holstege, 2005b; Johnson, 2006). Past studies have shown the existence of interneurons in the lumbosacral cord which mediate presynaptic inhibition of afferents, namely the sural and posterior biceps and semitendinosus nerves (Jankowska et al., 2000), although these interneurons were shown to be activated by neighboring afferents rather than supraspinal sites.

Primary afferent depolarization disrupts any incoming action potential by reducing the amplitude of the action potential (French et al., 2006). The existing depolarization at
the terminal does this by preventing much further cation influx. Thus, the voltage-gated calcium channels responsible for neurotransmission do not detect a large change in voltage, and are not as likely to get activated or, if activated, releases less excitatory neurotransmitter (French et al., 2006). Hence, presynaptic inhibition would depress discharges of pudendal motoneurons. PAD by itself does not trigger action potentials. However the depolarization can be inferred from an observed increase in axonal excitability during the administration of a “Wall Test” (Lidierth and Wall, 1998). Dorsal root potentials (DRPs) also are a sign that PAD may be occurring in the axons.

While our observed evidence of presynaptic inhibition is likely due to GABA-A receptor activation based on the latency of effect, the GABA-B receptor may also play a role in the regulation of sexual reflexes. GABA-B receptors are found on primary afferent terminals (Li et al., 2004). Studies have shown that lumbosacral cord injections of GABA-B agonists such as baclofen inhibit erection and ejaculation (Bitran et al., 1988; Leipheimer and Sachs, 1988; Vaidyanathan et al., 2004), but not when injections are performed at T8 levels (Bitran et al., 1988). Baclofen has been reported to depress the pudendal to pudendal nerve reflex, and is said to act centrally by potentiating presynaptic inhibition, so the GABA-B receptor also appears to be involved in presynaptic inhibition of pudendal afferents (Johnson, 2006; Teague and Merrill, 1978), although it most likely involves a mechanism other than PAD (Rudomin and Schmidt, 1999), since the GABA-B receptor is metabotropic and is not directly connected to an ion channel. GABA-A and 5-HT₂ receptor mechanisms appear to play roles in PAD-mediated presynaptic inhibition (Thompson and Wall, 1996), while GABA-B receptor manipulation was found to have no significant effect on DRPs. However, studies have
shown that 5-HT application produces a slow PAD (Lopez-Garcia and King, 1996) rather than the fast PAD seen in our system although it has been reported to increase the chloride current and depolarization caused by GABA (Akasu, 1988). GABA-B activation may cause presynaptic inhibition via modulation of calcium channels (French et al., 2006). This would result in inhibition of neurotransmission since calcium is essential for fusion of vesicles to the presynaptic membrane and release of their contents. Dolphin and Scott (1986) showed that a GABA-B agonist reduced voltage-dependent calcium currents in cultured rat dorsal root ganglion neurons. They later demonstrated that GABA-B receptors are directly coupled to calcium channels via G-proteins (Dolphin and Scott, 1989).

Use of Animal Models in Studying the Effects of Chronic Spinal Cord Injury on Sexual and Bladder Reflexes

The male rat is an appropriate animal model for these studies for several reasons including: 1) a substantial behavioral database exits for erectile and ejaculatory events in the normal and spinal cord-injured rat (Hart and Leedy, 1985), 2) normally appearing penile reflexes can be elicited in the urethane-anesthetized rat thereby providing strong rationale for detailed electrophysiological studies (McKenna et al., 1991), 3) the spinal cord-injured rat demonstrates similar erectile and ejaculatory signs to those of man with spinal cord injury (Sachs and Garinello, 1979; Johnson, 2006). 4) afferents and efferents in the pudendal nerve are segregated into the motor branch and sensory branch in the rat, making this model well suited for electrophysiological experiments.

Behavioral data has shown that ejaculation is dependent on intact pudendal nerve afferents. In the normal animal, the pudendal afferent facilitation of ejaculation requires the brainstem loop, much like the control of micturition (Mallory et al., 1989). The loss
of ejaculatory ability following severe spinal cord injury may be caused by the removal of this brainstem loop. Studies have shown that depressed ejaculatory reflexes after midthoracic spinal transection (Sachs and Garinello, 1979; Hart and Odell, 1981; Mas et al., 1987) are likely due to the loss of supraspinal control. The disruption of descending pathways that use monoamines (Mas et al., 1985; Svensson and Hansen, 1984), opiates (Wiesenfeld-Hallin and Sodersten, 1984), and other (Bitran and Hull, 1987) modes of neurotransmission which are know to affect sexual reflexes in the rat are likely involved in the decreased efficacy of penile sensory input. Normal micturition, which involves parasympathetic input to the bladder and simultaneous inhibition of motoneurons to the urethral sphincter, is similarly coordinated by a spino-bulbo-spinal reflex pathway (de Groat, 1995). Kakizaki and de Groat (1997) demonstrated that micturition reflexes mediated by lumbosacral parasympathetic pathways are unmasked after chronic spinal injury at T8. Thus, after spinal injury, a segmentally-based voiding reflex normally seen only in young animals re-emerges (Maggi et al., 1986), although bladder emptying may be incomplete due to detrusor-sphincter-dyssynergia.

Re-emergence of ejaculation does not, however, return following chronic spinalization possibly because of a difference in the input pathways (pudendal afferents versus pelvic afferents). Our previous data suggests that mid-thoracic spinal cord transection injury severs a pathway from the brain that acts to modulate the DNP-pudendal motoneuron reflex possibly through the initiation of segmentally-derived presynaptic inhibition of DNP afferents. At the segmental level, the polysynaptic efficacy of DNP afferents and associated interneurons onto pudendal motoneurons (Collins et al., 1991; McKenna and Nadelhaft, 1989) is progressively reduced in rats.
with chronic spinal cord transection (Johnson, 2006). In normal unoperated animals and in those with an acute 4-6 hour mid-thoracic transection, the magnitude of the DNP evoked reflex was significantly greater than that evoked by PN stimulation suggesting an acute loss of descending inhibition. In animals with a complete transection lesion of 3, 30, or 60 days, however, there was a progressive decrease in the ratio of reflex activation by DNP afferents to PN afferents. In particular, at 30 and 60 days after injury, the relative magnitude of PN-mediated activation was significantly greater than that generated by the DNP; the contralateral DNP-mediated activation was almost nonexistent. In addition, the degree of segmentally-derived presynaptic inhibition from stimulating DNP afferents was reduced.

These data are significant in that the spinal reflex circuitry for ejaculation, normally under supraspinal modulatory control, has undergone a reorganization following chronic complete spinal cord injury. The reduction in segmental reflex synaptic efficacy of pudendal nerve afferents suggests that some pudendal input reorganization has occurred after a long-term transection injury. Elimination of descending pathways after injury may have left pudendal reflex afferent circuits approachable by other segmental systems as described by Beattie et al. (1993) in the cat, possibly via the visceral afferent inputs originating in the pelvic nerve or by an increased distribution of segmental GABA-ergic interneurons on pudendal afferent terminals. In addition to this observed plasticity, Li and coworkers have shown that GABA-ergic input to pudendal motoneurons increases following SCI (1995). This may reflect the system’s attempt to restore inhibitory modulation to the pudendal reflex, as metabotropic GABA-ergic
receptors are thought to suppress plateau potentials that prolong a neuron’s output to a brief excitatory input (Derjean et al., 2003).

The loss of a descending GABA-ergic pathway that normally activates GABA-B receptors may contribute to the spasticity observed after SCI (Wang et al., 2002), since treatment with Baclofen, a GABA-B agonist, alleviates this condition. Both GABA-A and GABA-B receptors have important roles in micturition, as intrathecal application of agonists for either receptor results in urethral relaxation during isovolumetric bladder contractions in spinal cord injured rats (Miyazato et al., 2008). Other studies demonstrate that application of GABA-B agonists such as Baclofen to the lower cord inhibits erection (Bitran et al., 1988; Leipheimer and Sachs, 1988), suggesting that the GABA-B receptor is an important component in the segmental control of sexual function. The elimination of this descending system may account for the decrease in GABA binding sites seen after spinal transection (Charlton et al., 1981).

To determine where the pudendal reflex inhibitory pathway runs in the mid-thoracic spinal cord white matter, partial acute (4 hour) and chronic (30 day) lesions were performed in a series of rats (Hubscher and Johnson, 2000). LPGi microstimulation-induced inhibition of the DNP-elicited pudendal motoneuron reflex discharges was not seen in animals with a complete chronic dorsal hemisection or in animals where the central portion of both lateral funiculi was damaged. In Chapter 4, similar spinal lesions will be performed to assess the effect of lateral funiculus damage on bladder function.
Figure 1-1. Drawings of the ischiocavernosus (IC) and bulbospongiosus ventral (VBS) and dorsal (DBS) portions.
CHAPTER 2
THE PUDENDAL REFLEX: ELECTROPHYSIOLOGICAL STUDIES

Introduction

The neural control of the expulsive phase of ejaculation involves segmental circuitry at the lumbosacral level as well as descending modulation from the brainstem (Hubscher and Johnson, 2000; Johnson and Hubscher, 1998). Following the emission phase, semen is expelled from the urethra by coordinated and sequential contraction of somatic perineal muscles (Giuliano and Rampin, 2004; Holmes et al., 1991; McKenna, 2000a) innervated by pudendal motor neurons. Pudendal motor neurons innervate several perineal muscles (Chambille and Rampin, 2002) including the external urethral sphincter (EUS), external anal sphincter (EAS), ischiocavernosus muscle (IC), and bulbospongiousus muscle (BSM) (Coolen et al., 2004; McKenna and Nadelhaft, 1989; Pacheco et al., 1997). Contraction of different perineal muscles in the correct sequence may be accomplished by differences in their mean time-to-peak tension values (Bowen et al., 1984). Consistent with this, McKenna and Nadelhaft (1989) recorded from the pudendal motor branch while stimulating pudendal afferents and observed both early and late components to the pudendal reflex.

The bulbospongiosus muscle (BSM) surrounds the base of the penis (Figure 1-1) and is a frequently studied perineal muscle important to the expulsive phase of ejaculation (Giuliano and Rampin, 2004; McKenna, 2000a). In the male rat, the cell bodies of pudendal motoneurons are found in the L5-L6 spinal cord (Chambille and Rampin, 2002). Sensory input modulates these motor neurons via interneurons, which have been characterized electrophysiologically (Johnson, 2006) and by transneuronal tracing studies (Collins et al., 1991; Marson and McKenna, 1996). There are no
monosynaptic connections between sensory afferents and pudendal motor neurons (McKenna and Nadelhaft, 1989).

Two well-known sensory inputs to pudendal motoneurons are from dorsal nerve of the penis (DNP) afferents and from pelvic nerve (PN) afferents. Stimulation of either of these nerves causes pudendal motoneuron reflex discharges (PMRD) in the motor branch of the pudendal nerve (Johnson and Hubscher, 1998), a nerve containing motor axons terminating in the perineal muscles including the BSM. Observation of PMRD has been used as a measure of integrity of the spinal circuit (McKenna and Nadelhaft, 1989) for ejaculation in males after SCI as well a way to assess treatments for bladder control in females. Removal of any penile sensory (Hart and Leedy, 1985), lumbosacral spinal cord or motor limbs of this reflex prevents ejaculation (Everaert et al., 2010).

The pudendal nerve is a mixed nerve. It contains a primarily sensory branch containing DNP afferents which innervate the glans (Johnson and Murray, 1992; Giuliano and Rampin, 2004) and distal urethra (Kessler et al., 2007; Yang and Bradley, 1999), a superficial perineal branch which innervates the skin of the scrotum, and a primarily motor branch containing the axons of pudendal motor neurons. The pudendal nerve also carries sympathetic postganglionic nerves to the pelvic regions, though it is not thought to play a major role in the pudendal reflex (Johnson and Hubscher, 2000; McKenna and Nadelhaft, 1989). The pelvic nerve (PN) includes afferents that innervate regions such as the proximal urethra (Kessler et al., 2007), prostate, bladder (Shea et al., 2000), colon and rectum (Christianson et al., 2008; Feng et al., 2010). The pelvic nerve and pudendal nerve merge to form the L6-S1 trunk before once again splitting and entering the L6 and S1 dorsal roots. Some terminals contact interneurons in the
L6-S1 cord (Wiedey et al., 2008). One goal of our electrophysiological studies was to clarify to what extent each root carries these afferents.

Previous experiments by our laboratory demonstrate that stimulation of pudendal somatic afferents (DNP test stimulus) elicits a compound action potential in the motor branch of the pudendal nerve. DNP, glans, or distal urethral stimulation was also found by Yang and Bradley (2000) to elicit BSM contraction. Like the DNP, stimulation of pelvic nerve visceral afferents (PN test stimulus) also triggers the pudendal reflex (Johnson and Hubscher, 1998). Our electrophysiological studies of spino-bulbo-spinal connections to this circuit have found individual neurons in the gigantocellularis nuclear region of the medullary reticular formation (MRF) that respond to stimulation of both DNP and PN afferents, most of which were excitatory, some of which required wind-up from bilateral DNP stimulation to respond (Hubscher and Johnson, 1996). Compared to responses from DNP stimulation, responses of the neurons to PN stimulation were either similar or weaker (Hubscher and Johnson, 1996). Hence, DNP and PN afferents not only trigger the pudendal reflex at the segmental level, both send ascending projections to supraspinal regions including the MRF (Hubscher and Johnson, 1996).

Neurons from the MRF, particularly the LPGi, in turn send descending projections back to the segmental pudendal circuitry, forming a long-loop reflex (Hubscher and Johnson, 2000; Johnson and Hubscher, 1998; Marson and McKenna, 1990). One goal of our current study was to determine how these descending brainstem projections may be modulating the pudendal reflex at the segmental level.

Our laboratory has previously shown that a region of the brainstem, the lateral paragigantocellularis (LPGi), exerts descending inhibitory control of the pudendal reflex
circuitry. Lesion studies involving lateral hemisections have suggested that this descending inhibitory control runs through the central lateral funiculus of the cord, and the projections initially descend ipsilaterally, but project bilaterally in the lumbosacral cord somewhere below the T8 level (Hubscher and Johnson, 2000; Johnson et al., 2011). Microstimulation of the LPGi (conditioning stimulus) given just before a DNP test stimulus results in a depression of the pudendal reflex, as evidenced by a decrease in the evoked reflex compound action potential as recorded from the motor branch of the pudendal nerve. However, LPGi conditioning stimulation given just prior to the PN test stimulus does not result in a depression of the pudendal reflex (see Figure 2-1, adapted from Johnson and Hubscher, 1998). This suggested that the LPGi modulates the pudendal reflex by exerting selective presynaptic inhibition of pudendal somatic afferent inputs but not pelvic visceral afferent inputs.

Our previous studies concentrated on the reflex activation of pudendal motoneurons by stimulating the DNP in the ischiorectal fossa. At this site, the DNP includes afferents from the urethra and penile bulb in addition to the glans. Utilizing single-afferent recording techniques, Delcambre et al. (2011) recently confirmed the ability of DNP urethral afferents to respond to either fluid movement or pressure from fluid accumulation (the latter of which produced tonic firing) and for these DNP afferents as well as PN afferents to cause bilateral PMRD in response to such stimulation. Our interest in the present study was to determine the reflex effects of electrically stimulating the DNP afferents at the proximal border of the glans which only innervates the glans tissue and the most distal portions of the urethra.
Methods

Surgical Preparation for Terminal Electrophysiological Experiments

All procedures used for this study was approved by the Institutional Animal Care and Use Committee in accordance with USDA regulations. Anesthesia (urethane, 1.2 g/kg i.p.) was administered to mature male Wistar rats and they were given i.p. supplements as needed. The common carotid artery, jugular vein, and trachea were intubated in order to monitor blood pressure, infuse fluids via an i.v. route, and facilitate ventilation if needed. An esophageal thermistor and circulating water heating pad were used to measure and maintain body temperature at 37 degrees Celsius. The ventilatory state was monitored with an end tidal CO2 monitor and animals were artificially ventilated if necessary. Mean blood pressure was maintained at 75 mm Hg or above. The head was clamped in a stereotaxic holder at an approximate 30 degree angle, a dorsal mid-line incision was made and the occipital bone, dura mater, and atlantooccipital membrane was removed to expose the caudal medulla. In order to access the rostral medulla, a small caudal portion of the cerebellum was removed. In order to position the penis, ventral abdomen and perineum so that these areas could be exposed for stimulation, the ventral side of the pelvic area was swung caudally by pivoting the hindquarters around the axis through hip pins and tying the tail in an upward direction. Bipolar silicon cuff microelectrodes were placed either unilaterall or bilaterally around the DNP and the PN in the ischiorectal (IR) fossa and at the proximal border of the glans. The motor branch of the pudendal nerve containing the pudendal motorneuron axons was exposed, transected distally, stripped of epineurium, and surrounded by a small 36 gauge platinum bipolar recording electrodes in a pool of mineral oil. A T13-L2 laminectomy was made over the lumbosacral spinal cord and the
dura incised and reflected to form a mineral oil pool. The L6 and S1 dorsal roots were exposed at the dorsal root entry zone (DREZ) for recording of DRPs and stimulating afferent axons. After dorsally isolating the DNP and pelvic nerve (PN) in the ischiorectal fossa, their central cut ends were placed on bipolar recording electrodes distal to the microstimulation cuffs. To determine if the DRPs are in part due to PAD in the DNP or PN afferent terminals, the axonal excitability of central axons was tested by antidromically stimulating them before and after LPGi microstimulation using the technique of Lidierth (2006).

**Brainstem Microstimulation**

Fine gauge stainless steel microelectrodes attached to a stepping microdrive were used as previously described in Johnson and Hubscher (1998). Two microelectrodes were positioned for bilateral penetration of the brainstem in the same anterior/posterior plane, at equal distances from the midline. A monopolar cathodal stimulating current was passed through the microelectrodes either individually or simultaneously in order to stimulate the LPGi either unilaterally or bilaterally (Figure 2-2), a technique initially described in our lab (Johnson and Hubscher, 1998, 2000). To determine if specific sites of the LPGi were capable of inhibiting the segmental circuitry, pudendal motoneuron reflex discharges (PMRD) in response to DNP or PN stimulation (single 0.1 ms duration pulses at 3 x threshold and at 0.5 Hz) were quantified as averaged evoked responses (ten trials) before, during, and after LPGi stimulation. Current intensities of 15-20 mA were used in the brainstem. To determine whether this microstimulation-elicited inhibition of reflex discharge was due to presynaptic inhibition of L6-S1 afferent terminals, the L6 and S1 rootlets were placed over platinum hook electrodes to record the antidromic dorsal root potentials (DRPs) as described in our lab and others (Lidierth
and Wall, 1998; Cote and Gossard, 2003). In a few animals, we recorded DRPs in response to microstimulation of the white matter in the T8 spinal cord.

