Bilateral Bulbospinal Projections to Pudendal Motoneuron Circuitry after Chronic Spinal Cord Hemisection Injury as Revealed by Transsynaptic Tracing with Pseudorabies Virus

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Abstract

Complications of spinal cord injury in males include losing brainstem control of pudendal nerve-innervated perineal muscles involved in erection and ejaculation. We previously described, in adult male rats, a bulbospinal pathway originating in a discrete area within the medullary gigantocellularis (GiA/Gi), and lateral paragigantocellularis (LPGi) nuclei, which when electrically microstimulated unilaterally, produces a bilateral inhibition of pudendal motoneuron reflex circuitry after crossing to the contralateral spinal cord below T8. Microstimulation following a long-term lateral hemisection, however, revealed reflex inhibition from both sides of the medulla, suggesting the development or unmasking of an injury-induced bulbospinal pathway crossing the midline cranial to the spinal lesion. In the present study, we investigated this pathway anatomically using the transsynaptic neuronal tracer pseudorabies virus (PRV) injected unilaterally into the bulbospongiosus muscle in uninjured controls, and ipsilateral to a chronic (1–2 months) unilateral lesion of the lateral funiculus. At 4.75 days post-injection, PRV-labeled cells were found bilaterally in the GiA/Gi/LPGi with equal side-to-side labeling in uninjured controls, and with significantly greater labeling contralateral to the lesion/injection in lesioned animals. The finding of PRV-labeled neurons on both sides of the medulla after removing the mid-thoracic spinal pathway on one side provides anatomical evidence for the bilaterality in both the brainstem origin and the lumbosacral pudendal circuit termination of the spared lateral funicular bulbospinal pathway. This also suggests that this bilaterality may contribute to the quick functional recovery of bladder and sexual functions observed in animals and humans with lateral hemisection injury.

Key words: animal studies; electrophysiology; immunohistochemistry; *in vivo* studies; spinal cord injury

Introduction

SEXUAL DYSFUNCTION is a particularly disturbing complication of spinal cord injury (SCI), and is rated by many men with chronic SCI as having the most devastating impact on their quality of life, even surpassing locomotor dysfunction (Anderson, 2004; Anderson et al., 2006). Along with the control of micturition, erectile and ejaculatory function is severely disrupted and leads to infertility in men and male animals with chronic SCI (Elliot, 2003; Johnson, 2006). Compared to the locomotor system, there is relatively little known about the long spino-bulbo-spinal pathways involved in the control of sexual function, particularly regarding their integrity after

long-term incomplete SCI. In a rat model, we have previously shown that electrical microstimulation of discrete regions of the medullary gigantocellularis nuclear complex (Gi/GiA), and lateral paragigantocellularis nucleus (LPGi) of male rats produces a short-latency and short-duration inhibition of the dorsal nerve of the penis-elicited reflex discharge of pudendal motoneurons innervating the perineal striated muscles involved in male sexual function (Johnson and Hubscher, 1998; reviewed in Johnson, 2006). Unilateral microstimulation of this pathway produced bilateral spinal segmental effects, capable of modulating ipsilateral as well as contralateral lumbosacral reflex circuits. Ejaculatory expulsion of semen cannot occur without input from the dorsal nerve of the penis

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(DNP; Meisel and Sachs, 1994; Wieder et al., 2000), and the DNP-elicited reflex discharge of pudendal motoneurons is used as an assessment of lumbosacral ejaculatory reflex integrity in humans (Yang and Bradley, 2000), and animals (Guiliano et al., 2007; McKenna and Nadelhaft, 1989; Thor et al., 2002). The bulbospinal inhibitory pathway, proposed to be involved in the phasic coordination of segmentally-organized ejaculatory bursts of the bulbospongiosus muscle required to expel semen from the penis, is lost following a chronic symmetrical lesion of approximately the dorsal 60% of the mid-thoracic spinal cord, as described in electrophysiological and behavioral experiments (Hubscher and Johnson, 2000). Histological reconstruction of a variety of incomplete lesions showed the pathway to be located around and just below the level of the central canal in the lateral funiculus.

