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Anterograde labeling of ventrolateral funiculus pathways with spinal enlargement connections in the adult rat spinal cord

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Abstract

The ventrolateral funiculus in the spinal cord has been identified as containing important ascending and descending pathways related to locomotion and interlimb coordination. The purpose of this descriptive study was to investigate the patterns of axon termination of long ascending and descending ventrolateral pathways within the cervical and lumbar enlargements of the adult rat spinal cord. To accomplish this, we made discrete unilateral injections of the tracer biotinylated dextran-amine (BDA) into the ventrolateral white matter at T9. Although some BDA-labeled axons with varicosities were found bilaterally at all cervical levels, particularly dense BDA-labeling was observed in laminae VIII and IX ipsilaterally at the C6 and C8 levels. In the same animals, dense terminal labeling was found in the lumbar enlargement in medial lamina VII and ventromedial laminae VIII and IX contralaterally. This labeling was most apparent in the more rostral lumbar segments.

These observations continue the characterization of inter-enlargement (long propriospinal) pathways, illustrating a substantial and largely reciprocal inter-enlargement network with large numbers of both ascending and descending ventrolateral commissural neurons. These pathways are anatomically well-suited to the task of interlimb coordination and to participate in the remarkable recovery of locomotor function seen in the rat following thoracic spinal cord injuries that spare as little as 20% of the total white matter cross sectional area.

Keywords

propriospinal pathways; ventrolateral funiculus; biotinylated dextran-amine; interlimb coordination

1. Introduction

It is widely accepted that the central pattern generator (CPG) circuitry responsible for the alternating rhythmic bursting necessary for normal hindlimb locomotor activity in mammalian

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quadrupeds resides intrinsically within the spinal cord and is activated by reticulospinal pathways that reside largely within the ventrolateral funiculus (VLF; Jordan, 1991, 1998). In cats with chronic incomplete spinal cord lesions, bilateral disruption of pathways contained within the VLF abolishes all medullary locomotor region evoked locomotion (Eidelberg, 1980; Steeves and Jordan, 1980) leading to the suggestion that reticulospinal axons residing in the VLF comprise a critical locomotor command pathway. However, more recent studies suggest a redundancy among axons within the dorso- and ventrolateral white matter of the spinal cord capable of mediating overground locomotion (Sholomenko and Steeves, 1987; Vilensky et al., 1992; Brustein and Rossignol, 1998; Loy et al., 2002a, b; Schucht et al., 2002). The fact remains that preservation of a small percentage (20%) of white matter in the ventrolateral quadrant of the thoracic spinal cord following either laceration or contusive injuries is sufficient to permit not only the initiation of rhythmic stepping movements but also to provide for hindlimb weight support and hindlimb-forelimb coordination during open-field locomotion (Schucht et al., 2002; Basso et al., 2002) and alternating hindlimb kicking during swimming in the adult rat (Smith et al., 2006).

Rubrospinal, reticulospinal, vestibulospinal and long propriospinal axons have been shown to withstand damage sustained from contusion-type injuries much better than either corticospinal or short propriospinal axons (Hill et al., 2001; Basso et al., 2002; Conta and Stelzner, 2004). This innate differential sparing most likely results from several factors including the degree of axonal collateralization and their anatomical location within the spinal cord proper relative to the mechanical forces of the injury. Therefore, the meticulous task of determining the origin and termination of the minimal number of long descending and ascending axons contained within small amounts of white matter which are capable of subserving patterned movements becomes crucial to the development of therapeutic strategies aimed at restoration of locomotion following spinal cord injury (Behrmann et al., 1992; Basso et al., 2002, 1996, 1995; Schucht et al., 2002). Towards this goal, we recently used discrete unilateral injections of Fluorogold (FG) into ventral quadrant white matter at the thoracic (T9) segment paired with cervical and lumbar intraspinal (lamina VII) injections of Fluororuby (FR) to investigate inter-enlargement (long propriospinal) and reticulospinal pathways in the adult rat (Reed et al., 2006; 2008). We found substantial populations of both ascending and descending inter-enlargement axons that are commissural in nature, crossing the midline close to the cell bodies of origin (Reed et al., 2006). We also identified that approximately 30% of VLF-related reticulospinal neurons are commissural in nature and that the majority of neurons that were labeled from either lumbar or cervical gray matter were found in the gigantocellular group of nuclei. Of note, this study found substantial numbers of neurons labeled from both the cervical gray matter and the VLF at T9 suggesting that they project to both enlargements. These experiments were unable to delineate the patterns of innervation, however, prompting the current follow-up experiments focusing on VLF pathways using anterograde tracing.