**Axonal Excitability Tests for Primary Afferent Depolarization**

Using the “Wall test” technique of Lidierth (2006), the single shock “test” stimulus was applied to the DREZ of the left L6 or S1 dorsal root (0.5 ms duration pulse) before and after a “conditioning” train microstimulation (five 0.2 ms duration pulses) from the LPGi location generating the largest amplitude DRP. Recordings were made from the central cut end of the left DNP and left PN. Quantitation of axonal excitability changes after conditioning microstimulation from the LPGi were performed at 1.5 times threshold (T) for antidromic activation of DNP or PN afferents before and after LPGi conditioning microstimulation. Areas under the averaged potential curve were measured to assess the increase in number of DNP and PN axons recruited by LPGi microstimulation due to primary afferent depolarization (PAD).

**Results**

**DNP but not PN Afferents are Presynaptically Inhibited by the LPGi**

Primary afferent depolarization (PAD) can cause presynaptic inhibition in a spinal circuit by disrupting the synaptic efficacy of incoming action potentials. This happens because a preexisting, sub-threshold depolarization in an afferent terminal will lower the net change in terminal membrane potential from an incoming action potential. Summated PADs are electrotonically spread peripherally in the dorsal roots where it can be recorded as a dorsal root potential (DRP). To see if PAD might be occurring from LPGi stimulation, we recorded from an L6 dorsal root while stimulating the ipsilateral and contralateral LPGi. We observed DRPs from either ipsilateral or contralateral LPGi stimulation (Figure 2-3), supporting the bilateral nature of this modulatory pathway. The
DRPs elicited from the ipsilateral LPGi were stronger than those from the contralateral LPGi. The onset of the maximum DRPs occurred at approximately 20 ms with the peak potentials between 35-40 ms.

To determine whether LPGi stimulation causes PAD in DNP afferents, axonal excitability tests (“Wall test”) were performed. In this procedure, a test shock of 0.5 ms pulse was given to the L6 DREZ, and antidromic compound action potentials are recorded out in the periphery from the central cut end of the DNP. The test shock, when given at threshold, will cause only a few DNP afferents to antidromically fire. However if the afferent terminals are experiencing sub-threshold depolarization (such as from LPGi stimulation as in our hypothesis), this means that more of these afferents are already closer to threshold, and in this case the test shock would cause more afferents to antidromically fire. The results show that this is indeed what happened; LPGi conditioning stimulation given 35 ms (time frame chosen based on DRP tests) prior to the test shock resulted in an increase in the recorded compound antidromic action potential in the DNP, compared to the test shock alone (Figure 2-4). This demonstrated that the LPGi indeed exerts its presynaptic inhibitory effect on the pudendal reflex by causing primary afferent depolarization in DNP afferent terminals. On the contrary, there was no evidence for LPGi-induced PAD observed in PN afferent terminals as seen with simultaneous recordings of PN afferents (Figure 2-4). Figure 2-5 shows a quantification of the sizes of the DNP versus PN compound action potentials, each following LPGi conditioning and dorsal root test shock stimulation. There is a significant and quite profound increase in the size of the DNP compound action potential as compared to that of the PN.
Manipulation of the time interval between LPGi conditioning stimulation and the dorsal root test stimulus revealed that the optimal delay was approximately 35 ms. This delay resulted in the greatest increase in the axonal excitability (and therefore the maximum presynaptic inhibition) of DNP afferents (Fig. 2-6). The short time course of LPGi-induced PAD (over a 20-80 ms period) suggests the presence of a transient ionotropic GABA-A receptor mechanism versus the non-PAD based and long-lasting metabotropic GABA-B receptor mechanism.

Unilaterally stimulating the central lateral funiculus of the spinal cord at the T8 level resulted in bilateral DRPs similar to those caused by LPGi stimulation (Figure 2-7), suggesting this is the spinal location of this descending pathway, which agrees with lesion studies previously done in our lab.

**Effects of Glans DNP Afferent Stimulation on Pudendal Reflex**

To compare the efficacy of the glans DNP afferents (DNPg), the ischiorectal DNP (DNPi) and the PN to elicit the pudendal reflex when electrically stimulated, compound action potentials were recorded from the motor branch of the pudendal nerve as the PMRD. The DNPi and PN were accessed in the ischiorectal fossa and electrically stimulated with bipolar electrodes, and the DNP at the proximal base of the glans was stimulated bilaterally with a cuff electrode.

All three sources of afferents, when electrically stimulated, activated PMRD. Stimulation of either the glans or the DNPi elicited pudendal reflex responses of similar magnitude; however glans stimulation resulted in a response with longer latency (see top two traces in Figure 2-8), presumably because of the more central location of DNP stimulation in the ischiorectal fossa and thus shorter conduction distance. PN stimulation resulted in an even longer response latency (see bottom trace in Figure 2-8)
than either DNPi stimulation or DNPg stimulation. A quantification of the pudendal reflex response latencies from stimulation of the DNPg, DNPi, or PN is shown in Figure 2-9. DNP stimulation resulted in a significantly shorter response latency compared to glans stimulation. The longer response latency as a result of PN stimulation compared to either glans or DNPi stimulation was significant as well (P<0.001). We found that stimulation of glans DNP afferents induces depression of the pudendal reflex in a manner similar to stimulation of the DNP in the ischiorectal fossa. As we reported recently, if one side of the DNP is stimulated in the ischiorectal fossa to elicit the pudendal reflex (DNP test stimulus), DNP stimulation on the contralateral side results in transient depression of the reflex at an optimum condition-test interpulse interval of approximately 25 ms. The pudendal reflex inhibition is minimized with stimulation of only larger DNP afferents, and recruitment of smaller fibers through stronger stimulation increased the degree of inhibition (Johnson et al., 2012).

Similar to the effect of conditioning stimulation of DNP afferents in the ischiorectal fossa, conditioning stimulation administered to the DN Pg at the proximal border of the glans also produced an inhibitory effect on the pudendal reflex (Figure 2-10). Moreover, just as with conditioning stimulation from the DN Pi, the maximum depression of the reflex caused by DN Pg stimulation occurred at a condition-test interval of 25 ms. Different paired pulse intervals were tested, and the intervals of 500 and 100 ms produced significantly less (P<0.001) depression of DNP reflex inputs compared to intervals of 25 ms. Also, based on reflex latency time, the transient paired pulse-dependent inhibition of the reflex by glans afferents cannot be due to small myelinated or unmyelinated afferents; hence it must be due primarily to larger myelinated fibers.
However, strong stimulation to the glans which recruits the small fibers did increase the degree of inhibition.

Afferent volleys of glans DNP afferents were measured in the L6 and S1 spinal nerves. Analysis of the volleys demonstrated that in only some animals was a small but minimal projection seen in the S1 spinal nerve (see bottom trace in part A of Figure 2-12). However for most animals, the glans DNP afferents projected only through the L6 spinal nerve (see top trace in part A of Figure 2-12). This was corroborated in the anatomical tracing data as discussed in the next chapter.

Conduction velocities demonstrated waves for Aβ and Aδ myelinated fibers and unmyelinated C fibers.

**Discussion**

**Implications for the Differential Inhibition of PN versus DNP Afferents**

Results show that the pudendal reflex is under inhibitory control by both descending pathways from the LPGi and as well as segmental sources, namely DNP afferents from the glans and distal urethra. Any inhibitory control from PN afferents is not as substantial (Delcambre et al., 2011). While the inhibition from the LPGi is effective for the DNP-elicited pudendal reflex, it did not have an effect on the PN-elicited pudendal reflex. Thus, pelvic nerve afferents or the interneurons which synaptically connect them to bulbospongiosus motor neurons appear to be unresponsive to inhibition from the LPGi. On the other hand, the DNP afferents responsible for eliciting the pudendal reflex appear to be easily inhibited by either the descending influences of the LPGi or by contralateral DNP afferents. However, conditioning stimulation of PN afferents does not inhibit the DNP-elicited pudendal reflex (Johnson, 2006).
PN afferents therefore appear to be neither susceptible to or facilitators of presynaptic inhibition in the pudendal circuitry.

Specifically, the presynaptic inhibition produced from LPGi microstimulation involves a transient primary afferent depolarization (PAD), i.e. the inhibition is occurring directly at the DNP afferents’ terminals. The LPGi descending pathways appear to be exerting their inhibitory influence with a very high degree of specificity; DNP afferent terminals are inhibited without noticeable inhibition of PN afferents, excitatory PMRD interneurons, or the pudendal motoneurons themselves.

The pudendal reflex-eliciting DNP and PN afferents differ not only in their susceptibility to inhibition, but also in the regions of the urethra they innervate. DNP and PN afferents innervate the distal and proximal urethra, respectively. There is some overlap, which occurs at the proximal end of the penile bulb (Delcambre et al., 2011), approximately at the position of the BSM. Therefore, it would seem logical that these characteristics may be implicated in the timing of the repeated bulbospongiosus contractions and periods of quiescence between them that is necessary for complete ejaculatory expulsion to occur. Delcambre et al. (2011) has shown that afferents innervating the urethra indeed respond to movement of fluid flushed through the lumen (such as semen or urine in the physiological system). Such fluid movement (i.e., entry of semen into the proximal urethra) may be responsible for the uninhibited firing of PN afferents (and the most proximal DNP urethral afferents) leading to contraction of the BSM. Shafik and El-Sibai (2000) have also shown urethral stimulation elicits BSM contraction.
Such BSM contraction would (initially, during erection and emission) allow semen to build up in the proximal urethra behind the muscle, further increasing the activation of PN afferents. Neurons in the LPGi receiving convergent input from DNP and PN afferents that may have already undergone wind-up from bilateral DNP stimulation (Hubscher and Johnson, 1996, 1999b) as a result of copulatory behavior could then reach threshold with the additional input from such activation PN afferents. As discussed, the LPGi sends descending projections to inhibit DNP afferents presynaptically at their central terminals, which may be what happens next in this sequence. Once DNP afferents are inhibited, this source of input to the pudendal reflex is eliminated, which would allow the BSM to start to relax. Such an initial decrease in BSM contraction may allow semen to start to leak past the muscle. This would decrease the pressure in the proximal urethra and decrease stimulation of PN afferents, resulting in further relaxation of the BSM and a complete emptying of the proximal urethra. Meanwhile, the PN input to the LPGi would also decrease; hence the LPGi would cease its inhibition of DNP afferents.

Once past the BSM, semen next would enter the distal urethra. Such stimulation by this fluid of DNP afferents innervating the distal urethra, in conjunction with stimulation of glans afferents which may no longer be inhibited by the LPGi by that time, would cause these DNP afferents to fire and allow the bulbospongiosus to rapidly re-contract. This rapid re-contraction would forcibly expel the semen out of the distal urethra as well as allow more semen to accumulate behind the BSM, and the cycle would continue until there is no more semen to accumulate in the urogenital tract. This could be called Model 1.
Delcambre et al. (2011) has observed that urethral DNP stimulation causes a small degree of transient depression of the PN-elicited pudendal reflex (in contrast to the lack of such influence from the LPGi). This may fit into the physiological model in the following fashion: After the BSM starts to relax for the first time at the onset of the expulsion phase, as semen starts to leak past the BSM and contact the distal urethra, these DNP afferents may inhibit the proximal urethral PN afferents in order to more completely relax the BSM. Additionally, the urethral DNP afferents most proximal to the PN afferents (which may be just proximal to the BSM) may be stimulated immediately before the onset of expulsion, especially if very high pressures are reached in the proximal urethra before other inhibitory neural controls such as the LPGi can allow for BSM relaxation. This may eventually inhibit the PN afferents just enough to allow for adequate BSM relaxation to start expulsion. In addition, Delcambre et al. (2011) observed that PN stimulation causes an incomplete depression of the urethral DNP-elicited pudendal reflex (in contrast to the lack of PN influence on the reflex when the entire DNP is stimulated). However, PN stimulation paradoxically also causes PN afferents to trigger BSM contraction. Likewise, while it was discussed above that stimulation of distal urethral afferents inhibit proximal urethral afferents; stimulation of the same distal urethral afferents would also paradoxically cause BSM contraction. The key to understanding how these two functions are differentiated may lie in one particular anatomical trait: The area of overlap of PN and DNP afferents at the distal end of the pelvic (proximal) urethra and penile bulb observed by Delcambre et al. (2011). Given the finding that PN stimulation or urethral DNP stimulation can incompletely inhibit one another's elicitation of PMRD and hence BSM contraction, as semen reaches the point
of overlap of these afferents, it would logically follow that the simultaneous partial inhibition of each set of afferents creates a larger, complete, summed inhibition that results in maximal relaxation of the BSM at this point.

An alternative to Model 1 (which relies on urethral input) involves the sole influence of glans DNP afferents, and a less complex pathway involving the spino-bulbo-spinal loop (Model 2). While BSM contraction can be caused by either urethral or glans afferents (Carro-Juáreza and Rodríguez-Manzo, 2000), there has been little discussion in the literature concerning the relative necessity of each type of stimulus. While several sources have demonstrated that urethral stimulation is indeed sufficient to trigger BSM contractions (i.e., Delcambre et al., 2011), one source showed (via urethral anesthetization or prevention of emission with guanethidine) that urethral afferent stimulation (while sufficient for BSM contraction) is not necessary to trigger natural BSM contractile patterns during the expulsion phase in copulating male rats (Holmes and Sachs, 1991). Another study finding similar results soon followed (Carro-Juáreza and Rodríguez-Manzo, 2000). An analogous case in humans may be anejaculation, which may or may not be accompanied by orgasm (Wolters and Hellström, 2006). While apparently not completely necessary for the simple occurrence of BSM response, there is evidence that, during natural ejaculation, urethral afferents may help to coordinate the activity of the BSM or make the process more complete. For example, Carro-Juáreza and Rodríguez-Manzo (2000) did find slight differences in the duration of BSM response to penile versus urethral stimulation.

Model 2 may function as follows: Since neurons in the LPGi respond not only to inputs from PN but also DNP afferents (Hubscher and Johnson, 1999b), in the absence
of semen to activate PN afferents innervating the proximal urethra it is possible that LPGi neurons may still reach threshold if given enough input from DNP afferents. LPGi descending projections may then inhibit DNP afferents and the decrease in DNP activity would allow the BSM to relax. In this case (where there is a lack of urethral activation), re-contraction of the BSM might occur via continuing DNP afferent stimulation (by which time the inhibition of DNP afferents would have allowed neurons in the LPGi to cease firing and the process to begin again). This may be the neural basis for orgasm without ejaculation (Wolters and Hellstrom, 2006), or the basis for orgasm in the female where there is a lack of urethral stimulation via fluid accumulation or fluid movement yet there is contraction of perineal muscles/sphincters (Bohlen et al., 1982). Carro-Juárez and Rodríguez-Manzo (2003) demonstrated that an ejaculatory genital motor pattern could be provoked without the need for urethral stimulation if noradrenergic transmission was enhanced. Thus, while the sympathetic nervous system has long been known to be involved in ejaculation, its facilitory influence may be particularly important when urethral stimulation is absent, as in Model 2. Vasoconstriction caused by sympathetic influences, for example, may provide the additional required stimulation to DNP afferents, specifically those innervating small vessels as identified by Petruska (1997).

With regards to the supraspinal and segmental sources of inhibition of DNP afferents, these models only make use of the supraspinal source. The particular segmental source of focus- the inhibitory influence of DNP afferents on other DNP afferents- may be an equally important phenomenon for the timing of bulbospongiosus contractions during the expulsive phase of ejaculation, though it is not obvious how this might work. Supraspinal influences are definitely essential for normal sexual functions,
however, as evidenced by the relevant disabilities suffered by the spinal cord injured population (ver Voort, 1987; Seftel et al., 1991).

The proposed “spinal ejaculation center” at the L3-4 level of the spinal cord as discussed by Xu et al. (2005 and 2006) may be an intermediate player in any segmentally-based neural control of BSM contraction timing. This center may also be important for erection-related BSM contraction and prostatic fluid secretion since neural tracing studies have identified cells that make synaptic connections both with bulbospongiosus motoneurons and efferents leading to the prostate (Xu et al., 2005). BSM contraction not only occurs during expulsion but also aids erection (Giuliano and Rampin, 2004; Giuliano et al., 1995; Holmes et al., 1991; McKenna, 2000a; Rampin, 2004; Tang et al., 1998; Zempoalteca et al., 2008), particularly of the glans (Johnson, 1988), and aids and prostatic secretions are made during the emission phase of ejaculation (Bruschini et al., 1978); hence this “ejaculation center” in the lumbar cord may more likely be a coordinator of processes that occur prior to the expulsion phase, such as erection and emission.

Slightly different mechanisms may be at work for the onset of the ejaculatory expulsion phase compared to the middle of the expulsion process. Once the sequence has started, the BSM needs the necessary input at the correct time intervals to contract, relax, and re-contract again. The models discussed above have already described how this can occur. However, the start of the expulsion phase is marked by the sudden and complete relaxation of the BSM. How might this occur?

The contraction of the BSM during erection would allow semen to accumulate behind this muscle in the proximal urethra, just as described in Model 1. At this time,
just prior to expulsion, the pudendal motoneurons may be receiving excitatory inputs from two sources simultaneously since the both DNP (McKenna and Nadelhaft, 1989; Johnson and Hubscher, 1998) and proximal urethral (Delcambre et al., 2011) afferents are both known to activate pudendal motoneurons. Such simultaneous input may allow the BSM to reach maximal contraction intensity. Just as discussed in the two models for subsequent BSM contraction/relaxation cycles, the initial sudden relaxation of the muscle marking the onset of expulsion could be due to descending inhibition of DNP inputs by the LPGi once LPGi neurons reach threshold from adequate PN and/or DNP inputs, as LPGi neurons receiving convergent inputs from both sources have been found (Hubscher and Johnson, 1999b) as well as LPGi sites capable of exerting presynaptic inhibition onto DNP afferents as found in the present study. PN excitation may reach a level adequate to activate LPGi neurons due to gradually increasing pressure in the proximal urethra from accumulating semen, as proximal urethral afferents have been shown to respond to fluid pressure (Delcambre et al., 2011). DNP excitation may reach a level adequate to activate LPGi neurons due to peripheral glans stimulation (Hubscher and Johnson, 1999b) as well as stimulation of DNP afferents that are only capable of being activated during erection, such as those innervating blood vessels and erectile tissue (as found by Johnson, 1988; Johnson and Halata, 1991; and Petruska et al., 1997).

This might not be the only factor contributing to the initial ejaculatory expulsion event of BSM relaxation. There may be other supraspinal sites that influence this specific event. Other perineal muscles innervated by pudendal motoneurons such as the EUS and EAS (Floyd and Walls, 1953; Holstege, 2005a; Kegal, 1951) and generally
the pelvic floor muscles surrounding the vagina in females (van der Velde and Everaerd, 1999) are under voluntary control. Such voluntary control is present in animals as well as humans (Holstege, 2005a). Despite such control over these perineal muscles, any kind of voluntary influence has not yet been documented for the BSM or IC, and Giuliano (2006) agrees that there is a lack of convincing evidence for significant voluntary control over ejaculatory processes after their onset. However, Holstege (2005a) has described imaging studies that show increased activation of specific higher brain areas during ejaculation including the putamen and prefrontal cortex, both of which are involved in the control of movement. Also, the amygdala was found to be deactivated during any sexual behavior (Holstege, 2005a). Therefore, although not under significant voluntary control per se during ejaculation, the BSM may receive several complex go/no-go signals from supraspinal regions in addition to the LPGi. However, interestingly, the putamen and prefrontal cortex did not show significantly increased activation in rodents; and two areas that did show significantly increased activation during ejaculation that humans and rodents had in common were the midbrain lateral central tegmental field and the subparafascicular nucleus (Holstege, 2005a).