Our previous electrophysiological study demonstrated that this bulbospinal pathway descends unilaterally at least as far caudally as the caudal thoracic spinal cord (Hubscher and Johnson, 2000). This was demonstrated following an acute (1–2 h) lateral hemisection of the mid-thoracic cord, for which microstimulation of the lateral portions of the GiA and LPGi ipsilateral to the lesion had no effect on the segmental pudendal reflex of either side of the lumbosacral cord, suggesting that the bulbospinal pathway descends on the same side of the spinal cord before exerting an ipsilateral and contralateral inhibition of pudendal reflex circuitry below the level of T8. However, microstimulation following a chronic lateral hemisection lesion (1–2 months) revealed a weak but definitive inhibitory effect from the GiA-LPGi on the same side as the lesion, suggesting the development or unmasking of an injury-induced crossed bulbospinal pathway crossing the midline to bypass the lesion somewhere cranial to T7–T8. These crossed connections may contribute to the quick functional recovery seen in rats with unilateral SCI.

In the present study, transsynaptic tracing of the pathway with pseudorabies virus (PRV) injected unilaterally into the bulbospongiosus muscle was used to answer the following questions. First, is there anatomical evidence of synaptically-coupled PRV-positive cell bodies in the gigantocellularis complex on the same side of a chronic unilateral hemisection lesion? Second, does the bulbospinal pathway at the mid-thoracic level descend predominantly from cell bodies located on the same side of the medulla? In studies by others, PRV has been shown to have a strong affinity for motoneurons in rats when injected into muscle (Card et al., 1990). The virus is transported to motoneuron somata in the spinal cord, replicates, and sequentially infects only those neurons synaptically and functionally connected to the primarily-infected motoneurons (Aston-Jones and Card, 2000; Card et al., 1990, 1993; Enquist, 2002; Rinaman et al., 1993), making this a useful methodology for visualization of a neuronal control circuit to a specific muscle. The PRV method has been used in normal rats to identify spinal and brainstem neurons involved in sexual and bladder functions. Using the attenuated Bartha strain of PRV, many labeled neurons in the gigantocellularis nuclear complex have been identified following injection, in spinally-intact animals, of the bulbospongiosus muscle (Marson and McKenna, 1996), male reproductive organs (Marson and Carson, 1999; Marson et al., 1993), female reproductive organs (Lee and Erskine, 2000; Marson and Murphy, 2006), and urinary tract (Nadelhaft and Vera, 1996; Vizzard et al., 1995).

We hypothesized that following a chronic lateral hemisection injury, transsynaptic tracing of the neural pathways converging on ipsilateral bulbospongiosus motoneurons would reveal labeled neurons bilaterally in the gigantocellularis nuclear complex, but predominantly on the side contralateral to the lesion, thereby providing neuroanatomical evidence that the descending bulbospinal pathway on each side of the cord synapses with both ipsilateral and contralateral pudendal motoneuron circuits, and as predicted by physiological microstimulation experiments, includes input from medullary neurons on the side ipsilateral to the lesion in chronically-injured, but not acutely-injured, animals.

Methods

A total of 35 male Wistar rats approximately 90 days old were used in this study. All animal procedures were approved by the institutional animal care and use committee in accordance with USDA regulations. To determine if PRV transsynaptic tracing would reveal crossed connections from the medullary GiA/Gi and LPGi nuclei above and below a mid-thoracic spinal cord injury, eight animals were given a unilateral hemisection with a chronic post-injury recovery period (1–2 months). The lateral hemisection was made in the T8 spinal cord ipsilateral to a subsequent unilateral PRV injection into the bulbospongiosus muscle (given 4.75 days prior to euthanasia). As explained in more detail below, control groups consisted of unlesioned ($n=4$), chronic hemisection contralateral to the injection ($n=4$), chronic spinal transection ($n=4$), chronic symmetrical dorsal hemisection ($n=4$), acute (5-day injury) hemisection ipsilateral ($n=4$) or contralateral ($n=1$) to the injection, and non-muscle PRV injection controls ($n=6$). Immunohistochemical analysis of transverse sections through the rostral medulla determined the presence and number of PRV-immunoreactive (PRV-IR) cells in the gigantocellularis complex on each side of the midline (see details below).

Spinal lesion surgeries

Lesion surgeries were performed under aseptic conditions following anesthesia induced with a mixture of ketamine (80 mg/kg IP) and xylazine (10 mg/kg IP). A long-acting antibiotic (0.5 mL of penicillin G) was administered subcutaneously before surgery. The spinal cord was exposed at the T8 level via removal of the caudal two-thirds of the overlying T6 vertebral lamina. Lesions were made through a longitudinal dural incision using a pair of microdissecting scissors (Hubscher and Johnson, 1999, 2000). Thrombin-soaked absorbable gelatin sponge was placed into the vertebral defect. The surrounding musculature and subcutaneous tissue was sutured in layers with 4-0 monofilament and the skin was closed with Michel clips. The animals recovered in a temperature-controlled environment while housed singly in plastic cages with wood shavings. Analgesia, with ketoprofen (5 mg/kg) and/or buprenex (0.04 mg/kg), was administered for 1–2 days, then as needed to alleviate post-operative discomfort. Throughout the recovery period the animals were examined three times daily, 7 days a week. At these times, the animals were observed for evidence of infection or other complications, and the bladder was manually expressed or voided reflexively until automatic voiding function returned.

