

Descending Propriospinal Axons in the Hindlimb Enlargement of the Red-Eared Turtle: Cells of Origin and Funicular Courses

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ABSTRACT

Spinal neurons with descending axons are important components of spinal sensorimotor networks. We used an anatomical tracing technique to study the distribution of descending propriospinal axons and cell bodies in red-eared turtles. We injected horseradish peroxidase into a portion of one funiculus in the middle of the hindlimb enlargement and examined six spinal segments rostral to the injection site (dorsal 3 through dorsal 8) for labeled neuronal cell bodies.

Injections into each region of the white matter labeled substantial numbers of descending propriospinal neurons. Each injection labeled cell bodies over most of the six spinal segments examined. Each injection also labeled cell bodies in the ipsilateral dorsal horn, intermediate zone, and ventral horn as well as the contralateral intermediate zone and ventral horn. Injections into each of four regions of the white matter, the dorsal funiculus, the medial part of the lateral funiculus, the lateral part of the lateral funiculus, and the ventral funiculus reliably gave rise to a distinct distribution of labeled cell bodies.

These experiments establish that descending propriospinal axons in red-eared turtles are found in all regions of the spinal white matter. This finding contrasts with a popular contemporary view of the organization of descending propriospinal axons in mammals. These experiments also demonstrate that neurons in each region of the gray matter give rise to a different distribution of descending, funicular axons, although these distributions are widely overlapping. Different funicular axon distributions could be associated with different sets of synaptic contacts with the white-matter dendrites of spinal neurons. © 1994 Wiley-Liss, Inc.

Key words: spinal cord, interneurons, neural pathways, horseradish peroxidase, motor activity

In both mammals and nonmammalian vertebrates, the spinal cord is able to generate the complex patterns of muscle activity that underlie withdrawal reflexes, scratching, and locomotion (Paton, 1846; Bickel, 1897; Sherrington, 1906, 1910; Grillner, 1981; Stein, 1983, 1984, 1989; Gelfand et al., 1988). Propriospinal neurons (i.e., neurons with a spinal cell body and an axon that terminates within the spinal cord) and their projections are an important component of the spinal circuitry that mediates these behaviors. Surprisingly little is known about the cells of origin and funicular courses of propriospinal fibers in vertebrates, including mammals. We have focused on the spinal control of scratching in red-eared turtles as a convenient system for elucidating principles of neural organization that underlie selection and generation of particular limb movements (Stein, 1989). Spinal turtles exhibit three distinct forms of scratching by a hindlimb in response to tactile stimulation of distinct regions of the body surface (Stein, 1989). Propriospinal circuitry responds to tactile

afferent input from the body surface by selecting and generating an appropriate form of scratching to bring a hindlimb into contact with the stimulated site. Descending propriospinal neurons may play particularly important roles in this behavior, because the afferent signals that evoke two forms of scratching (Mortin et al., 1985; Mortin and Stein, 1990) enter the spinal cord rostral to the spinal segments crucial for hindlimb motor pattern generation (Mortin and Stein, 1989).

Successive anterograde degeneration experiments (see Discussion) have demonstrated that descending propriospinal axons are distributed throughout the spinal white matter in dogs (Sherrington and Laslett, 1903). Nevertheless, a popular contemporary view (Brodal, 1981; Carpenter and Sutin, 1983; Carpenter, 1991) holds that both ascend-

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ing and descending propriospinal axons are found predominantly or exclusively in fasciculi proprii immediately adjacent to the gray matter (see Discussion). This view is based on interpretations (Nathan and Smith, 1959; Brodal, 1981) of experiments (Tower, 1937; Tower et al., 1941) in which the lumbosacral spinal cord of a dog or monkey was isolated by dorsal root and spinal cord transections. After a few weeks or months, the locations of the remaining funicular fibers were assessed to determine the normal locations of propriospinal axons. To our knowledge, the present study is the first to use focal injections of a retrograde tracer in the spinal white matter, allowing a clear demonstration of the locations and relative numbers of propriospinal neurons that project a descending axon into each region of the white matter.

