

Research report

Propriospinal projections to the ventral horn of the rostral and caudal hindlimb enlargement in turtles

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Available online 1 June 2004**Abstract**

In limbed vertebrates, the capacity to generate rhythmic motor patterns for locomotion and scratching is distributed over spinal cord segments of the limb enlargement (e.g., lumbosacral segments), but within this region, rostral segments are more rhythmogenic than caudal segments. The underlying reasons for this rostrocaudal asymmetry are not clear. One possibility is that rostral and caudal segments receive distinct sets of propriospinal projections. To test this hypothesis, I injected horseradish peroxidase (HRP) into the ventral horn unilaterally in a rostral or caudal segment of the turtle hindlimb enlargement. I quantitatively assessed the distributions of retrogradely labeled neurons in six hindlimb enlargement and pre-enlargement segments. The cross-sectional distribution did not depend on which segment was injected. Ipsilateral labeling occurred predominantly in the deep dorsal horn, the lateral part of the intermediate zone, and the dorsal two-thirds of the ventral horn, while contralateral labeling occurred mainly in the medial part of the ventral horn and the lateral part of the intermediate zone. This cross-sectional distribution is similar to what has been seen in mammals. The rostrocaudal distribution of labeled cells, however, depended on which segment was injected. Rostral injections gave rise to rostrally skewed distributions, dominated by descending propriospinal neurons. Caudal injections gave rise to caudally skewed distributions, dominated by ascending propriospinal neurons. Thus, rostral segments of the hindlimb enlargement received more propriospinal inputs from immediately rostral than immediately caudal segments, while the reverse was true for inputs to caudal segments. This anatomical asymmetry may contribute to known functional asymmetries within the enlargement.

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Theme: Motor systems and sensorimotor integration*Topic:* Spinal cord and brainstem*Keywords:* Spinal cord; Interneuron; Motoneuron; Locomotion; Hindlimb; Horseradish peroxidase**1. Introduction**

The basic motor patterns underlying vertebrate rhythmic limb movements, such as locomotion and scratching, can be generated by the spinal cord, even in the absence of input from the brain and movement-related sensory feedback [22,38,58,68]. The capacity to generate rhythmic limb motor patterns is distributed over several segments of the spinal cord enlargement and pre-enlargement for each limb (e.g., the lumbosacral spinal cord for mammalian hindlimb movements). Within this group of segments, however, rostral segments are more rhythmogenic than caudal segments, in cats [6,19], neonatal rats [16–18,41], chicks [26],

turtles [53], and mudpuppies [72]. What mechanisms underlie this rostrocaudal difference in rhythmogenicity? One possibility is that there are intrinsic differences between rostral and caudal segments, such as a gradient of excitability [26] or a greater percentage of rhythmogenic cells (e.g., endogenous or conditional pacemaker cells) in more rostral segments. A second possibility is that rostral and caudal segments receive distinct sets of propriospinal inputs, which impose a functional difference in rhythmogenicity. (“Propriospinal” is used here to include all neurons having their soma and an axonal termination within the spinal cord.) These two possibilities are not mutually exclusive.

Numerous physiological and anatomical studies have demonstrated that mammalian propriospinal neurons projecting to limb motoneurons (i.e., premotor interneurons) or to their vicinity in the spinal cord ventral horn are located mainly in laminae V–VII ipsilaterally and in lamina VIII

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contralaterally [1–3,5,13–15,20,21,23–25,27–29,31–36,39,40,50–52,60,69,70,73]. Only one of these studies, however, compared the distributions of premotor or ventral horn-projecting propriospinal neurons for projections to rostral versus caudal segments of a limb enlargement; this study reported some differences in the cross-sectional distribution of labeled cells within ipsilateral laminae V–VII following rostral versus caudal injections, but no clear differences in the shape of the segmental distributions [60].

The turtle spinal cord is a convenient model system to examine the neural control of vertebrate limb movements [68]. The turtle spinal cord can generate three forms of scratching, forward swimming, and hindlimb withdrawal motor patterns, even in the absence of input from the brain and movement-related sensory feedback [8,37,62,67]. The turtle's unusual resistance to anoxia is a major advantage for physiological studies [30,47]. The organization of the spinal cord has largely been conserved in evolution, making turtles a suitable model system for limbed vertebrate spinal cords generally [46,57]. The turtle hindlimb enlargement comprises five spinal segments (as in cats): dorsal 8–10 (D8–D10) and sacral 1–2 (S1–S2). The rostral enlargement and pre-enlargement segments, D7–D10, are more important for scratch rhythmogenesis than the caudal segments, S1–S2 [53]. The cross-sectional distribution of descending propriospinal neurons is similar to the distribution of premotor interneurons in mammals [12,45]. Injections of a retrograde tracer specifically into the ventral horn, however, have not yet been reported for turtles.

In this study, I injected horseradish peroxidase (HRP) unilaterally into the ventral horn of the D9, D10, or S1 segment of the turtle hindlimb enlargement and quantitatively assessed the distributions of retrogradely labeled neuronal somata in the D7–S2 segments. Results have been reported previously in an abstract [10].

2. Materials and methods

2.1. Surgical procedures and tracer injection

Red-eared turtles, *Trachemys scripta elegans* ($n=10$; 600–1000 g; both sexes) were used for all experiments. Hypothermic anesthesia was induced by placing the animal in ice for 2 h prior to surgical dissection [48,49]. The animal was kept partly immersed in ice throughout the dissection and labeling. The first procedure in each case was a dorsal laminectomy and complete transection of the spinal cord between the D2 and D3 dorsal roots. The D6–caudal 1 (Ca1) spinal cord was then exposed and the meninges were torn on the dorsal surface of the segment to be injected. A glass micropipette (15–20 μm tip) containing a solution of 10% HRP (Roche Diagnostics, Mannheim, Germany) in 1 M KCl was lowered to a depth of 1100 μm (D9 and D10 injections) or 1000 μm (S1 injections), 80% of the way laterally from the midline to Lissauer's tract, to target the ventral horn,

using a piezoelectric microdrive (Burleigh Instruments, Fishers, NY). [Biotinylated tracers are inadvisable for retrograde labeling in this tissue because some spinal interneurons show endogenous biotin staining [9].] The HRP was ejected via iontophoresis (15 μA continuously for 10–20 min) using a precision current source (Stoelting, Wood Dale, IL). Injections were into the rostral D9, the mid-D10, or the mid-S1 segment, in three sets of animals (Fig. 1A). Following injection, the exposed spinal cord was covered with saline-moistened Gelfoam and the exposure sealed with dental wax.