Pseudorabies virus injections

Approximately 104 h (4.75 days) prior to euthanasia, unilateral injections of the attenuated Bartha strain (6×10^8 pfu/mL) of PRV (courtesy of Dr. L.W. Enquist) were made into the ventral head of the bulbospongiosus muscle (BSM). We found in our pilot studies, as have others, that the attenuated Bartha strain infects a few medullary neurons as early as 4 days post-injection of the BSM region, and there are no ill effects from the virus for at least 6 days post-injection. In 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. Moreover, dorsal root ganglia were not labeled at short survival times verifying the affinity of the Bartha strain of PRV for motor axons as described by others (Aston-Jones and Card, 2000; Card, 1993; Loewy, 1998).

Under aseptic conditions, the animals in the present study were anesthetized with ketamine/xylazine and a unilateral para-midline incision was made in the perineal/scrotal skin. Using a 33-gauge Hamilton syringe, five injections of PRV (5 μ L 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 microdrop of cyanoacrylate glue immediately following the withdrawal of the injection needle. In animals with a chronic spinal lesion, the injection was made near the end of the recovery period. In animals with an acute spinal lesion, the injection was made immediately (20–30 min) 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 4.75-day survival period after PRV injection, the rats were euthanized and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde.

Immunohistochemistry and histology

Protocols for immunohistochemistry were adapted from our previously published methodologies (Petruska et al., 2000; Rau et al., 2006). Brainstem and spinal cord tissues were sectioned with a cryostat at 18 μ m thickness, mounted on electrostatically charged slides, and processed for immunohistochemical localization of virus by using rabbit anti-PRV polyclonal antibody (100 μ g of protein A-purified IgG in PBS; Affinity BioReagents, Golden, CO). The PRV primary antibody was labeled with a biotinylated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and an avidin-biotin complex (Elite standard Vectastain ABC kit; Vector Laboratories, Burlingame, CA), and detected using diaminobenzidine (DAB) for 6 min as the chromogen (20 mg in 50 mL PBS; Sigma-Aldrich, St. Louis, MO). Brainstem sections were viewed with light microscopy. Total counts were made of PRV-IR neurons by an investigator blinded to the lesion status in every sixth 18- μ m-thick section (108 μ m spacing) through the rostral part of the gigantocellularis nuclear complex (Gi/ GiA and LPGi). As outlined in Figure 1A, the distinct orientation, morphology, cell clustering, and distribution of neurons defined the borders be-

tween Gi/ GiA and LPGi using the coordinates of Paxinos and Watson (1998) as a guide. Only neuronal somata with cytoplasmic PRV-positive granules were counted (manually), and all neurons satisfying these criteria were included in the count for that section (a mean of 13.9 sections were counted per animal). The most important quantitative measure was the ratio of labeled cells on the side contralateral to the lesion over the ipsilateral side. Examples of labeled versus non-labeled neurons are shown in Figure 1B (see inset). Section spacing of 108 μ m prevented double-counting of cells. Verification of successful PRV infection was determined by visualizing labeled pudendal motoneurons and adjacent interneurons in the L5–L6 spinal cord. The maximal extents of the spinal cord lesions were determined with careful post-mortem analysis of paraffin-embedded transverse sections of the T6–T9 spinal cord cut at 10 μ m on a rotary microtome. Sections were stained with Luxol fast blue and cresyl violet (Klüver-Barrera stain), and the composite area of damaged spinal cord, typically spread over a 5-mm segment of T7, T8, and T9, was drawn on a spinal cord template by an investigator blinded to both the surgical procedure and the results of the PRV counts in the brainstem. This reconstruction method reduced the possibility of underestimating the size of the lesion, while also verifying that specific spared white-matter regions, particularly in the lateral funiculus, were continuous throughout the mid-thoracic spinal cord.