In the present study, we describe the white-matter distribution of descending propriospinal axons and the locations of their cell bodies, in order to lay the groundwork for further study of propriospinal circuitry in turtles. We injected horseradish peroxidase (HRP) unilaterally into a different region of the spinal white matter in each of several turtles. All HRP injections were made in the middle of the hindlimb enlargement, at the caudal end of the ninth postcervical segment (the dorsal 9 or D9 segment), a region crucial for spinal motor pattern generation (Mortin and Stein, 1989). Spinal motoneurons in turtles have extensive dendritic fields in the white matter (Banchi, 1903; Ruigrok et al., 1984), so it is likely that funicular axons make many synaptic contacts in the white matter. Hence the funicular courses of descending propriospinal axons in turtles may have a bearing on the synaptic outputs of descending propriospinal neurons. The rostrocaudal location injected with HRP in this study (the caudal end of the D9 segment) is the same location from which descending propriospinal axons were recorded during fictive scratching in parallel studies (Berkowitz and Stein, 1990, 1991, 1994a,b; Berkowitz, 1993). We examined six segments (D3–D8) rostral to the injected segment for labeled cell bodies; these segments innervate all of the rostral scratch and pocket scratch receptive fields (Mortin et al., 1985; Mortin and Stein, 1990). These experiments have been reported previously in abstract form (Berkowitz and Stein, 1992) and as a chapter in a doctoral thesis (Berkowitz, 1993).

MATERIALS AND METHODS

The methods used for injection of HRP into the spinal white matter, histological processing of the tissue, and analysis of the neuronal labeling, respectively, are described below.

HRP injections

Thirty-two adult red-eared turtles, *Trachemys scripta elegans* (formerly *Pseudemys scripta elegans*), with a plastron length of 12–16 cm (weighing 300–500 g) were injected with HRP (Boehringer-Mannheim). Each turtle was placed in crushed ice for at least 1 hour prior to surgery to induce hypothermic analgesia (Marcus, 1981; Melby and Altman, 1974). The turtle was kept partly immersed in crushed ice throughout surgery. A dorsal laminectomy was performed to expose the spinal segments dorsal 2 through sacral 2 (D2–S2). The spinal cord was completely transected just caudal to the forelimb enlargement, midway between the D2 and the D3 dorsal roots. The HRP injection was made in the middle of the hindlimb enlargement, midway between

the D9 and the D10 dorsal roots (Fig. 1). Each HRP placement was made by one of two methods. 1) For most HRP placements, a microscalpel was used to make a small transverse cut in a region of the white matter; a tiny pledget of Gelfoam that had been soaked in a concentrated solution of HRP in 0.1 M phosphate buffer (pH 7.4) was then inserted into the lesion site. For HRP placements in the ventral white matter, the D9 and D10 dorsal and ventral roots were cut, the spinal cord was completely transected between the D10 and the S1 dorsal roots, and the D9–D10 cord was bent back upon itself, exposing the ventral D9–D10 cord to a dorsal approach. After the HRP placement, the spinal cord was returned to its normal position. 2) For HRP placements into an inner region of the white matter, an insect pin was glued to the end of a glass micropipette and the micropipette clamped in a micromanipulator. The insect pin was lowered into the region of white matter in which an injection was intended and then moved up and down, to damage axons. Waterproof ink on the tip of the insect pin marked the insertion site. Then, the insect pin-micropipette was removed and replaced with a glass micropipette (10–15 μ m inner diameter), filled with a 10% solution of HRP in phosphate buffer. The HRP microelectrode was introduced with the same micromanipulator, to the same site, along the same trajectory, and to the same depth. HRP was ejected iontophoretically (+5–6 μ amps, 8 seconds on/8 seconds off, for 4–10 minutes), using a Midgard constant current source (Transkinetics). The electrode was left in place for an additional 5–10 minutes after iontophoresis.

In one experiment, D9 motoneurons were unilaterally labeled. The D9-caudal 1 (Ca1) dorsal and ventral roots were cut, the spinal cord was transected between the Ca1 and the Ca2 dorsal roots, and the cord was bent back upon itself. The D9 ventral rootlets were freed from meninges, and each was placed in contact with a tiny piece of Gelfoam soaked in a concentrated solution of HRP in phosphate buffer, inside a short length of polyethylene tubing (0.7 mm inner diameter), the other end of which had been heat sealed. After 2.5 hours, the tubing was removed, the area was thoroughly rinsed with turtle saline, and the spinal cord was returned to normal position.

Following every injection, the injected region was monitored for bleeding or HRP leakage for 15–30 minutes, and any fluid in the region was drawn off with a small piece of Kimwipes. The region was monitored until any bleeding had stopped. Then, the exposed spinal cord was covered with strips of Gelfoam soaked in turtle saline, and the entire region of the laminectomy was sealed over with dental wax. The turtle was removed from ice and allowed to warm to room temperature.

