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# Inter-enlargement pathways in the ventrolateral funiculus of the adult rat spinal cord

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## Abstract

The ventrolateral funiculus (VLF) in the spinal cord contains important ascending and descending pathways related to locomotion and interlimb coordination. The primary purpose of this descriptive study was to investigate the distribution of inter-enlargement pathways in the adult rat spinal cord with an emphasis on the VLF.

We made discrete unilateral injections of Fluoro-Gold (FG) into the right VLF at T9, and either unilateral or bilateral injections of Fluoro-Ruby (FR) into the intermediate gray matter at the C5-6, C7-8, or L2 segmental levels. Inter-enlargement neurons with ascending axons in the right VLF were found bilaterally in laminae VII and VIII throughout the rostral lumbar spinal cord (L1-L3) and predominately contralaterally in the caudal lumbosacral (L4-S1) spinal cord. Following left unilateral FR injections at C5-6 or C7-8 and right unilateral VLF injections of FG at T9, very few double-labeled neurons could be found anywhere in the lumbar spinal cord. Similar injections of FR at L2 revealed an almost symmetrical bilateral distribution of double-labeled neurons throughout the cervical spinal cord (C1-C8).

These results describe ascending and descending pathways within the spinal cord that interconnect the two enlargements and involve both commissural and ipsilateral interneurons. The majority of inter-enlargement neurons had axons within the ventrolateral funiculus at T9. These observations support the hypothesis that the VLF contains long ascending and descending axons with propriospinal inter-enlargement, commissural and ipsilateral connections that are anatomically well-suited to mediate interlimb coordination.

### Keywords

propriospinal pathways; interlimb coordination; Fluoro-Gold; Fluoro-Ruby

There is renewed interest of late in propriospinal pathways due in part to reports of the spontaneous formation of new functional intraspinal circuitry following spinal cord injury (Bareyre et al., 2004) and the potential these pathways have in combinatorial repair strategies involving cellular transplantation and neurotrophin delivery (Jordan and Schmidt, 2002; Conta and Stelzner, 2004; Fouad et al., 2005). While many regions of the spinal cord possess the neural circuitry necessary to produce rhythmic activity, in the mammalian quadruped, separate central pattern generator (CPG) networks are thought to exist for the hindlimbs and forelimbs (Cazalets et al., 1995, 1996; Magnuson and Trinder, 1997;

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Magnuson et al., 1999; Ballion et al., 2001; Juvin et al., 2005). In the rat, intraspinal Kainic Acid injections have profound effects on locomotor function when delivered to the intermediate gray matter at L2 (Magnuson et al., 1999) and locomotor deficits resulting from moderately-severe contusion injuries are greater when the contusion is centered on the more rostral lumbar segments (T13/L1 or L2) compared to injuries centered on L3/4 (Magnuson et al., 2005). These findings suggest that the more rostral segments of the adult rat lumbar spinal cord contain critical CPG circuitry. A number of studies using the neonatal rat pup spinal cord, *in vitro*, have concluded that a rostrocaudal gradient in rhythmogenic potential also exists in the lumbar enlargement of the immature spinal cord (Cazalets et al., 1995, 1996; Kjaerulff and Kiehn, 1996). In contrast to the lumbar enlargement, Ballion et al. (2001) found a caudo-rostral gradient for rhythmogenesis in the cervical enlargement of the isolated neonatal rat spinal cord and using the same preparation, Juvin et al. (2005) concluded that while segments C5 through C8 are rhythmogenic, they can be driven by the lumbar pattern generating circuitry in a longitudinally intact preparation. A number of authors have proposed that long propriospinal axons are likely candidates for the functional coupling of the cervical and lumbar CPG networks and the mediation of interlimb coordination (Miller et al., 1973, 1975; English et al., 1985, Cazalets and Bertrand, 2000; Juvin et al., 2005). This category of neuron has previously been described in the spinal cord of the rat, cat, and monkey (Matsushita et al., 1979; Menetrey et al., 1985; Verburgh et al., 1989; Matsushita 1998; Miller et al., 1998).

In the cat, a subgroup of long ascending propriospinal fibers in the ventrolateral funiculus (VLF) projects to a confined ventral portion of the C8 and T1 lateral motoneuronal cell groups, thus implying a direct influence on forelimb motor control (Sterling and Kuypers, 1968; Giovanelli and Kuypers, 1969). Descending axons in the VLF are part of an important (but not solitary) command pathway for the initiation of locomotor-related hindlimb or locomotor-like rhythmic activity (Steeves and Jordan 1980; Yamaguchi, 1986; Garcia-Rill and Skinner, 1987; Noga et al., 1991; Shik, 1997), and lesions of the mid-thoracic ventral or ventrolateral white matter induces deficits in forelimb-hindlimb coordination that persist long after the recovery of gross locomotor and postural deficits (Brustein and Rossignol, 1998). Webb and Muir (2004) demonstrated that fibers in the VLF contribute to limb movements and to locomotion, but the immediate functional losses were overcome by spared pathways following unilateral VLF lesions at the C3 segmental level. However, it is important to recognize that in Webb and Muir's model of spinal cord injury, the contralateral VLF remained intact and fibers therein may have been responsible for much of the compensatory capacity demonstrated. In fact, remarkable compensatory capacity is clearly shown by Schucht et al., (2002) who demonstrated that rats with as little as 20% of unilateral peripheral white matter sparing in the ventral and ventrolateral funiculi exhibited intact hindlimb-forelimb coordination, clear plantar stepping and hindlimb weight support. Thus, a small amount of spared ventrolateral white matter can have profound effects on the coordinated limb activity necessary for basic locomotion following spinal cord injury. It appears that the minimal required circuitry that can both initiate locomotion and modulate interlimb coordination is located in the peripheral white matter of the unilateral VLF.

We hypothesize that a sub-population of neurons with axons or axon collaterals in the adult rat mid-thoracic VLF conveys CPG-related information between the two enlargements of the spinal cord allowing normal interlimb coordination to occur. By definition, these neurons would need to be a part of, or receive direct input from the hindlimb and forelimb CPGs and project to regions containing limb-related interneurons and motoneurons. As an initial step in the identification of neuronal subpopulations of interest, the present descriptive study investigated cells with long ascending and descending VLF axons with propriospinal connections using discrete intraspinal injections of two retrograde tracers (Fluoro-Ruby (FR) and Fluoro-Gold (FG)).