Regarding Model 1, the proposal of the inhibition of DNP afferents by the LPGi as being the triggering event for initial BSM relaxation raises another question: Why would sudden cessation of peripheral glans stimulation fail to have the same effect as inhibition of DNP afferents by the LPGi? Intact sensation to the glans is, in fact, necessary for ejaculation to occur (Hart and Leedy, 1985), and there is definitely no known instance of cessation of peripheral glans stimulation being cited as the cause for
onset of ejaculation. If Model 1 is correct, this would imply that removing some DNP inputs via, for example, interruption of copulation, would not be sufficient to relax the BSM enough to allow semen to flow through to start the contraction/relaxation cycle. A possible answer to this may be the influence on the BSM of other DNP inputs, such as those innervating blood vessels, erectile tissues, and other regions affected by the processes of erection that are more difficult to manipulate in an experimental setting. When activated at the onset of expulsion, the LPGi may more completely and suddenly inhibit several types of DNP afferents, including those which are activated by the effects of erection alone.

**Characteristics of DNP Glans Afferents that Impact the Pudendal Reflex**

We found nearly all glans DNP (DNPg) afferents to be in the L6 dorsal root with only a few in S1, and only in a few animals. This was corroborated in the anatomical tracing data as discussed in Chapter 3. This further clarifies the innervation patterns of the DNP in the rat model.

DNPg stimulation resulted in a qualitatively similar PMRD to that elicited by stimulation of the DNPi. However, stimulation of the entire DNPi produced somewhat larger PMRDs, perhaps from the additional influences of non-glans DNP afferents such as those innervating the distal urethra. The pudendal reflex response latency was significantly longer for DNPg versus DNPi stimulation. This was presumably due to the greater conduction distance for the glans afferents, as they were stimulated further out in the periphery than the entire DNP in the ischiorectal fossa. However, stimulation of the pelvic nerve, also isolated in the ischiorectal fossa, resulted in a pudendal reflex response of significantly longer latency than either glans or entire DNP stimulation. Since the conduction distances of PN and DNP afferents were both equal, the
difference in response latencies is most likely due to smaller myelinated fibers and DRG cell sizes (characteristics which are correlated with conduction velocity- see Lawson and Waddell, 1991; Lee et al., 1986; and Sakai and Woody, 1988) of PN afferents compared to DNP afferents. Further clarification of these possibilities is presented in Chapter 3.

Regarding conduction velocities, as measured in spinal nerve volleys (Figure 2-12), there were three groups visible: A group between 12 and 40 m/s consistent with the conduction velocity of the myelinated Aβ fiber type, a group between 11.9 m/s and 2 m/s consistent with the myelinated Aδ fiber type (similar to results of McKenna and Nadelhaft, 1989), and the slowest group below 2 m/s consistent with the unmyelinated C fiber type. While these conduction velocities and fiber type correlations are roughly consistent with other sources (i.e., Rindos et al. 1984), they are not exact. This is not surprising, since while correlations can generally be made for larger and small fiber types, those of intermediate size, conduction velocity or myelination statuses often do not reliably fall neatly into certain categories (Bosco et al., 2010; Lee et al., 1986).

**Conclusions**

Recordings of afferent volleys in L6 and S1 revealed the majority of glans afferents to be traveling in the L6 dorsal root, with very few in S1 in some animals. Conduction velocities suggested that the population of glans afferents consists of Aβ, Aδ and C fibers. Stimulation of the glans, entire DNP or entire PN elicited PMRD and thus is capable of driving the pudendal reflex. These reflex discharges can be depressed by conditioning stimulation of glans or DNP afferents given an optimal paired-pulse interval. The DNP-driven reflex (but not the PN-driven reflex) also can be depressed (via PAD of DNP afferent terminals- presynaptic inhibition) by conditioning
stimulation of the LPGi. Projections from this supraspinal region have been shown to descend through the central lateral funiculus at the T8 level. The nature of the described inhibitory mechanisms may have implications in the coordination and timing of BSM contractions during the expulsive phase of ejaculation.
Figure 2-1. Pudendal motoneuron reflex discharges recorded in the motor branch of the pudendal nerve elicited by stimulating afferents in either the DNP or the PN. The top trace in each of the two sets is from the test stimulation alone with subsequent traces following conditioning train stimulation to the LPGi bilaterally (microelectrode depth increments in µm shown at right). Note the presence of reflex depression in the DNP afferent-elicited reflex circuit but not the PN afferent-elicited reflex circuit. Bar = 10 ms. (Adapted from Johnson and Hubscher, 1998).
Figure 2-2. Schematic representation of the experimental set-up. Our previous studies have shown that the most robust medullary site (gray region) for inhibitory modulation (depression) of pudendal motoneuron reflexes were obtained by simultaneous bilateral electrical microstimulation of the lateral paragigantocellularis (LPGi) nucleus and the adjacent parts (GiA, Gi) of the gigantocellularis nuclear complex, dorsolateral to the pyramidal (py) tracts and lateral to the raphe magnus (RMg). The location of the reticulospinal pathway containing axons from this microstimulation site was determined to be in the central lateral funiculus of T8 following acute and chronic spinal lesions (Hubscher and Johnson, 2000). S, stimulation; R, recording. (Adapted from Johnson, 2006).
Figure 2-3. A typical set of DRPs recorded from the L6 dorsal root after microstimulation of either the ipsilateral or contralateral LPGi at the microelectrode depths listed. Although weaker than the ipsilateral side, contralateral LPGi microstimulation is effective in producing a DRP. Microelectrode tracks were 2000 µm from midline and 2400 µm rostral to obex. Train: Five 0.2 ms, 15 µA pulses.
Figure 2-4. Recordings from axonal excitability tests. The single shock test stimulus was applied to the left L6 dorsal root at the DREZ (0.5 ms pulse) with (right traces) or without (left traces) conditioning train stimulation from the LPGi (delay of 35 ms). Recordings were made from the central cut end of the left DNP and PN. Note that the conditioning stimulus produced PAD in the DNP afferents (but not PN afferents) and caused many medium to large diameter myelinated afferent fibers to be recruited with the test stimulus. (N=4 animals).
Figure 2-5. Quantitation of axonal excitability changes after conditioning microstimulation from the LPGi. Data was taken from 4 animals at 1.5-2 times threshold for antidromic activation of DNP or PN afferents before and after LPGi conditioning microstimulation. Areas under the curve were measured to assess the increase number of axons recruited by LPGi microstimulation using the method described in Lidierth (2006). The data demonstrated a significant increase in DNP axonal excitability (P<0.001) due to PAD but a lack of effect in PN afferent axons. Excitability changes were also seen in single DNP afferent fibers (data not shown). Standard error bars are shown.
Figure 2-6. Typical examples of axonal excitability tests in the DNP and PN from test stimuli on L6 where the condition-test interval was varied. Note the optimum delay was 35 ms for PAD in the DNP afferents. In 6 animals tested, the optimum delay was between 32-45 ms. Also note the lack of LPGi-induced PAD on PN afferents. Traces shown are from the same animal.
Figure 2-7. Microstimulation of the lateral white matter at spinal segment T8 produced DRPs bilaterally in L6 and S1. Traces shown are DRPs in ipsilateral L6 from one animal. Stimulation parameters were the same as used in the LPGi. DRPs were produced by stimulation of the central portion of the lateral funiculus which corresponds to the likely pathway location as suggested by lesion studies.
Figure 2-8. Stimulation of glans afferents elicits a pudendal reflex response of similar magnitude as compared to when the entire DNP is stimulated in the IR fossa. Stimulation of the glans bilaterally activates PMRD with a longer latency when compared to the DNP in the IR fossa (expected based on longer conduction distance) but a significantly shorter latency when compared to the PN which has a much shorter conduction distance. This is likely due to the smaller diameter afferent and slower conducting afferents in the PN. The pudendal motoneuron reflex activation by glans afferents is not due to small myelinated or unmyelinated afferents based on reflex latency time.
Figure 2-9. Pudendal reflex latency measurements for the DNP-glans, DNP-IR fossa, and PN. Reflex latencies from stimulating the DNP-IR fossa and PN are significantly (P<0.001) shorter and longer, respectively when compared to the DNP-glans. Note that the latency from stimulation of the PN is significantly longer than the DNP-IR fossa despite an identical conduction distance. This reflects the smaller axonal caliber of the PN afferents compared to the DNP afferents. Standard error bars are shown. (N=16).
Figure 2-10. Stimulation of the DNP afferents at the proximal border of the glans (DNPg) induces a transient reflex depression of DNPi inputs to reflex firing of pudendal motoneurons. As was true for DNP afferent conditioning stimulation at the ischiorectal site, the maximum depression occurred at a condition-test interval of 25 ms.
Figure 2-11. Paired pulse intervals of 500 and 100 ms produced significantly less (P<0.001) depression of DNP reflex inputs compared to intervals of 25 ms. The occurrence of transient inhibition of other pudendal afferents by glans afferents is not due to small myelinated or unmyelinated afferents based on reflex latency time. However, strong stimulation to the glans which recruits the small fibers did increase the degree of inhibition. Standard error bars are shown. (N=8 animals).
Figure 2-12. Afferent volleys of glans DNP afferents recorded in the L6 and S1 spinal nerves. A) Some animals had a small but minimal projection in the S1 spinal nerve, but in most animals, the glans DNP afferents projected solely through the L6 spinal nerve. B) Enlarged image of A. Conduction velocities of the different peaks demonstrated the presence of large Aβ and small Aδ myelinated fibers as well as unmyelinated C fibers. The data show that the pudendal motoneuron reflex activation by glans afferents is not due to small myelinated or unmyelinated afferents based on reflex latency time. Traces are average of 25 stimulations at 1 Hz (0.2 ms pulse duration).
CHAPTER 3
THE PUDENDAL REFLEX: ANATOMICAL STUDIES

Introduction

The pudendal reflex is a polysynaptic segmental reflex in the lumbosacral cord that controls the expulsive phase of ejaculation (Giuliano and Rampin, 2004; Johnson, 2006), observed in the laboratory as firing of pudendal motor neurons that innervate the bulbospongiosus muscle (BSM) (Pacheco et al., 1997). This reflex is modulated by several segmental and supraspinal influences (Hubscher and Johnson, 2000; Johnson and Hubscher, 1996, 1998), as described in detail in Chapter 2. Supraspinal regulation is very important to the expulsive phase. For example, regions within the medullary reticular formation (MRF) such as the lateral paragigantocellularis (LPGi) are known to receive input from penile (Cothron et al., 2008; Hubscher and Johnson, 1996, 1999b) and pelvic nerve (PN) afferents (Hubscher and Johnson, 1996) as well as send descending projections to regulate the pudendal circuitry (Hubscher and Johnson, 2000; Johnson and Hubscher, 1998). Ejaculation and other sexual reflexes are modulated by several other brain regions as well (Kelly and Jennions, 2011; Steers, 2000). For most spinal cord injured patients with lesions cranial to T10, even with intact segmental reflex arcs, ejaculation is severely impaired or impossible (ver Voort, 1987; Seftel et al., 1991).

The previous chapter discussed supraspinal inputs more extensively. While the current chapter is primarily focused on the segmental level, it is equally important to explore this aspect since very little is known about the general normal functioning of this reflex. It is possible that unexpected findings may shed light on integration of supraspinal modulation.
The BSM is a perineal muscle which surrounds the base of the penis (Giuliano and Rampin, 2004). Stimulation of either dorsal nerve of the penis (DNP) or pelvic nerve (PN) afferents can trigger the pudendal reflex and contraction of the BSM (Johnson and Hubscher, 1998) which expels semen from the urethra (Giuliano and Rampin, 2004). Appropriate timing and sequence of BSM contractions is essential for complete ejaculation to occur (McKenna, 2000a; Johnson, 2006). DNP afferents innervate structures such as the glans (Johnson and Halata, 1991; Giuliano and Rampin, 2004; Pacheco et al., 1997; Yang and Bradley, 1999) and distal urethra (Kessler et al., 2007). In contrast, PN afferents innervate a variety of regions including pelvic viscera (Martinez-Gomez et al., 1998) such as the proximal urethra (Kessler et al., 2007), prostate, bladder (Shea et al., 2000), colon, rectum (Christianson et al., 2008; Feng et al., 2010) and the midline peri-anal and dorsal perineal regions (Martinez-Gomez et al., 1998; Cruz et al., 2004). DNP or PN afferents synapse onto interneurons in the cord (McKenna and Nadelhaft, 1989); some of these interneurons then synapse onto pudendal motor neurons in the ventral horn (McKenna and Nadelhaft, 1989). In the male rat, which is our model of choice, the cell bodies (soma) of pudendal motor neurons are found at the L5-L6 levels (McKenna and Nadelhaft, 1986). Pudendal motoneurons innervate perineal (pelvic floor) muscles that control eliminative functions including the BSM. The BSM is frequently studied in the field of urogenital function and in the rat is involved in micturition as well as ejaculation (Cruz and Downie, 2005; Yasuhiro et al., 2004).

The electrophysiological studies presented in the previous chapter from our lab as well as many others have shown that the pudendal reflex can be triggered by DNP
afferents (i.e., Yang and Bradley, 2000) or PN afferents (i.e., Delcambre et al., 2011; McKenna and Nadelhaft, 1989). Our studies presented in Chapter 2 also showed that stimulation of the lateral paragigantocellularis (LPGi) inhibits the reflex presynaptically by causing bilateral primary afferent depolarization (PAD) specifically in DNP afferents but not PN afferents. The fact that individual cells in regions of the MRF such as the LPGi also receive convergent input from both from the DNP and PN (Hubscher and Johnson, 1996), provide evidence of a spino-bulbo-spinal long-loop pathway for the regulation of the pudendal reflex. Selective presynaptic inhibition onto DNP afferents similar to that from the LPGi can also be caused at the segmental level, by stimulation of other DNP afferents, as suggested in the previous chapter. Studies in Chapter 2 focusing on pudendal reflex response latencies in response to, and conduction velocities of, peripheral nerves suggest that the PN contains relatively small and slowly conducting afferent fibers that trigger the pudendal reflex compared to larger and faster conducting myelinated fibers in the DNP. Our goal for the studies presented in this chapter was to use transsynaptic tracing and immunohistochemical techniques to characterize the DRG neurons synaptically connected to the BSM spinal circuitry and thus confirm or expand upon the electrophysiological findings as presented in the previous chapter.

Double-labeling is a very useful technique for identifying neurons in a particular circuit. For example, a peripheral afferent terminal may be exposed to one neural tracer and the effector to a different tracer, and synaptic connectivity between afferents, efferents or interneurons may be established. Pseudorabies virus (PRV) has been used extensively in other studies of this system as a transsynaptic tracer (Marson and
McKenna, 1996; Johnson et al., 2011; Vera and Nadelhaft, 2000), being taken up selectively by motor neurons, replicating inside the cell, and traveling to other neurons via synaptic connections. If the virus is sufficiently weak, such as the Bartha strain of PRV we use in our experiments, it will only infect neurons at the injection site and not directly infect the DRG neurons innervating the injected organ (Loewy, 1998). The virus can spread transsynaptically not only to spinal interneurons, but to even further regions such as the brainstem and primary afferents after injection of PRV to the BSM (Johnson et al., 2011), for example.

In the studies presented in this chapter, we used two neural tracers to first identify neurons in dorsal root ganglia (DRG) that are primary afferents innervating the glans, and then to identify those DRG neurons which are synaptically connected to BSM spinal circuitry. To label glans afferents we used 1,1'-dilinoleyl-3, 3', 3', 3' tetramethylindocarbocyanine perchololate (DiI) a fluorescent dye applied to the glans penis and visualized in DRG neurons after 11 days of uptake and transport. Hence, regardless of target circuitry, every DiI labeled DRG cell should be a glans afferent. To identify afferents that are synaptically connected to the BSM, we injected the Bartha strain of PRV into the left BSM. Hence, any PRV labeled DRG cell, regardless of origin, is an afferent that makes synaptic connections with the BSM circuitry. Such PRV labeled cells could therefore be afferents originating in the glans, urethra, or perineum (Yang and Bradley, 2000), or they could be Group II muscle spindle afferents in the BSM (Yang and Bradley, 2000). Any DRG neurons that were double-labeled for both Dil and PRV are glans afferents which are part of the pudendal reflex circuit (glans
pudendal circuit afferents). Such double-labeled afferents from here on will be referred to as glans pudendal circuit afferents (GPAs).

Once identified, our next goal for studying the GPAs was to determine their cellular characteristics including their soma size, neurofilament-M (NFM) and substance P (SP) content, and their ability to bind isolectin I-B4 (IB4). Based on electrophysiological data showing larger peaks at faster conduction velocities, we expected GPAs to be primarily larger, as well as most GPAs to be positive for neurofilament-M (NFM), a 160 kDa cytoskeletal component commonly used as a marker to identify myelinated afferents (Mata et al., 1992). We also hypothesized that most GPAs may be positive for SP based on the fact that SP has been implicated in sexual reflex components in several other studies (i.e., Gu et al., 1983). Finally, we hypothesized that only a minority of GPAs would stain positively for IB4, since modulation of the pudendal reflex by faster-conducting afferents was more easily apparent in our electrophysiological studies, and IB4 is associated with slower-conducting, smaller fibers.

Neurons positive for neurofilament are usually myelinated and have large somas, large axon diameters, and fast conduction velocities; studies have shown correlations between several of these factors: There are positive correlations between axon diameter, conduction velocity and soma size (Lee et al., 1986; Sakai and Woody, 1988). Lawson and Waddell (1991) showed correlations between NFM immunoreactivity, myelination state and fiber type as well as looser positive correlations between soma size, NFM immunoreactivity and fiber conduction velocity. However, such correlations are not quite as reliable for intermediate-sized fibers such as Aδ fibers (Bosco et al., 2010; Lee et al., 1986). Unmyelinated axons are often identified as polymodal, carrying
information relevant to more than one modality such as pressure, temperature, etc. (Schmidt et al., 1995; Torebjörk, 1974; Torebjörk and Hallin, 1974).