Histological processing

Following a 6–8 day survival period, deep anesthesia was induced by intraperitoneal injection of sodium pentobarbital (0.4–2.0 ml; 60 mg/ml). Surgical procedures were carried out with the turtle partly immersed in crushed ice to provide mechanical stability and to keep the spinal cord cold once the perfusion was completed. The heart was exposed and perfused with 100–300 ml cold turtle saline (usually including 1% sodium nitrite to reduce clotting), followed by 70–120 ml cold fixative (1% paraformaldehyde, 1.25% glutaraldehyde, in 0.1 M phosphate buffer, pH 7.4). The D3–D10 spinal cord was removed, pinned out in a dish of Sylgard to maintain its morphology, and postfixed for 2

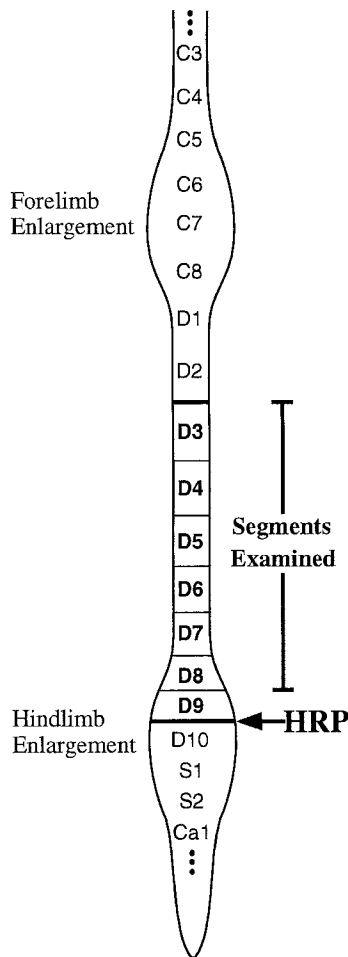


Fig. 1. Schematic illustration of the experimental paradigm. Horseradish peroxidase (HRP) was injected into the white matter at the caudal border of the D9 spinal segment of the red-eared turtle. Six spinal segments were examined for labeled cell bodies. Four of these segments (D3–D7) are located in the middle of the body between the limb enlargements; two of these segments (D8–D9) are the most rostral segments of the five-segment hindlimb enlargement (D8–D10, S1–S2). C, cervical; D, dorsal; S, sacral; Ca, caudal.

hours at 4°C. The solution was then changed to 20% sucrose in phosphate buffer; after several hours, the cord was unpinned. When the tissue sank, the spinal cord was removed and cut into blocks, which were placed in an embedding medium (0.25% gelatin, 20% powdered egg albumin, 10% sucrose, in 0.1 M phosphate buffer, pH 7.4) for 12–36 hours at 4°C. The embedding medium was then hardened by addition of glutaraldehyde to a final concentration of 1.25%. The embedded tissue was frozen and serially sectioned on a sliding microtome. Some cases were sectioned transversely at 50 μ m, with every twentieth section kept. Other cases were sectioned horizontally at 80 μ m, with all sections kept; in these cases, the region of the injection site was usually sectioned transversely at 80 μ m for easier identification of the injection site. Sections were processed with diaminobenzidine and hydrogen peroxide, using cobalt-nickel intensification (Adams, 1981). Sections were dipped in Albrecht's solution (0.75% gelatin in 40% ethanol) to increase adhesiveness and mounted on gelatin-

coated slides. Mounted sections were air dried, fixed (to bind the gelatin), dehydrated through graded ethanols, cleared in xylenes, and coverslipped with DPX mountant. Some horizontally sectioned sections were also lightly counterstained with cresyl violet.

Analysis

Each effective injection site within the white matter was defined by the bundle of labeled axons just rostral to the injection. In some cases, injection sites were reconstructed in the transverse plane from horizontal sections. Labeled cell bodies in the D3–D8 segments were marked or counted with darkfield illumination, using brightfield illumination to settle ambiguities. A cell body was counted only if it was clearly labeled and if it had a labeled dendrite connected to it. Transversely sectioned cases were used for illustration of the precise location of labeled cell bodies (Figs. 2–4), with all labeled cell bodies in every twentieth section marked on standard segmental transverse sections (Fig. 4). Labeled cell bodies could not be precisely localized within cytoarchitectonic "laminae" or "areas" in the transverse plane. Previous investigators have found laminar distinctions to be unclear and inconsistent in the spinal cords of turtles (Kusuma et al., 1979; Kusuma and ten Donkelaar, 1980). We have also found laminar distinctions to be unclear and inconsistent in red-eared turtles; thus we provide only a broad description of the locations of labeled cell bodies in terms of cross-sectional areas. Horizontally sectioned cases were used for the quantitative descriptions (Figs. 5, 6), with all labeled cell bodies in all sections counted. Each segmental boundary was defined as the point midway between the midpoints of segmental groups of dorsal and ventral rootlets. Sections were mounted in alignment to one another to facilitate the definition of segmental boundaries in sections that did not contain dorsal or ventral roots. The intermediate zone was defined as the gray matter region surrounding the central canal and its extension directly lateral to the edge of the gray matter (Fig. 6). The dorsal horn and the ventral horn were defined as the gray matter regions above and below the intermediate zone, respectively. For several cases, the dorsal horn, intermediate zone, and ventral horn on each side were further divided into medial and lateral halves for separate counts.