2.2. Perfusion and histology

After a survival period of 12–14 days at room temperature, animals were deeply anesthetized with sodium pentobarbital (390 mg, i.p.). [A survival period of 7 days is sufficient to label propriospinal neurons as far as 4 cm away [12], but in pilot experiments, it was found that a 12–14 day survival increases the intensity of labeling in distant somata.] The heart was exposed and perfused with: (1) 800 ml turtle saline containing 0.1% sodium nitrite, 10 units/ml heparin, and 39 mg pentobarbital; (2) 300 ml cold 4% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 (PB); and (3) 200 ml cold 20% sucrose/PB. The D6–Ca1 spinal cord was embedded in a gelatin–albumin–PB medium,

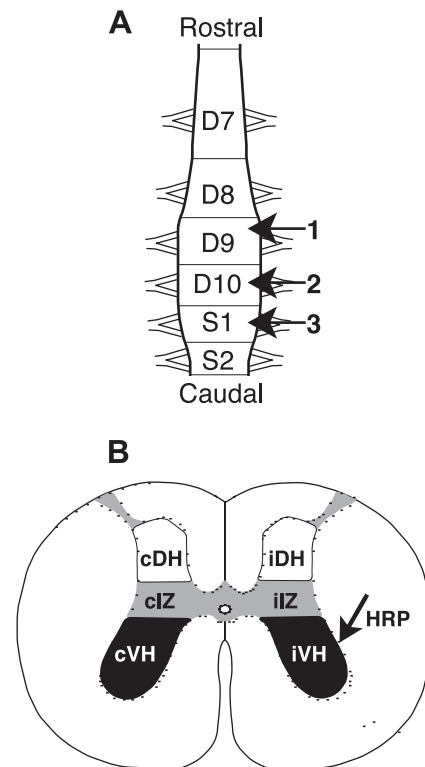


Fig. 1. Schematic illustrations of the experimental design. (A) Segmental locations of the ventral horn injection sites (1–3) in the three groups of animals and locations of the spinal cord segments of the hindlimb enlargement (D8–S2) and pre-enlargement (D7) in which retrograde labeling was assessed. (B) Definitions of cross-sectional regions: c: contralateral; DH: dorsal horn; i: ipsilateral; IZ: intermediate zone; VH: ventral horn.

with glutaraldehyde added to 2.5% to harden it [12]. Frozen sectioning was conducted either horizontally or transversely at 60 μm . All horizontal sections were kept; every 4th transverse section was kept. Sections were reacted with VIP or SG reagent (Vector Laboratories, Burlingame, CA) as the chromagen, mounted with a gelatin–ethanol–water solution (Albrecht's solution) onto gelatin-coated slides, dehydrated in graded ethanols, cleared in xylenes, and coverslipped [12].

All animal procedures conformed to NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma.

2.3. Analysis of labeling

Cases were analyzed if and only if sufficient HRP had been ejected and the injection site was entirely or primarily within the ventral horn on one side. For transversely sectioned cases, all labeled cells in each of the D7–S2 segments were marked on a drawing of a representative section from the middle of that segment (Fig. 4), using a camera lucida mounted on an Optiphot-2 microscope (Nikon, Melville, NY). In all cases, all labeled neuronal somata within the D7–S2 segments were counted, except those within the injection site itself. Labeled somata were counted by segment, as ipsilateral or contralateral, and as dorsal horn (DH), intermediate zone (IZ), or ventral horn (VH), as defined in Fig. 1B. [These divisions provide a reliable means of comparison [12]. The turtle spinal cord does not contain distinct laminae [46].] The amount of labeling in each cross-sectional region of each segment was then expressed as a percentage of the total labeling in the D7–S2 segments of that animal (Figs. 6–8). To further compare labeling between segments, each value of segmental labeling was then normalized by an estimate of the relative gray matter volume of that segment, and the percentage of total labeling recalculated, to obtain a measure of the relative density of labeling in each segment (Fig. 9). Each segment's relative gray matter volume was estimated as the mean cross-sectional gray matter area for that segment (measured from camera lucida drawings onto graph paper of representative transverse sections through the middle of each segment; each value was an average from four sections, two from each of two animals), multiplied by the mean rostrocaudal length of each segment (measured from horizontal sections from two animals, with each segmental border defined as the midpoint between adjacent sets of ventral roots).

3. Results

3.1. Injection sites

In successfully injected cases ($n = 10$; see Materials and methods), all cells were darkly labeled within a sphere at the

ventral horn injection site (e.g., Figs. 2 and 3). These cases included three with injections in the rostral D9 segment (two sectioned horizontally and one transversely), two with

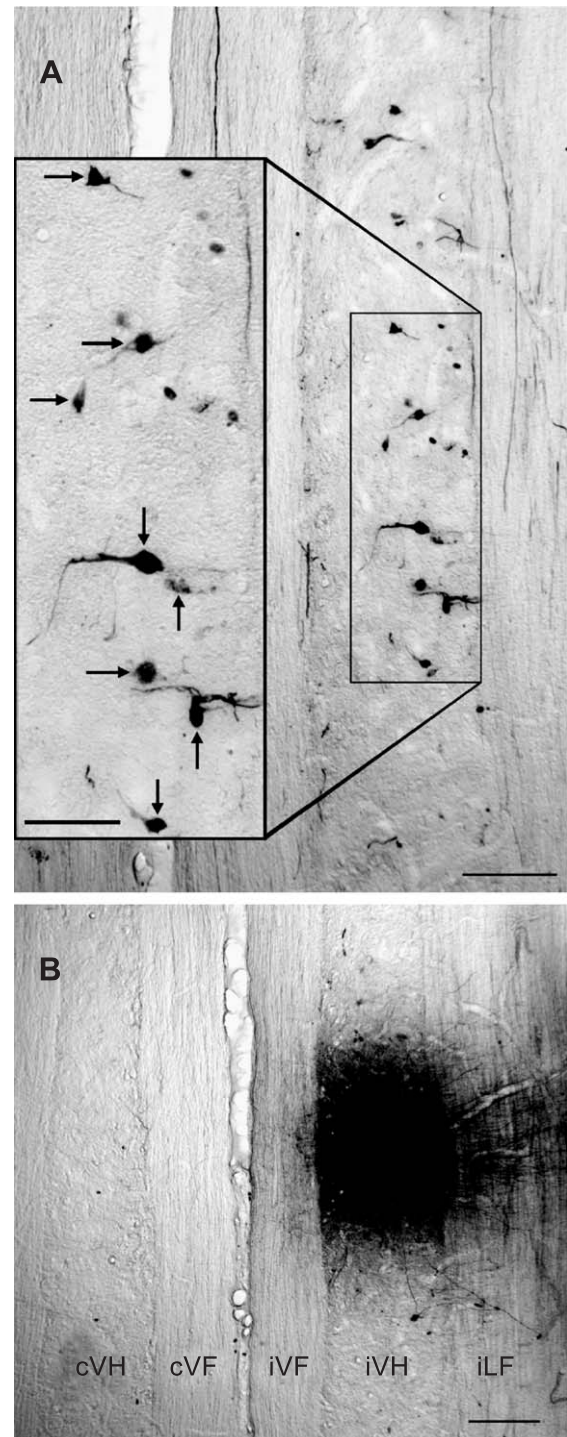


Fig. 2. Photomicrographs of horizontal sections showing (A) descending propriospinal neurons in the ipsilateral D8 ventral horn that were retrogradely labeled by (B) an HRP injection into the rostral D9 ventral horn. Inset in (A) shows labeled neuronal somata (arrows) at higher magnification; unmarked stained cells are erythrocytes. Rostral is up. LF: lateral funiculus; VF: ventral funiculus. Scale bars: (A and B) = 200 μm ; (A) inset = 100 μm .