Substance P (SP) is a neuropeptide most widely known for its modulation of pain and nociception processes (Dirajlal et al., 2003; Öztürk et al., 2010; Russell et al., 1994). However, these are not its only functions. SP has also been implicated in other phenomena as varied as depression (Bondy et al., 2003; Kramer et al., 1998, 2004), cardiovascular response (Potts et al., 1999), vasodilation (Bombardi et al., 2010) and vomiting. Vomiting is recognized as an eliminative process, and like ejaculation, requires complex, coordinated control of striated muscle contraction sequence including brainstem regulation. SP modulation in brain regions has been implicated in mating behavior (Damalama and Swann, 1993; Dornan and Malsbury, 1989; Swann and Macchione, 1992; Swann and Newman, 1992). There is also evidence that SP is involved more specifically in ejaculation: SP immunoreactive cells have been found in the LPGi (Menetrey and Basbaum, 1987) and in proximity to the BSM motor neuron pool in the lower lumbar segments (Micevych et al., 1986). Micevych et al. (1986) also found depletion of this SP in the cord following spinal cord injury, suggesting that SP modulatory pathways are one of the functions that are sensitive to SCI. SP immunoreactivity has also been observed in the glans (Yucel and Baskin, 2003) and specifically, localized in nerves around corpuscular receptors (Gu et al., 1983). Other studies have proposed a putative “ejaculatory center” in the L3-L4 spinal cord (Truitt and Coolen, 2002; Xu et al., 2005, 2006). Xu et al. (2006) used double labeling to identify individual interneurons that sent axons to both the BSM and the prostate.
These neurons were discovered to be positive for the neurokinin -1 (NK-1) receptor, a target for SP (Xu et al., 2006).

The glycoprotein isolectin I-B4 (IB4) from *Griffonia simplicifolia* binds specifically to the α-D-galactose residue (Bennett et al., 1998; Benton et al., 2008; Fullmer et al., 2004). IB4 is commonly recognized as a marker for a subpopulation of small unmyelinated afferents (Petruska et al., 1997; Wang et al., 1994), however it also binds to certain populations of small myelinated afferents in the glans penis (Petruska et al., 1997). Use of IB4 as a marker for a specific group of nociceptors is also widespread in the literature (Bennett et al., 1998; Dirajlal et al., 2003; Genzen et al., 2001; Hind et al., 2005; Woodbury et al., 2004). While it is known that IB4 labels various types of cells including microglia, some sympathetic efferents, visceral and cutaneous afferents, Petruska et al. (1997) demonstrated that IB4 reactive axons also extend to the glans penis and distal urethral epithelium. Cell adhesion may be the factor important for IB4’s putative function of neural recovery after injury; IB4 also labels non-neural cells such as endothelial cells (Benton et al., 2008; Galili et al., 1988) and has been implicated in angiogenesis following SCI as well (Benton et al., 2008).

Both SP and IB4 are reported to be markers for nociceptors, and are part of the criteria commonly used to divide C fiber nociceptors into two groups (Dirajlal et al., 2003): One group is composed of peptidergic (i.e., SP+) neurons that are dependent on NGF, and the other composed of non-peptidergic (often IB4+) neurons responsive to GDNF. These two groups differ in other ways as well including response to protons and/or capsaicin (Dirajlal et al., 2003). However, as presented further below, not all
neurons with particular SP or IB4 profiles fall neatly into one of these two categories of nociceptors.

The glans contains a unique complement of receptors whose associated cell bodies may be labeled by the Dil procedure. Ruffini corpuscles have been observed in small numbers in the glans penis of the rat (Johnson and Halata, 1991) and are understood as slowly adapting receptors responding to skin stretch. Many receptors are classified as free nerve endings (Johnson and Halata, 1991) which have either thinly myelinated Aδ or C fibers (Johnson and Halata, 1991; Munger and Ide, 1988; Steers, 2000). Another receptor, less abundant than the free nerve endings is unique to the glans and is called the genital corpuscle (or lamellated corpuscle) which has the appearance of a group of primarily unmyelinated axons (Corona et al., 1991) whose terminals exhibit a bundled configuration usually enclosed by a perineural capsule (Corona et al., 1991; Halata et al., 1997; Johnson and Halata, 1991; Munger and Ide, 1988) and often reside within the erectile tissue (Johnson and Halata, 1991) where they are likely the afferents that have been shown to fire during penile erection (Johnson, 1988). The different receptors in the glans may have various functions which depend not only on the structure of their axon, but also the targets for their projections. For example Hubscher et al. (2010) described multiple ascending pathways from the male genitalia to the MRF which may correspond to different reflexive and perceptive functions.

In addition to obtaining the unique histochemical signatures and size characteristics of GPAs, we were also interested in clarifying other anatomical details of the pudendal circuit components. For instance, DNP afferents are usually stated to
enter the cord and contact interneurons at the L6-S1 levels (Wiedey et al., 2008), while other sources mention L5-L6 as the segments that receive DNP afferent terminals (Coolen, 2011), and cell bodies of DNP afferents have been found in L6 and S1 DRGs (McKenna and Nadelhaft, 1986). Our electrophysiological data showed evidence of DNP afferents running through L6 dorsal roots, however very few DNP afferents were found to run in S1, and some animals had none at all in S1. Hence, we expected to find glans afferents positive for Dil in L6 DRGs, but fewer in S1 DRGs. Also, since the pudendal reflex circuit is bilaterally organized as shown by anatomical and electrophysiological studies (Johnson et al., 2011; McKenna and Nadelhaft, 1989) we hypothesized that we would find GPAs in DRGs both ipsilateral and contralateral to PRV injection.

**Methods**

**Neuroanatomical Tracing**

All procedures used for this study was approved by the Institutional Animal Care and Use Committee in accordance with USDA regulations. A solution of the fluorescent tracer Dil (Fast Dil oil; 1,1’-dilinoleyl-3, 3, 3’, 3’ tetramethylindocarbocyanine percholate; dissolved in 0.5 ml methanol) was applied to the surface of the glans and external urethral orifice to label DNP neurons in the DRG and was performed under isofluorane anesthesia. Initially 5% isofluorane was used and then 1.5 to 2% isofluorane was used to maintain the animal in an unconscious state throughout the procedure. Care was taken to prevent the spread of dye to surrounding skin structures.

For some of these same animals (N=12), approximately one week following Dil application, pseudorabies virus (PRV) was injected unilaterally into the BSM as described below. At the end of a 3.75-4.75 day survival period after PRV injection
(10-11 days after Dil application), rats were euthanized with an overdose of urethane administered i.p. and perfused transcardially with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS (pH 7.4).

**Pseudorabies Virus Injections**

Approximately 3.75-4.75 days prior to euthanasia, unilateral injections (6 x 10^8 pfu/ml) of the attenuated Bartha strain of PRV (from Dr. L. W. Enquist) were made into the ventral BSM. We found in our pilot studies that the attenuated Bartha strain infects medullary neurons as early as 4 days post-injection of the BSM, and for at least 6 days post-injection, animals do not display any adverse reactions to the virus. In our other pilot studies, we verified the specificity of the BSM injection by finding only labeled motoneurons in the dorsomedial nucleus of the L5-L6 ventral horn at short survival times of 2-3 days. On the other hand, at these short survival times, DRG cells were not labeled, which confirms the affinity of the Bartha strain of PRV for motor axons, which has been described by others as well (Loewy, 1998; Aston-Jones and Card, 2000). Control studies have verified the bilateral nature of pudendal motoneuron circuitry; as shown in Figure 3-1, ipsilateral PRV injection into the BSM resulted in bilateral PRV labeling in the brainstem (Johnson et al., 2011). Similar experiments by Johnson et al. (2011) involving ipsilateral and contralateral hemisection lesions revealed that the supraspinal pathway that descends to the pudendal segmental circuitry first descends ipsilaterally but becomes bilateral somewhere below the T8 level, where the lesions were made. In this same collection of studies, lack of neuron labeling following PRV injection into surrounding tissue confirmed specificity of PRV for motor neurons.

Under aseptic conditions, animals in the present study were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg) administered i.p., and a
unilateral para-midline incision was made in the perineal/scrotal skin. Using a 33 gauge Hamilton syringe, 5 injections of PRV (5 µl each) were made unilaterally into five equally spaced sites in the ventral head of the BSM (Marson and McKenna, 1996). Utmost care was taken to prevent leakage of virus into adjacent tissue by sealing the muscle injection site with a drop of cyanoacrylate glue immediately following the withdrawal of the injection needle. In animals with chronic spinal lesion (see below), injections were made near the end of the recovery period. In animals with acute spinal lesions, injections were made immediately (20-30 minutes) after the spinal cord was injured. For non-muscle PRV controls, injections were made into the surrounding tissue external to the connective tissue (epimysium) around the muscle. These injection sites included the subcutaneous tissue under the skin of the scrotal incision site, the deeper adipose and connective tissue adjacent to the muscles and on the outside of the fascial layer over the BSM. At the end of the survival period after PRV injection, rats were euthanized with an overdose of urethane and perfused transcardially with phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS.

**Immunohistochemistry and Dorsal Root Ganglia Tissue Processing**

The day after sacrifice, the left and right L5, L6, and S1 DRGs were dissected out, post-fixed in 4% paraformaldehyde overnight and then cryoprotected in a 30% sucrose solution until equilibrated. DRGs were then embedded in OCT compound (Baxter), serial sectioned on a cryostat at 14 µm, and thaw-mounted on poly-L-lysine double-subbed slides. Nucleated Dil-positive DRG neurons could be directly visualized via fluorescence microscopy and digitally imaged with Zeiss Axioskop/ImagePro software. Tissue from animals that were not injected with PRV was then processed with toluidine blue counterstain. The cells were re-imaged, and their diameters/circumferences
measured. To identify the glans/distal urethral afferents in the DRGs which are connected to the BSM, PRV was also detected in the cell bodies for those animals that had received injections. Tissue from these animals was immunohistochemically processed using polyclonal antiserum directed against multiple PRV proteins (obtained from Affinity BioReagents, Golden CO, host- rabbit), diluted 1:5000. The PRV primary antibody was labeled with the fluorescent secondary antibody goat-anti-rabbit-AlexaFluor-488 (green; 1:100). Transverse sections of the L5-L6 spinal cord were processed to visualize PRV within the cord to confirm the success of PRV transport to the pudendal motoneurons into the bulbospongiosus motoneuron pool (Figure 3-2).

To detect neurofilament-M (NFM) in the DRG cell bodies, a marker for myelinated neurons, the tissue was immunohistochemically processed using mouse anti-neurofilament-M antibody, diluted 1:500. The neurofilament-M primary antibody was labeled with the fluorescent secondary antibody anti-mouse-350 (blue), diluted 1:100. Alternate slides were similarly processed with the same fluorophore to detect substance P in the cell bodies, using mouse anti-substance P antibody diluted 1:40. All primary antibodies were applied to the tissue for 12-18 hours, and secondary antibodies were applied for 4 hours. Nucleated DRG cells that co-labeled for DiI and PRV were visualized and determined to be positive or negative for neurofilament-M or for substance P, and were digitally imaged with Zeiss Axiophot/ImagePro software. To detect a population of cells that bind the lectin IB4 (a marker for cells with unmyelinated axons), the tissue on all slides was then incubated for 12-18 hours in biotinylated lectin IB4 followed by an avidin-biotin complex for 1 hour prepared at half concentration (Elite standard Vectastain ABC kit; Vector Laboratories, Burlingame, CA) and detected using
DAB (diaminobenzidine) as the chromogen (20 mg in 50 mls PBS; Sigma). Following application of DEPEX mounting media, the slides were coverslipped. The previously identified Dil/PRV co-labeled cells were then re-imaged and identified as positive or negative for IB4, and their diameters/circumferences were measured. The section thickness of 14 µm and exclusion of cell images that were not nucleated, or those which fell on the border of the image, ensured avoidance of double counting of the cells.

**Statistics**

Statistics were performed utilizing the SigmaStat program. Significant differences between percentages of populations were determined using a Chi squared test. Significant differences between mean diameters of groups of cells were determined using the Mann-Whitney Rank Sum Test. Significance level was set to P<0.05. Data on cell diameter is expressed as the mean ± SEM.

**Results**

**Successful Dil and PRV Labeling of DRG Cells**

Dil applied to the entire glans 10-11 days prior to sacrifice successfully labeled DRG cell somas bilaterally. PRV injected into the left BSM was successfully transported ipsilaterally to motor neurons in the dorsomedial nucleus of the L5-L6 spinal cord and bilaterally to DRG cell somas. A total of 1215 labeled cells were measured for somal diameter and further processed and determined to be positive or negative for IB4, as well as for NFM or SP.

**Characteristics of Glans Afferents as Identified with Dil**

In a first experiment utilizing 8 animals receiving only Dil application to the glans (but not PRV injection), morphometric analysis of glans afferents as visualized with Dil revealed cell bodies primarily in L6 DRGs bilaterally. Very few labeled neurons were
found in S1 DRGs (specifically, two Dil-labeled cells were found in each of two animals). Dil labeled soma had both large and small diameters, ranging in sizes from 15 to 65 µm (Figure 3-3), supporting the electrophysiological data showing the existence of Aβ, Aδ, and C fiber glans afferent volleys running through mainly L6 spinal nerves with only a few seen in S1.

**Multi-Labeling of DRG Cells**

Afferents that were double-labeled for both Dil (glans afferents) and PRV (afferents synaptically connected to the BSM spinal circuitry) from here on will be referred to as glans pudendal circuit afferents (GPAs). GPAs were found in L6 DRGs both ipsilateral and contralateral to PRV injection, demonstrating the bilateral nature of the system; ipsilateral and contralateral DNP afferents are trans-synaptically connected to the left BSM. Control experiments showed no direct PRV infection of the DRG peripherally via the spinal nerves.

Quadruple-labeling of L6 DRG cells allowed for identification of glans pudendal reflex circuit afferents, and the discovery that these afferents are composed of several different cell populations, each population with its own distinct immunohistochemical characteristics and soma size range. First, Dil (directly visualized via fluorescence as red/orange) and PRV (visualized via a green fluorophore) co-labeling allowed glans pudendal reflex circuit afferents to be identified. Subsequent labeling for neurofilament-M and for substance P was performed on alternate slides since both were visualized with a blue fluorophore, yielding two separate data sets. Finally, glans pudendal reflex circuit afferents were determined to be positive or negative for IB4 following a DAB reaction (visualized as brown). Hence, each glans pudendal reflex circuit DRG cell identified could be classified as positive or negative for neurofilament-M
or substance P, and positive or negative for IB4, as well as measured for soma diameter. In looking at the group of afferents tested for neurofilament-M and that for substance P separately, for each of these two data sets there exist four populations of glans pudendal circuit afferents with distinct immunohistochemical signatures, soma size distributions, and prevalence in the GPA group.

Following the discovery of these four distinct cell populations in each data set that comprise the group of GPAs, it was speculated that the prevalence or characteristics of these populations might differ for other groups of afferents. By utilizing the same photographs that were taken to analyze the double-labeled cells, IB4 and either NFM or SP immunoreactivity as well as cell diameter were recorded from cells labeled with Dil but not PRV, and for cells labeled with PRV but not Dil. The singly labeled Dil cells are glans afferents that are not synaptically connected to the BSM. These could be described as glans non-pudendal circuit afferents (glans non-PAs). The singly labeled PRV cells are afferents that are connected to the BSM but are not from the glans. These could be described as non-glans pudendal circuit afferents (non-glans PAs).

Results from analysis of these additional two groups of afferents indeed revealed several differences. These differences are described in further detail in the sections below those discussing GPAs in each data set.

**Characteristics of GPAs, Non-GLANS PAs, and Glans Non-PAs: NFM Data Set**

In the sections tested for neurofilament M (NFM) in 12 animals, 26% of glans pudendal reflex afferents (GPAs) were positive for NFM and negative for IB4 (e.g., Figure 3-4). This population had a mean cell diameter of $44.3 \pm 1.10 \, \mu m$. A population of GPAs that tested negative for NFM and positive for IB4 (e.g., Figure 3-5) made up 42% of GPAs. This population had a mean cell diameter of $32.6 \pm 0.47 \, \mu m$. A
population of GPAs that was positive for both NFM and IB4 (e.g., Figure 3-6) comprised 7% of the population, with a mean diameter of 31.4 ± 1.06 µm. Finally, a population of GPAs that tested negative for both NFM and IB4 (e.g., Figure 3-7) made up 25% of total GPAs, with a mean diameter of 30.9 ± 0.60 µm. The soma size distributions of these four populations are shown in Figure 3-8. The NFM+/IB4- population is the only one that is comprised of two subpopulations of visibly different sizes, possibly reflecting a bimodal distribution.

When comparing non-glans afferents connected to the BS muscle (non-glans PAs) (Figure 3-9) to glans afferents which are connected to the BS muscle (GPAs) (Figure 3-8), a noticeable difference is the size distributions of their NFM+/IB4- populations. While double-labeled cells (GPAs) include a bimodal distribution (both larger and smaller) of NFM+/IB4- afferents, for non-glans afferents leading to the BS muscle (non-glans PAs), this population mostly matches the size range of the smaller subpopulation of NFM+/IB4- cells seen for double-labeled cells. In fact, the mean diameter of NFM+/IB4- non-glans PAs (40.7 ± 0.54 µm) is significantly less (P=0.003) than that of NFM+/IB4- GPAs (44.3 ± 1.10µm).

When comparing glans afferents specifically NOT connected to the BS muscle (glans non-PAs) (Figure 3-10) to glans afferents which are connected to the BS muscle (GPAs) (Figure 3-8), just as when comparing to non-glans PAs, a noticeable difference in their cell populations (of the NFM data set) is the size distributions of their NFM+/IB4-populations. While double-labeled cells (GPAs) appear to include a bimodal distribution (both larger and smaller) of NFM+/IB4- afferents, for glans afferents not connected to the BS muscle, this population mostly matches the size range of the larger
subpopulation of NFM+/IB4- cells seen for double-labeled cells. In fact, the mean
diameter of NFM+/IB4- glans non-PAs (47.0 ± 0.83 µm) is significantly greater
(P=0.049) than that of NFM+/IB4- GPAs (44.3 ± 1.10 µm).

**Characteristics of GPAs, Non-Glans PAs, and Glans Non-PAs: SP Data Set**

In the sections tested for substance P (SP) in 12 animals, 3% of total GPAs were
positive for SP and negative for IB4 (Figure 3-11) with a mean diameter of
32.6 ± 1.08 µm. A population negative for SP and positive for IB4 (Figure 3-12)
composed 41% of total GPAs with a mean cell diameter of 33.3 ± 0.51 µm. A
population positive for both SP and IB4 (Figure 3-13) composed 15% of the total with a
mean diameter of 33.4 ± 0.86 µm. Finally a population negative for both SP and IB4
(Figure 3-14) composed 41% of total with a mean diameter of 42.2 ± 0.96 µm.

Soma size distributions of these four cell populations for GPAs are shown in
Figure 3-15. The soma size distribution of the SP-/IB4- cells overlaps considerably with
the size distribution of NFM+/IB4- cells, particularly the larger subpopulation of
NFM+/IB4- cells, so it is quite possible that some, if not most, of the largest GPAs may
be positive for NFM, negative for IB4, and negative for SP.