Each injection was made at the border between the D9 and D10 spinal segments; the D3–D8 segments were examined for labeled neuronal cell bodies. All, or nearly all, of the labeled D3–D8 neurons can be assumed to be propriospinal neurons, rather than motoneurons or preganglionic neurons. Labeling of spinal segmental nerves with HRP (Ruigrok, 1984) and intracellular labeling of motoneurons with HRP (Ruigrok et al., 1984) have demonstrated that motoneuron axons exit the spinal cord via the nearest ventral root in turtles, and motoneuron axon collaterals are rare (Ruigrok et al., 1984). In addition, the dendrites of spinal motoneurons in turtles do not extend more than 1.5 mm rostrally or caudally from the cell body (Ruigrok et al., 1984), whereas the D9 segment alone has a rostrocaudal length of 3–5 mm. Thus few if any motoneurons outside the D9 and D10 spinal segments would have been labeled in this study.

The axonal projections of preganglionic neurons have been studied only briefly in turtles (Kusuma and ten Donkelaar, 1979). However, it has been demonstrated in a variety of vertebrates that sympathetic preganglionic axons exit the spinal cord through the ventral root of the same

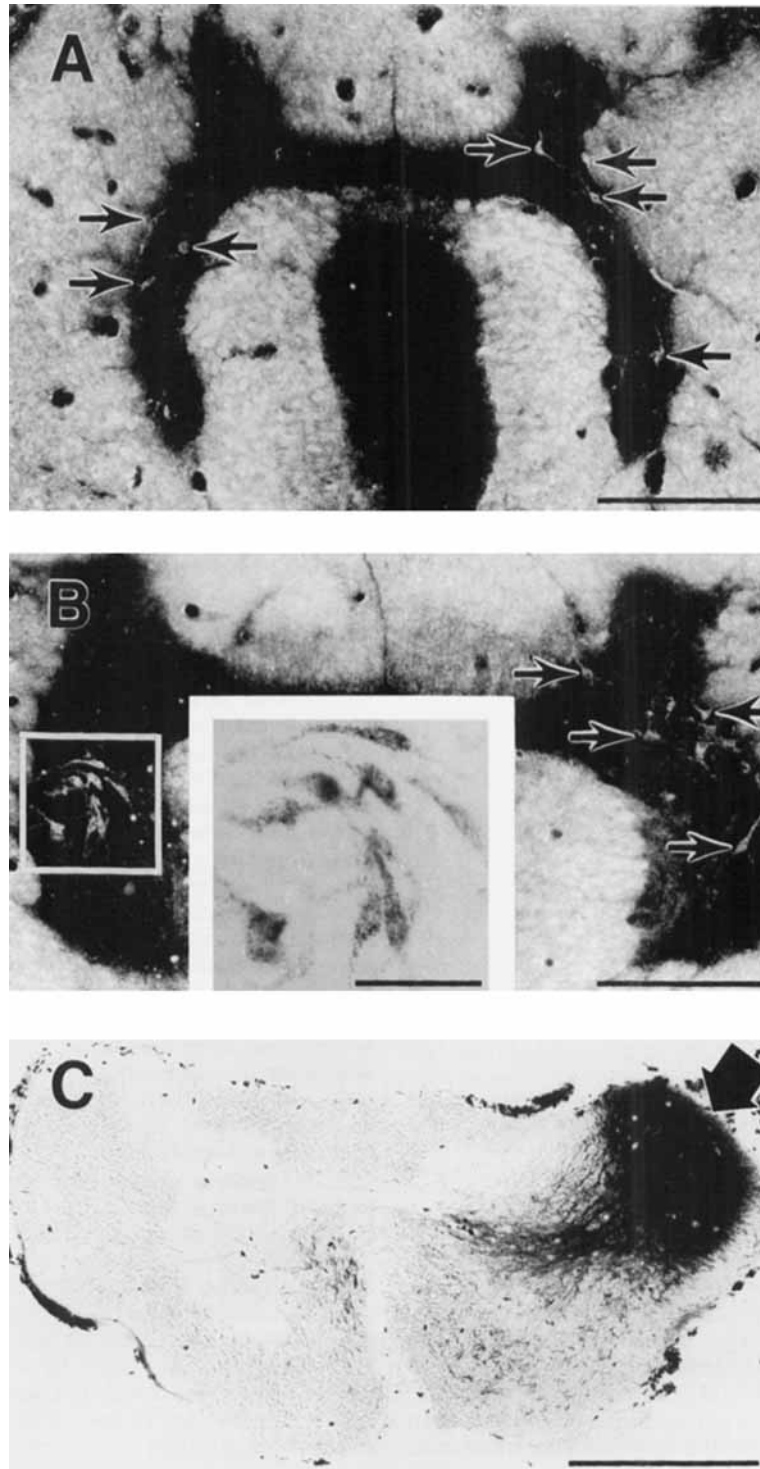


Fig. 2. Examples of labeled cell bodies following an HRP injection into the lateral part of the lateral funiculus. **A,B:** Labeled cell bodies in transverse sections through the D5 (A) and D7 (B) segments, with darkfield illumination. The arrows indicate labeled cell bodies. In B, for clarity, arrows indicate only four of the cell bodies that were labeled

ipsilaterally. **Inset** in B shows labeled cell bodies in the contralateral ventral horn at higher magnification, with brightfield illumination. **C:** Transverse section just rostral to the injection site in caudal D9, with brightfield illumination. The arrow indicates the effective injection site. Scale bars = 200 μm in A and B, 500 μm in C, 50 μm in inset.

segment or occasionally an adjacent segment (frogs: Szekely, 1976; Horn and Stofer, 1988; mammals: Cabot, 1990; Forehand, 1990). Before reaching the ventral root, pregan-

glionic axons travel through the ventral horn or along the border of the ventral horn and the lateral funiculus (Rethelyi, 1972; Szekely, 1976; Dembowski et al., 1985; Horn and