injections in the mid-D10 segment (both sectioned horizontally), and five with injections in the mid-S1 segment (three sectioned horizontally and two transversely). Thus, there were a total of five injections within the more rhythmogenic rostral segments (rostral D9 and mid-D10) and five injections within the less rhythmogenic caudal segments (mid-S1). Each injection site included most of the ventral horn on the right side; six (one rostral D9, one mid-D10, and four mid-S1 injections) also included a part of the adjacent intermediate zone; five (one rostral D9, one mid-D10, and three mid S1 injections) also included a small part of the adjacent lateral funiculus. Away from the injection site, neuronal somata were clearly labeled, usually including proximal dendrites (e.g., Figs. 2 and 3), in all segments examined. Labeled somata included a variety of sizes and morphologies (e.g., Figs. 2 and 3). The total number of labeled somata varied considerably from case to case but did not vary significantly as a function of which segment was injected [means $\pm$ S.D.: rostral D9 cases,

956 $\pm$ 222; mid-D10 cases, 1110 $\pm$ 1293, rostral D9 + mid-D10 cases collectively, 1017 $\pm$ 671; mid-S1 cases, 591 $\pm$ 372; $p > 0.25$ for (rostral D9 + mid-D10) versus mid-S1, Student's *t*-test].

3.2. Cross-sectional labeling distributions

In each case, ipsilateral labeled cells were concentrated in the deep dorsal horn, the lateral part of the intermediate zone (i.e., at the same mediolateral level as the adjacent dorsal horn and ventral horn, not surrounding the central canal), and the dorsal two-thirds of the ventral horn (Figs. 3 and 4). Ipsilateral ventral horn labeling was concentrated centrally and laterally but not medially. In contrast, contralateral labeling was concentrated medially within the dorsal two-thirds of the ventral horn and to a lesser extent in the lateral part of the intermediate zone. Ipsilateral labeling was consistently greater than contralateral labeling. Labeled somata were rarely seen in the contralateral dorsal horn or

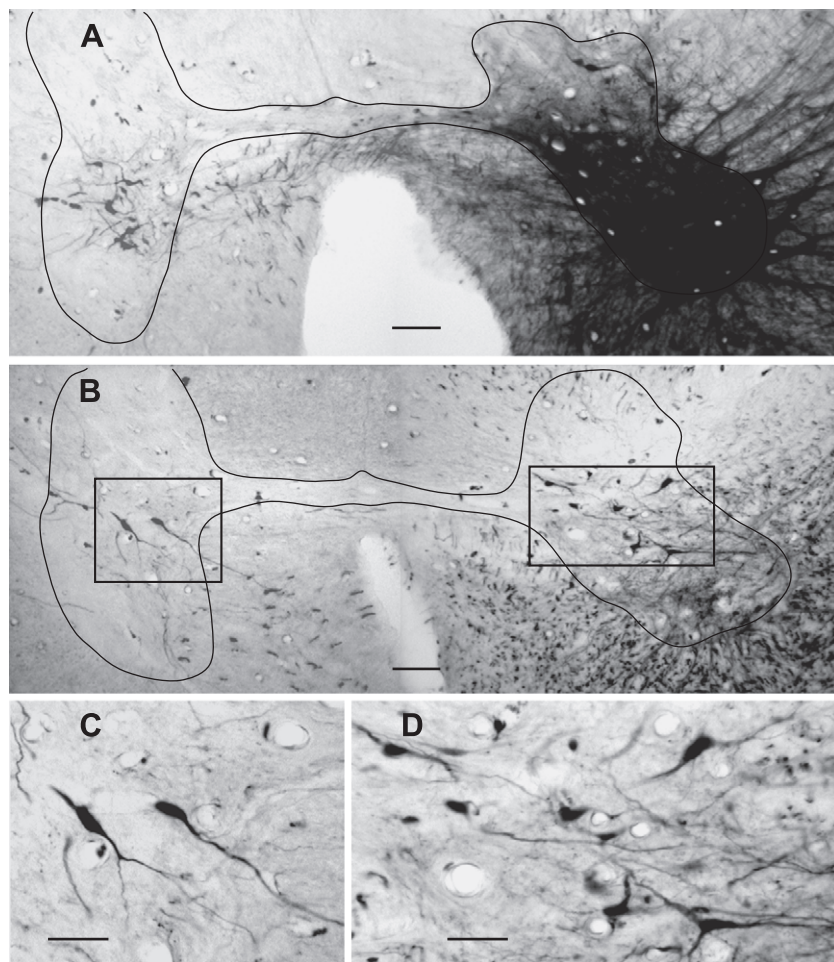


Fig. 3. Photomontages (A and B) and photomicrographs (C and D) of transverse sections showing (A) an HRP injection site into the right rostral D9 ventral horn and (B) retrogradely labeled neuronal somata in the D8 segment. (C and D) Labeled somata from the boxed areas in (B) shown at higher magnification contralaterally (C) and ipsilaterally (D). The gray matter is outlined for clarity in (A) and (B). Note in (A) that the injection site involved nearly all of the ventral horn but none of the intermediate zone; motoneurons were darkly labeled along with their extensive white matter dendrites. Scale bars: (A and B) = 100 μ m; (C and D) = 50 μ m.