As can be visualized in Figure 3-16, there were significantly fewer (P<0.001) SP+
cells in the population of non-glans PAs (6%) than there are in GPAs (18%). This is
evidence that there are few; if any, SP+ afferents leading to the bulbospongiosus circuit
that are non-glans in origin. However, significantly more SP+ cells (32%) were found in
the glans non-PA group (P<0.001). Soma size distributions of all four cell populations
from the SP data set for glans non-PA afferents are displayed in Figure 3-17.
Overall Percentages of GPAs, Glans Non-PAs and Non-Glans PAs that are Positive for SP, NFM, or IB4

Overall, GPAs consisted of a minority (18%) of SP positive cells (Figure 3-18). GPAs were also composed of a minority (33%) of NFM+ cells. However, IB4+ cells comprised approximately half (54%) of all GPAs. Non-glans PAs consisted of significantly fewer (P<0.001) IB4+ cells (23%) compared to IB4+ cells for GPAs (52%).

Glans non-PAs consisted of 32% of SP positive cells (Figure 3-19). Glans non-PAs were composed of 36% of NFM+ cells. IB4+ cells comprised approximately half (49%) of all glans non-PAs.

Non-glans PAs consisted of very few SP positive cells (6%) as can be visualized in Figure 3-20. Non-glans PAs were composed of 53% of NFM+ cells. IB4+ cells comprised 23% of all non-glans PAs, which is significantly less (P<0.001) than the percentage of IB4+ cells for either group of glans afferent.

Soma Diameters for Different Immunohistochemical Populations for GPAs, Glans Non-PAs and Non-Glans PAs

As diagramed in Figure 3-21, the mean soma diameter for all non-glans PAs (37.7 ± 0.34 µm) was significantly larger than that for all GPAs (36.0 ± 0.37 µm) or glans non-PAs (36.5 ± 0.60 µm). The mean diameter for the general population of NFM+ glans non-PAs was significantly larger than that for NFM+ GPAs or NFM+ non-glans PAs (see Table 3-1 for data on cell diameters for such general populations). In contrast, the mean diameter for SP+ glans non-PAs was significantly smaller than that for SP+ GPAs or SP+ non-glans PAs. Similarly, the mean diameter for IB4+ glans non-PAs was significantly smaller than that for IB4+ GPAs or IB4+ non-glans PAs, and the mean diameter for IB4+ non-glans PAs was significantly larger than GPAs.
Regarding specific immunohistochemical cell populations from the NFM data set, as diagramed in Figure 3-22, the mean soma diameter for NFM+/IB4- non-glans PAs (40.7 ± 0.54 µm) was significantly smaller than the diameters of either GPAs (44.3 ± 1.10 µm; P=0.003) or glans non-PAs (47.0 ± 0.83 µm) with the NFM+/IB4- signature. The mean diameter of NFM+/IB4- glans non-PAs was significantly larger than that of NFM+/IB4- GPAs. The mean diameter of NFM-/IB4+ glans non-PAs (29.3 ± 0.53 µm) was significantly smaller than that of GPAs (32.6 ± 0.47 µm) or non-glans PAs (33.2 ± 0.61 µm) with the NFM-/IB4+ signature. The mean diameter of NFM+/IB4+ non-glans PAs (38.3 ± 0.70 µm) was significantly larger than that of GPAs (31.4 ± 1.06 µm) or glans non-PAs (35.2 ± 0.11 µm) with the NFM+/IB4+ signature. Similarly, the mean diameter of NFM-/IB4- non-glans PAs (33.7 ± 0.64 µm) was significantly larger than that of GPAs (30.9 ± 0.60 µm) or glans non-PAs (29.8 ± 1.48 µm) with the NFM-/IB4- signature.

Regarding specific immunohistochemical cell populations from the SP data set, as diagramed in Figure 3-23, the mean soma diameters for SP+/IB4- GPAs (32.6 ± 1.08 µm), SP+/IB4- glans non-PAs (29.7 ± 1.19 µm), and SP+/IB4- non-glans PAs (33.8 ± 0.93 µm) were not significantly different from one another. The mean diameter of SP-/IB4+ non-glans PAs (35.4 ± 0.78 µm) was significantly larger than that of GPAs (33.3 ± 0.51 µm) or glans non-PAs (32.6 ± 0.86 µm) with the SP-/IB4+ signature. The mean diameter of SP+/IB4+ glans non-PAs (27.9 ± 0.58 µm) was significantly smaller than that of SP+/IB4+ GPAs (33.4 ± 0.86 µm). There was only a single SP+/IB4+ non-glans PA, with a soma diameter of 35.8 µm. The mean diameter of SP-/IB4- glans non-PAs (46.5 ± 1.10 µm) was significantly larger than that of SP-/IB4-
GPAs (42.2 ± 1.00 µm). The mean diameter of SP-/IB4- non-glans PAs (39.5 ± 0.71) was significantly smaller than that of GPAs or glans non-PAs with the SP-/IB4-signature.

Discussion

Immunohistochemical Phenotypes of GPA Neurons

Contrary to what was expected, only 33% of GPAs were found to be positive for NFM. Only a minority (19%) of GPAs were found to be positive for SP. This alone does not confirm nor rule out the possibility that modulation by SP plays a role in the pudendal reflex. For example, regarding the proposed ejaculatory center at L3-L4 with interneurons containing the NK-1 receptor as described by Xu et al. (2006), it is possible that the SP+ GPAs make connections with the BSM circuitry selectively via this L3-L4 center. These SP+ GPAs may also have a function at the L6-S1 level since interneurons here were found to have NK-1 receptors colocalized with c-fos from pudendal sensory nerve stimulation (Wiedey et al., 2008).

It does not seem likely that most SP+ GPAs would be nociceptors, since most SP+ GPAs co-labeled with IB4, and nociceptors are generally recognized as being positive for SP or IB4 but not both (Bennett et al., 1998; Matsuka et al., 2007). Singly positive IB4+ nociceptors and SP+ nociceptors have been reported to be segregated in their characteristics (Dirajlal et al., 2003), for example terminating in different lamina of the cord; SP+ afferents in Lamina I and IB4+ afferents in Lamina II (Braz et al., 2005). IB4 has even been referred to as a label for non-peptidergic C fibers (Kang et al., 2010). However, such segregation does not exist for all neurons containing these markers. For example, Wang et al. (1994) demonstrated that there is actually a significant overlap of peptidergic and non-peptidergic markers. The SP+/IB4+ GPAs, therefore, may serve a
function that is non-nociceptive in nature. It is interesting to note that SP+/IB4- cells, which do fit into one of the defined categories for nociceptors, only comprised 3% of the GPAs but 8% of glans non-PAs. These SP+/IB4+ GPA cells however, may be specific to the glans penis (see discussion below).

The 52% of IB4+ cells comprising the GPA group was greater than expected based on the hypothesis. All IB4+ cells were either small or medium sized neurons, and there were very few that did co-label for NFM (7% of total GPAs). In a study by Wang et al. (2004), a similarly small proportion of NFM+/IB4+ neurons were also found to comprise a very small proportion of sciatic nerve afferents. However, as with non-glans PAs and glans non-PAs, nearly all IB4+ GPAs were evidently unmyelinated. In addition, there was a population of IB4-/NFM- GPAs that were presumably unmyelinated (25% of total GPAs). These two groups together compose most of the total GPAs (77%) and are presumably unmyelinated. The conclusion follows that that smaller, unmyelinated glans afferents most likely have a role of at least equal importance to their large, myelinated counterparts in influencing the pudendal reflex circuitry.

Regarding possible nociceptive input, it was already alluded to how only 3% of all GPAs were composed of SP+/IB4- cells. This profile agrees with the description of one immunohistochemical category of nociceptors (Dirajlal et al., 2003); however, if these GPAs are indeed nociceptors, they do not appear to play a large role. It is interesting to note that there appears to be a larger proportion of such possible SP+/IB4- nociceptors in the glans non-PA group of afferents compared to the GPA group. The other category of putative nociceptors is said to have the SP-/IB4+ signature (Bennett et al., 1998; Matsuka et al., 2007). IB4+ neurons have been suggested to be (based on factors such
as spinal cord terminal distribution patterns) thermoreceptors (Molliver et al., 1995),
chemoreceptors and/or nociceptors (Dirajlal et al., 2003; Streit et al., 1985). SP-/IB4+
cells do comprise a substantial portion of GPAs at 41% of the total. Hence, whatever
the modality or modalities of these receptors may be, they appear to play a larger role in
regulation of the pudendal reflex.

Our GPAs consist of afferents from the glans and external urethral orifice but
probably not many from the distal urethra within the body of the penis. Nevertheless,
the percentage of IB4+ cells found in our GPAs (52%) is comparable to the 49% found
in distal urethral afferents in other studies (Yoshimura et al., 2003). Hence, this is at
least one cellular characteristic that glans and distal urethral DNP afferents may have in
common.

**Differences between PN and Glans/DNP Cell Diameters is likely Related to
Conduction Velocities**

As can be seen in Figure 3-9, the size distribution of NFM+/IB4- non-glans PAs,
compared to the size distributions of two subgroups of NFM+/IB4- GPAs (Figure 3-8), is
skewed toward the size distribution of the smaller-diameter subgroup of NFM+/IB4-
GPAs. While GPAs are limited to include only glans afferents, non-glans PAs could
include any type of afferent (except glans) that makes synaptic connections with the
BSM circuitry. These most likely include proximal urethral PN afferents and distal
urethral DNP afferents, since both are known to trigger the pudendal reflex and thus
would be anatomically connected to the BSM (Delcambre et al., 2011). However, the
non-glans PA group is the only one (versus GPAs and glans non-PAs) that could
possibly include PN afferents since DNP afferents only run in the pudendal nerve
(Steers, 2000), so in looking at non-glans PAs, we will address the putative PN
afferents. The fact that the NFM+/IB4- population of non-glans PAs has a significantly smaller mean diameter than that of the GPAs and glans non-PAs is consistent with the electrophysiological data demonstrating different pudendal reflex latencies following PN versus glans/DNP stimulation (Figures 2-8 and 2-9); the significantly longer latency of PN stimulation to trigger the reflex does indeed appear to be due, in part, to smaller fiber diameter. Since cells with the NFM+/IB4- signature are the only population to show a visible difference in size distribution between GPAs and non-glans PAs, it would be logical to propose that this is the specific cell type responsible for the difference in PN versus glans/DNP difference in reflex latency. Hence, the decreased conduction velocity of PN afferents as a whole is likely primarily due to the smaller average axon diameter of the population of myelinated, IB4- afferents. Such smaller myelinated afferents of the PN fit the description of Aδ fibers; and the larger myelinated afferents of the DNP, Aβ fibers.

**Substance P Immunoreactivity as another Distinguishing Factor between the DNP and Putative PN Afferents Involved in the Pudendal Reflex**

The percentage of SP+ cells in our identified GPAs (19%) is comparable to that of DNP afferents in general (21%) found by Kawatani et al. (1986), suggesting that SP is equally prevalent among glans and distal urethral afferents. However, while Kawatani et al. (1986) found a total of 24% of pelvic nerve afferents to be SP positive via direct fluorescent dye application to the ends of the whole cut nerve, our only 6% of our non-glans PAs were SP+ (Figures 3-16 and 3-20). Furthermore, less than 1% of non-glans PAs were members of the SP+/IB4+ cell population in particular (one single cell, in fact). This suggests that nearly all SP+ PN afferents lead to destinations other than the BSM circuitry, possibly for purposes such as processing of bladder pain (Andersson, 2002).
Pelvic nerve afferents are known to innervate the bladder (Sengupta and Gebhart, 1994), it is recognized that bladder afferents are primarily peptidergic (Hwang et al., 2005), and SP has been implicated in bladder dysfunction (Marchand et al., 1998). However, it can be concluded that PN afferents do not use SP specifically to regulate the pudendal reflex, as glans DNP afferents apparently do. This is yet another difference between these two afferent inputs, in addition to the findings that PN inputs to the pudendal reflex are not susceptible to presynaptic inhibition as are DNP inputs.

With regards to the DNP afferents that connect to the segmental pudendal circuitry, they may use SP to modulate L3-L4 interneurons in the putative spinal ejaculatory center described by Coolen (2011) and other research groups (i.e., Xu et al., 2005, 2006), and/or use SP to modulate interneurons in the L6-S1 cord. Interneurons in both of these locations have been found to express the NK-1 receptor as well as to receive DNP inputs (Coolen, 2011; Xu et al., 2006; Wiedey et al., 2008). As reviewed by Coolen (2011), ablation of the putative spinal ejaculatory center selectively abolishes ejaculation. This therefore makes it likely that, if the above-described L6-S1 interneurons are involved in ejaculation, they may first synapse onto L3-L4 neurons. There may be not just one, but two, interconnected ejaculatory centers in the spinal cord. Holstege (2005a) has also made such a proposition. The center in L3-L4 is unique in that Xu et al. (2005, 2006) has observed double-labeled interneurons from BSM and prostate retrograde tracing.

**The Role of SP in Glans Non-PAs**

Compared to the 19% of SP+ GPAs, glans non-PAs included a significantly greater percentage of SP+ cells at 32% (P<0.001). This suggests that more glans afferents projecting to regions other than the BSM (such as the brain) have a function
that involves SP signaling; possibly pain sensation in the brain but, like the SP+ cells of GPAs, most of these co-labeled for IB4 and thus may not be nociceptors. Alternatively, SP+ glans non-PAs could be projecting to the LPGi to regulate ejaculation, as this brain center does receive ascending DNP input (Hubscher and Johnson, 1999b). SP signaling has been documented to regulate other complex reflexes via the LPGi such as emesis (Felipe et al., 1998). In fact, LPGi neurons regulating emesis (Felipe et al., 1998) and ejaculation involve wind-up (Hubscher and Johnson, 1996, 1999b), which as previously discussed may be a common factor to a variety of SP-related processes; SP is implicated in a variety of processes that require “wind-up” (Felipe et al., 1998). In comparing the 21% of SP+ DNP afferents observed by Kawatani et al. (1986) to our observed 32% of SP+ glans non-PA afferents, this may indicate that more SP+ DNP afferents are from the glans compared to from other DNP territories such as the distal urethra, prepuce and penile body.

**Proposed Contribution of Proximal versus Distal Urethral Afferents to the Pudendal Reflex**

The non-glans (Dil-negative) afferents labeled by our PRV injections into the BSM (non-glans PAs) may include both proximal and distal urethral afferents, since we know from Delcambre et al. (2011) that both can drive BSM contractions, and from our other electrophysiological data that PN and DNP stimulation can drive BSM contractions. The afferents labeled by our PRV injection into the BSM included a population of IB4+ cells that comprised 23% of the PRV-labeled population (significantly less than the percentage of IB4+ cells in our glans populations). Yoshimura et al. (2003) found 20% of proximal urethral afferent C fibers to be IB4+ versus 49% for distal urethral afferent C fibers. However, their data was based solely on C fibers while our non-glans PAs
included both unmyelinated and myelinated cells. When analyzing just the NFM-
(unmyelinated) C fiber population of our non-glans PAs, it was found that 47% of these were IB4+. This more closely matches the proportion of IB4+ C fibers found in distal urethral afferents by Yoshimura et al. (2003). At first, this suggests that distal (versus proximal) urethral afferents may play a greater role in BSM contraction; however, this would only be for C fiber afferents, and as Delcambre et al. (2011) found, C fibers are a minority in distal urethral afferents and so this does not clarify the significance of the contribution of distal urethral afferents as a whole.

The finding by Delcambre et al. (2011) that most distal urethral afferents are myelinated A fibers combined with the fact that approximately only half of our non-glans PAs were NFM+ would at first suggest that our non-glans PAs probably also include smaller proximal and distal urethral afferents. However our NFM data for our non-glans PAs matches closely with neurofilament data for distal urethral afferents gathered by Yoshimura et al (2008) and, in combination with the role of myelinated afferents in the activation of the PMRD, our non-glans PAs in fact may be primarily a reflection of distal urethral afferents. Despite this specific evidence that distal urethral afferents may make more connections to the pudendal circuitry than do proximal urethral afferents, electrophysiological and immunohistochemical studies presented earlier as well as other findings by Delcambre et al. (2011) indicate that proximal urethral afferents do play a substantial role in regulation of the pudendal reflex. Hence, these findings together are consistent with the assumption that our non-glans PAs do include both proximal and distal urethral afferents, and while both appear to contribute to the pudendal reflex, it is still not confirmed as to how equally they contribute.
Possible Cell Types Contributing to Soma Size Differences between Glans Afferents and Non-Glans PAs

DRG neurons innervating the glans (i.e., Dil+ cells, regardless of PRV labeling) are overall significantly smaller (P<0.001) in diameter than PRV+ non-glans pudendal circuit afferents (non-glans PAs), as shown in Figure 3-21. This may be due to important roles of certain small afferents from the glans in particular in regulating the pudendal reflex in ways other than simple excitation of pudendal motoneurons, as well as regulating other processes such as cortical sensory perception pathways. The data also showed that myelinated SP-/IB4- glans non-PA neurons were significantly larger than both GPAs and non-glans PAs, and suggests that these may be part of a PMRD-independent tactile perception pathway. Regarding GPAs in particular versus non-glans PAs, such a size disparity may also reflect functional differences between the regulation of urethral- versus glans-elicited contraction of the BSM: For example it may be important for certain populations of urethral afferents to be larger and faster, in order to effectively transmit signals in response to the location of semen along the genitourinary tract so that the BSM will contract (or relax) at the correct time.

When broken down by cell types based on histochemical labeling, the overall size difference between glans afferents and non-glans PAs appears to be in part due to the significantly smaller (P<0.001) IB4+ cell diameters of the glans non-PA group (29.8 ± 0.40 μm) compared to those of the non-glans PA group (34.3 ± 0.48 μm). IB4+ cell diameters were also significantly smaller for the GPAs compared to non-glans PAs. IB4+ cell sizes were even significantly different between the two glans groups: IB4+ glans non-PAs were significantly smaller than IB4+ GPAs. To summarize, IB4+
afferents were smallest for glans non-PAs, largest for non-glans PAs, and intermediate in size for GPAs.

SP+ cells also were significantly smaller for glans non-PAs than those for non-glans PAs. This proposition may be supported by the fact that SP+ non-glans PAs and SP+ GPAs were not significantly different in size. Regarding afferents containing SP in abundance, SP+ cells were significantly smaller for glans non-PAs than for GPAs. Hence, SP+ glans non-PAs are smaller, SP+ GPAs are larger, and there were only a minimal number of SP+ non-glans PAs (which were of similar size to GPAs).

In contrast to IB4+ and SP+ cells, NFM+ cells were significantly larger for GPAs when compared to NFM+ non-glans PAs, and NFM+ glans non-PAs were significantly larger than both of the other groups. Hence, these results would not contribute to the overall size differences observed for glans afferents versus non-glans PAs.