Statistical analysis

Distribution normality tests were conducted for each set of grouped data, and since normal distributions were not verified in all cases, the more appropriate (and more conservative) non-parametric Mann-Whitney *U* test was used to determine the significance between groups with the significance level set at $p < 0.05$. Within the chronic lesion groups, we combined the data for animals with chronic lesions of 1 or 2 months because the datasets were not significantly different from each other, most importantly between the labeling ratios of contralateral to ipsilateral brainstem.

Results

In summary, strong bilateral descending connections from the Gi/ GiA/ LPGi nuclear complex to the lumbosacral spinal cord were found in non-injured controls after unilateral PRV injection into the bulbospongiosus muscle. In addition, there was no difference in PRV-IR labeling frequency between the two halves of the brainstem in non-injured controls, suggesting an equivalent projection from each bulbospinal pathway to the bulbospongiosus circuitry in the lumbosacral spinal cord. The results also showed that after chronic SCI, the bulbospinal pathway, descending within a spared mid-thoracic lateral funiculus, originated from Gi/ GiA/ LPGi neurons on both sides with an ipsilateral predominance, as seen by chronically lesioning the lateral funiculus on the opposite side. Bilateral labeling, however, was also found after a 5-day lesion, thereby providing no direct evidence for our hypothesized injury-induced plasticity resulting from a chronic but not an acute 5-day lesion.

In non-lesioned control animals, neurons in the gigantocellularis nuclear complex of the medullary reticular formation (MRF) were found with PRV-IR labeling after a post-infection period of 4.75 days. Potential leakage of PRV into

FIG. 1. Examples of pseudorabies virus-immunoreactive (PRV-IR) neurons in the gigantocellularis nuclear complex approximately 2700 μm rostral to the obex. **(A)** Unilateral photomicrograph (left) and schematic diagram (right) of the rostral portion of the gigantocellularis nuclear complex demonstrates the boundaries of the subnuclear regions used in the cell-counting process. **(B)** Photomicrograph from a chronically-injured animal in which the lateral funiculus was severed ipsilateral to the PRV injection site. The PRV-IR neurons on the side contralateral to the lesion/injection in the medullary gigantocellularis (GiA/Gi) and lateral paragigantocellularis (LPGi) nuclei can be seen on the left side of the image. Only a few neurons ipsilateral to the lesion/injection were labeled in this section. The inset to the upper left is a magnified image from the area shown, demonstrating neurons with (thick arrows) and without (thin arrows) PRV-IR labeling. The inset to the upper right is a diagram of the total area of damage (gray shading) from the reconstruction of the T8 lesion (Gi, gigantocellularis; GiA, gigantocellularis pars alpha; LPGi, lateral paragigantocellularis; Py, pyramid; ROb, raphe obscurus; RMg, raphe magnus; 7, facial nucleus; DPGi, dorsal paragigantocellularis; IRt, intermediate reticular; PCrt, parvocellular reticular nucleus). The template of the medullary section was modified from Paxinos and Watson (1998).

surrounding tissue was tested in non-muscle injection control animals in which injections were made into the tissue external to the connective tissue (epimysium) around the BSM, including the subcutaneous tissue under the skin of the scrotal incision site, the deeper adipose and connective tissue adjacent to the muscles, and over the BSM epimysium. No labeled lumbosacral motoneurons or gigantocellularis subnuclei neurons were found in these animals, suggesting that viral leakage into non-specific tissue surrounding the bulbospongiosus muscle was not a likely complication. In four animals with a chronic T8 complete transection, no labeling was found in the rostral medulla despite successful PRV infection (as evidenced by labeled bulbospongiosus motoneurons in the L5–L6 spinal cord). Counts of PRV-labeled neurons in non-lesioned control animals (Fig. 2) revealed no significant difference between the two sides of the gigantocellularis subnuclear regions ipsilateral and contralateral to the unilateral PRV injection, confirming the strength of the bilateral lumbosacral connections. Consistently, more neurons were labeled in GiA compared to LPGi, as expected due to the larger area of the GiA subnucleus.

A chronic mid-thoracic spinal lesion of the lateral funiculus on the side ipsilateral to the PRV injection resulted in labeling

of the contralateral gigantocellularis subnuclei (Fig. 3), which demonstrated the bilateral connections of the bulbospinal pathway below the mid-thoracic level. An example of PRV labeling in one cross-section is shown in Figure 1B. The presence of PRV-labeled cells on the side ipsilateral to the lesion, while significantly fewer in number compared to the contralateral side, was consistent with the presence of electrophysiological connections above the level of the lesion reported in our previous study of chronic spinal lesions (Hubscher and Johnson, 2000). Lesion reconstructions in this group (Fig. 3 inset) showed the loss of the ipsilateral dorsal column and lateral funiculus in all eight cases, with partial sparing of the ipsilateral ventral funiculus in three cases.