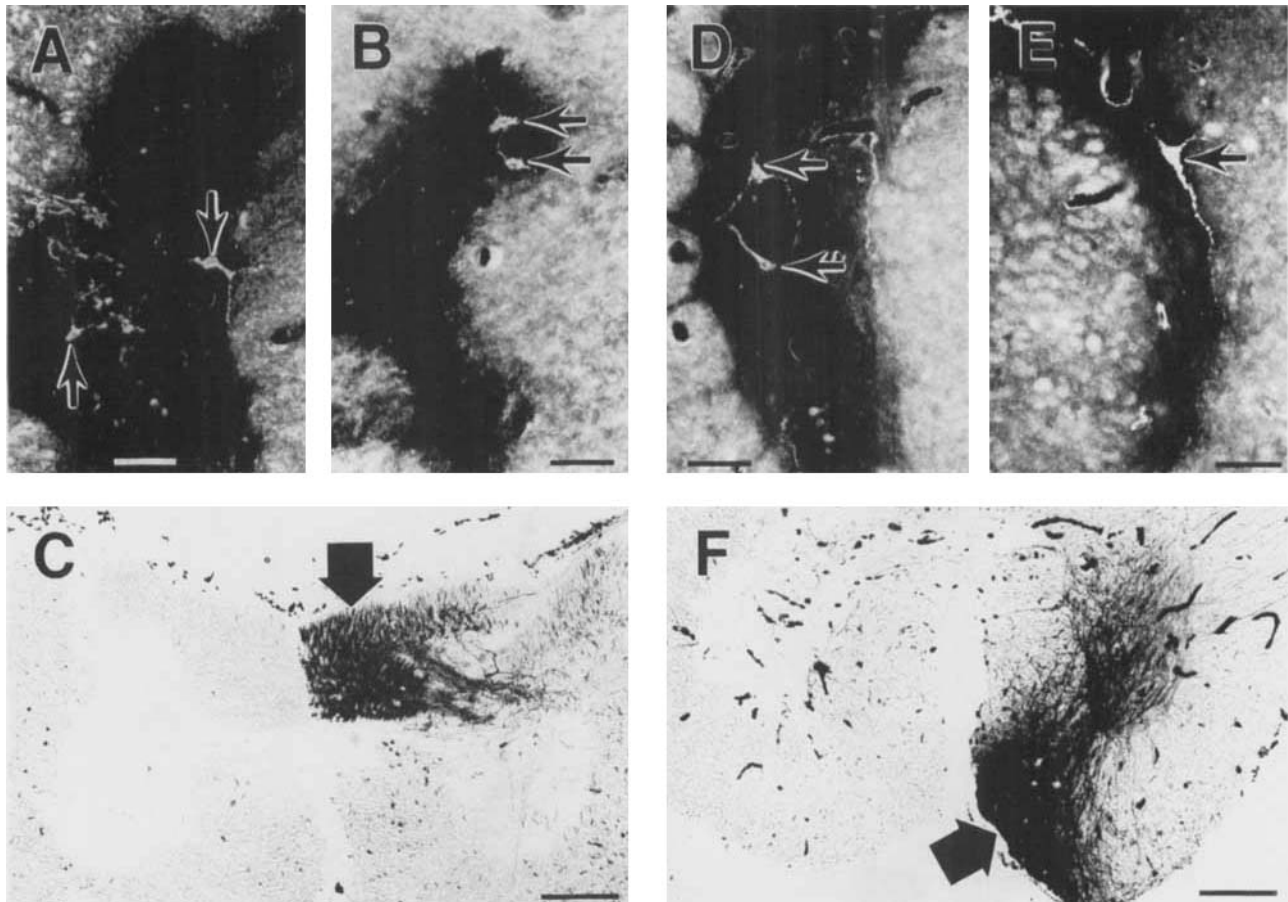


Fig. 3. Examples of labeled cell bodies following HRP injections into the dorsal funiculus (A–C) and ventral funiculus (D–F). A, B, D, and E are transverse sections, with darkfield illumination. A and B show labeled cell bodies in the ipsilateral dorsal horn of the D7 and D5 segments, respectively. D and E show labeled cell bodies in the D7

contralateral ventral horn and the D5 ipsilateral ventral horn, respectively. Thin arrows indicate labeled cell bodies. C and F are transverse sections just rostral to the injection sites in caudal D9, with brightfield illumination. Thick arrows indicate the effective injection sites. Scale bars = 50 μ m in A, B, D, and E, 200 μ m in C and F.

Stofer, 1988; Forehand, 1990); only one HRP injection (case H in Figs. 5–6) was made into this region in the current study. Although sympathetic preganglionic neurons occasionally have axon collaterals, these collaterals are found locally in the gray matter and nearby white matter (Cabot and Bogan, 1987; Bogan and Cabot, 1991). Thus, data from other vertebrates strongly suggest that D3–D8 preganglionic neurons would not have been labeled by an HRP injection more than one segment away. If any D3–D8 motoneurons or preganglionic neurons were labeled, they would almost certainly have been in the D8 segment. Because the labeling patterns in the D8 segment were very similar to the labeling patterns in the D3–D7 segments (see Fig. 4), it is unlikely that substantial numbers of motoneurons or preganglionic neurons were labeled in the D8 segment either.

RESULTS

The appearances of injection sites and labeled neurons are described below, followed by accounts of the locations of labeled cell bodies, the existence of labeled white-matter dendrites, and the courses of labeled axons.

General characteristics of labeling

Injection sites. Thirty-one animals received HRP injections to the spinal cord. In 20 cases, the injections were restricted or nearly restricted to a small region of the white matter. These included eight transversely sectioned cases, three of which are presented in Figures 2–4, and 12 horizontally sectioned cases. Ten of these horizontally sectioned cases were analyzed quantitatively, with all labeled cell bodies in the D3–D8 segments counted (Figs. 5, 6). In the remaining five transversely sectioned cases and two horizontally sectioned cases, the distributions of labeled cell bodies were qualitatively consistent with the patterns described in Figures 4–6. In the other 11 cases, the injection sites appeared to involve substantial amounts of gray matter. These cases were only qualitatively examined for labeled cell bodies. The distributions of labeled cell bodies in these 11 cases appeared to be consistent with the distributions seen for cases with similar injections confined to the white matter.

Each effective injection site in the white matter (Figs. 4–6) was defined by the bundle of labeled axons seen just rostral to the injected area. In most cases, this appeared to