To summarize the characteristic differences of populations between these three groups of afferents (glans non-PAs, GPAs and non-glans PAs), non-glans PAs tend to have the largest diameter IB4+ cells and the smallest diameter NFM+ cells. This statement of IB4+ cell size could be generalized to unmyelinated cells, since IB4+ and IB4- unmyelinated non-glans PAs were of very similar sizes. As alluded to earlier, the relatively large size of such unmyelinated non-glans PAs could possibly be useful, for example, for relatively fast signaling of urethral afferents (compared to this cell type from glans afferents) to the pudendal circuitry (i.e. changes in BSM activity in response to semen pressure/flow). The role of these neurons may be inhibitory; these may very well be the inhibitory afferents responsible for the proposed model in Chapter 2 that patterns of mutual inhibition between proximal and distal urethral afferents are
necessary for maximal BSM relaxation as semen activates both of these afferents at
their area of overlap at the BSM region of the urethral bulb. The excitatory pathway
from urethral afferents leading to BSM contraction, on the other hand, may be regulated
by the myelinated NFM+ population. GPAs (compared to non-glans PAs), tend to have
smaller-diameter unmyelinated cells; meaning perhaps their regulatory role need not
require conduction velocity as fast as the non-glans PA population. On the other hand,
their myelinated cell populations include smaller-diameter and larger-diameter
subpopulations, possibly mediating different excitatory functions. Glans non-PAs
(compared to GPAs) tended to have smaller-diameter unmyelinated cells, smaller-
diameter SP+ cells, and larger-diameter NFM+ cells. The larger size of this last
population compared to those of GPAs and non-glans PAs may be for quick conduction
of information to regions such as the somatosensory cortex, for example. However, it
appears that signaling by the unmyelinated and SP+ populations by glans non-PAs
would be slower than that by this population of GPAs. As stated, this may be adaptive
to coordination of the pudendal reflex. For example, if SP is indeed a neuromodulator
necessary for DNP-responsive LPGi neurons to reach threshold and fire, such an
ascending pathway from the glans need not be as rapid as for similar signaling
pathways to the cord. As mentioned in the previous chapter, we found that descending
pathways from the LPGi presynaptically inhibit myelinated DNP afferents, presumably to
allow for BSM relaxation (i.e., at the onset of the expulsive phase of ejaculation).
Logically, BSM contraction would be sustained to allow semen to collect in the proximal
urethra; hence, inhibition from the LPGi is likely to be a relatively delayed process. On
the other hand, whatever role SP has in transmitting information from the glans to the
segmental circuitry appears to be faster. The NK-1 immunoreactive interneurons in the L3-L4 cord could be candidates for such a pathway; these neurons are shown to be connected, via PRV tracing studies, to both the prostate and BSM (Xu et al., 2006) and when ablated eliminates only ejaculation but not any other sexual behaviors (Xu et al., 2005, 2006). If SP has a role in this circuitry, it is not clear what it would be. However it can be proposed that, based on the divergent targets of prostate and BSM (Xu et al., 2005, 2006), this circuitry is most likely to be active during the emission phase or just before expulsion.

Support for Existence of Unmyelinated SP+/IB4+ Glans non-PAs versus Myelinated SP+/IB4+ GPAs

Interestingly, GPAs include a unique population of SP+/IB4+ cells, as well as a unique population of NFM+/IB4+ cells which fell within the size range of the SP+/IB4+ cells. Glans non-PAs were also observed to include a unique population of SP+/IB4+ cells; however unlike the GPAs, they did not include a substantial proportion of myelinated IB4+ cells. Furthermore, SP+/IB4+ glans non-PAs were smaller than this same population in the GPA group. These data together suggest the likelihood for the existence of a unique NFM-/SP+/IB4+ population for glans non-PAs, and a unique NFM+/SP+/IB4+ population for GPAs. This may be the start of a description of two SP signaling pathways utilizing IB4+ neurons; one segmental and supraspinal, fast and slow respectively; the segmental pathway employing larger myelinated fibers and the supraspinal employing smaller unmyelinated fibers. For the latter cell type, patch clamp studies performed by our laboratory have described an immunohistochemical identical Type 7 cell, unique to the glans penis (Johnson et al., 2005).
Possible Receptor Structure and Functional Roles of Glans Receptors

In general, it is difficult to propose the functions of the observed populations of GPAs based solely on anatomical characteristics when exact synaptic connectivity is not known. However, since it can be concluded that all GPAs are indeed more or less directly synaptically connected to the BSM circuitry, it would seem logical that each GPA population is somehow involved in modulating the pudendal reflex by influencing the BSM.

While one-third of the GPAs were NFM+ cells including some with large diameters, the majority of GPAs had small soma diameters, positive IB4 reactivity, negative NFM immunoreactivity, or a combination of these characteristics. Small soma size, lack of myelination and the ability to bind IB4 have traditionally been used to label neurons as nociceptors (Bennett et al., 1998; Dirajlal et al., 2003; Genzen et al., 2001; Woodbury et al., 2004). However, one must be cautious if considering the conclusion that most GPAs are nociceptors based on this. For example, not all nociceptive cells have the “telltale” signs (Woodbury et al., 2004) and myelinated nociceptors have been described (Kruger et al., 1981; Silbert et al., 2003) as important for quick response to new injury. Moreover, the glans penis is classified as mucocutaneous tissue, anatomically and embryologically different from skin and viscera, with unique sensory receptor endings (Johnson and Halata, 1991).

In contrast, a population of unmyelinated C fibers has been described as coding innocuous, pleasant touch, termed C-tactile fibers in humans (Andrew, 2010; Löken et al., 2009; Olausson et al., 2008, 2010; Vallbo et al., 1999) and C fiber low threshold mechanoreceptors (C-LTMR) in animals (Seal et al., 2009). These fibers respond to an optimum velocity of slow, soft stroking (Löken et al., 2009). The literature
reports these receptors to be found solely in hairy skin (Löken et al., 2009; Liu et al., 2007; Olausson et al., 2008; Vallbo et al., 1999) including on the face (Johansson et al., 1988; Nordin, 1990). C-tactile fibers are reported to be involved in emotional, non-sexual tactile processing, and project to limbic brain regions (Löken et al., 2009).

Reportedly no C-LTMRs have been found in the male genitalia of mice according to one source (Liu et al., 2007), however they failed to specify if this was specifically tissue from the glans, or some other region of the genitalia. C-tactile fibers, while apparently not likely to innervate the glans, cannot be absolutely ruled out at this point as a type of glans receptor. More likely however is the possibility that an analogous group of non-nociceptive C fibers that, while not matching all the characteristics of C-tactile fibers, play a similarly unique role in the pudendal reflex circuitry.

As alluded to earlier, each of the main two nociceptor populations is often cited as positive for SP or IB4, but not both (Dirajial et al., 2003). The existence of the observed SP+/IB4+ population of GPAs (and glans non-PAs) therefore suggest that these may not be nociceptors. It is indeed plausible that even the other group of small SP-/IB4+ neurons may not be predominantly composed of nociceptors, especially since glans sensory receptors in general are known to be highly unique compared to those innervating other areas of the body. One possible function for singly-labeled IB4+ or co-labeled SP+/IB4+ glans afferents may be to contribute to BSM contraction in response to pressure in the small vessel vasculature during erection, since SP+ neurons have been implicated in vasculature of other tissues (Walsh et al., 1992) and some IB4+ axons have been found to innervate vessels of the penis (Petruska et al., 1997). Regarding the latter, it has been suggested that at least some of these may be
sensory in nature, especially those innervating smaller surface capillaries (Petruska et al., 1997).

Of course, it is quite possible that most of the observed unmyelinated fibers simply reflect a large number of nociceptors, which may be needed to inhibit the pudendal reflex when appropriate. Our laboratory has indeed observed increased inhibition of the pudendal reflex with glans stimulation strong enough to recruit smaller fibers. It is unclear what the function of such inhibition might be in the natural setting; whether to transiently allow for BSM relaxation between phasic contractions during the expulsion phase, or cessation/prevention of BSM contraction. Regardless, we have evidence only of an inhibitory role of these small afferents. Of course, while pudendal reflex inhibition as well as activation can be seen by stimulation of large fibers, any activation caused by small fibers would be masked by activation caused by simultaneous stimulation of large fibers by stronger stimuli. Hence an additional, excitatory, role by small glans afferents has yet to be ruled out.

Aside from functionality, the large proportion of small-diameter afferents as seen for GPAs in particular may hold promise for elucidating possible mechanisms of functional recovery after spinal cord injury. The small IB4+ glans afferents in particular could be a carry-over from a developmental role earlier in life of establishing proper connectivity and undergo sprouting post injury during adulthood; they have been implicated in establishing connectivity during early development (Molliver et al., 1997). C and Aδ fibers have been observed to undergo collateral sprouting (Diamond et al., 1992). It is known that recovery of bladder function is dependent, in part, on unmasking
of reflexes triggered by C fiber bladder afferent neurons (de Groat and Yoshimura, 2006). Therefore, such small-diameter afferents are particularly worthy of future study.

Regardless of function, the anatomical characteristics of our NFM-/IB4- small-diameter glans afferents best fit the description of having unmyelinated C fibers with free nerve endings. Genital corpuscles are innervated by myelinated fibers (as shown by Johnson and Halata in 1991). It is interesting to note that there exists a greater proportion of these NFM-/IB4- cells in the glans non-PA group (64%) compared to the GPA group (25%), although it is difficult to deduce the contributions of each possible fiber/receptor type. NFM-/IB4+ GPAs most definitely have unmyelinated C fibers with free nerve endings, since IB4 has been shown by Petruska et al. (1997) to fail to bind axons with specialized receptors. This population is found in equal proportions in both glans afferent groups (52-53%). Glans afferents of the NFM+/IB4+ population likely have Aδ myelinated axons with free nerve endings.

More specific deductions concerning structure and function can be made based on well-established correlations; for example, NFM immunoreactivity is a reliable sign of myelination and hence fast conduction velocity. The subpopulation of NFM+/IB4- GPAs with larger soma diameters most likely have larger axon diameters that are myelinated with faster conduction velocities, based on relatively well-established correlations (Lawson and Waddell, 1991; Lee et al., 1986; Sakai and Woody, 1988). Hence, they are the ones most likely to be classified as Aβ fibers to carry mechanoreceptive information about touch. Their receptors may include Ruffini and genital (lamellated) corpuscles in the glans (Johnson and Halata, 1991; Halata et al., 1997) consistent with our findings that the larger-diameter NFM+ cells compose a minority of GPAs. While
the subpopulation of larger-diameter NFM+/IB4- GPAs likely have Aβ fibers and are myelinated, the subpopulation of smaller-diameter NFM+/IB4- GPAs may have thinly myelinated Aδ fibers or genital corpuscles for receptors. These NFM+ subpopulations may perform different roles in the segmental regulation of the pudendal reflex. Petruska et al. (1997) reports that IB4 fails to label myelinated axons of lamellated corpuscles lining the corpus spongiosum cavernous spaces, which is consistent with the proposal that such receptors would reflect the NFM+/IB4- signature, and which also suggests that our smaller population of GPAs double-positive for both NFM and IB4 probably innervates areas other than corpus spongiosum tissue. Glans non-PAs - which, by our methods and definition, project to regions other than the BSM - have a predominance of larger-diameter NFM+/IB4- cells. The larger-diameter NFM cells of glans non-PAs likely form pathways that do not involve the BSM, including the brainstem, thalamus (Hubscher and Johnson, 2003) and cortex.

To summarize deductions utilizing the NFM data set: The smaller-diameter NFM+/IB4- subpopulation may consist of afferents with genital corpuscles which may have a unique function that is specific to regulation of the pudendal reflex at the segmental level. The larger NFM+/IB4- myelinated afferents, as well as cell populations with putative free nerve endings, appear to regulate the pudendal reflex on the segmental and possibly supraspinal levels as evidenced by projections to both the BSM motor neuron circuitry and non-BSM targets. Such projections by the unmyelinated free nerve endings in particular is consistent with the finding by Petruska et al. (1998) that medullary Gi complex neurons respond to C fiber electrical stimulation.
The function of the NFM+/IB4+ afferents with unmyelinated free nerve endings is not as clear, but incorporating data from the SP data set reveals some interesting deductions. To start, as mentioned previously, the soma size distribution of NFM+/IB4+ GPAs fits completely within that of the SP+/IB4+ cell population and hence they may be at least a subset of this population. This would fit the description of myelinated, SP-signaling Aδ fibers with free nerve endings. This is more likely the case than if either double positive IB4+ group were to simply contribute to the singly IB4+ population of each opposite data set, since the NFM-/IB4+ and SP-/IB4+ populations comprises equal percentages of their respective totals. Along with Aδ afferents with the putative NFM+/SP+/IB4+ signature, the SP+/IB4+ population may also include unmyelinated C fibers. Coincidentally, such a resulting NFM-/SP+/IB4+ signature is consistent with that of a proposed subtype of C-tactile afferent (Liu et al., 2007); however these glans afferents may be analogs at best, since C-tactile afferents have been reported only in hairy skin (i.e., Liu et al., 2007). As mentioned in the previous section, soma size distributions make it apparent that the putative Aδ NFM+/SP+/IB4+ cells are more likely to project to the segmental pudendal circuitry. On the other hand, putative C fiber NFM-/SP+/IB4+ cells are more likely to project to non-BSM targets. Hence, there seems to be a bias in targets for these two putative populations, and possible functions for these were discussed in the previous section. Given the associations of SP processing with reproductive-related neural processes as well as the inhibitory role of the LPGi onto pudendal circuitry, such a bias in targets for such SP-containing fibers would be a logical contribution to the control of timing of events necessary for ejaculation.
Further deduction is not yet possible due to the several remaining unknown variables such as the electrophysiology of certain glans populations with specific immunohistochemical signatures. Also, while single unit recordings from urethral afferents have clarified the role that they play in the pudendal reflex (Delcambre et al., 2011) and proximal versus distal urethral afferents were differentiated electrophysiologically, the retrograde tracing technique utilized was unable to allow for such unequivocal differentiation of the proximal versus distal urethral afferents’ immunohistochemical signatures. The multi-labeling immunohistochemical technique was, however, very valuable in differentiating the characteristics and projection pathways of specific populations of glans and urethral afferents that simple NFM, SP, or IB4 immunoreactivity alone would have been unable to identify. As the conceptualization of pudendal circuitry becomes clearer, possible targets for therapies become apparent. For example, the two spinal centers which may combine to form the proposed ejaculation pattern generator could be targeted for stimulation in spinal cord injured patients who cannot use input from supraspinal or even segmental afferents.

**Conclusions**

As suggested by the electrophysiological evidence presented in Chapter 2, DiI tracing has confirmed that glans afferents travel primarily in the L6 dorsal root, with their somas located predominantly in L6 dorsal root ganglia with very few glans cells found in S1 DRGs in some animals. Glans afferents (GPAs) were found to be synaptically connected to the ipsilateral and contralateral BSM circuitry, with GPAs identified in left and right L6 DRGs.

GPAs consisted of four different cell populations for each of two data sets, having immunohistochemical signatures of the following: NFM+/IB4-, NFM-/IB4+, NFM+/IB4+,
NFM-/IB4- SP+/IB4-, SP-/IB4+, SP+/IB4+, and SP-/IB4-. Regarding the NFM data set, of note was the smaller average diameter of the NF+/IB4- population for non-glans PAs compared to glans afferents, which likely accounts for the slower conduction velocities for pudendal reflex-integrated PN afferents compared to DNP afferents as presented in Chapter 2.

Glans cells not connected to the BSM, like GPAs, also consisted of cell populations with the same signatures listed above; however non-glans cells connected to the BSM (e.g., urethral afferents) were virtually devoid of SP. As alluded to earlier, these non-glans PAs are probably not bladder afferents, since most bladder afferents have been shown to be peptidergic (Hwang et al., 2005). Comparison of information from the NFM data set with that from the SP data set suggests that GPAs that are SP+ are likely myelinated and larger, while glans non-PAs that are SP+ are likely unmyelinated and smaller. This may be implicated in the coordination between spino-bulbo-spinal loops and segmental circuits in the timing of BSM contraction during the expulsive phase of ejaculation. The larger size of unmyelinated afferents for non-glans PAs (e.g., urethral afferents) compared to glans afferents may be another factor in the coordination of the expulsive phase.
Table 3-1. Comparison of DRG cell diameters between groups

<table>
<thead>
<tr>
<th>Population</th>
<th>GPA (N=493)</th>
<th>Glans non-PA (N=302)</th>
<th>Non-glans PA (N=420)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFM+</td>
<td>41.3 ± 0.30</td>
<td>46.6 ± 0.84*#</td>
<td>40.6 ± 0.52</td>
</tr>
<tr>
<td>NFM-</td>
<td>31.9 ± 0.37**</td>
<td>29.4 ± 0.52**</td>
<td>33.5 ± 0.44**</td>
</tr>
<tr>
<td>SP+</td>
<td>33.3 ± 0.75</td>
<td>28.4 ± 0.55*#</td>
<td>34.0 ± 0.86</td>
</tr>
<tr>
<td>SP-</td>
<td>37.8 ± 0.63**</td>
<td>42.0 ± 1.05**</td>
<td>38.5 ± 0.59**</td>
</tr>
<tr>
<td>IB4+</td>
<td>32.9 ± 0.31</td>
<td>29.8 ± 0.40*#</td>
<td>34.3 ± 0.48*</td>
</tr>
<tr>
<td>IB4-</td>
<td>39.3 ± 0.64**</td>
<td>42.8 ± 0.82**</td>
<td>38.7 ± 0.40**</td>
</tr>
</tbody>
</table>