In the current study we have used discrete unilateral injections of biotinylated dextran-amine (BDA) into the VLF white matter at the thoracic (T9) segment to examine the patterns of innervation of long ascending and descending axons with terminal connections in the cervical and lumbar spinal cord. A better understanding of the anatomy combined with electrophysiological and behavioral investigations will reveal which long propriospinal pathways are most important for favorable functional outcomes and thus become targets for combinatorial neuroprotective or neuroregenerative strategies following spinal cord injury.

2. Results

Cervical BDA-labeling

BDA injections into the thoracic (T9) VLF white matter yielded anterograde BDA-labeling of axons with and without varicosities throughout the entire cervical spinal cord. BDA-labeled

axons with varicosities were heavily distributed bilaterally in the intermediate and ventral gray matter following single unilateral injections of BDA ($10\% \times 0.33\mu\text{l}$) in the VLF at T9 (Figure 2). The gray matter ipsilateral to the injection site typically had more labeling than the contralateral side and labeled commissural fibers were commonly seen crossing the midline both dorsal and ventral to the central canal. Numerous varicosities were visible just lateral to the central canal in lamina X bilaterally throughout the cervical spinal cord (Figure 2). Diffusion of BDA following the $0.33\mu\text{l}$ BDA-injections resulted in some labeling of axons in the ventral column and lateral funiculus, making it likely that these axons contributed to the pattern of BDA labeling. The experiments were repeated with a reduced volume ($0.18\mu\text{l}$) of 10% BDA which improved the specificity of fiber labeling as shown in Figure 3. The density of BDA-labeled axons with varicosities also decreased but the distribution patterns were largely retained (Figure 3). For example, the $0.33\mu\text{l}$ injections (BDA 4) yielded a high density of axons with varicosities in lamina IX of the C6 segment (Figure 2). This higher density of lamina IX labeling was also present following the low ($0.18\mu\text{l}$) volume injections (Figure 3).

Lumbar BDA-labeling

In addition to the labeling of axons with varicosities within the cervical spinal cord, the unilateral thoracic (T9) BDA-injections also resulted in labeling of axons with varicosities within the lumbar spinal cord. Similar to the cervical spinal cord, BDA-labeled axons were found bilaterally within the lumbar intermediate and ventral gray matter throughout the entire enlargement (Figure 4). In all the animals that received $0.18\mu\text{l}$ injections, numerous BDA-labeled fibers were seen crossing just ventral to the central canal and smaller numbers were also seen dorsally. There were numerous axons with varicosities located just lateral to the central canal, in lamina X, bilaterally throughout the lumbar spinal cord. There appeared to be more defined and concentrated densities of BDA-labeled axons with varicosities among rostral lumbar segments (L1–L3), particularly in lamina VIII contralaterally, compared to that of the caudal lumbar segments (L4–L6; Figure 4). Following $0.33\mu\text{l}$ injections (BDA 4; Figure 4B), there was some spread of labeled axons at L2 into the lateral funiculus and ventral column, as was predicted by the labeling observed at the T9 injection site and at C8 (Figure 2). This spread appears to have resulted in a pattern of labeling emphasizing the intermediate gray matter and deep dorsal horn ipsilateral to the injection site at L2, while the labeling at L4 is similar to that following a $0.18\mu\text{l}$ injection (Figure 4A).