# EXPERIMENTAL PROCEDURES

## Dissection and tracer injections

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Louisville. Sixteen adult female Sprague-Dawley rats were anesthetized with sodium pentobarbital (50mg/kg) and a single-level laminectomy was performed at C5, C7 or T13. The vertebral column was stabilized by clamps applied to the spinous processes, and the entire column was lifted 1-2cm to reduce the movement of the spinal cord associated with breathing/heartbeat. Each animal received two injections of Fluoro-Ruby (FR), separated by 1.5 mm rostrocaudally, either unilaterally or bilaterally into the deep intermediate gray matter (lamina VII) at C5-6, C7-8 or L2. Injections were made from a glass micropipette, beveled to a 25μm tip diameter (o.d.) using pressure pulses of 20-40mm Hg delivered from a PV800 Picopump (WPI, FL; Table 1). FR was injected as a 10% solution and either 0.5 or 0.25μl volumes at each site (Figure 1; Table 1). Three weeks later each animal received a single injection of Fluoro-Gold (FG; 0.3μl of 0.5%) into the right VLF at T9. After each injection, the micropipette remained in place for five minutes 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). After a survival period of 72 hours, 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. These volumes, concentrations and survival times were chosen following a series of preliminary experiments designed to determine the optimal parameters for these tracers and injection sites. None of the animals showed even transient locomotor deficits following tracer injection.

Labeled neurons were identified, photographed and counted in sections taken from the lumbar segments (L1 through L6) of six of the ten preparations that received FR injections into the cervical spinal cord. Cells containing FR had axons or collaterals terminating in the lower cervical enlargement (C5-6, C7-8). Cells containing FG had axons that passed through the right VLF at T9 and double-labeled cells had both characteristics. Labeled neurons were also identified, photographed and counted in sections taken from the cervical segments (C1 through C8) of three of the six preparations that received FR injections into the L2 spinal cord. Cells containing FR had axons or collaterals terminating in the intermediate gray matter of L2. Cells containing FG had descending axons that passed through the right VLF at T9 and double-labeled cells had both characteristics. In seven preparations the FR injection sites were outside of the intermediate gray matter and/or the FG labeling near the injection site was not limited to the ipsilateral ventrolateral funiculus. These seven preparations were excluded from further analysis.

## Histochemical procedures

Transverse spinal cord sections were prepared at 30μm on a cryostat. Sections were mounted onto glass slides (Fisher Scientific, PA) in five sets. One set of slides representing every fifth section (150μm) was hydrated and directly coverslipped with DPX medium (Fluka, UK). Adjacent sections were stained with cresyl violet to determine segmental and laminar levels according to the cytoarchitectural descriptions of Molander et al. (1984, 1989) and Paxinos and Watson (1986).

## Double FR-FG retrograde labeling

In total, nine animals were evaluated for the presence of labeled neurons. Photomicrographs were taken of every fifth section with a Spot RT CCD digital camera (Diagnostic Instruments, MI) on a Nikon Eclipse microscope using ultraviolet (UV) or rhodamine

epiflourescent filters. Photomicrographs were optimized for brightness and contrast using Adobe Photoshop (Adobe Systems, CA) running on a Macintosh G4 computer. Photomicrographs were opened in Appleworks 6.0 and fully labeled cell bodies were counted and then traced using a Wacom drawing tablet (Vancouver, WA). Drawings from each segmental level (14-17 sections) were then superimposed to illustrate the rostral-caudal distribution of labeled neurons in the spinal cord. Single FG-labeled neurons were counted but not individually traced. Cells lacking a well-defined shape and perimeter were not counted or traced.

Total counts of double FR-FG, single FR, and single FG-labeled neurons were compared using the appropriate two-way ANOVA analyses. Comparisons made were: (rostral-caudal [L1-L3, L4-L6] and side [right, left]).

## RESULTS

### Ascending inter-enlargement neurons

Lumbar neurons with long ascending axons in the right VLF (FG – blue) and/or terminal connections in the intermediate gray matter of C5-6 or C7-8 (FR – red) were assessed in six animals (Fig. 1C). Neurons with ascending axons in the right VLF at T9 and terminals in the intermediate gray matter at C5-6 or C7-8 were pink in color (Fig. 1C). Single FR-labeled lumbar neurons possessed long ascending axons in areas other than the right VLF (including those with axons ascending in the left VLF) and terminal connections at C5-C6 or C7-C8. Single FG-labeled (blue) lumbar neurons possessed long ascending axons in the right thoracic VLF and terminal connections at segmental levels other than C5-6 or C7-8. Cell counts are given as totals per spinal cord segment, unless indicated otherwise. Cells were counted in one set of slides that represents one-fifth of the total number of sections.

Following bilateral FR injections (FR-FG 1, 2, 3) into the cervical gray matter at C5-C6, FR-labeled inter-enlargement neurons were found bilaterally throughout the lumbar enlargement. These neurons were generally large and multipolar in shape and were relatively sparse with up to 4 or 5 per section or 30-40 per segment (Table 2). Double-labeled inter-enlargement neurons with axons in the right VLF at T9 made up the majority of FR-labeled neurons on the side contralateral to the FG injection site (62 - 70%; Fig 1C, Table 2A) and a substantial minority, ipsilaterally (25%; Table 2). Comparison of the right and left sides from all four preparations that received bilateral FR injections (FR-FG 1,2,3 and FR 6) showed that there were significantly more double-labeled cells on the left side, contralateral to the FG injections, than on the right ($p<.05$). Single FR-labeled cells were more numerous on the right side, ipsilateral to the FG injection ($p<.05$).

Segments L1-L3 had somewhat greater numbers of FR-labeled inter-enlargement neurons compared to L4-L6, however this difference did not reach statistical significance (129 vs 82; Table 2A). In these cords, double FR/FG-labeled neurons were also more numerous in L1-L3 when compared to L4-L6 (69 vs 46; Table 2A; $p<.05$), however the percentage of FR-labeled cells that were also filled with FG was similar (53 vs 56; Table 2A). In L1-L3, double-labeled cell bodies were distributed bilaterally throughout laminae VII and VIII, while in L4-L6 these neurons were confined almost exclusively to lamina VIII (Fig. 2). Very few double-labeled neurons were seen anywhere in the lumbar enlargement following left unilateral FR-injections at C5-C6 (FR-FG 7) or C7-C8 (FR-FG 10; Table 2B; Fig. 2B, D). Nearly twice the number of double-labeled cells were seen in FR 6, a preparation that received bilateral injections of FR at C7-C8, compared to the three preparations that received injections at C5-C6 (FR-FG 1, 2 and 3; Table 2A).