While the results in chronically injured animals supported our hypothesis, we tested whether the presence of PRV-IR neurons on the side ipsilateral to the lesion/injection was also seen following an acute lesion. Accordingly, counts were made in four animals with a 5-day acute lesion of the lateral funiculus ipsilateral to the PRV injection. In contrast to our conclusions regarding a lack of an ipsilateral modulatory effect from microstimulation studies performed in animals with an acute (1–2 h) lesion, PRV-IR neurons were found in the gigantocellularis

FIG. 2. Bilateral transsynaptic connections to the gigantocellularis nuclear region in four non-lesioned control animals after unilateral injection of pseudorabies virus (PRV) into the bulbospongiosus (BSM) muscle. Mean numbers of PRV-labeled neurons per section ($\pm$ standard error of the mean) showed no significant differences in labeling frequency between the ipsilateral and contralateral sides of the different subnuclei (Gi, gigantocellularis; GiA, gigantocellularis pars alpha; LPGi, lateral paragigantocellularis; py, pyramid).

subnuclei on the ipsilateral side after a 5-day lesion (Fig. 4). In another control group, chronic lesions of the lateral funiculus contralateral to the PRV injection resulted in bilateral labeling of the GiA/Gi/LPGi, and demonstrated that the bilateral labeling pattern was not dependent on the side of the lesion (Fig.

5). This was also confirmed in one animal with an acute lesion of the lateral funiculus contralateral to the injection site.

The level of PRV labeling in the gigantocellularis nuclear region was somewhat variable among animals in all groups, likely related in part to different degrees of PRV infection in

FIG. 3. Effects of chronic spinal hemisection and injection of pseudorabies virus (PRV) into the ipsilateral bulbospongiosus muscle (BSM) in eight animals. The mean number of PRV-labeled neurons per section ($\pm$ standard error of the mean) shows that while there were PRV-positive neurons found on the side ipsilateral to the lesion, there were significantly more neurons labeled on the contralateral side in all subnuclei ($p < 0.05$). The diagram of the spinal lesion shows the damaged regions (shaded) common to all eight lesion reconstructions (Gi, gigantocellularis; GiA, gigantocellularis pars alpha; LPGi, lateral paragigantocellularis; py, pyramid).

FIG. 4. Control for chronic versus acute lesion. Pseudorabies virus (PRV)-labeling after acute spinal hemisection and injection of PRV into the ipsilateral bulbospongiosus muscle (BSM) in four animals. As in the chronically injured animals, an acute 5-day lesion also resulted in PRV-labeled neurons on the side ipsilateral to the lesion. While the mean number of PRV-labeled neurons per section ($\pm$ standard error of the mean) shows a trend toward more neurons labeled on the contralateral side compared to the ipsilateral side in all subnuclei, the mean values were not significantly different. The diagram of the spinal lesion shows the damaged regions (shaded) common to all four animals in this group (Gi, gigantocellularis; GiA, gigantocellularis pars alpha; LPGi, lateral paragigantocellularis; py, pyramid).

FIG. 5. Control for potential effects by the side of muscle injection. Pseudorabies virus (PRV)-labeling after chronic spinal hemisection and PRV injection into the contralateral bulbospongiosus muscle (BSM) in four animals. There was a similar trend toward more neurons labeled on the side ipsilateral to the PRV injection compared to the contralateral side, but the mean values were only significantly different in the LPGi. The diagram of the spinal lesion shows the damaged regions (shaded) common to all four animals in this group (Gi, gigantocellularis; GiA, gigantocellularis pars alpha; LPGi, lateral paragigantocellularis; py, pyramid).

individual animals, including the uninjured control animals. To reduce this potential virulence effect and also to eliminate any accidental subnuclear assignment of a labeled neuron on or near a cytoarchitectural border, we recalculated the data for each animal as a PRV-IR labeling ratio. For each animal, this consisted of combining the PRV-IR counts of all subnuclei (combined Gi, GiA, and LPGi) on that side of the brainstem, calculating the mean numbers of labeled cells per section, and then deriving a PRV-positive labeling ratio of the side contralateral to the lesion to the side ipsilateral to the lesion. In other words, an equal labeling frequency on each side of the brainstem would yield a labeling ratio near 1.0. This analysis, graphically depicted in Figure 6, showed that compared to non-lesioned controls in which there was no side-to-side difference (despite a unilateral PRV injection into the BSM), unilateral chronic and acute lesions of the lateral funiculus resulted in significantly greater labeling in the Gi-GiA-LPGi on the side contralateral to the spinal lesion. A symmetrical and bilateral chronic lesion of the dorsal half of the lateral funiculus, however, did not result in any side-to-side difference in labeling (far right bar in Fig. 6), presumably because the pathway was incompletely damaged in a similar manner on both sides of the T8 cord.