3. Discussion

For this study, used discrete unilateral injections of BDA into thoracic (T9) ventrolateral white matter to examine the patterns of innervation of the cervical and lumbar enlargements by axons traveling in the VLF at this level. This study represents a follow-up of our recently published work using retrograde tracers to identify spinal cord interneurons with long propriospinal (inter-enlargement) connections. We identified lumbar and cervical neurons with commissural and ipsilateral axons in the VLF at T9 that were double-labeled by tracer injected into the intermediate gray matter of C5–C8 or L2, ipsilateral to the VLF injection site (Reed et al., 2006). We also identified a previously unreported class of long descending propriospinal neurons with axons that cross the midline rostral to T9 and axons or collaterals that re-cross the midline caudal to T9 (Reed et al., 2006). Thus, the goal of the current study was to identify the patterns of termination within the cervical and lumbar enlargements of axons labeled from the thoracic VLF. The labeled terminals will include those from the populations of interneurons previously identified in the adult, *in vivo* preparation (Reed et al., 2006) and the neonatal, *in vitro* preparation (Antonino-Green et al., 2002). We used discrete injections of the anterograde tracer, BDA (Veenman et al., 1992; Rajakumar et al., 1993), into the VLF at T9 to focus on long ascending and descending propriospinal pathways, or projection axons with propriospinal collaterals. Short propriospinal neurons, with projections of 3–4 segments in length, were

excluded. Our procedures resulted in very little non-specific BDA-labeling either locally or at the level of the enlargements. The BDA-labeled axons certainly include reticulospinal (Reed et al., 2008) in addition to propriospinal axons (Reed et al., 2006), but may also include fibers from the spinocerebellar, spinoreticular and vestibulospinal tracts.

Cervical distribution patterns

The patterns of BDA labeling were grossly similar in the cervical enlargement when comparing small and large volume BDA-injections (0.18 μ l vs 0.33 μ l) in the VLF (Figure 2 & Figure 3). In all 5 preparations included, BDA-labeled axons with varicosities were present bilaterally throughout the entire length of the cervical spinal cord. This finding is consistent with earlier Nauta technique and cholera toxin beta-subunit (CTB) investigations examining long ascending propriospinal pathways (Matsushita and Ueyama, 1973; Giovanelli Barilari and Kuypers, 1969; Dutton et al. 2006). Also common to all preparations were BDA-labeled commissural axons crossing the midline both dorsal and ventral to the central canal throughout the cervical region. Cowley and Schmidt (1997) have shown in the neonatal rat spinal cord that only a small portion of intact commissural white matter is required to retain left-right coordination of activity along the length of the spinal cord. Therefore, functional redundancy (interneuronal activation) among these long ascending cervical commissural axons is both plausible and likely.

As represented by BDA 15 (0.18 μ l; Figure 3) all the preparations had relatively dense labeling in the deep ventral horn (laminae VIII and IX) of the C6 segment, ipsilateral to the injection site, a pattern that likely includes motoneurons and premotoneurons associated with muscles of the elbow and lower forelimb (McKenna et al., 2000), in addition to the ventral motor nucleus in segments C7 to T1 (VMN; Giovanelli Barilari and Kuypers, 1969; Sterling and Kuypers, 1968; Matsushita and Ikeda, 1973; Matsushita and Ueyama, 1973). The low volume of our BDA-injections and the inclusion of collaterals from projection neurons likely accounts for the less defined concentration of labeling associated with the VMN compared to the classical degeneration studies that involved strictly propriospinal pathways (Giovanelli Barilari and Kuypers, 1969; Matsushita and Ueyama, 1973). These findings suggest that ascending VLF-related fibers, some of which originate from the intermediate gray matter of the lumbar enlargement (Reed et al., 2008), have direct monosynaptic access to the motoneurons or premotoneurons within the caudal cervical segments (C6–C8; Fig. 5B) responsible for the muscles of the elbow and lower forelimb (English et al., 1985; Alstermark et al. 1987, 1990; McKenna et al. 2000; Ballion et al. 2001).