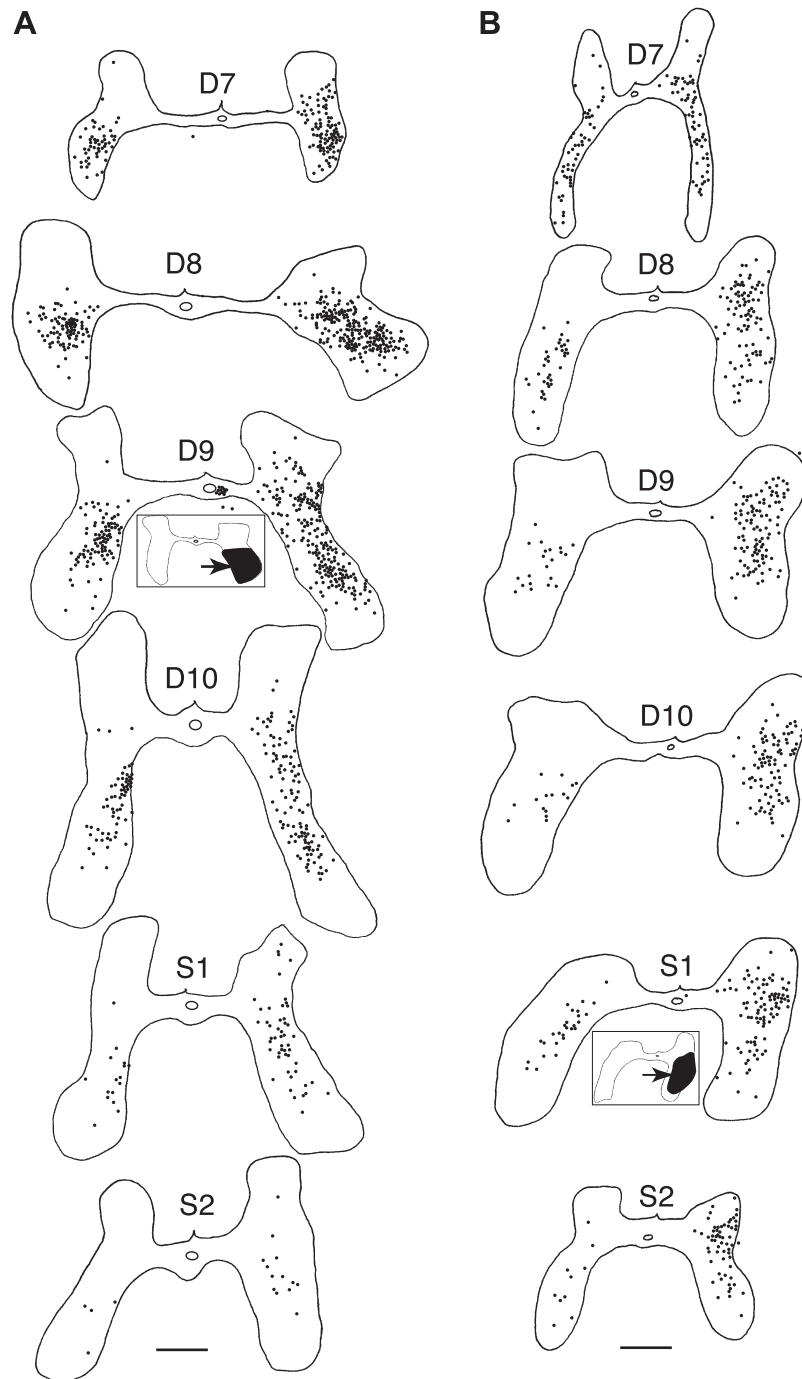


Fig. 4. Retrogradely labeled neuronal somata in transverse sections through the D7–S2 spinal segments, following ventral horn HRP injections into the (A) rostral D9 or (B) mid-S1 segment. All labeled neurons from every 4th section were marked on a camera lucida drawing of a representative section through the middle of each segment (see Materials and methods). Insets illustrate injection sites. Panel (A) is from the same case as in Fig. 3. Scale bars = 200 μ m.

within motoneuron areas. Labeled somata also were rare in the medial part of the intermediate zone, except for a small cluster of cells near the central canal which were only seen at the rostrocaudal level of the injection site and only in some cases (e.g., Fig. 4A).

This cross-sectional distribution of labeled somata was relatively consistent, regardless of the segment examined or the segment injected (Figs. 4–7). For example, labeled somata from the case illustrated in Fig. 2 were predominantly in the ipsilateral intermediate zone and ventral horn,

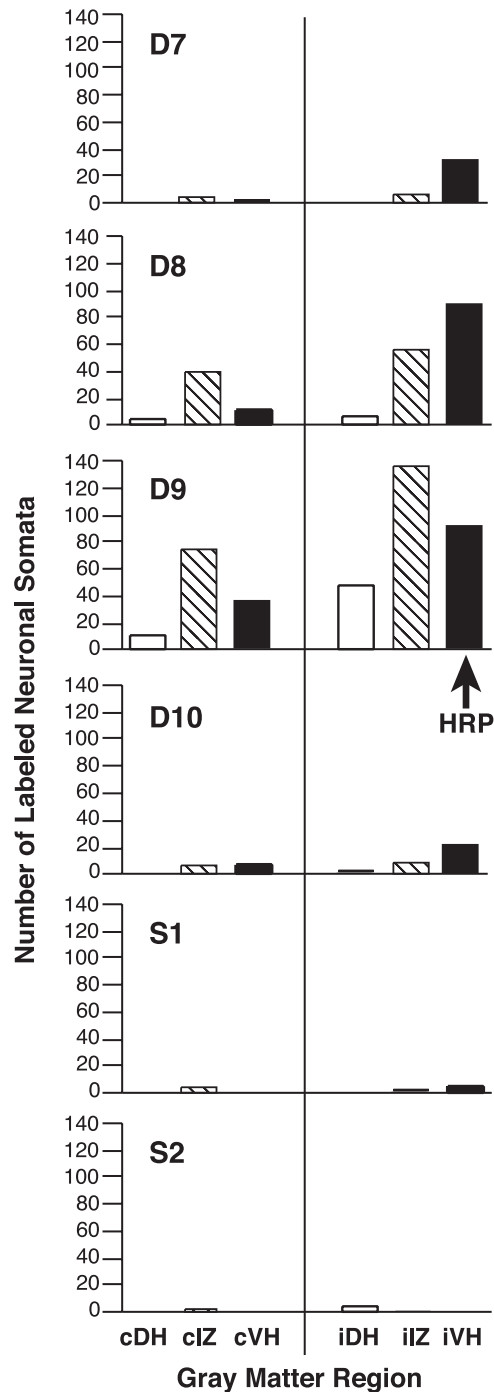


Fig. 5. Total numbers of labeled somata in each cross-sectional region of each of the D7–S2 spinal segments, following an HRP injection into the rostral D9 ventral horn. Open bars, dorsal horn; hatching, intermediate zone; filled bars, ventral horn.

and to a lesser extent, in the contralateral intermediate zone, the contralateral ventral horn, and the ipsilateral dorsal horn, in each of the six segments examined (Fig. 5). A comparison of the mean labeling percentages for each cross-sectional region of each segment shows that a similar pattern occurred consistently in multiple animals and occurred whether a rostral (rostral D9) or a caudal (mid-S1) segment was

injected (Fig. 6). This analysis also shows that, in the D7–D10 segments, the intermediate zone ipsilaterally and the ventral horn bilaterally contained a substantial number of cells that projected to the rostral D9 ventral horn as well as a substantial number of cells that projected to the mid-S1 ventral horn (Fig. 6). If one averages across all six segments examined, a similar pattern of cross-sectional labeling was seen whether the injection site was in the rostral D9 (Fig.