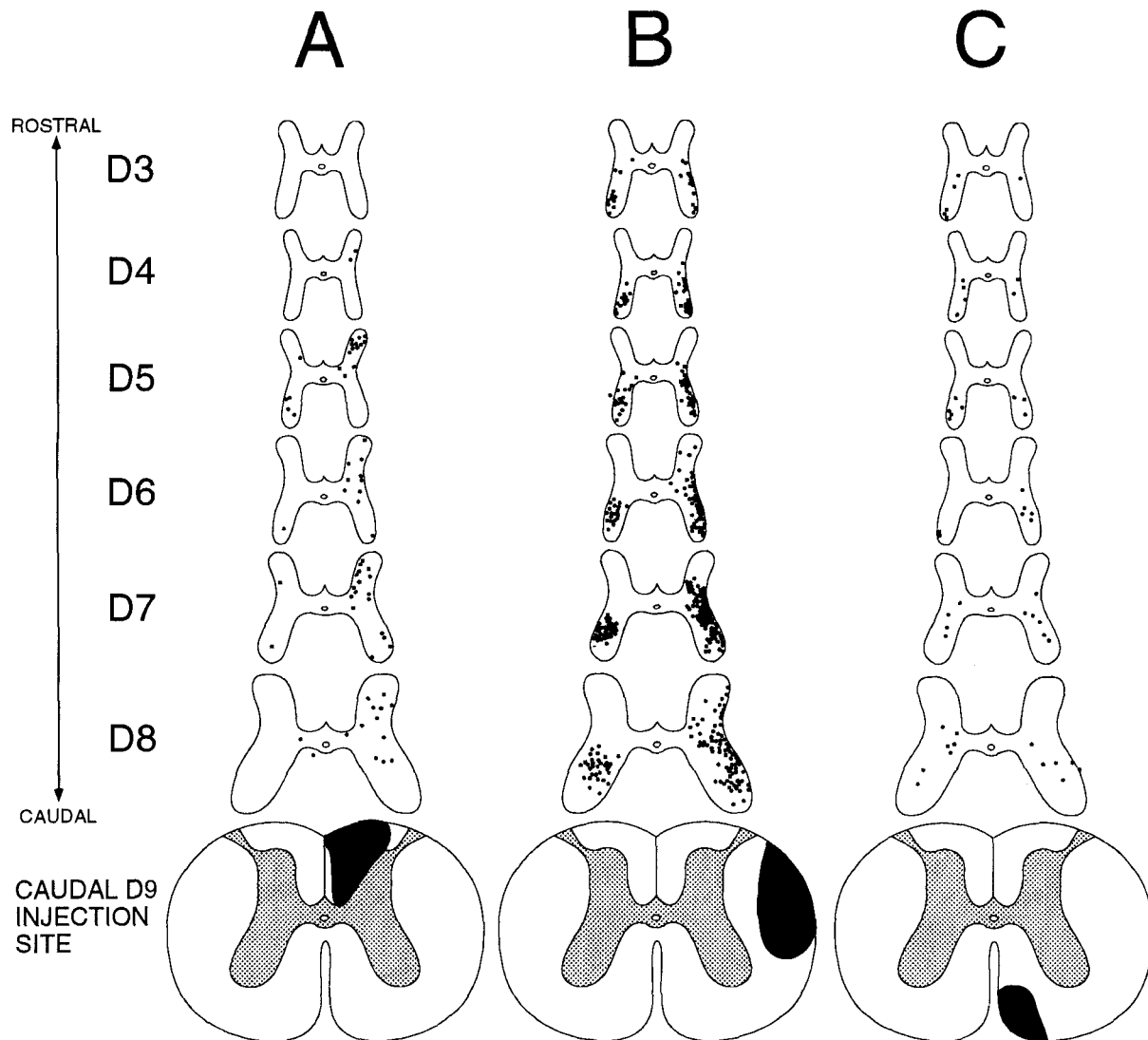


Fig. 4. Localization of descending propriospinal cell bodies labeled by HRP injections into the dorsal funiculus (A), lateral funiculus (B), and ventral funiculus (C). All labeled cell bodies in every twentieth 50 μ m transverse section were plotted on sketches of segmental cross

sections. Each solid circle represents one cell body. The black regions on the D9 sections represent the effective HRP injection sites. Photomicrographs from the cases illustrated in A–C are shown in Figures 3A–C, 2A–C, and 3D–F, respectively.

be the same region of white matter in which reaction product could be seen in sections through the middle of the injection site. However, spinal motoneurons (Fig. 7C; see also Banchi, 1903; Ruigrok et al., 1984), unidentified spinal neurons (Banchi, 1903), and propriospinal neurons (Fig. 7A,B) in turtles have extensive dendritic fields in the white matter; in sections through the injection site, most neuronal cell bodies in the nearby gray matter appeared to be heavily labeled, along with their dendrites (data not shown). Most were probably labeled via their dendrites. These labeled dendritic fields often obscured the regions of reaction product and labeled axons, making the determination of an effective injection site unreliable in these sections. About 1 mm rostral to the center of the injection, the bundle of labeled axons could be seen and described reliably.

In some iontophoretically injected cases, the bundle of labeled axons was substantially smaller than the apparent

region of reaction product, especially in early experiments in which less attention was given to damaging axons at the injection site. Thus the effective injection site may have been influenced more by the region in which axons were damaged than the region through which HRP diffused. This inference is supported by the results of a pilot experiment (data not shown) in which the spinal cord was completely transected between the D9 and D10 dorsal roots and a tiny pledget of HRP-Gelfoam inserted into a small region of white matter in the cut face. In this case, axons were labeled throughout the ipsilateral white matter and much of the contralateral white matter, with the density of labeling declining progressively away from the HRP-Gelfoam site.