We also observed substantial labeling of the contralateral intermediate and ventral gray matter, illustrating the pattern of innervation provided by VLF-related commissural axons that cross the midline rostral to T9 (Fig. 5B). Our recent retrograde tracing study (Reed et al., 2006) suggested that the majority of lumbar commissural neurons that project to the cervical enlargement have axons that cross caudal to T9, at the level of the cell bodies. Thus, the source of the contralaterally located BDA-labeled terminals identified here must be from some other population of neurons, as outlined above (Giovanelli Barilari and Kuypers, 1969; Matsushita and Ueyama, 1973).

Largely absent from the 4 preparations that received 0.18 μ l injections, were BDA labeled axons with varicosities in the intermediate gray matter at C2 and C4. This observation suggests that these terminals arise largely from axons labeled outside the main body of the VLF, in either the lateral funiculus or ventral column, following a 0.33 μ l injection of tracer. Thus, the cervical component of the inter-enlargement propriospinal network carried in the VLF itself appears to involve primarily the more caudal cervical segments (Reed et al., 2006) that innervate the lower forelimb (McKenna et al., 2000).

Lumbar distribution patterns

BDA-labeled axons with varicosities were found bilaterally throughout the intermediate and ventral gray matter of the entire lumbar spinal cord following either the 0.33 or 0.18 μ l injections. This finding is consistent with earlier reports of Giovanelli Barilari and Kuypers (1969) who investigated long descending propriospinal pathways which appeared to terminate in the central part of the ventral horn in the more rostral lumbar segments and the medial and dorsomedial ventral horn in the more caudal lumbar segments. These authors also noted the striking similarities in the patterns of termination of reticulospinal and long propriospinal fibers, both of which are included in the population of axons labeled in the current study. These descending axons or their collaterals are likely responsible for the labeling of terminals in the deep dorsal horn of the lumbar spinal cord that is largely absent in the cervical segments. Whether or not a shift in either ascending or descending VLF altered labeling in the deep dorsal horn is impossible to determine.

Within the enlargements, laminae VII and VIII have been implicated as containing the interneurons and premotoneurons responsible for generating and disseminating the alternating rhythmic pattern of activity during locomotion. While the functional and anatomical organization of the central pattern generator (CPG) is still not clear, the concept of localized rhythmogenic “lead segments” residing in the rostral lumbar spinal cord is fairly well supported (Cazalets et al., 1992; 1995; Kjaerulff and Kiehn, 1996; Magnuson and Trinder, 1997; Magnuson et al., 1999; Marcoux and Rossignol, 2000; Cazalets and Bertrand, 2000; Hadi et al., 2000; Ballion et al., 2001). While still controversial, the dominant rhythmogenic circuitry appears to reside in T13 through L2 (Cazalets et al., 1995; Kjaerulff and Kiehn, 1996; Cowley and Schmidt, 1997; Cazalets and Bertrand, 2002), and in adult rats, at least, excitotoxic lesions centered on L1/2 induce dramatic and persistent locomotor deficits (Magnuson et al., 1999; Hadi et al., 2000). In the present study, BDA-injections into the VLF resulted in substantial bilateral labeling of the ventromedial lumbar gray matter (laminae VIII/IX and medial lamina VII) that appears to peak at L1 and L2 (Figure 4). This observation suggests that ipsilateral and commissural long descending propriospinal neurons, with cell bodies in the cervical enlargement (Reed et al., 2006) have direct synaptic access to interneurons, premotoneurons and motor neurons in L1 and L2 (Fig. 5A). In addition to being considered the rhythmogenic lead segments, L1 and L2 are also home to the motor neuron pools mediating hip and knee flexion (Nicolopoulos-Stournaras and Iles, 1983). The preferential ventromedial and contralateral termination pattern may help explain the remarkable capacity for locomotor recovery in rats following severe spinal cord injuries sparing only small portions of the ventral quadrant white matter. Thus, the noted rostro-caudal differences in the pattern of BDA-labeling may reflect the functional rostrocaudal gradient in rhythmogenesis as described earlier (Cazalets et al., 1995; Kjaerulff and Kiehn, 1996; Cazalets and Bertrand, 2000).