These results illustrate two populations of lumbo-cervical inter-enlargement neurons with axons in the VLF at T9. The largest group is commissural with cell bodies in the intermediate gray matter and axons that cross the midline caudal to T9 and ascend in the VLF to terminate contralaterally in the intermediate gray matter of the cervical enlargement (Fig. 4). A second, smaller population is entirely ipsilateral. Both these populations are distributed rostrocaudally throughout the lumbar enlargement with greater numbers being found in L1-L3 than in L4-L6. Larger numbers of neurons were labeled from C7-C8 than from C5-C6 (Fig. 4).

### Cervical spinal cord

To determine the cervical origin of neurons with long descending axons in the right VLF and terminal connections at the L2 segmental level, three additional animals were evaluated after receiving either a bilateral or unilateral pair of FR injections into the intermediate gray matter at L2, followed by a unilateral injection of FG into the right VLF at T9.

Neurons that possessed long descending axons in the right VLF (FG – blue) and terminal connections in the intermediate gray matter at L2 (FR – red) contained both FR and FG (double-labeled) and were pink in color (Table 3). Single FR-labeled (red) cervical neurons possessed long descending axons in areas other than the right VLF (including those with axons descending in the left VLF) and terminal connections at L2.

Two animals received a pair of FR injections delivered bilaterally in the intermediate gray matter at L2 (FRL 3, 4; Table 1, Table 3). FR-labeled cervical neurons were found distributed bilaterally throughout the cervical spinal cord. They ranged from 4 to 48 per segment with greater numbers being found in the caudal cervical spinal cord than in the more rostral segments (136.5 vs 80.5; C5-C8 vs C1=C4).

Similar to the double-labeled lumbar cells, double-labeled cervical neurons were multipolar in shape and widely distributed within the intermediate gray matter (laminae VII and VIII; Fig 4A-B). Double–labeled cells were found bilaterally throughout the entire length of the cervical enlargement (Fig. 4A, Table 3), with slight biases towards the side contralateral to the FG injection and towards the caudal segments. Overall, slightly more than 50% of the FR labeled cells were also labeled with FG indicating that these neurons have axons descending in the right VLF. The single animal (FRL-2) that received a left unilateral FR-injection at L2 had more double-labeled cells located contralateral to the FG injection than were found ipsilaterally (73 vs 38; Fig. 4B, Table 3).

These results describe at least two different populations of cervico-lumbar inter-enlargement neurons. Approximately 80% of FR labeled neurons on the left side of FRL 2 were also filled with FG, indicating that these neurons have axons that descended within the right VLF. This preparation received a pair of FR injections into the left intermediate gray matter at L2, thus, the double-labeled neurons appear to be commissural (axons in the right VLF) with axons that re-cross the midline caudal to T9, to terminate ipsilateral to the cell body location. Double-labeled cells on the right side of FRL 2 must also be commissural, but with axons that cross the midline caudal to the VLF injection site at T9. This pattern is distinct from that of the cervical C5-C6 FR-injected preparations in which only the left side of the lumbar spinal cord, contralateral to the FG VLF injection, had a single FR to double FR/FG ratio greater than 50% (Figs. 3 & 4; Table 2B).

## DISCUSSION

The majority of spinal cord tract tracing studies utilize relatively large volumes of injected tracer in an attempt to label all, or the majority of fibers ascending or descending from/to an

entire spinal enlargement or hemicord. With the objective of examining the anatomical relationships between the ventrolateral funiculus (VLF), the intermediate gray matter of the L2 segment and the intermediate gray matter of the cervical enlargement, we chose to use small gray matter injections of the retrograde tracer Fluoro-Ruby (FR) in combination with discrete, low-volume injections of Fluoro-Gold (FG) into the VLF unilaterally at T9. This strategy has allowed us to specifically examine the distribution of neurons with long ascending or descending axons traveling through a relatively small and defined component of the unilateral ventral quadrant white matter. A unilateral approach was undertaken to better understand the inter-enlargement connections of long ascending and descending, commissural and ipsilateral VLF fibers.

More specifically, we used multiple (2 or 4) injections of FR into the intermediate gray matter to maximize axon terminal uptake at the respective segmental levels, combined with single discrete thoracic FG-injections in order to minimize the number of axons exposed to the tracer and to limit FG-labeling of undamaged axons of passage (Schmued and Fallon 1986, Burstein et al., 1990). We chose to inject FG into the thoracic white matter at T9 to reduce labeling of short (3-4 segment) propriospinal pathways and to focus our observations on long ascending/descending pathways with terminals in the intermediate gray matter at locomotor-relevant spinal cord segments. The T9 FG-injection site also reduced the inclusion of neurons concerned primarily with cardiovascular or respiratory functions while at the same time being well rostral to the thoracolumbar junction and the hindlimb locomotor circuitry. Our focus specifically on the VLF was driven by earlier work relating this area of white matter to both the initiation of locomotor-like rhythmic activity (Kjaerulff and Kiehn, 1996, Magnuson and Trinder, 1997) and to the normal performance and/or recovery of interlimb coordination, plantar foot placement and locomotion following spinal cord injury (Steeves and Jordan 1980, Noga et al., 1991, Brustein and Rossignol 1998, Schucht et al., 2002, Loy et al., 2002a, Webb and Muir 2004). Using this strategy, cell bodies containing both FR and FG included long ascending or descending propriospinal neurons as well as projection neurons (spinoreticular, spinocerebellar or other) with collaterals terminating in the cervical (C5-C6, C7-C8) or lumbar (L2) intermediate/ventral gray matter. No attempts were made to isolate pure propriospinal neurons from projection neurons with collaterals because our primary interest was in labeling any neurons with long axons traveling within the unilateral VLF with inter-enlargement connections.

## Long propriospinal pathways: Ascending

Approximately one-third of fibers in the rat ventral and lateral spinal cord are propriospinal in nature (Chung and Coggeshall 1983), and while the majority of these are short in length traversing four segments or less, the concept of multi-segmental or long propriospinal neuron involvement in the coupling of enlargement-centered rhythmic activity is supported by both anatomical and electrophysiological studies, *in vivo* and *in vitro* (Menetrey et al., 1985; Cazalets and Bertrand 2000; Ballion et al., 2001; Conta and Stelzner, 2004; Juvin et al., 2005).