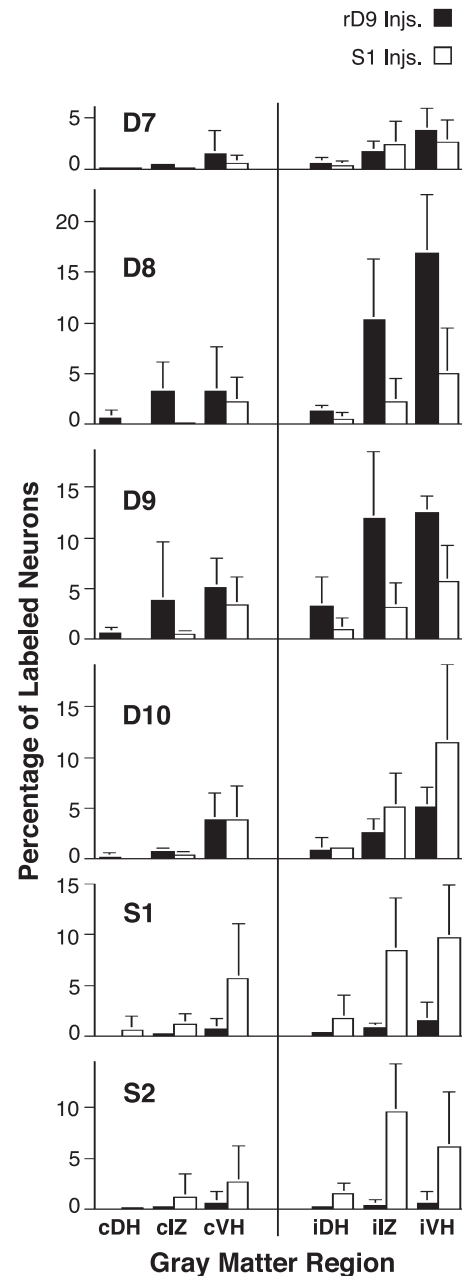


Fig. 6. Mean percentages of total labeling for each cross-sectional region and each segment, following rostral D9 (black bars) and mid-S1 (white bars) ventral horn HRP injections. Error bars are standard deviations. rD9: rostral D9.

7A), the mid-D10 (Fig. 7B), or the mid-S1 (Fig. 7C) segment.

3.3. Segmental labeling distributions

The segmental distribution of labeled cells, however, differed depending on which segment was injected (Figs.

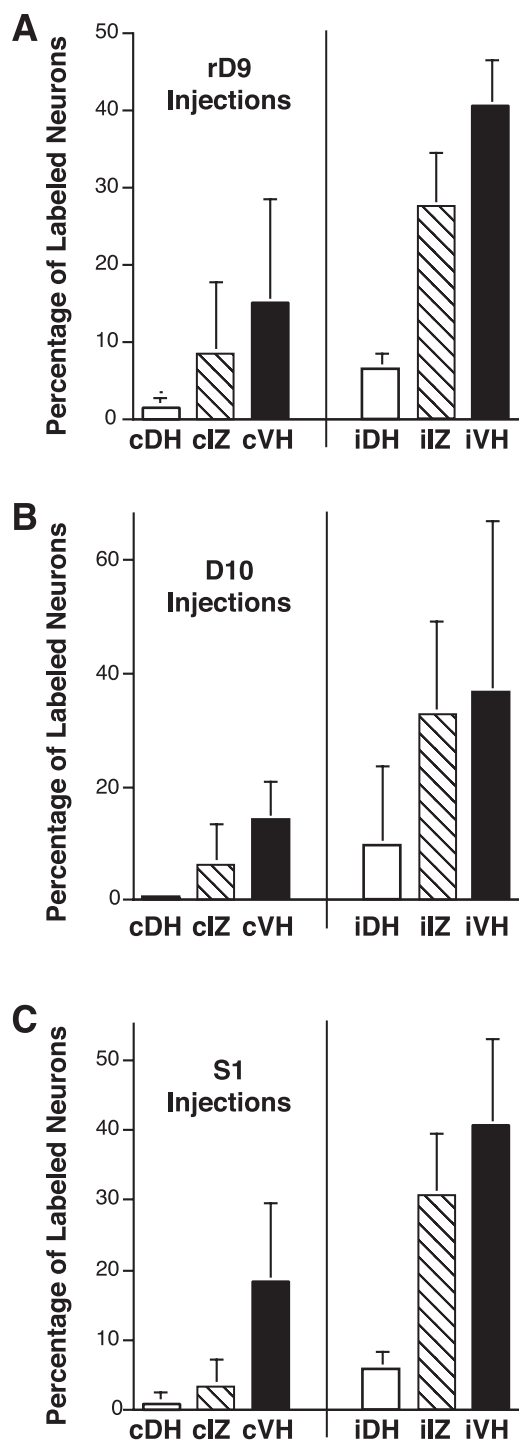


Fig. 7. Mean percentages of total D7–S2 labeling for each cross-sectional region, following (A) rostral D9, (B) mid-D10, and (C) mid-S1 injections.