Mean cell diameter in µm ± SEM; *, significant from GPA; #, significant from non-glans PA; **, significant from+, P<0.05
Figure 3-1. Bilateral transsynaptic connections to Gi nuclear regions in 4 non-lesioned control animals after unilateral injection of PRV into the BSM. Mean number of PRV labeled neurons per section (± SEM) showed no significant differences in labeling frequency between ipsilateral and contralateral sides of the different subnuclei. There is an equal bilateral representation. (Adapted from Johnson et al., 2011).
Figure 3-2. Photomicrograph of putative pudendal motoneuron (white arrow) and synaptically connected putative interneuron (green arrow) in a transverse L5 spinal cord section. Neurons were identified as synaptically connected to the BSM via PRV injection into the left BSM; PRV was detected with anti-PRV followed by a DAB reaction to visualize cells of interest as brown. Photograph was taken at 10x (x1.25).
Figure 3-3. Soma size distribution of L6 DRG cells labeled with Dil. Cells positive for Dil (N=8 animals) were identified as glans afferents following application of this anterograde tracer to the glans. The soma size distribution has a noticeable peak at 25-30 µm. Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-4. Four photomicrographs of the same NFM+/IB4- cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is positive for NFM (blue) but negative for IB4 as can be seen by the absence of brown chromagen. Photos were taken at 20x (x1.25).
Figure 3-5. Four photomicrographs of the same NFM-/IB4+ cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is negative for NFM as can be seen by the absence of blue fluorophore, but is positive for IB4 (brown). Photos were taken at 20x (x1.25).
Figure 3-6. Four photomicrographs of the same NFM+/IB4+ cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is positive for NFM (blue) and also positive for IB4 (brown). Photos were taken at 20x (x1.25).
Figure 3-7. Four photomicrographs of the same NFM-/IB4- cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is negative for NFM as can be seen by the absence of blue fluorophore, and is also negative for IB4 as can be seen by the absence of brown chromagen. Photos were taken at 20x (x1.25).
Figure 3-8. Soma size distributions of four populations of GPAs from the NFM data set. The NFM+/IB4- population was the only one to appear to have a bimodal distribution of soma sizes. Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-9. Soma size distributions of four populations of non-glans PAs from the NFM data set. Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-10. Soma size distributions of four populations of glans non-PAs from the NFM data set. The NFM+ population had a peak at a much larger size (45 µm) compared to the unmyelinated populations (25 µm). Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-11. Four photomicrographs of the same SP+/IB4- cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is positive for SP (blue) but negative for IB4 as can be seen by the absence of brown chromagen. Photos were taken at 20x (x1.25).
Figure 3-12. Four photomicrographs of the same SP-/IB4+ cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is negative for SP as can be seen by the absence of blue fluorophore, but is positive for IB4 (brown). Photos were taken at 20x (x1.25).
Figure 3-13. Four photomicrographs of the same SP+/IB4+ cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is positive for SP (blue) and also positive for IB4 (brown). Photos were taken at 20x (x1.25).
Figure 3-14. Four photomicrographs of the same SP+/IB4+ cell identified with arrows. This glans pudendal circuit afferent (as identified by Dil/PRV co-labeling) is negative for SP as can be seen by the lack of blue fluorophore, and also negative for IB4 as can be seen by the absence of brown chromagen. Photos were taken at 20x (x1.25).
Figure 3-15. Soma size distributions of four populations of GPAs from the SP data set. Note that SP+ cells were predominantly IB4+. Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-16. Soma size distributions of four populations of non-glans PAs from the SP data set. There was only one SP+/IB4+ cell counted in this group of afferents. Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-17. Soma size distributions of four populations of glans non-PAs from the SP data set. The SP-/IB4- population has a peak at a larger size (50 µm) than do the other populations (25-30 µm). The SP+/IB4+ population has a particularly large peak at 25 µm. Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-18. Soma size distributions of GPAs positive or negative for SP, NFM, and IB4. SP+ cells totaled 19% of all GPAs. NFM+ cells totaled 33% of all GPAs. IB4+ cells totaled 52% of all GPAs. The mean soma diameter for each group negative for each marker was significantly different from each group positive for each marker (Table 3-1). Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-19. Soma size distributions of glans non-PAs positive or negative for SP, NFM, and IB4. SP+ cells totaled 32% of all glans non-PAs. NFM+ cells totaled 36% of all glans non-PAs. IB4+ cells totaled 49% of all glans non-PAs. The mean soma diameter for each group negative for each marker was significantly different from each group positive for each marker (Table 3-1). Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-20. Soma size distributions of non-glans PAs positive or negative for SP, NFM, and IB4. SP+ cells totaled 6% of all non-glans PAs. NFM+ cells totaled 53% of all non-glans PAs. IB4+ cells totaled 23% of all non-glans PAs. The mean soma diameter for each group negative for each marker was significantly different from each group positive for each marker (Table 3-1). Numbers on the x-axis represent bin sizes ranging from the stated number to the number plus 5 µm.
Figure 3-21. Mean soma diameters (+/- SEM) for all cells and overall immunohistochemical populations between GPAs, glans non-PAs and non-glans PAs. The mean diameter for all non-glans PAs was significantly larger than those of GPAs or glans non-PAs. The mean diameter for NFM+ glans non-PAs was significantly larger than GPAs and non-glans PAs. In contrast, the mean diameter for SP+ glans non-PAs was significantly smaller than GPAs and non-glans PAs. Similarly, the mean diameter for IB4+ glans non-PAs was significantly smaller than GPAs and non-glans PAs, and the mean diameter for IB4+ non-glans PAs was significantly larger than GPAs. *, significant from GPAs; # significant from glans non-PAs.
Figure 3-22. Mean soma diameters (+/- SEM) for specific immunohistochemical populations from the NFM data set between GPAs, glans non-PAs and non-glans PAs. The mean diameter for NFM+/IB4- non-glans PAs (40.7 ± 0.54 µm) was significantly smaller than the diameters of either GPAs (44.3 ± 1.10 µm) or glans non-PAs (47.0 ± 0.83 µm) with the NFM+/IB4- signature. The mean diameter of NFM+/IB4- glans non-PAs was significantly larger than that of NFM+/IB4- GPAs. The mean diameter of NFM-/IB4+ glans non-PAs (29.3 ± 0.53 µm) was significantly smaller than that of GPAs (32.6 ± 0.47 µm) or glans non-PAs (33.2 ± 0.61 µm) with the NFM-/IB4+ signature. The mean diameter of NFM+/IB4+ non-glans PAs (38.3 ± 0.70 µm) was significantly larger than that of GPAs (31.4 ± 1.06 µm) or glans non-PAs (35.2 ± 0.11 µm) with the NFM+/IB4+ signature. Similarly, the mean diameter of NFM-/IB4- non-glans PAs (33.7 ± 0.64 µm) was significantly larger than that of GPAs (30.9 ± 0.60 µm) or glans non-PAs (29.8 ± 1.48 µm) with the NFM-/IB4- signature. *, significant from GPAs; #, significant from glans non-PAs.
Figure 3-23. Mean soma diameters (+/- SEM) for specific immunohistochemical populations from the SP data set between GPAs, glans non-PAs and non-glans PAs. The mean diameter for SP-/IB4+ non-glans PAs (35.4 ± 0.78 µm) was significantly larger than that of GPAs (33.3 ± 0.51 µm) or glans non-PAs (32.6 ± 0.86 µm) with the SP-/IB4+ signature. The mean diameter of SP+/IB4+ glans non-PAs (27.9 ± 0.58 µm) was significantly smaller (P<0.001) than that of SP+/IB4+ GPAs (33.4 ± 0.86 µm). The mean diameter of SP-/IB4- glans non-PAs (46.5 ± 1.10 µm) was significantly larger (P=0.003) than that of SP-/IB4- GPAs (42.2 ± 1.00 µm). The mean diameter of SP-/IB4- non-glans PAs (39.5 ± 0.71 µm) was significantly smaller than that of GPAs or glans non-PAs with the SP-/IB4- signature. *, significant from GPAs; #, significant from glans non-PAs.
CHAPTER 4
EFFECTS OF LATERAL FUNICULUS SPARING AND SPINAL LESION LEVEL ON RECOVERY OF BLADDER VOIDING REFLEXES AND HEMATURIA IN RATS

Introduction

While loss of bladder function is just one of several functional deficits resulting from spinal cord injury (SCI), this impairment greatly reduces the quality of life for and regaining bladder function is of highest priority for this population (Anderson, 2004; Anderson et al., 2007), second only to regaining hand and arm function for quadriplegics and sexual function for paraplegics (Anderson, 2004). Therefore, elucidating the neural circuitry and effects of spinal cord injury on pelvic reflexes such as micturition and ejaculation are of great clinical importance. However, they are relatively poorly understood compared to other functions such as locomotion.

Bladder function is controlled at the segmental and supraspinal levels via both autonomic and somatic mechanisms. Regarding segmental control, activity of sacral parasympathetic efferent neurons leads to contraction of the detrusor musculature to permit bladder voiding, and sympathetic neurons at T10-L2 are responsible for contraction of the internal urethral sphincter to allow the bladder to fill. Sensory afferents communicate information about bladder distension, which accordingly leads to appropriate coordination of autonomic output to cause retention or voiding of urine. Micturition can also be voluntarily regulated via the activity of lumbosacral somatic motor neurons which contract the external urethral sphincter to delay voiding until conditions are appropriate.

There are several supraspinal sites involved in the coordination of micturition, some of which send descending projections to the segmental circuitry (Tai et al., 2009; Fowler et al., 2008; Sugaya et al., 1998; Noto et al., 1991; Nishizawa et al., 1987;
Holstege et al., 1986). One of the most heavily studied regions is the pontine micturition center (PMC), which has been reported to send fibers bilaterally to inhibitory interneurons to likely relax the external urethral sphincter (Blok et al., 1997) and to the spinal parasympathetic nucleus to contract the detrusor (Ding et al., 1995; Benevento and Sipski, 2002). Several cortical areas are also implicated in the voluntary regulation of bladder voiding (Athwal et al., 2000; Benevento and Sipski, 2002), sending descending projections to regulate the external urethral sphincter. In addition to descending pathways, bladder sensory afferents communicate with interneurons which in turn send ascending projections to brain regions such as the periaqueductal gray and on to the PMC. Thus, the micturition reflex is dependent on a spino-bulbo-spinal loop (Fowler et al., 2008; Noto et al., 1991; de Groat and Yoshimura, 2006; Blok and Holstege, 1994; Blok et al., 2005; Athwal et. al, 2000; de Groat and Yoshimura, 2010).

Spinal lesions that disrupt descending pathways from such areas as the PMC result in severe dysfunction of the micturition reflex (David and Steward, 2010; Sugaya et al., 1998; de Groat and Yoshimura, 2010). Micturition controlled by purely segmental reflexes is inefficient (Pikov et al., 2007), and injury-induced neuroplasticity can exacerbate such problems (Tai et al., 2006) as urinary incontinence resulting from detrusor contraction at even small bladder volumes or loss of descending regulation from voluntary control centers. Also, incomplete bladder emptying can result when the external urethral sphincter contracts at the same time as the detrusor, known as detrusor-sphincter dyssynergia (Kruse et al., 1993; Benevento and Sipski, 2002; Tai et al., 2004) which can lead to complications such as urinary tract infections.
While injury to segmental micturition circuitry often results in permanent loss of bladder function (Hoang et al., 2006) following SCI of upper spinal levels there is, to some extent, recovery of micturition reflexes. Studies have demonstrated the existence of an “unmasking” of segmental micturition reflexes often seen in young animals (de Groat and Yoshimura, 2006, 2010), in which cutaneous stimulation of the perineal area triggers micturition. Other studies have shown that cutaneous-based micturition (triggered by scratching the skin) is possible after surgically grafting thoracic or lumbar ventral roots onto ventral roots containing efferents acting on the lower urinary tract (Lin et al., 2011; Lin et al., 2010; Liu et al., 2005; Wang et al., 2011; Xiao et al., 1999).

Maladaptive neuronal changes after SCI result in detrusor hypertrophy and overactivity (de Groat, 1998; de Groat and Yoshimura, 2010; Radziszewski et al., 2009; Tai et al., 2004;) which leads to the aforementioned problems of incontinence and detrusor-sphincter dyssynergia. After SCI, there have been reported increases in numbers of motor neurons and numbers/size of DRG cells in the lumbosacral region which could contribute to the causes for such detrusor hypertrophy and overactivity, and observed increases in numbers of dorsal horn cells could result from unmasking of a C-fiber based micturition reflex (de Groat, 2006; Yu et al., 2003). Changes in trophic factors have been implicated in the post-SCI plasticity of bladder afferents (Seki et al., 2004; de Groat and Yoshimura, 2010).

This study utilizes a rat model of SCI to investigate the importance of white matter sparing to the integrity of the spino-bulbo-spinal loop and differential effects on behavioral recovery of micturition reflexes following spinal injury. Focus was placed on the lateral funiculus at the mid-thoracic level, since there is evidence that descending
pathways coordinating micturition run through this white matter (Fedirchuk and Shefchyk, 1991; Sugaya et al., 1999; Thor et al., 1989) and previous studies performed by this lab (Hubscher and Johnson, 2000; Johnson, 2006) have shown that descending pathways regulating a similar reflex (ejaculation) also travel through this area at T8. Lesions made at this level can sever long-loop ascending and descending pathways without damaging segmental micturition circuits. As with the bulbospinal loop involved in ejaculation, descending pathways from supraspinal sites such as the PMC regulating micturition are known to exert a bilateral influence on the segmental circuitry (Benevento and Sipski, 2002; Blok et al., 1997; Ding et al., 1995) therefore special attention was given to behavioral results of lateral hemisections versus bilateral lesions. We hypothesized that the speed of recovery of bladder voiding reflexes would be correlated with lateral funiculus sparing.

Another urinary tract complication of SCI is hematuria, which likely results from changes in the integrity of the uroepithelium (Acharya et al., 2004; Apodaca et al., 2003; Herrera et al., 2010; Tiebosch et al., 1989), allowing blood to escape into the urine. There are many factors involved in hematuria, including hormonal (Chaovipoch et al. 2006; Rodriguez Luna et al., 1992) and neurogenic influences (Acharya et al., 2004; Apodaca et al., 2003), although definite causes remain unknown. Accordingly, this study assessed the effects of lateral funiculus damage on the duration of hematuria.

Methods

Spinal Lesions

All procedures used for this study was approved by the Institutional Animal Care and Use Committee in accordance with USDA regulations. Bladder and urinary tract data were collected for two weeks following the spinal lesion. All of the animals used in
the present study were also used for other studies involving a terminal
electrophysiological or neuroanatomical procedure. Adult Wistar rats, approximately 90
days of age, were anesthetized for surgery using a mixture of ketamine (80 mg/kg i.p.)
and xylazine (10 mg/kg i.p.). Surgeries were done under aseptic conditions. Prior to
surgery, the long-acting antibiotic Flo-Cillin (0.5 ml; Fort Dodge Laboratories, Fort
Dodge, IA) was administered subcutaneously. In male rats, laminectomies were
performed to expose one segment of the T6-T9 spinal cord and one of the following
types of lesions was made through a longitudinal dural incision using a pair of
microdissecting scissors: Complete transection, a lesion destroying the bilateral dorsal
columns, lateral hemisection, dorsal hemisection, or a lesion completely destroying the
bilateral lateral funiculus in addition to bilateral dorsal columns. After each lesion was
performed, the dura, overlying musculature and tissue was sutured, and the skin was
closed with Michel clips.

In a separate control experiment used to assess the impact of manual bladder
expression on the duration of hematuria, both male and female rats received a ‘severe’
contusion injuries to the T8 spinal cord via rapid compression with a concave probe
having the same radius and size of the T7 lamina (as described in the methods section
of Hubscher and Johnson, 1999a). The probe was attached to a displacement-
controlled device driven with a trapezoid waveform generator (2.0 mm displacement for
5 seconds).

Post-operative Treatment and Data Collection

Following surgery, the animals received the analgesic buprenorphine for two days
and subsequently on an as-needed basis to alleviate post-operative discomfort, as well
as a 9 cc daily injection of sterile lactated Ringers solution (3 cc injected s.c. every 8
hours) until water consumption returned to normal (approximately 40 cc/day). The
antibiotic gentamicin sulfate was administered s.c. for the first five post-operative days,
and likely significantly reduced the severity of hematuria. Hong and Henderson (1989)
showed that gentamicin significantly reduced the mortality rate of rats with SCI
compared to penicillin/streptomycin administration or no antibiotic treatment.
Approximately every 8 hours, bladder voiding function was evaluated by manually or
reflexively (requiring gentle pressure or stimulation of the perineum) voiding the bladder
if it was distended, or recording that “automatic” voiding had occurred (triggered via
contact with cage bedding or otherwise without caretaker assistance). The presence or
absence of visible blood in the urine (hematuria) was also noted. In all animals across
groups, bladder voiding function had developed automatic status by 2 weeks.

**Spinal Cord Lesion Reconstructions**

At 30 to 60 days post injury, each animal was anesthetized for an
electrophysiological experiment and then euthanized with an overdose of urethane and
transcardially perfused with 4% paraformaldehyde. Specific spinal cord segment blocks
from the T6-T9 spinal cord were removed (after confirmation with spinal nerve cadaveric
dissection), embedded in paraffin, transversely sectioned at 8 µm with a rotary
microtome, and stained with luxol fast blue and cresyl violet (Klüver-Barrera stain).
Precise reconstruction drawings were made of the injured cord to confirm the exact and
total extent of the damage along the length of the entire lesion (in addition to the
epicenter), by an observer blinded to both the surgical procedure and behavioral results.
The lesions were then grouped into specific categories, eliminating those from analysis
that did not fit the criteria. The categories were: Complete transection, in which the cord
was severed completely; dorsal columns lesion, which destroyed the dorsal columns
bilaterally; lateral hemisection, which eliminated the entire left or right side of the cord (see Figure 4-1); dorsal hemisection, which ablated the dorsal columns and the more dorsal portions of the lateral funiculus bilaterally; and a lesion including both the dorsal columns and lateral funiculi bilaterally. The severity of the contusion injuries used in the second experiment resembled that of complete transections although there was sometimes a thin rim of spared axons in some portions of the peripheral white matter. Contusion injuries were classified as severe.

Data Analysis

Each pair of groups was compared using a Mann-Whitney Rank Sum Test to determine significant differences between groups (significance level was set to $P<0.05$). Data are presented as mean ± SEM.

Results

Effects of Lateral Funiculus Sparing

Following chronic mid-thoracic SCI in male rats, functional recovery of bladder voiding (for both reflexive and automatic voiding measures) was fastest in animals with a significant degree of lateral funiculus sparing when compared to animals with complete transection lesions and bilateral lesions of the lateral funiculus (Figure 4-2). Those with dorsal column lesions ($N=7$) recovered bladder function quickly ($1.07 \pm 0.48$ days for reflexive voiding and $1.67 \pm 0.67$ days for automatic voiding), as did those with lateral hemisections ($N=11$) in which one side of the lateral funiculus was left intact ($1.86 \pm 0.41$ days for reflexive voiding and $2.91 \pm 0.63$ days for automatic voiding). Rats with lesions that completely ablated the lateral funiculus bilaterally ($N=8$), classified as “dorsal columns plus lateral funiculus” lesions, recovered reflex voiding ($4.25 \pm 0.36$ days) and automatic voiding ($6.31 \pm 0.45$ days) significantly more slowly than those with
lateral hemisections or dorsal column lesions (P<0.001) but significantly faster than animals with complete transections [5.89 ± 0.30 days (reflexive); 9.50 ± 0.43 days (automatic)] possibly because of the sparing of the most ventral portions of the lateral funiculus and ventral funiculus in the former group. The lesions classified as dorsal hemisections (N=7) included ablation of dorsal columns and more dorsal parts of the lateral funiculus bilaterally. The times for this group to recover reflexive (3.52 ± 0.48 days) and automatic (4.84 ± 0.54 days) bladder voiding were intermediate to the recovery times for the dorsal columns plus lateral funiculus group and the lateral hemisection group. This may have been due to some of the dorsal hemisection lesions more closely mimicking those classified as dorsal columns plus lateral funiculus lesions, versus other dorsal hemisections failing to ablate as much of the lateral funiculus.

Duration of hematuria roughly paralleled the bladder function data (Figure 4-2); hematuria resolved more quickly for animals with a greater degree of lateral funiculus sparing. Specifically, animals with at least one side of the lateral funiculus left intact (lateral hemisection) evidenced faster resolution of hematuria than those with bilateral ablation of the dorsal half of the lateral funiculus (dorsal hemisection) as well as faster than those with dorsal hemisections which incompletely destroyed the lateral funiculus bilaterally. However, unlike the voiding onset results, time to resolution of hematuria was similar (not significantly different) between those with dorsal columns plus lateral funiculus lesions and complete transections.