Discussion

Utilizing the transsynaptic tracer pseudorabies virus injected into the bulbospongiosus muscle of male adult rats

with and without chronic SCI, the present study has provided neuroanatomical evidence for bilateral connections between the gigantocellularis/lateral paragigantocellularis nuclei in the medulla and the lumbosacral circuitry synaptically associated with the bulbospongiosus, a muscle mediating the propulsive ejaculation of semen. Strong bilateral descending connections to the lumbosacral spinal cord were verified in uninjured controls. The results also showed that after chronic SCI, the bulbospinal pathway, descending within a spared mid-thoracic lateral funiculus, originated from Gi/GiA/LPGi neurons on both sides with an ipsilateral predominance, as seen by chronically lesioning the lateral funiculus on the opposite side. These anatomical results are consistent with those of our previous electrophysiological studies using microstimulation of this bulbospinal pathway to phasically inhibit DNP-elicited pudendal motoneuron reflexes in uninjured controls and in animals with chronic incomplete spinal injury (Johnson and Hubscher, 1998; Hubscher and Johnson, 2000), and in particular are consistent with the quick recovery of bladder control and sexual reflexes in rats with hemisection injury (Johnson and Hubscher, 2000), in which the pathway is spared on one side. In addition, the results demonstrate that the attenuated Bartha strain of PRV can be successfully used to transsynaptically trace central ejaculatory pathways following long-term incomplete SCI.

In our uninjured controls, bilateral labeling of GiA, Gi, and LPGi seen following unilateral PRV injection into the BSM was consistent with a previous PRV study of the BSM in

FIG. 6. Increased numbers of pseudorabies virus-immunoreactive (PRV-IR) neurons on the side contralateral to a unilateral lesion of the lateral funiculus. For each animal, the labeling ratio was computed for the combined Gi-GiA-LPGi region by dividing the labeling frequency contralateral to the lesion by the frequency ipsilateral to the lesion. For the four non-lesioned control animals, the labeling ratio was computed relative to the side of the PRV injection into the bulbospongiosus muscle. Note that despite a unilateral PRV injection, the mean labeling ratio for the unlesioned controls was near unity (mean = 1.07), as expected considering the bilateral lumbosacral connections for both the ipsilateral and contralateral descending pathways. However, chronic and acute unilateral lesions of the lateral funiculus resulted in significantly higher labeling ratios compared to controls ($p < 0.05$), and demonstrated the increased number of labeled neurons in the Gi-GiA-LPGi region contralateral to the lesion, regardless of the side of PRV injection. In four animals with a chronic dorsal hemisection, a partial, but relatively equal, lesion to the lateral funiculi on both sides resulted in a mean labeling ratio near 1 (Gi, gigantocellularis; GiA, gigantocellularis pars alpha; LPGi, lateral paragigantocellularis).

spinally-intact animals (Marson and McKenna, 1996), in which the authors described significant labeling at a post-injection period of 5 days, but only limited labeling at 4 days, consistent with our optimum period of 4.75 days. While our data are consistent with anterograde tracing studies from the gigantocellularis region to the pudendal motoneuron pool (Hermann et al., 2003; Marson and McKenna, 1990; Monaghan and Breedlove, 1991; Shen et al., 1990), the PRV method employed here labels several synaptically-coupled neurons in a sequential manner, retrogradely from the BSM. The specificity of labeling circuit elements synaptically and/or functionally connected to BSM motoneurons was tested in our control experiments, in which we observed no labeling of lumbosacral motoneurons or medullary neurons when the PRV was injected into tissue surrounding the BSM connective tissue. In controls in which the BSM was injected after a chronic T8 spinal transection, no labeling was seen in the medulla, suggesting that the escape of virus into the bloodstream or CSF was not a complicating factor. This is consistent with the view that the Bartha strain of PRV is only mildly cytopathic, leaving the primary infected motoneurons intact for several days before lysis of the cell membrane (Loewy, 1998). Studies by others have utilized PRV tracing following chronic SCI (Kim et al., 2002; Yu et al., 2003), and demonstrated that chronic spinal transection did not reduce the ability of PRV to transsynaptically spread in the spinal cord below the lesion (Yu et al., 2003), although the situation may be different in sympathetic circuits (Duale et al., 2009).