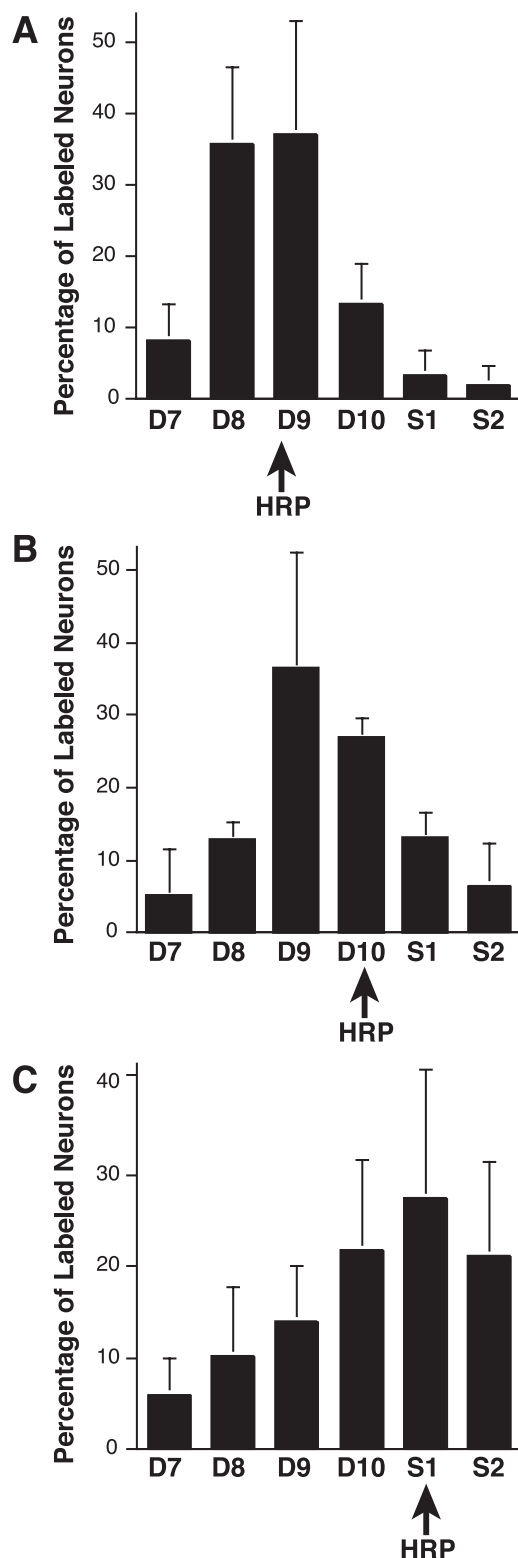


Fig. 8. Mean percentages of total D7–S2 labeling for each spinal segment, following (A) rostral D9, (B) mid-D10, and (C) mid-S1 injections.

4, 6, 8–10). In general, each injection gave rise to more labeled cells in nearby segments than in distant segments (Figs. 4–6, 8). Beyond this reduction in labeling with

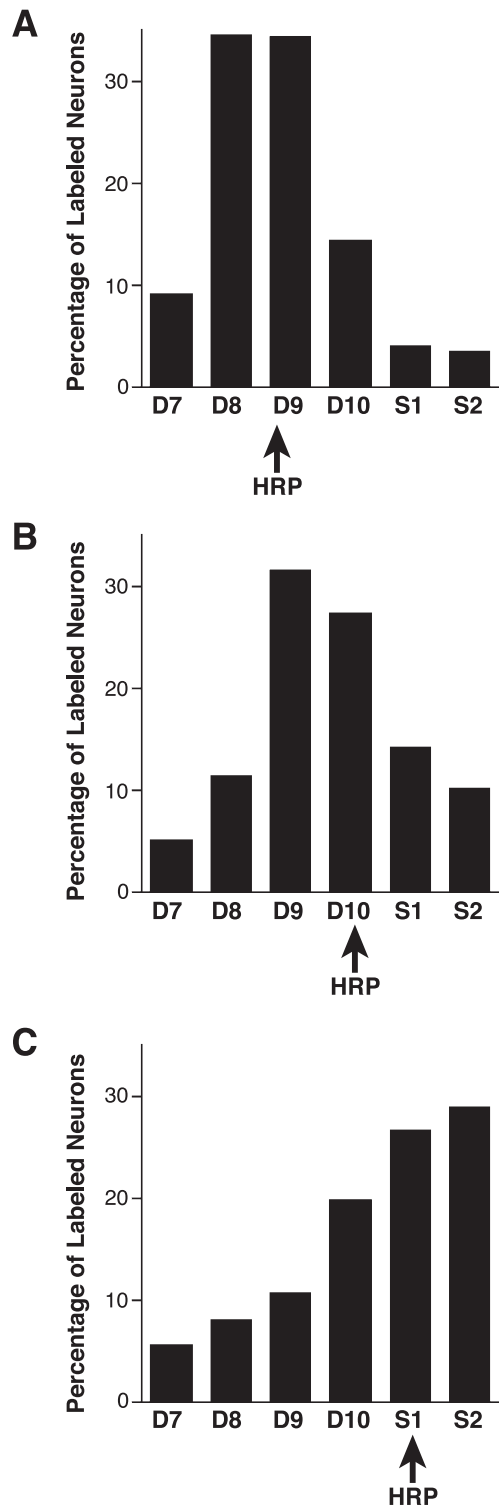


Fig. 9. Mean percentages of total D7–S2 labeling for each spinal segment, normalized to estimates of relative segmental gray matter volume (see Materials and Methods and Results), following (A) rostral D9, (B) mid-D10, and (C) mid-S1 injections.

distance, however, the shape of the segmental distribution depended on the segment injected. For example, rostral D9 or mid-D10 injections labeled more cells in the two seg-

ments rostral to the injection site than in the two segments caudal to it (Figs. 4–6, 8). In other words, more descending propriospinal neurons than ascending propriospinal neurons were labeled with rostral injections. In contrast, mid-S1 injections did not give rise to rostral skewing (Figs. 6, 8).

A possible confounding factor, however, is the volume of each spinal cord segment. That is, one might expect there to be more labeled cells in whichever segments contain more gray matter. To assess the relative density of labeled somata in each segment, it was first necessary to estimate the relative gray matter volume of each of the D7–S2 segments (see Materials and methods). The S2 segment was found to have the lowest gray matter volume; thus, S2 was arbitrarily assigned a normalization factor of 1.00. Estimates of the relative gray matter volumes of the other segments, relative to S2, were: D7 = 1.45, D8 = 1.71, D9 = 1.79, D10 = 1.52, and S1 = 1.41. Normalized segmental labeling percentages were then calculated using these factors as divisors and recalculating labeling as a percentage of the total (Fig. 9). The normalized distributions showed rostral skewing following rostral D9 (Fig. 9A) and mid-D10 (Fig. 9B) injections but caudal skewing following mid-S1 injections (Fig. 9C). These three distributions are superimposed in Fig. 10A and are aligned by the injection sites in Fig. 10B. This

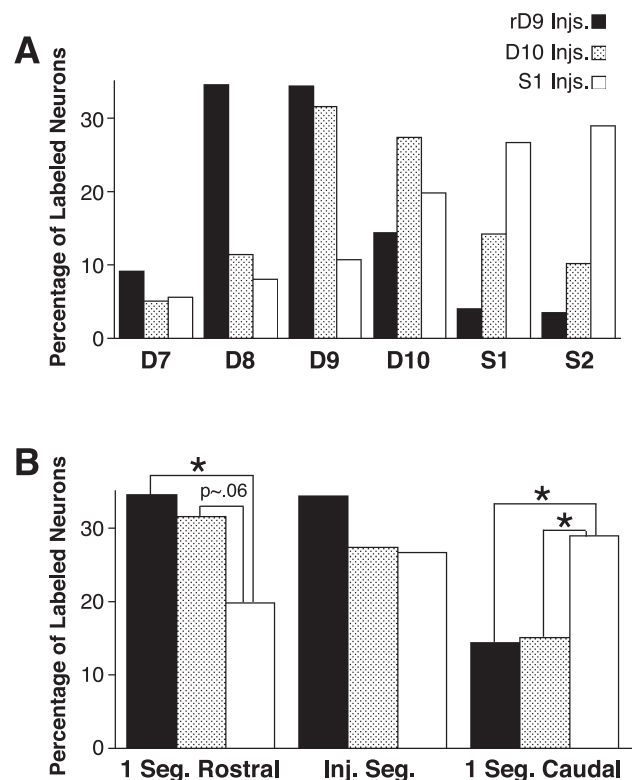


Fig. 10. Comparisons of normalized mean percentages of segmental labeling for injections into rostral D9 (black bars), mid-D10 (shaded bars), and mid-S1 (white bars). (A) Distributions across the D7–S2 segments. (B) Distributions for the injected segment and the immediately adjacent segments, aligned by the segment of injection. Asterisks indicate statistically significant differences ($p < 0.025$; G -test of independence with Williams's correction); unmarked differences were not significant.