In the present study, greater numbers of double-labeled neurons were found in lamina VIII and medial lamina VII contralaterally within the lumbar enlargement while more single FR-labeled cell bodies were found in these laminae, ipsilaterally (Table 2, Figure 2). This mirror-like distribution of single FR and double FR/FG-labeled cells suggests that the majority of single FR-labeled cells most likely have axons that ascend in the contralateral VLF rather than in other non-VLF tracts. The population of contralateral double-labeled cells represents a novel category of commissural, inter-enlargement neurons, numbering in the hundreds, that project to C5-C6 and/or C7-C8 (Table 2; Fig. 4). By definition, the axons of this category of neuron must cross the midline caudal to T9, and we observed many FR

axons in the ventral commissure of L1, L2 and L3, in particular, suggesting that they are crossing at level.

Our results also illustrate a second smaller population of inter-enlargement lumbar neurons with axons that ascend in the ipsilateral VLF to terminate in the ipsilateral intermediate gray matter of C5-C6 and/or C7-C8 (Table 2, Fig. 4A). Following unilateral injections of FR, however, only a few double-labeled neurons were observed indicating that the vast majority of lumbo-cervical inter-enlargement neurons are either ispilateral or commissural with axons that cross the midline caudal to T9 (Fig. 4). This circuitry pattern allows lumbar commissural neurons to innervate neurons located contralaterally in the lumbar spinal cord (via axon collaterals) en route to the VLF, as well as neurons in the contralateral cervical gray matter.

### Long propriospinal pathways: Descending

Double-labeled neurons were also found bilaterally throughout the cervical enlargement with only a slight bias in numbers for the side contralateral to the FG injection. The results from the animals that received bilateral injections of FR at L2 illustrate two significant populations of double-labeled neurons, one ipsilateral and one commissural, each involving a column of several hundred neurons extending the entire length of the cervical enlargement (Fig. 4A). The commissural neurons necessarily have axons that cross the midline rostral to T9 and therefore appear to be the descending equivalent of the commissural neurons described in the lumbar enlargement. However, the results from the single animal that received unilateral injections of FR into the left intermediate gray matter at L2 illustrates a very different population of commissural neurons. In contrast to the lumbar enlargement, it appears that there is a population of cervical neurons with axons that cross the midline rostral to T9, descend in the VLF and re-cross the midline to terminate in the intermediate gray matter of the L2 segment, ipsilateral to the cell body (Fig. 4B). While we cannot rule out the possibility that our unilateral FR injections at L2 were not completely restricted to the side of the injection, we used very strict inclusion criteria and saw no evidence of direct FR labeling contralateral to the injection site in this preparation. In contrast, the unilateral injections into the cervical enlargement resulted in very few double-labeled lumbar neurons (~ 1 per segment) suggesting that the FR injections are site-restricted. There also appears to be a population of cervical commissural neurons with axons that descend in the VLF ipsilateral to the cell body and cross the midline caudal to T9 (Fig. 4B). This pattern of innervation allows both ipsilateral and commissural cervical inter-enlargement neurons to innervate the intermediate gray matter of L2 on both sides of the spinal cord.

### Functional Considerations

In keeping with the distribution of double-labeled cervical neurons found in the current study, Menetrey et al. (1985) reported bilateral labeling of propriospinal cell bodies following unilateral injections of tracer into the lumbar enlargement of adult Sprague-Dawley rats. They identified these long descending propriospinal fibers as originating in medial lamina VII, lamina VIII, and the reticular extension of the neck of the dorsal horn throughout the cervical spinal cord (Menetrey et al., 1985). An earlier study by Giovanelli and Kuypers (1969) used fiber degeneration in combination with spinal cord hemi-sections in the cat to show that neurons with long ascending and descending propriospinal axons are distributed preferentially in the ventromedial gray matter (lamina VIII and medial aspects of lamina VII) which is also in agreement with the current study. Within the spinal enlargements, inter- and pre-motor neurons thought to be responsible for generating and disseminating locomotor activity are also located in laminae VII and VIII in the rabbit (Viala et al., 1991), cat (Shefchyk et al., 1990; Noga et al., 1995), mouse (Hinckley et al., 2005), and the neonatal rat (Kjaerulff et al., 1994; MacLean et al., 1995; Kjaerulff and

Kiehn, 1996; Kiehn and Kjaerulff, 1998, Antonino-Green et al., 2002, Butt and Kiehn, 2003). The current study describes columns of neurons, numbering in the hundreds in the L1, L2 and L3 segments, that overlap with these regions of functional interest (laminae VII and VIII). By virtue of their double-labeling, these neurons are characterized as ascending inter-enlargement (lumbo-cervical) and likely include true long propriospinal neurons in addition to projection neurons (spinoreticular, spinothalamic) with collaterals at C5-C6 or C7-C8.

Using the neonatal rat spinal cord *in vitro* preparation, Ballion and colleagues (2001) showed that the cervical and lumbar enlargements exhibit separate locomotor-like rhythms when disconnected and that either enlargement is capable of driving or entraining thoracic circuitry. Juvin et al. (2005) extended on those findings by showing that the lumbar circuitry can drive or entrain the cervical enlargement, however the reverse is not true. Interestingly, Juvin et al (2005) also showed that the lumbar generators could impose left-right alternation on the cervical circuitry even after a midsagittal lesion was performed from C1 to T7. They concluded that the caudorostral drive from the thoraco-lumbar spinal cord appeared to be mediated principally by homolateral excitatory pathways, and that an asymmetrical caudorostral propriospinal excitability gradient mediates interlimb coordination and not differences in the inherent rhythmogenic capacities of the two spinal enlargements. The novel population of commissural and ipsilateral inter-enlargement (lumbo-cervical) neurons identified in the present study are anatomically well-suited to this task in particular since very few appear to have axons that would be interrupted by a complete cervical mid-sagittal lesion.

Numerous studies involving tract tracing and/or genetic characterization of spinal cord commissural interneurons (Stokke et al, 2002; Hinckley et al., 2005; Nissen et al., 2005) have been used to describe short and “long” (greater than one and a half segments) projecting commissural neurons located in the most rostral segments of the lumbar enlargement. These studies illustrated that the majority of neurons that project two segments or more rostrally do not have axon collaterals that also project caudally, and vise verse (Stokke et al., 2002). These studies have also shown that, at least in early postnatal spinal cord, there are distinct domains in the intermediate gray matter where short and long ipsilateral and short and long commissural interneurons reside (Stokke et al., 2002; Butt et al., 2005), and that they share many features with locomotor-related interneurons that can be identified using the marker Eph4a. While the neurons in these studies were labeled from two or three segments away, by definition these populations include inter-enlargement neurons.