Taken together, our findings using retrograde (Reed et al., 2006, 2008) and anterograde tracing (current study) suggest that the spinal cord contains a substantial population of ipsilateral and commissural propriospinal neurons that interconnect the two enlargements via the VLF (Figure 5). In addition to ipsilateral neurons, the descending component appears to include a majority of commissural neurons with axons that cross at the level of the cell body (Skinner et al., 1979), and/or caudal to T9 with terminal patterns that emphasize the intermediate and ventral gray of the most rostral lumbar segments. The ascending projections appear to involve fewer commissural neurons with the ipsilateral component emphasizing direct ventral horn input into the most caudal (C6 and C8) cervical segments. These observations confirm and extend the findings originally made by Miller et al. (1973), Matsushita and Ikeda (1973), English et al. (1985) and many others, who collectively show that long propriospinal neurons in the mammalian spinal cord preferentially interconnect the rostral lumbar segments (L1-3) and the more caudal cervical segments (C5-T1). We have extended these findings by demonstrating

that 30% or more of these neurons are commissural in nature and that they can be labeled successfully from the VLF at T9 (Reed et al., 2006; 2008). Clearly, these populations of neurons are anatomically well suited to mediate interlimb coordination during locomotion in the intact animal and the dramatic functional recovery observed following incomplete contusive or laceration spinal cord injuries. In future experiments, larger studies combining behavioral assessment and quantitative analysis of anterograde/retrograde labeling following spinal cord injury will help to further delineate the functional roles of propriospinal pathways and their potential as targets for neuroprotective or neuroregenerative strategies (Jordan and Schmidt, 2002) following spinal cord injury.

4. Experimental Procedure

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. Eight adult female Sprague-Dawley rats were anesthetized with sodium pentobarbital (50mg/kg) and a T8 laminectomy was performed. BDA (lysine-fixable, mw 10/1000; Molecular Probes, Oregon) was dissolved to 10% in 0.05M TRIS-HCL buffer (pH 7.4) for pressure injection (Table 1). The dura was opened and a single unilateral injection of BDA (0.33 μ l or 0.18 μ l) was made into the thoracic white matter using a glass micropipette beveled to a 25 μ m (O.D.) tip diameter and 20–40mmHg of pressure delivered by a PV 800 pneumatic picopump (WPI, FL). The micropipette remained in place for five minutes post-injection to reduce leakage of tracer into the pipette track. Spinal cord injection site coordinates were similar to those described by Loy et al. (2002a, 2002b). Preparations in which the BDA injection site was not solely within VLF were excluded from further evaluation (n=3). Table 1 shows the details of each of the 5 preparations that were included in the analysis. After survival periods of 21–27 days, the animals were euthanized by anesthetic overdose (sodium pentobarbital, 90mg/kg) and transcardially perfused with 500ml of 0.1M phosphate buffer, pH 7.4, containing 4% paraformaldehyde. The spinal cords were removed, post-fixed overnight and transferred to 30% sucrose for 2–4 days at 4°C. Transverse sections were cut on a cryostat (30 μ m) and mounted onto glass slides (five sets). For histochemical detection of BDA, sections were hydrated in 0.1M PBS, rinsed in sodium acetate followed by an avidin-biotinylated peroxidase complex (ABC, 1:100 Vector laboratories, CA) with nickel ammonium sulfate (1.5g/50ml) enhanced 3,3' diaminobenzidine (80mg/50ml DAB, 30% H₂O₂). The sections were processed through a series of alcohol and xylene washes prior to being coverslipped with Permount neutral medium (Fisher Scientific, PA). Adjacent sections on one set of slides were 150 μ m apart. One set of slides from each preparation was stained with cresyl violet to determine segmental level and laminar distribution of BDA-labeled axons with varicosities. Injection sites were determined by examining one complete set of slides to find visual evidence of the pipette track and/or the section with the largest amount of local BDA labeling (Figure 2). The chosen sections were compared with adjacent sections stained with cresyl violet to confirm the injection site by the appearance of mild inflammation.