Our previous chronic spinal lesion studies demonstrated that following a chronic symmetrical lesion of approximately the dorsal 60% of the mid-thoracic spinal cord, GiA-LPGi microstimulation-induced inhibition of pudendal motoneuron reflexes were lost. Lesion reconstructions of these and other partially lesioned animals showed the pathway to be located in the central portion of the lateral funiculus (Hubscher and Johnson, 2000). Unilateral microstimulation of the GiA and LPGi following an acute (1–2 h) lateral hemisection of the mid-thoracic cord demonstrated that the bulbospinal pathway descends ipsilaterally in the mid-thoracic spinal cord and exerts a bilateral inhibition of pudendal reflex circuitry by crossing to the contralateral side somewhere below the level of T8 (Hubscher and Johnson, 2000). However, microstimulation following a chronic lateral hemisection of at least 30 days revealed an inhibitory effect from both sides of the brainstem, suggesting an injury-induced development of a bulbospinal pathway crossing the midline somewhere cranial to the spinal lesion. In the present study, following an asymmetrical chronic lesion of the lateral funiculus ipsilateral to PRV injection into the BSM, labeled neurons were found on both sides of the gigantocellularis nuclear complex, supporting the electrophysiological evidence for a crossed connection above the lesion, and the location of this pathway in the lateral funiculus of the mid-thoracic spinal cord. In addition, the finding of significantly more neurons on the spinally intact side of the brainstem was consistent with the stronger inhibitory effect via the uncrossed bulbospinal pathway compared to the crossed pathway after chronic lateral hemisection (Hubscher and Johnson, 2000). Injury-induced development or unmasking of a crossed bulbospinal pathway has also been described in phrenic motoneuron control circuitry following lateral hemisection of the cervical spinal cord (Goshgarian, 2003). In the phrenic system, the crossed pathway decussates

at the supraspinal level and remains unilateral until reaching the phrenic motoneuron spinal segments. We suggest that the bulbospinal pathway to the pudendal motoneuron circuitry has a similar crossing pattern, although in the present study we did not specifically address the (1) specific level of crossing, or (2) the possibility of multiple decussations.

The finding of a similar crossed pathway in animals with an acute (5-day) lesion of the lateral funiculus, however, is somewhat contradictory to our hypothesis and previous electrophysiological data (Hubscher and Johnson, 2000), in which the crossed pathway was not evident immediately (a few hours) following a similar lesion. While the anatomical results reported here do not provide direct evidence for injury-induced plasticity, it is important to discuss several possibilities of interpretation. First, the time between a few hours and 5 days (the minimum time necessary for PRV infection) may have been enough to develop a new connection or strengthen an existing or latent connection. Second, the effects of spinal shock in the 1–2 h lesion (Holmes et al., 1998) may have masked an existing normally active crossed connection, that became physiologically functional once spinal shock had subsided (before 5 days). Third, the neurons labeled with PRV in the brainstem contralateral to the spinal lesion are not involved specifically in the pudendal reflex depression circuit. Relative to the first two possibilities, it is interesting that bladder and sexual reflexes normally recover by 5 days after lateral hemisection injury, but not after a few hours (Hubscher and Johnson, 2000). Putative new or latent connections from the side of the medulla opposite the lesion may have been triggered by expansion of the terminal field in the lumbosacral cord, which provided PRV access from additional pudendal reflex neurons, a form of SCI plasticity seen in the pudendally-innervated anal sphincter of rats (Holmes et al., 2005). On the last point regarding pathway specificity, while the possibility exists that sympathetic postganglionic efferent fibers in the BSM were infected with PRV (Marson and McKenna, 1996), and subsequently labeled some putative cardiovascular neurons in the ventrolateral medulla, as also described by others following PRV injection into other striated muscles (Daniels et al., 2001; Kerman et al., 2003), we believe the vast majority of the neurons labeled in our study were not labeled as a result of transsynaptic connections via pudendal sympathetic axons. First, the pronounced unilateral PRV labeling predominance in the medulla contralateral to the lesion (Fig. 6) would not be predicted based on PRV labeling in medullary cardiovascular neurons following unilateral injection. Second, electrophysiological data on the firing in the pudendal nerve sympathetic postganglionic fibers elicited by LPGi-GiA microstimulation would also predict relatively equal labeling on each side of the medulla (Hubscher and Johnson, 2000; Johnson and Hubscher, 2000).