The concept of long propriospinal fibers traveling in the outermost rim of white matter instead of directly adjacent to the gray matter is supported by closely examining the spinal cord lesions created by Schucht et al. (2002). Among the lesions they created were several preparations in which the medial white matter surrounding the gray matter was lesioned bilaterally and yet inter-limb coordination remained intact with less than 20% white matter sparing. This suggests the participation of long propriospinal fibers traveling in the peripheral white matter, a finding that has also been noted by others (Busch, 1964; Conta and Stelzner, 2004). Multisegmental and long-propriospinal neurons have already become important targets for neuroprotection, regeneration and/or transplantation combinatorial strategies due to their ability to grow through Schwann cell bridges (Xu et al., 1995, 1997) and their potential role as relay or detour circuits in the injured spinal cord as alluded to by Fouad et al. (2005) and demonstrated by Baryere et al. (2004). This pattern of long ascending and descending propriospinal circuitry could easily account for the rapid compensatory adaptations observed to occur naturally following a variety of experimental spinal cord injury models (Magnuson et al., 1999; Schucht et al., 2002; Loy et al., 2002a; Webb and Muir, 2004). The identified ascending and descending inter-enlargement circuitry

also provides an innervation pattern ideally designed to mediate interlimb coordination during normal locomotion.

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## Abbreviations

| | |
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| **VLF** | ventrolateral funiculus |
| **FG** | Fluoro-Gold |
| **FR** | Fluoro-Ruby |
| **CPG** | central pattern generator |
| **o.d.** | outside diameter |
| **UV** | ultraviolet |
| **L#** | # lumbar segment(s) |
| **C#** | # cervical segment(s) |
| **T#** | # thoracic segment(s) |

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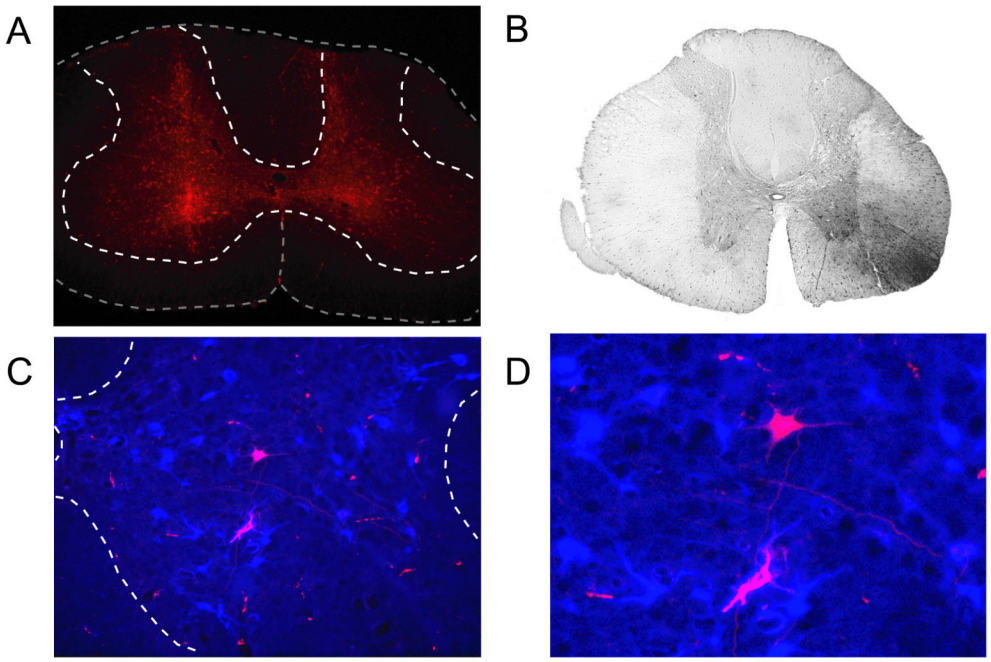


**Fig. 1.**

Shown is an example of a bilateral cervical (C5-C6) intermediate gray matter FR injection site (A), and an inverted image of a thoracic T9 FG injection site (B). In C, a section from the L2 segment shows a single FR-labeled neuron at the level of the central canal (red) and a double-labeled neuron in lamina VIII (pink). In D, the single and double-labeled neurons are shown at higher magnification.

Figure 2

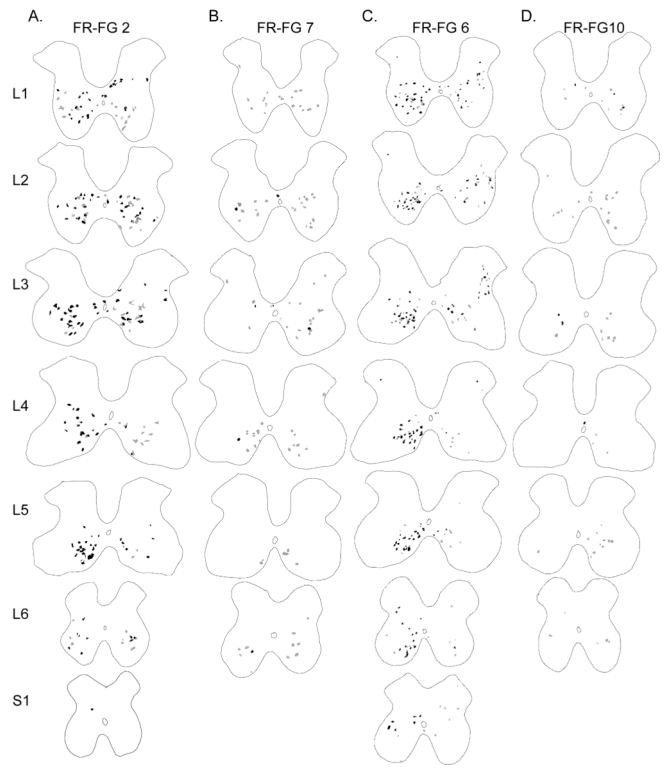


**Fig. 2.**

Superimposed composite drawings of 14-17 sections from each lumbosacral segmental level (L1-S1) of double FR/FG (black) and single FR-labeled cells (gray) from preparations with the following injections.