**Effects of Spinal Lesion Level**

Since it is known that spinal lesions at different levels may have differential effects on disruption of reflexes such as micturition (David and Steward, 2010), we then analyzed the results from animals based on the segmental lesion level as confirmed
postmortem. The lesions ranged from T6 to T9, and the levels did not substantially vary between the groups of different lesion types. To determine if differences in lesion level were correlated with a difference in bladder functional recovery or duration of hematuria, we compared the data between three subgroups within the complete transection lesion group; those with lesions at levels T6/T7 (N=26), T8 (N=13), and T9 (N=8). Results revealed significant differences in certain bladder voiding onset measures between the three subgroups (Figure 4-3). Specifically, those with T9 lesions had significantly slower reflex voiding onset compared to those with T6/T7 (7.97 ± 0.84 days versus 5.42 ± 1.02 days; P<0.002) or T8 lesions (7.97 ± 0.84 days versus 5.34 ± 0.33 days). Also, those with T9 and T8 lesions had significantly slower automatic bladder voiding onset than those with T6/T7 lesions, with mean differences of 3.91 days and 2.84 days, respectively (P<0.001). These differences may be due to the presence, in the T10-L2 spinal segments, of spinal neuronal circuits which control 1) sensory input from the upper urinary tract and 2) the sympathetic neurons to the bladder and lower urinary tract. In contrast to the effects on bladder voiding, hematuria duration was not significantly different between the three lesion level subgroups.

While it has been suggested that the manual pressure used to express the bladder may cause hematuria, we do not believe it was an important variable in this study because hematuria was observed even upon the first bladder expression, and the same minimal pressure was used across all groups and across all time points. While the first set of results suggests that severity of injury or degree of white matter sparing influences the duration of hematuria post SCI, the proximity of the lesion to T10 had no correlation with the duration of hematuria.
**Sex Differences in Hematuria Recovery**

To investigate other factors that may have played a role in the hematuria results, we compared male versus female animals, since hormonal (Chaovipoch et al., 2006; Rodriguez Luna et al., 1992) and other sex-related differences such as the length of the urogenital tract may differentially affect hematuria duration. As can be seen in Figure 4-4, following severe contusion injuries to the T8 spinal level, hematuria took a significantly shorter time to resolve for females than for males (1.33 ± 0.19 days vs. 3.73 ± 0.30 days; P<0.001). However there was no significant difference in bladder voiding onset between these two groups, for either the perineal reflex-based voiding or the automatic voiding measures, meaning that since the duration (in days) of manual bladder expression was the same in both groups, the physical squeezing of the bladder was unlikely to be the cause of hematuria.

**Discussion**

The results of this study suggest that the preservation of the mid-thoracic lateral funiculus, particularly with at least one side left intact, is important for the quick recovery of bladder reflexes following SCI, likely through preservation of descending regulatory pathways running through this region. The fact that animals with lesions to only one side of the lateral funiculus recovered function faster than those with complete or incomplete bilateral lesions of this white matter supports the bilateral, and somewhat redundant, nature of these descending pathways. The similar recovery speed of the lateral hemisection group as compared to the dorsal columns group indicates that one side of this descending pathway is capable of exerting sufficient bilateral effect at the lumbosacral segmental level. Lesions of only the dorsal columns serve as a good control because, while these regions may transmit bladder sensation to the cerebral
cortex, they are not essential for reflex micturition (Sugaya et al., 1999). The relatively slower recovery for animals with complete transections compared to those with bilateral ablation of the lateral funiculus and dorsal columns is consistent with reports of descending micturition pathways traveling in the ventral columns to some extent (Inatomi et al., 1998).

Both behavioral measures (reflexive and automatic) of bladder voiding recovery were significantly delayed in animals with transections closer to the T10 level. This is in partial agreement with those of another study which compared the effect of moderate contusion injuries at T9 with more cranial levels (T1 and T4) by measuring amount of retained urine to indicate decreased bladder function (David and Steward, 2010). Although they found significantly greater bladder impairment for T9 contusion compared to T1, they found no difference between T9 and T4 lesions. However, their study was different from ours in the following ways: Only females were tested (versus only males), moderate contusion injuries were given (versus complete transection), bladders were expressed every 12 hours (versus every 8 hours), the bladder functional measure was different (urine volume) and was obtained under anesthesia. Preliminary data from our lab has shown that a T9 transection causes secondary damage in T10 and T11. This may impinge on essential circuitry governing urinary tract function, or there may be localized spinal centers that regulate micturition (David and Steward, 2010; Pikov et al., 2007).

Hematuria was equally prolonged in groups receiving total or partial bilateral damage to the lateral funiculus. However, hematuria resolved significantly more quickly in animals with at least one side of the cord spared. One possible explanation is that a
more severe lesion (total volume of spinal tissue damaged) results in significantly prolonged hematuria. While upon casual observation the lesion extent of two particular lesion types appears similar, the dorsal hemisection lesions may have in fact destroyed more neural tissue than the lateral hemisection lesions. The possibility of hematuria being a function of lesion severity (volume) is supported by studies demonstrating neurogenic contributions to hematuria (Acharya et al., 2004; Apodaca et al., 2003). In those studies it was suggested that cutting the spinal cord could stimulate neural release of norepinephrine and other stress hormones. It was not proposed exactly how or at what spinal level such hormones may get released. However, if they are released into spinal cord circuits and they are able to directly reach the bladder through the circulatory system, may affect the bladder uroepithelium. Apodaca et al. (2003) has shown that intravesicular application of norepinephrine indeed significantly reduces epithelial viability. In addition, other studies have shown that norepinephrine results in a desquamation of the bladder epithelium, and Veranic and Jezernik (2000) suggest this effect may be due to alternations in the tight junction, possibly through nitric oxide release, which Apodaca et al. (2003) has shown to occur in uroepithelial cells when exposed to norepinephrine. The exact mechanism for this, however, is not yet known. Thus, this neurogenic component to hematuria could be a possible cause for the differences seen with different degrees of overall sparing.

Although the exact cause or causes of SCI-induced hematuria (also known as hemorrhagic cystitis) is not known (Apodaca et al., 2003), the longer duration we have demonstrated in males compared to females may be due to the longer length of the lower urinary tract in males. The observed sex difference may also be due to a
potential protective effect of circulating ovarian steroid hormones since estradiol has been shown to have a neuroprotective effect (Chaovipoch et al., 2006). The lack of correlation of hematuria duration with lesion level within the T6 to T9 range suggests a lesser importance on proximity of the lesion to urinary thoracic segmental circuitry/spinal micturition centers compared to the disruption of descending pathways as factors in the persistence of hematuria.

In conclusion, the results of these lesion studies support our hypothesis that the lateral funiculus is the white matter spinal location at the thoracic level for descending pathways from supraspinal regions to the segmental micturition circuitry. In addition, these studies support the bilateral nature of the pathway below the thoracic level; sparing of at least one side of this bilateral pathway is sufficient for quick recovery of voiding reflexes. Behavioral data gathered after administration of lesions at different levels within the mid-thoracic region also demonstrate the potential importance of sparing the circuitry immediately caudal to T9 for the rapid recovery of bladder voiding reflexes, possibly due to the preservation of lower thoracic neural circuits important for micturition. The prolonged duration of hematuria for animals with a greater extent of overall damage is consistent with propositions made by other studies concerning the possible response of the sympathetic nervous system to such damage and subsequent uroepithelium breakdown, and the quicker resolution of hematuria for females versus males may be due to protective hormonal or anatomical factors.
Figure 4-1. Photomicrograph of a typical T8 lateral hemisection lesion at the end of a one month recovery period after SCI. White matter was stained with luxol fast blue and cells in gray matter with cresyl violet. The darker color signifies spared tissue. Photo was taken at 4x (x1.25).
Figure 4-2. The post injury onset (in days) of both reflex voiding and automatic voiding behavior in male rats was correlated with the degree of bilateral damage to the lateral funiculus. Compared to total transection lesions in which the recovery of reflex voiding was the slowest among all groups (*; P<0.01), bladder function recovered fastest in animals with dorsal column lesions or lateral hemisections and slower (#; P<0.05) with increased bilateral damage to the lateral funiculus (dorsal hemisection; dorsal columns/lateral funiculus). The duration of hematuria was significantly shorter than complete transection (*; P<0.01) only in animals with dorsal column lesions or lateral hemisections. The Y-axis represents the number of days necessary for resolution of hematuria and for perineal reflex-based and automatic bladder voiding onset in rats after receiving the following categories of spinal lesions: Complete transection, dorsal columns lesion, lateral hemisection, dorsal hemisection, and dorsal columns plus lateral funiculus lesions. Standard error bars are shown.
Figure 4-3. Transection of the spinal cord closer to T10 resulted in a slower recovery of bladder voiding behavior. Compared to lesions at T6-T7 (N=26), lesions at T8 (N=13) and T9 (N=8) showed a significantly slower onset (in days) of automatic voiding (*; P<0.05) and reflex voiding (for T9 lesions only). The duration of hematuria, however, was not affected by the lesion level. Standard error bars are shown.
Figure 4-4. The onset of bladder voiding (in days) following severe contusion injury of T8 was not significantly different between male rats (N=48) and female rats (N=19) but the duration of hematuria was significantly shorter (*, P<0.05) in female rats. Note that the duration of manual bladder expression was the same in both groups, making it an unlikely cause of hematuria. Standard error bars are shown.
CHAPTER 5
CONCLUDING REMARKS

The neural circuitry of the normal function (and recovery after spinal cord injury) of the processes of micturition and ejaculation is relatively under-studied compared to that of other processes such as locomotion. However, the fact that recovery of these functions is of even greater importance to the spinal cord-injured population (Anderson, 2004; Anderson et al., 2007b) accentuates the importance of conducting the basic research necessary to build a knowledge base complete enough to address such issues.

Common characteristics of the processes of micturition and the expulsive phase of ejaculation make it most efficient to study these two processes together. The segmental circuitry for both processes is found at the same spinal cord levels (Vera and Nadelhaft, 2000), including the pudendal motor neurons in the L5-L6 ventral horn which innervate perineal muscles required for both processes, i.e., the external urethral sphincter (EUS) and the bulbospongiosus muscle (BSM). While, in the rat, the EUS and BSM motor neurons are segregated respectively into the dorsolateral and dorsomedial pools of motor neurons, in the dog, monkey and human, these two pools are combined into a single nucleus called Onuf’s nucleus. The BSM itself functions in both micturition and ejaculation, at least in the rat model, to completely empty the urethra (of urine or semen) for both processes (Cruz and Downie, 2005). The EUS also functions in both processes; to exert conscious control over micturition and to prevent semen backflow during ejaculation. The pelvic nerve carries afferents utilized for both processes; i.e., proximal urethral afferents to regulate the pudendal reflex, and bladder afferents to regulate micturition.
The similarities extend beyond the segmental circuitry. Both micturition and ejaculation are complex reflexes under supraspinal control; i.e., micturition by the pontine micturition center (PMC) (Holstege, 2005b) and ejaculation by the lateral paragigantocellularis (LPGi). Supraspinal areas have been shown by our work to exert of bilateral modulation onto their appropriate segmental circuitries and to send their descending projections through similar areas in the lateral funiculus in the spinal cord. This agrees with observed similar recovery curves for eliminative and reproductive reflexes following SCI (Holmes et al., 2005). The electrophysiological studies in Chapter 2 as well as our PRV tracing/lesion studies (Johnson et al., 2011) reveal that the descending presynaptic inhibitory pathway from the LPGi projects first ipsilaterally through the lateral funiculus, then becomes bilateral below the thoracic level as it approaches the segmental circuitry. Lateral hemisection lesions do not prevent ipsilateral brainstem stimulation from having a bilateral presynaptic inhibitory effect on the pudendal circuitry. Similarly in the behavioral bladder studies presented in Chapter 4, animals with lesions that spared at least one side of the lateral funiculus regained bladder function more quickly than those with bilateral damage. This indicates that descending projections from supraspinal regions controlling micturition, as well as ejaculation, run in the lateral funiculus region and exert a bilateral effect on the lumbosacral circuitry. Taken together, these results indicate that if there is sparing of one side of the lateral funiculus pathway, recovery of function will be optimal for either the process of micturition or ejaculation, due to compensation by the intact side. Therefore it may be helpful for clinical translation to focus on SCI treatments that spare at least one side of the cord.
With regard to the segmental control of the pudendal reflex, our studies suggest that the dorsal nerve of the penis (DNP) afferents which have an excitatory influence on the reflex include relatively larger fibers (Aβ), and pelvic nerve (PN) afferents which have an excitatory influence on the reflex include relatively smaller fibers (Aδ). This is consistent with the observed shorter and longer reflex response latencies in response to DNP versus PN stimulation, respectively.

Pudendal reflex-triggering afferents in the DNP versus PN have distinguishing characteristics that suggest they may work together in a coordinated fashion to initiate and carry through the expulsion phase of ejaculation. The DNP afferent terminals were susceptible to presynaptic inhibition from both supraspinal and segmental sources, while the PN afferents were not. Also, in contrast to the relative abundance of SP in glans DNP afferents, putative urethral afferents (e.g., PN afferents) that make synaptic connections with the BSM circuitry are devoid of substance P. As discussed in Chapter 2, the differential presynaptic inhibition of pudendal reflex afferent inputs fits with a model of the ejaculatory expulsive phase in which inhibition of DNP afferents is necessary to allow the BSM to relax while uninhibited PN activity functions to quickly recontract the muscle in response to the flow of the next pulse of semen into the proximal urethra. If single neurons were isolated intracellularly for electrophysiological recording and then marked for immunohistochemical profiling, the distinction of possible function of PN versus DNP afferents could be clarified by performing axonal excitability tests on more specific populations of DNP or PN afferents with LPGi conditioning stimulation. Such studies may reveal whether the differential inhibition from the LPGi is specific to
particular afferents; for example, distal urethral SP- afferents or glans SP+/IB4+ afferents innervating the epidermis.

Electrical stimulation and recording (i.e., of the LPGi) could be done in awake, behaving rats as has been done in the study of other systems (Fontanini and Katz, 2006; Gutierrez et al., 2006; Lyness et al., 1979; Stapleton et al., 2006). Such experiments with the LPGi would reveal information on whether this region is important for the onset of (or, alternatively, coordination during) ejaculation, as well as possibly other information about the timing of specific events as they occur in the natural setting.

Taken together, our discoveries have clarified previously poorly understood physiological processes on primarily a basic science level but which has potential for clinical translation. Most promising are the findings that the urogenital reflexes of micturition and ejaculation are dependent on descending pathways from supraspinal regions that which, if spared on one side, may allow for compensation by the intact side and retention of function or potential for more complete recovery of function. These phenomena may provide the impetus for treatments for spinal cord injured patients that spare at least one side of this important pathway. In addition, the potential for sprouting by IB4+ C or Aδ pudendal or PN afferents could be explored further and possibly harnessed for recovery of reflexes in the clinical setting.

Certain difficulties that SCI patients often encounter such as bladder incontinence, detrusor-sphincter dyssynergia and erectile/ejaculatory dysfunction, involve inappropriate perineal muscle activity. Plateau potentials are sustained depolarizations that can occur in motor neurons following brief sensory input, via the opening of noninactivating L-type calcium channels (Thor and de Groat, 2010). Even long after the
sensory stimulus is removed, such sustained, threshold-level depolarizations can lead to spontaneous, repeated action potentials. This may be the mechanism for increased spasticity/hyperreflexia observed after SCI (Bennett et al., 2001; Yates et al., 2008) including in the motoneurons innervating the striated muscles involved in urogenital functions. Under normal circumstances, inputs that trigger plateau potentials are likely modulated by descending pathways from supraspinal sites. This may be via serotonergic or norepinephrine signaling through GABA-ergic interneurons in the spinal cord (Heckman et al., 2008). Severing these descending pathways likely leads to increased incidence of plateau potentials and thus hyperreflexia following SCI. Activation of adaptive spinal plasticity by rehabilitative therapies such as passive exercise before the onset of hyperreflexia has been demonstrated in the context of limb movement (Yates et al., 2008). Similar approaches may hold promise for developing therapies for incontinence, detrusor-sphincter dyssynergia and sexual dysfunction disorders caused by SCI. Investigation of 5-HT receptors (i.e., using selective 5-HT antagonists) would likely be helpful to determine the exact mechanism of their regulation of plateau potentials and possibly lead to a pharmacological therapy.

Baclofen is a GABA-B receptor agonist that is effective in treating spasticity in SCI patients (Jones et al., 2008; Lewis and Mueller, 1993). Unfortunately, Baclofen has deleterious effects on erectile function in SCI men when given in high doses (Jones et al., 2008). This is probably due to the long-acting inhibitory effects of GABA-B receptor activation. This effect on sexual function appears to be reversible upon lowering of the dose. Oral administration of Baclofen is usually preferred; however this route requires larger doses which sometimes cause adverse effects (Lewis and Mueller, 1993).
intrathecal route is more effective with smaller doses; however, tolerance can develop with prolonged treatment (Heetla et al., 2009; Nielsen et al., 2002).

Ejaculation involves activation of the sympathetic nervous system. Hence, pharmacological modulation of sympathetic effectors may be beneficial for ejaculatory disorders, including those caused by spinal cord injury. Midodrine, for example, is an alpha-adrenergic drug shown to be effective in eliciting ejaculation for patients with central neurologic injuries (Blanchard-Dauphin et al., 2005; Courtois et al., 2008; Soler et al., 2007; Staerman et al., 2001) and increasing intensity of the sensation of orgasm (Riley and Riley, 1982). Other methods to stimulate the sympathetic nervous system such as exercise may also be beneficial as therapies. Despite therapies to elicit ejaculation, semen quality is often poor due to events following SCI (Staerman et al., 2001); hence, therapies that elicit ejaculation should be used in conjunction with treatments that address the injury itself.
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BIOGRAPHICAL SKETCH

Sunny Ferrero first took an interest in biology long ago; her hobbies as a youngster included reading about animals and their phylogenetic relationships, studying/experimenting with wild food plants, and fishing (occasionally dissecting fish saved for food). Her passion for biology as an academic subject became particularly apparent in 11th grade when most of her courses consisted of biology classes, her favorite of which was human anatomy and physiology.

Rensselaer Polytechnic Institute was where Sunny obtained her Bachelor of Science dual degrees in biology and psychology. Her courses varied widely, from biochemistry laboratory to behavioral neuroscience to origins of life seminar. At Berry College in Georgia, she also obtained some experience in fish ecology, and very much enjoyed working in the field.

However, Sunny’s most pointed interest surfaced and solidified when she entered the University of Florida’s IDP program, or Interdisciplinary Program in Biomedical Sciences. Even with six concentrations offered, she knew she would be heading into neuroscience. Within this sub-discipline, her interests again varied widely, from learning and memory to addiction to…spinal reflexes that are very rarely studied. This last topic is what finally got her more permanent attention. Nothing interests her as strongly as what is not yet known, especially when not mere details but entire neuroscience textbook chapters appear to be missing from the literature. This appeared to be the case for pelvic and reproductive reflexes, namely, micturition and ejaculation, and it was interest of this relatively poorly-understood field that led Sunny to receive her Ph.D. from the University of Florida in the summer of 2012.