analysis shows that at one segment rostral to the injection, there was a substantially higher percentage of labeling for rostral D9 and mid-D10 than for mid-S1 injections. This difference between rostral and caudal injections was statistically significant for rostral D9 versus mid-S1 ($p < 0.025$; G -test of independence with Williams's correction) and approached significance for mid-D10 versus mid-S1 ($p = \sim 0.06$; Fig. 10B). Conversely, one segment caudal to the injection, there was a substantially higher percentage of labeling for mid-S1 than for either rostral D9 or mid-D10 injections (Fig. 10B); both of these differences were statistically significant ($p < 0.025$).

4. Discussion

This study directly compared propriospinal projections to the ventral horn of rostral versus caudal segments of a spinal cord limb enlargement. Following ventral horn tracer injections into a more rhythmogenic rostral segment (rostral D9 or mid-D10) or a less rhythmogenic caudal segment (mid-S1), retrogradely labeled neuronal somata were quantitatively assessed throughout the turtle D7–S2 spinal cord. Cross-sectional labeling distributions were independent of the segment injected and the segment examined. Segmental labeling distributions, however, were rostrally skewed following rostral D9 and mid-D10 injections but were caudally skewed following mid-S1 injections. This finding is consistent with the hypothesis that the greater rhythmogenicity of rostral versus caudal segments of spinal cord limb enlargements is partly due to rostrocaudal differences in the sets of propriospinal projections received.

4.1. Cross-sectional labeling distributions

Ventral horn-projecting cells were concentrated ipsilaterally in the deep dorsal horn, the intermediate zone, and the dorsal two-thirds of the ventral horn. Contralaterally, labeled cells were concentrated in the dorsal two-thirds of the medial ventral horn and to a lesser extent in the intermediate zone. Although laminae are indistinct in turtles [46], this distribution corresponds approximately to mammalian spinal laminae V–VIII (especially V–VII) ipsilaterally and laminae VII–VIII (especially VIII) contralaterally [46,61].

The cross-sectional distribution found here for turtle ventral–horn projecting propriospinal cells is similar to those of mammalian spinal premotor interneurons and ventral–horn projecting propriospinal cells studied physiologically and anatomically. The somata of cat Renshaw cells [21,33,70,73] and cells mediating Ia reciprocal inhibition [34] have been localized to ipsilateral lamina VII, adjacent to motor nuclei, by antidromic activation from motor nuclei or ventral roots. Other cat propriospinal interneurons projecting to limb motor nuclei (identified either by antidromic activation or by spike-triggered averaging) have been found

mainly in ipsilateral laminae V–VIII, especially V–VII [14,15,20,27,28,31,35,39,40,50,69], and in contralateral lamina VIII [35]. Primate ipsilateral spinal “premotor” neurons, identified by their short-latency spike-triggered average effects on forelimb muscles, were similarly found mainly in the deep dorsal horn and lateral parts of the intermediate zone and ventral horn [59].

Retrograde tracer injections have also been used to examine the distributions of mammalian propriospinal neurons that project to limb motor nuclei or to the ventral horn generally. Injections of retrograde tracers into the ventral horn in the hindlimb enlargement labeled cells mainly in ipsilateral laminae V–VIII (especially V–VII) and contralateral lamina VIII, in cats [23,29,51,52] and rats [13,60]. Injections into the L4–L5 lateral motor column labeled cells throughout the mediolateral extent of ipsilateral laminae V–VII in rats, while injections into the L1–L2 lateral motor column labeled cells centrally and laterally but not medially within laminae V–VII [60]. Transynaptic retrograde labeling of premotor neurons with wheat germ agglutinin-conjugated HRP (which preferentially labels highly active neurons) from particular hindlimb or forelimb muscles or muscle nerves also gave rise to labeling mainly in ipsilateral laminae V–VII and contralateral lamina VIII, in cats [1–3,24,25,32,36,56] and rats [5,24].

The similarity in the cross-sectional distributions of ventral–horn projecting propriospinal cells in turtles and mammals suggests that information gained on the organization of such circuitry in turtles is likely to be informative for mammals as well.

The small cluster of ipsilaterally labeled somata near the central canal, seen only at the rostrocaudal level of the injection (Fig. 4A), may be autonomic preganglionic neurons whose axons traverse the ventral horn en route to the ventral root, as suggested by Kusuma and ten Donkelaar [43].

4.2. Segmental labeling distributions

The retrograde labeling of ventral–horn projecting propriospinal cells was also examined as a function of the spinal segment of the labeled somata, for each of the D7–S2 segments and for each set of injection sites Figs. (6,8–10). Labeling was quantified as a percentage of the total labeling in that case and was also normalized to an estimate of the relative gray matter volume of each segment. In contrast to the consistent cross-sectional distributions of labeling, the segmental distributions depended on which segment was injected. In each case, labeling declined with distance from the injection site. More surprisingly, segmental distributions were skewed. Injections into a rostral segment of the hindlimb enlargement (rostral D9 or mid-D10) gave rise to rostrally skewed distributions, in which the segments immediately rostral to the injection site had greater labeling than the segments immediately caudal to the injection site. In contrast,

injections into a caudal segment of the hindlimb enlargement (mid-S1) gave rise to caudally skewed distributions, in which the segment immediately caudal to the injection site had greater labeling than the segment immediately rostral. Differences between the rostral D9 and mid-S1 injections were statistically significant both one segment rostral and one segment caudal to the injection site (Fig. 10B). One might object that rostral D9 injections could lead to rostral skewing simply because these injections were into the rostral part of the segment, rather than into the middle of the segment (for motivation for this experimental design, see Section 4.3 below). This objection, however, does not apply to the mid-D10 injections. Differences between the mid-D10 and mid-S1 injections were statistically significant ($p < 0.025$) one segment caudal to the injection site and approached statistical significance ($p = 0.06$) one segment rostral to the injection site (Fig. 10B), despite the fact that the D10 and S1 segments are adjacent (see Fig. 1).