A. Bilateral C5-C6 FR and right unilateral T9 VLF FG (FR-FG 2)

B. Left unilateral C5-C6 FR and right unilateral T9 (FR-FG 7)

C. Bilateral C7-C8 FR and right unilateral T9 VLF FG (FR-FG 6)

D. Left unilateral C7-C8 FR and left unilateral T9 FG (FR-FG10)

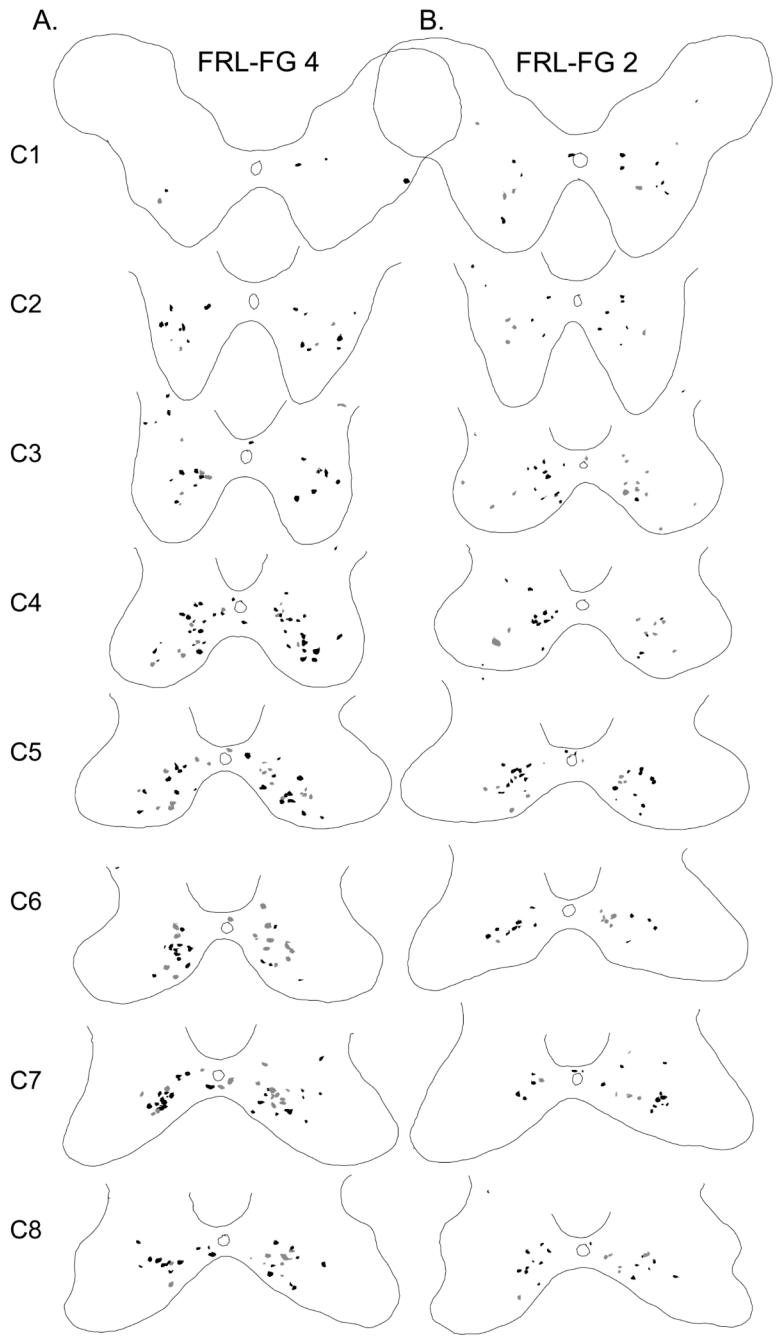


**Fig. 3.**

Superimposed composite drawings of 14-17 sections from each cervical segmental level (C1-C8) of single FR (gray) and double FR/FG (black) labeled cells from preparations with the following injections.

A. Bilateral L2 FR and right unilateral T9 FG (FRL-FG 4)

B. Left unilateral L2 FR and right unilateral T9 FG (FRL-FG 2)

Note the bilateral nature of the double-labeled (black) cell bodies throughout the cervical spinal cord regardless of whether a unilateral or bilateral FR-injection was performed.