BDA-labeled axons with varicosities

Photomicrographs (10 $\times$) of five non-consecutive tissue sections (150 μ m apart) from the cervical (C2,C4,C6,C8; n=20) and the lumbar (L1–L6; n=30) spinal cord were taken and assembled using a Spot RT CCD digital camera (Diagnostic Instruments, MI) and Adobe Photoshop (Adobe Systems, CA) running on a Macintosh G4 computer. Photomicrographs were optimized for brightness and contrast using Adobe Photoshop (Adobe Systems, CA) and were then opened in Appleworks 6.0 (Apple Computer, CA) and magnified (200 $\times$ zoom) allowing axons with varicosities to be traced using a Wacom (Vancouver, WA) drawing tablet (Figure 1). Five drawings from each segmental level evaluated were then superimposed to determine the distribution of BDA-labeled axons with varicosities resulting from thoracic (T9) VLF white matter injections. Cytoarchitectural descriptions of Molander et al. (1984,1989) and Paxinos

and Watson (1986) were used, with reference to the adjacent section stained with cresyl violet, to determine spinal levels and laminae that were innervated by BDA-labeled axons with varicosities.

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Abbreviations used

VLF, ventrolateral funiculus; BDA, biotinylated dextran-amine; CPG, central pattern generator; CTB, cholera toxin beta-subunit.

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Figure 1.

(A) Shown is an example BDA-labeled axons with varicosities (arrowhead), from ipsilateral lamina VII, at C6, which were magnified and traced as well as axons without varicosities (arrow) which were not traced in an animal that received a 0.18 μ l injection of BDA into the VLF at T9. Shown in (B) is an example of BDA-labeled axons with varicosities in ipsilateral laminae VIII and IX in close proximity to motoneurons in an animal that received a 0.33 μ l injection of BDA into the VLF.

Figure 2.

Shown are sections taken from the T9 VLF injection site (upper left, asterisk; 0.33 μ l; BDA 4) and corresponding camera lucida drawings of BDA-labeled axons in the white matter at C8 and BDA-labeled axons with varicosities from five superimposed, 30 μ m sections, 150 μ m apart, taken from each of the cervical segmental levels examined (C2,C4,C6,C8).

Figure 3.

Shown are sections taken from the T9 VLF injection site (upper left, asterisk; 0.18 μ l; BDA 15) and corresponding camera lucida drawings of BDA-labeled axons in the white matter at C8 and BDA-labeled axons with varicosities from five superimposed, 30 μ m sections, 150 μ m apart, taken from each of the cervical segmental levels examined (C2,C4,C6,C8).

Figure 4.

A. Camera lucida drawings of BDA-labeled axons with varicosities from five sections (150 μ m apart) superimposed from each of the lumbar segmental levels (L1–L6) from the same preparation (0.18 μ l; BDA 15) shown in Fig. 3. Shown on the left are camera lucida drawings (five sections superimposed) of BDA-labeled fibers in the ventrolateral white matter at the L2 level. B. Camera lucida drawings of BDA-labeled axons with varicosities from five sections (150 μ m apart) superimposed from L2 and L4 from the same preparation (0.33 μ l; BDA 4) shown in Fig. 2. Shown on the left are camera lucida drawings (five sections superimposed) of BDA-labeled fibers in the ventrolateral white matter at the L2 level.

Figure 5.

Shown are summary diagrams illustrating the primary findings of this work based on the preparations that received 0.18µl injections of BDA into the VLF at T9. The shaded areas represent BDA filled axons with terminals, and the segments shown are those for which the pattern is most representative. Shown in A are the patterns of descending axons or collaterals. Shown in B are the patterns of ascending axons or collaterals. The cell body locations are taken from our previously published work using retrograde tracing (Reed et al., 2006). The question marks (?) indicate non-enlargement sources which would include reticulospinal, long-propriospinal and others.

Table 1**BDA Injections**

Rat ID #	Volume	Time
BDA 4	0.33 μ l	21d
BDA 15	0.18 μ l	21d
BDA 22	0.18 μ l	27d
BDA 23	0.18 μ l	27d
BDA 24	0.18 μ l	27d

Experimental details for all included preparations. Each received a single, unilateral injection of BDA into the ventrolateral funiculus at T9. Data from animals in which the BDA injections were not completely restricted to the VLF were not included in the final descriptions.