Thus, propriospinal inputs to the ventral horn of rostral enlargement segments were weighted toward descending propriospinal projections, while inputs to caudal segments were weighted towards ascending propriospinal projections. The change from mainly descending to mainly ascending propriospinal inputs occurred between the D10 and S1 segments. This suggests that there are more propriospinal projections within the D7–D10 rostral segments and within the S1–S2 caudal segments than between the D7–D10 and S1–S2 segments. While the observed differences in propriospinal inputs are quantitative, not qualitative, they could underlie functional differences between these segments. In rats, no differences were reported in the shapes of the segmental distributions of labeled cells following retrograde tracer injections into the L1–L2 versus L4–L5 lateral motor column; these distributions, however, were apparently assessed qualitatively and not quantitatively [60].

One type of functional difference between rostral and caudal segments of a limb enlargement is in the capacity to generate rhythmic motor patterns for locomotion and scratching. Rostral segments are more rhythmogenic than caudal segments in a variety of vertebrates examined. The five-segment lumbar 4 (L4)–S1 hindlimb enlargement in cats corresponds to the five-segment D8–S2 hindlimb enlargement in turtles [63,64]. In cats, experiments in which segments were functionally removed by lesion [6,19] or by cooling [4,19] have shown that the L3–L5 segments are necessary for approximately normal fictive scratching, but more caudal segments are not. In chicks, lesion experiments have shown that the capacity to generate rhythmic motor output is distributed over the lumbosacral enlargement, but the L1–L4 rostral segments are more important than the L5–L9 caudal segments [26]. In mudpuppy fictive forelimb locomotion, lesion experiments have shown that the cervical 2 (C2)–C3 rostral segments are more important for rhythmogenesis than the C4–C5 caudal segments [72]. In neo-

natal rats, the capacity to generate locomotor-like rhythms under pharmacological stimulation is distributed over thoracic and lumbosacral segments [18,41], but the L1–L2 rostral enlargement segments are more rhythmogenic than the L3–L5 caudal enlargement segments [16,17,41]. In turtle fictive scratching, the D7–D10 rostral enlargement segments are more rhythmogenic than the S1–S2 caudal enlargement segments, with the D8 segment having the greatest rhythmogenicity [53]. The differences in propriospinal inputs to the D9–D10 versus S1 segments observed here may contribute to these previously observed rostrocaudal differences in rhythmogenicity. The greater proportion of descending propriospinal inputs to rostral segments may facilitate their rhythmogenicity, instead of, or in addition to, any intrinsic differences between these segments. It will be of interest to see whether similar rostrocaudal differences in the distribution of propriospinal inputs occur in the other limbed vertebrates displaying a rostrocaudal gradient of rhythmogenicity.

Another type of rostrocaudal difference within the hindlimb enlargement is in the control of hip musculature. Hip flexor motoneurons are located in rostral segments and hip extensor motoneurons in caudal segments of the hindlimb enlargement (with some overlap) in a variety of vertebrates [63,64,71]. In turtles, hip flexor motoneurons are in the D8–D9 segments while hip extensor motoneurons are in the D9–S2 segments [64]. The interneuronal control of hip flexor and hip extensor motoneuron activity may also show a rostrocaudal gradient. For example, fictive scratch motor patterns in spinal turtles can include multiple cycles (called hip extensor deletions) in which rhythmic hip flexor motoneuron activity occurs in the absence of hip extensor motoneuron activity [53,65,66]. This implies that scratch pattern-generating circuitry for hip flexors is to some degree separable from scratch pattern-generating circuitry for hip extensors. In addition, a turtle spinal cord preparation containing only the D3–D8 segments produces a normal hip flexor phase, but a reduced hip extensor phase, during fictive rostral scratching [53]. Conversely, a preparation containing only the D9 and more caudal segments produces a normal hip extensor phase, but a reduced hip flexor phase, during fictive caudal scratching [53]. This suggests that rostral segments may play a relatively larger role in hip flexor rhythmogenesis and caudal segments in hip extensor rhythmogenesis [53]. The differences in propriospinal inputs to the D9–D10 versus the S1 segments seen here might contribute to this differential ability to generate rhythmic hip flexor versus hip extensor motoneuron activity. However, the division between “rostral-like” and “caudal-like” appears to occur approximately between D8 and D9 for rhythmic control of hip flexors versus hip extensors [53] but between D10 and S1 for rostral-skewed versus caudal-skewed propriospinal projections.

An additional possibility is that the rostrocaudal difference in propriospinal inputs may be wholly or partly due to differential relaying of cutaneous sensory information to the

hindlimb enlargement. Rostral scratching and pocket scratching are initiated by cutaneous stimulation of the midbody and hindlimb pocket regions [55], via sensory inputs to the D3–D8 spinal segments [54]. Caudal scratching is instead initiated by stimulation of the region behind the hindlimb [55], via sensory inputs to the caudal spinal segments [54], which are immediately caudal to the S2 segment. Most turtle primary afferents terminate within the spinal segment of entry [43,44], although some may travel several segments [42]. Thus, the preponderance of descending propriospinal inputs to the rostral enlargement and ascending propriospinal inputs to the caudal enlargement might reflect the relaying of sensory signals from their segment of entry to pattern-generating circuitry in the enlargement.

4.3. Dual-projecting propriospinal neurons?

A model for the selection and generation of turtle rostral and pocket scratch motor patterns predicts that some broadly tuned and rhythmically active spinal interneurons project to two functionally distinct sets of limb motoneurons, thus generating particular knee–hip muscle synergies [7,11]. This model predicts that one group of spinal interneurons projects to both knee extensor motoneurons and hip extensor motoneurons. Turtle knee extensor motoneurons are in the D8–D9 segments, while hip extensor motoneurons are in the caudal D9–S2 segments [64]. Thus, tracer injections into the rostral D9 ventral horn should label projections to knee extensor but not hip extensor motoneurons, while injections into mid-S1 should label projections to hip extensor but not knee extensor motoneurons. If there are spinal interneurons that project to both knee extensor motoneurons and hip extensor motoneurons, some of these interneurons should have been labeled by both the rostral D9 and the mid-S1 injections. A comparison of these two sets of injections shows that the D8–D10 ipsilateral intermediate zone and ventral horn and contralateral ventral horn had substantial numbers of cells labeled following both rostral D9 and mid-S1 injections (Fig. 6). These regions may contain individual interneurons that project to both knee extensor and hip extensor motoneuron areas, as predicted by the model. Alternatively, separate sets of interneurons within each region may project to these two motoneuron areas. Future studies may resolve this issue by injecting two different fluorescent retrograde tracers into these two motoneuron areas and looking for double-labeled neurons.

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