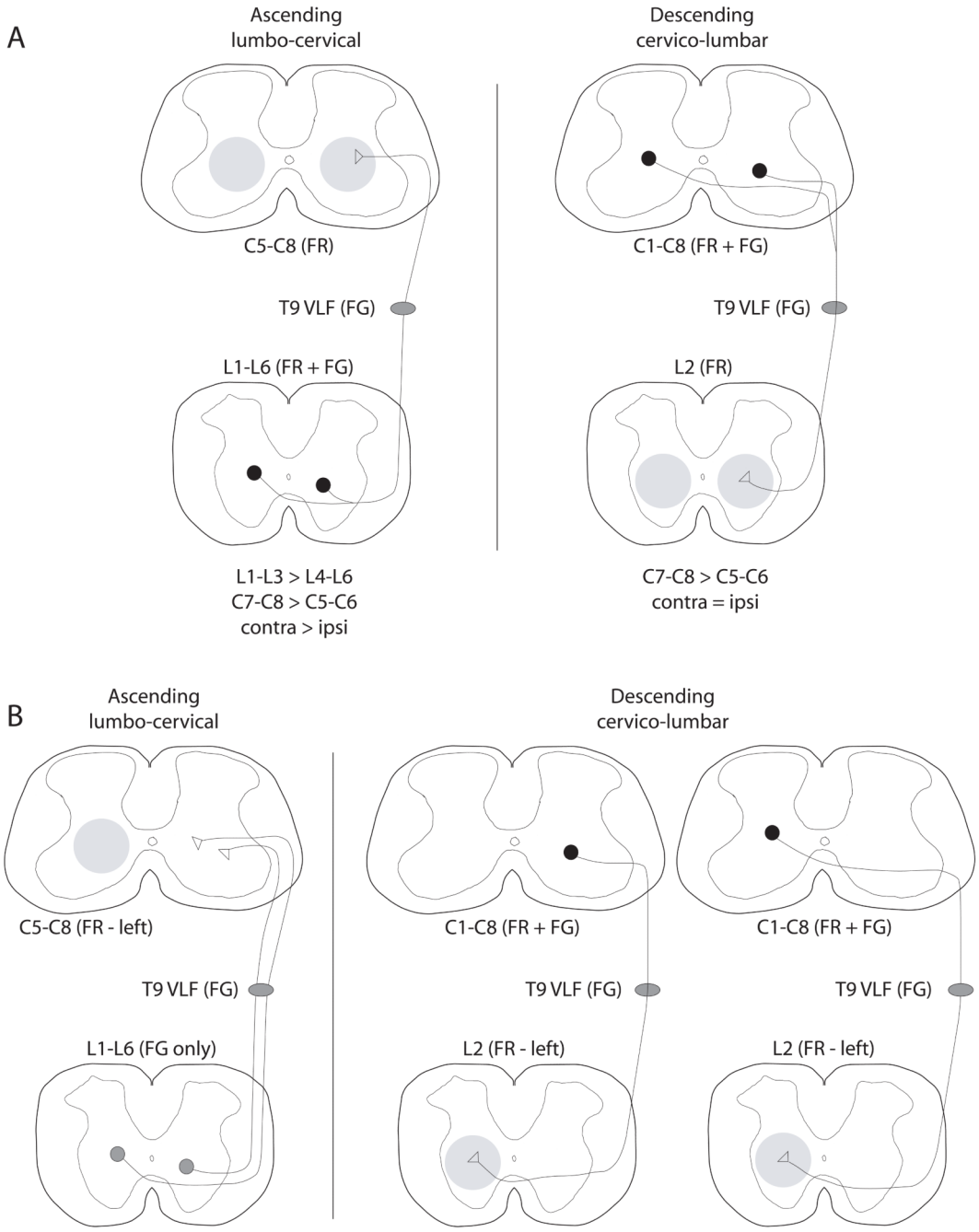


**Fig. 4.**

Shown are diagrams summarizing the major findings of the paper. In A, the results of the bilateral injections of FR into the intermediate gray matter at L2, C5-C6 or C7-C8 are shown. In B the results of unilateral injections of FR into the intermediate gray matter at L2, C5-C6 or C7-C8 are shown. FR is illustrated as light gray, FG as medium gray and populations of double-labeled cell bodies as black.

## Table 1

Detailed injection information for all the animals included in the study.

| | FR-FG injections | | | | | | |
|---|---|---|---|---|---|---|---|
| | Bi-(B) Unilateral (U) | | | | Unilateral | | |
| **ID #** | **%FR** | **Inj. Vol/Total** | **FR Inj Site** | **Time** | **%FG** | **Inj Vol/Site** | **Time** |
| FR-FG 1,2 | 10% | 0.5μl × 4 (2.0μl) | (B) C5-C6 | 21d | 0.5% | 0.3μl/VLF | 72hr |
| FR-FG 3 | 10% | 0.25μl × 2 (1.0μl) | (B) C5-C6 | 21d | 0.5% | 0.3μl/VLF | 72hr |
| FR 7 | 10% | 0.5μl × 2 (1.0μl) | (U) C5-C6 | 21d | 0.5% | 0.3μl/VLF | 72hr |
| FR 6 | 10% | 0.5μl × 4 (2.0μl) | (B) C7-C8 | 21d | 0.5% | 0.3μl/VLF | 72hr |
| FR 10 | 10% | 0.5μl × 2 (1.0μl) | (U) C7-C8 | 21d | 0.5% | 0.3μl/VLF | 72hr |
| FRL 2 | 10% | 0.5μl × 2 (2.0μl) | (U) L2 | 21d | 0.5% | 0.3μl/VLF | 72hr |
| FRL 3,4 | 10% | 0.5μl × 4 (2.0μl) | (B) L2 | 21d | 0.5% | 0.3μl/VLF | 72hr |

## Table 2

Numbers of single FG-labeled neurons counted in sections of the L1–S1 spinal cord (A) and C1–8 spinal cord (B) following injection of FG into the right VLF at T9

**A**

| | Bilateral C5–6 FR-injections | | | | | | Bilateral C7–8 FR-injections | | | | Unilateral C5–6 FR-injections, FR 7 | | Unilateral C7–8 FR-injections, FR 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FR-FG 1 | | FR-FG 2 | | FR-FG 3 | | FR 6 | | Means | % FR | | | | |
| | (L) Means | (R) % FR | (L) | (R) | (L) | (R) | (L) | (R) | | | (L) | (R) | (L) | (R) |
| L1 | 147 | 98 | 273 | 246 | 236 | 191 | 248 | 224 | L1–L3 | | 230 | 228 | 133 | 89 |
| L2 | 310 | 213 | 215 | 167 | 235 | 154 | 178 | 155 | 1574 | 4.4 | 306 | 195 | 159 | 80 |
| L3 | 271 | 145 | 232 | 175 | 218 | 109 | 285 | 119 | | | 339 | 153 | 174 | 80 |
| L4 | 298 | 144 | 199 | 100 | 244 | 104 | 254 | 112 | L4–L6 | | 303 | 105 | 173 | 79 |
| L5 | 334 | 164 | 219 | 100 | 203 | 100 | 337 | 164 | 1201 | 2.9 | 216 | 101 | 137 | 121 |
| L6 | 322 | 197 | 222 | 145 | 212 | 132 | 319 | 178 | | | 113 | 56 | 72 | 45 |
| S1 | 147 | 101 | 144 | 65 | 168 | 132 | 114 | 58 | | | — | — | — | — |
| Totals | 1829 | 1062 | 1504 | 998 | 1516 | 922 | 1735 | 1010 | | | 1234 | 738 | 848 | 494 |
| % FR | 4.5 | 2.4 | 3.7 | 1.9 | 3.9 | 3.0 | 8.2 | 5.9 | | | 0.16 | 0.14 | 0.35 | 0.81 |

**B**

| | Bilateral L2 FR-injections | | | | | | Unilateral L2 FR-injections | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | FRL 3 | | FRL 4 | | Means | % FR | FRL 2 | | Total | % FR |
| | (L) | (R) | (L) | (R) | | | (L) | (R) | | |
| C1 | 28 | 15 | 48 | 39 | C1–4 | | 41 | 37 | C1–4 | |
| C2 | 34 | 27 | 36 | 37 | 760 | 13.4 | 40 | 40 | 342 | 13.1 |
| C3 | 47 | 24 | 86 | 46 | | | 55 | 44 | | |
| C4 | 71 | 50 | 100 | 72 | | | 58 | 27 | | |
| C5 | 44 | 23 | 131 | 60 | C5–8 | | 67 | 44 | C5–8 | |
| C6 | 58 | 33 | 100 | 58 | 1023 | 12.3 | 55 | 22 | 416 | 15.8 |
| C7 | 65 | 49 | 104 | 67 | | | 61 | 37 | | |
| C8 | 77 | 43 | 70 | 41 | | | 66 | 64 | | |
| Totals | 424 | 264 | 675 | 420 | | | 443 | 315 | | |
| % FR | 9.9 | 11.4 | 11.7 | 18.3 | | | 16.5 | 12.1 | | |

Percentage FR indicates the percentage of FG-labeled cells that also contained FR.