The present study did not address the specific transsynaptic path of the PRV infection in the lumbosacral spinal cord. Unilateral injection of a more limited retrograde transsynaptic tracer, wheat germ agglutinin, into the BSM (Collins et al., 1991) revealed interneurons on both sides of the lower lumbar spinal cord. We saw the same pattern in our uninjured control spinal sections following unilateral injection of PRV. The possible cross-contamination of the contralateral BSM motoneurons cannot be easily controlled for because of probable gap junctions between their spinal processes, and the high potential for single interneurons innervating BSM

motoneurons on both sides of the cord. However, the results of the present study demonstrate that a unilateral descending bulbospinal pathway ultimately has access to both pools, shown now with tracer data and previously with physiological data. The termination of the PRV-labeled bulbospinal neurons is likely to be near interneurons on both sides of the spinal cord, in addition to the motoneuron pool, based on our previous electrophysiological microstimulation studies. Microstimulation (conditioning stimulus alone) never produced direct firing or postsynaptic inhibition of pudendal motoneurons. The latency and duration of the reflex depression effect strongly suggests that the lumbosacral site of action involves the triggering of presynaptic inhibition in dorsal nerve of the penis afferent terminals (Ferrero et al., 2007; Johnson and Hubscher, 1998; Johnson, 2006).

From a functional perspective, a fully bilateral bulbospinal pathway modulating each side of the pudendal motoneuron pool in synchrony would be the most efficient mechanism for control of a midline perineal muscle that surrounds an unpaired organ, the bulb of the penis. The bilateral spinal connections of each pathway, demonstrated in the present neuroanatomical study and previously with selective microstimulation, provides a level of overlap. Accordingly, elimination of one side of the pathway through chronic lateral hemisection does not produce a prolonged loss of sexual and bladder reflexes as is true for bilateral chronic lesions of at least 60% of the dorsal spinal cord (Hubscher and Johnson, 2000). This is likely the result of (1) sufficient synaptic integrity of the spared pathway, (2) connections to both sides of the pudendal motoneuron circuitry, and (3) the possible development of new complementary crossed connections above the level of the spinal lesion. The efficacy of the bulbospinal pathway for bulbospongiosus function (propulsive ejaculation for the rapid expulsion of semen) has been demonstrated in spinally intact animals with bilateral lesions that included the ventral Gi, GiA, and LPGi, and were required to affect ejaculatory bursts (Marson and McKenna, 1990), and smaller bilateral lesions had significant effects on pudendal reflexes (Holmes et al., 2002) and ejaculatory behavior (Yells et al., 1992). Our previously reported electrophysiological data (Hubscher and Johnson, 2000; Johnson and Hubscher, 1998) demonstrated that the ipsilateral bulbospinal pathway produces greater reflex inhibition than the contralateral pathway, but that they were additive in nature. In the present study, the finding of PRV-labeled neurons descending in the crossed pathway provides neuroanatomical evidence for the bilateral reflex modulation of the spared bulbospinal connections following chronic spinal hemisection injury, and implicates their potential role in the rapid recovery of sexual and bladder function seen in animals with lateral hemisection injury (Hubscher and Johnson, 2000; Little et al., 1991; Pritchard et al., 2010). A lateral hemisection injury in humans, sometimes referred to as Brown-Séquard syndrome, also results in a good prognosis for pudendal function, with return to voluntary voiding and less occurrence of detrusor-sphincter dyssynergia (Scivoletto et al., 2008). As is true for locomotor ability after hemisection, only a percentage of the pathway fibers in the spared lateral funiculus appear to be necessary for functional recovery (Schucht et al., 2002), particularly if the spared pathway is strengthened through plasticity (Babu and Namasivayam, 2008). Therefore the development of therapeutic strategies to strengthen or minimize damage to a

spared portion of one of the lateral funicular bulbospinal ejaculatory pathways, as well as those spinobulbar lateral funicular pathways we have described that transmit the penile sensory limb of the spino-bulbo-spinal loop (Hubscher et al., 2010), would have strong merit considering the bilateral connections in the spinal cord and brainstem.

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