**Table 3**

Numbers of single FR (A) and double FR/FG (B) labeled neurons counted in sections of the L1–S1 spinal cord from animals that received bilateral injections of FG into the intermediate gray matter of the cervical spinal cord (C5-6 or C7-8) and FG into the right VLF at T9

**A**

| | C5–6 FR-injections | | | | | | C7–8 FR-injections | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | FR-FG 1 | | FR-FG 2 | | FR-FG 3 | | FR 6 | | Means | % FR |
| | (L) | (R) | (L) | (R) | (L) | (R) | (L) | (R) | | |
| FR-labeled cells | | | | | | | | | | |
| L1 | 18 | 13 | 20 | 15 | 22 | 22 | 41 | 34 | L1–L3 | |
| L2 | 20 | 19 | 20 | 17 | 15 | 22 | 28 | 23 | 129 | |
| L3 | 23 | 15 | 14 | 15 | 16 | 19 | 37 | 27 | | |
| L4 | 19 | 12 | 9 | 8 | 12 | 12 | 25 | 17 | L4–L6 | |
| L5 | 13 | 11 | 10 | 4 | 11 | 12 | 36 | 13 | 82 | |
| L6 | 22 | 17 | 7 | 11 | 14 | 9 | 17 | 6 | | |
| S1 | 3 | 10 | 1 | 4 | 5 | 13 | 14 | 6 | | |
| Total | 118 | 97 | 81 | 74 | 95 | 109 | 198 | 126 | | |
| FR-FG labeled cells | | | | | | | | | | |
| L1 | 11 | 0 | 11 | 4 | 8 | 6 | 26 | 17 | L1–L3 | |
| L2 | 16 | 10 | 14 | 6 | 4 | 3 | 19 | 13 | 69 | 53.4 |
| L3 | 19 | 8 | 11 | 6 | 14 | 6 | 26 | 16 | | |
| L4 | 17 | 1 | 8 | 0 | 10 | 4 | 19 | 10 | L4–L6 | |
| L5 | 13 | 4 | 8 | 1 | 9 | 3 | 33 | 3 | 46 | 56.0 |
| L6 | 5 | 2 | 4 | 2 | 10 | 3 | 12 | 1 | | |
| S1 | 2 | 0 | 0 | 0 | 4 | 3 | 8 | 0 | | |
| Total | 63 | 25 | 56 | 19 | 59 | 28 | 143 | 60 | | |
| % FR | 70.3 | 25.8 | 69.1 | 25.7 | 62.1 | 25.7 | 72.2 | 47.6 | | |

**B**

| | FR labeled | | | | Means | FR-FG labeled | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Unilateral C5–6 FR-injections, FR 7 | | Unilateral C7–8 FR-injections, FR 10 | | | Unilateral C5–6 FR-injections, FR 7 | | Unilateral C7–8 FR-injections, FR 10 | |
| | (L) | (R) | (L) | (R) | | (L) | (R) | (L) | (R) |
| L1 | 7 | 12 | 4 | 10 | L1–L3 | 0 | 1 | 1 | 2 |
| L2 | 7 | 9 | 5 | 9 | 41 | 2 | 0 | 0 | 0 |
| L3 | 3 | 7 | 3 | 6 | | 0 | 0 | 2 | 0 |
| L4 | 3 | 5 | 1 | 4 | L4–L6 | 0 | 0 | 0 | 1 |
| L5 | 5 | 8 | 1 | 10 | 25 | 0 | 0 | 0 | 0 |
| L6 | 3 | 6 | 1 | 2 | | 0 | 0 | 0 | 1 |
| Total | 28 | 47 | 15 | 41 | | 2 | 1 | 3 | 4 |

Totals are given for one set of slides representing one-fifth of the total enlargement tissue. Percentage FR is calculated as the number of FR-FG double-labeled cells divided by the total number of FR labeled cells multiplied by 100.

**Table 4**

Numbers of single FR (A) and double FR/FG (B) labeled neurons counted in sections of the C1–C8 spinal cord from animals that received bilateral injections of FG into the intermediate gray matter of the lumbar spinal cord (L2) and FG into the right VLF at T9

| | Bilateral L2 FR-injections | | | | | | Unilateral L2 FR-injection | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | FRL 3 | | FRL 4 | | Means | % of FR | FRL 2 | | Means | % FR |
| | (L) | (R) | (L) | (R) | | | (L) | (R) | | |
| FR-labeled cells | | | | | | | | | | |
| C1 | 2 | 2 | 2 | 3 | C1–4 | | 7 | 7 | C1–4 | |
| C2 | 5 | 9 | 10 | 9 | 80.5 | | 5 | 3 | 70 | |
| C3 | 10 | 9 | 16 | 12 | | | 17 | 14 | | |
| C4 | 11 | 14 | 23 | 25 | | | 11 | 6 | | |
| C5 | 19 | 11 | 15 | 22 | C5–8 | | 22 | 12 | C5–8 | |
| C6 | 10 | 20 | 16 | 16 | 136.5 | | 13 | 10 | 96 | |
| C7 | 21 | 21 | 16 | 24 | | | 6 | 12 | | |
| C8 | 19 | 14 | 12 | 17 | | | 9 | 12 | | |
| Total | 97 | 99 | 110 | 128 | | | 90 | 76 | | |
| FR-FG labeled cells | | | | | | | | | | |
| C1 | 0 | 2 | 1 | 3 | C1–4 | | 5 | 4 | C1–4 | |
| C2 | 0 | 2 | 8 | 7 | 51 | 63.4 | 1 | 3 | 45 | 67.1 |
| C3 | 6 | 5 | 11 | 10 | | | 15 | 4 | | |
| C4 | 4 | 5 | 17 | 21 | | | 9 | 4 | | |
| C5 | 10 | 2 | 8 | 12 | C5–8 | | 17 | 6 | C5–8 | |
| C6 | 4 | 5 | 12 | 4 | 63 | 46.2 | 12 | 5 | 66 | 68.8 |
| C7 | 8 | 5 | 12 | 12 | | | 6 | 6 | | |
| C8 | 10 | 4 | 10 | 8 | | | 8 | 6 | | |
| Total | 42 | 30 | 79 | 77 | | | 73 | 38 | | |
| % FR | 43.3 | 30.3 | 71.8 | 60.2 | | | 81.1 | 50.0 | | |

Percentage FR is calculated as the number of FR-FG double-labeled cells divided by the total number of FR labeled cells multiplied by 100.