Labeled cell bodies. Labeled cell bodies in the D7–D9 spinal segments were either darkly and diffusely labeled (Figs. 7A,B, 8C) or lightly and granularly labeled (Figs. 2B, 3A,D). Labeled cell bodies in the D3–D6 segments were

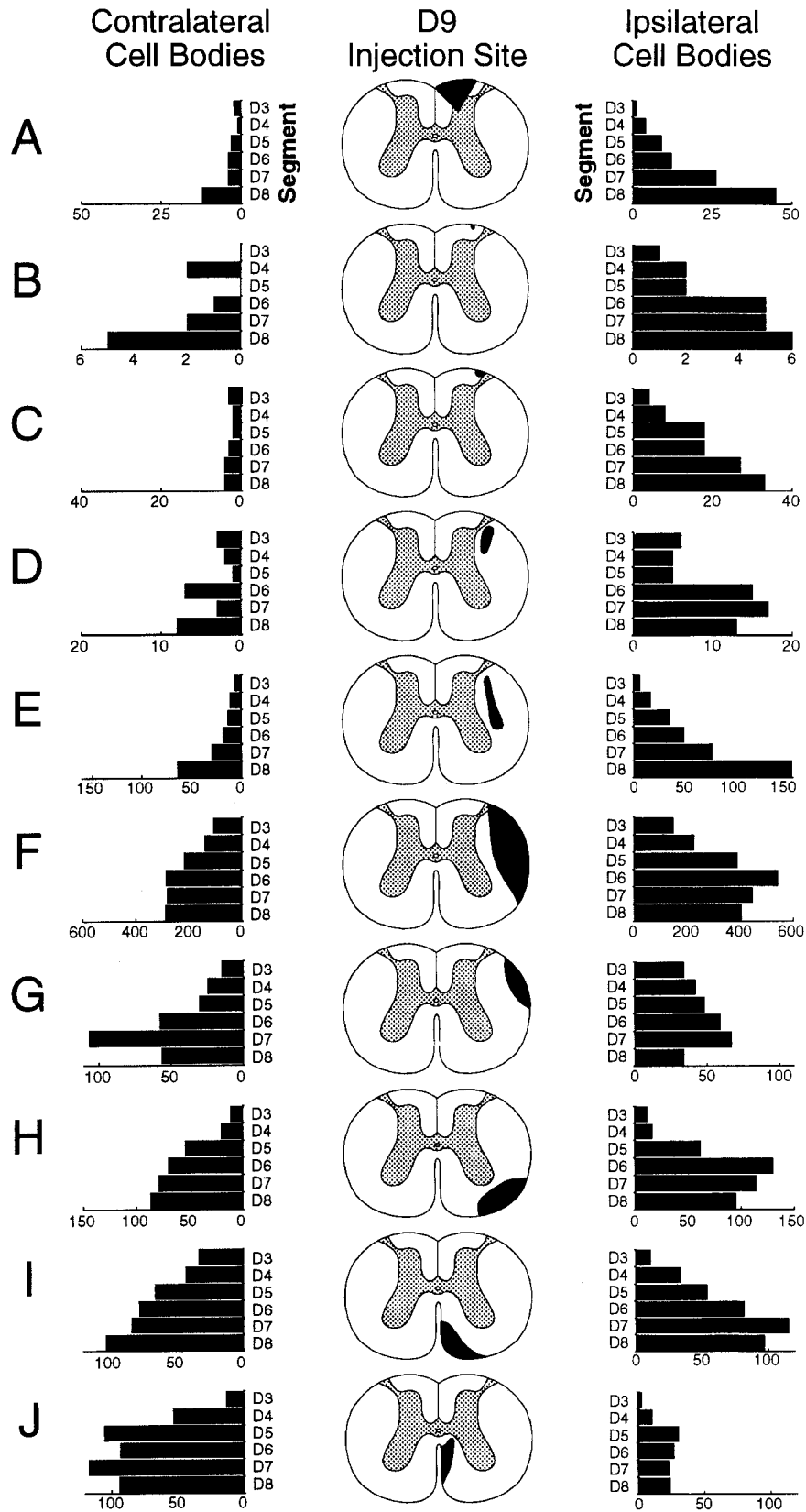


Fig. 5. Segmental distributions of labeled cell bodies following white-matter HRP injections in ten animals (A–J). The center column depicts the caudal D9 effective injection sites on sketches of D9 cross sections, from dorsomedial (top) clockwise to ventromedial (bottom).

The black regions show the effective injection sites. For each case, the total number of labeled cell bodies ipsilaterally (right column) and contralaterally (left column) is given for each of the D3–D8 segments. All labeled cell bodies were counted in all 80 μ m horizontal sections.

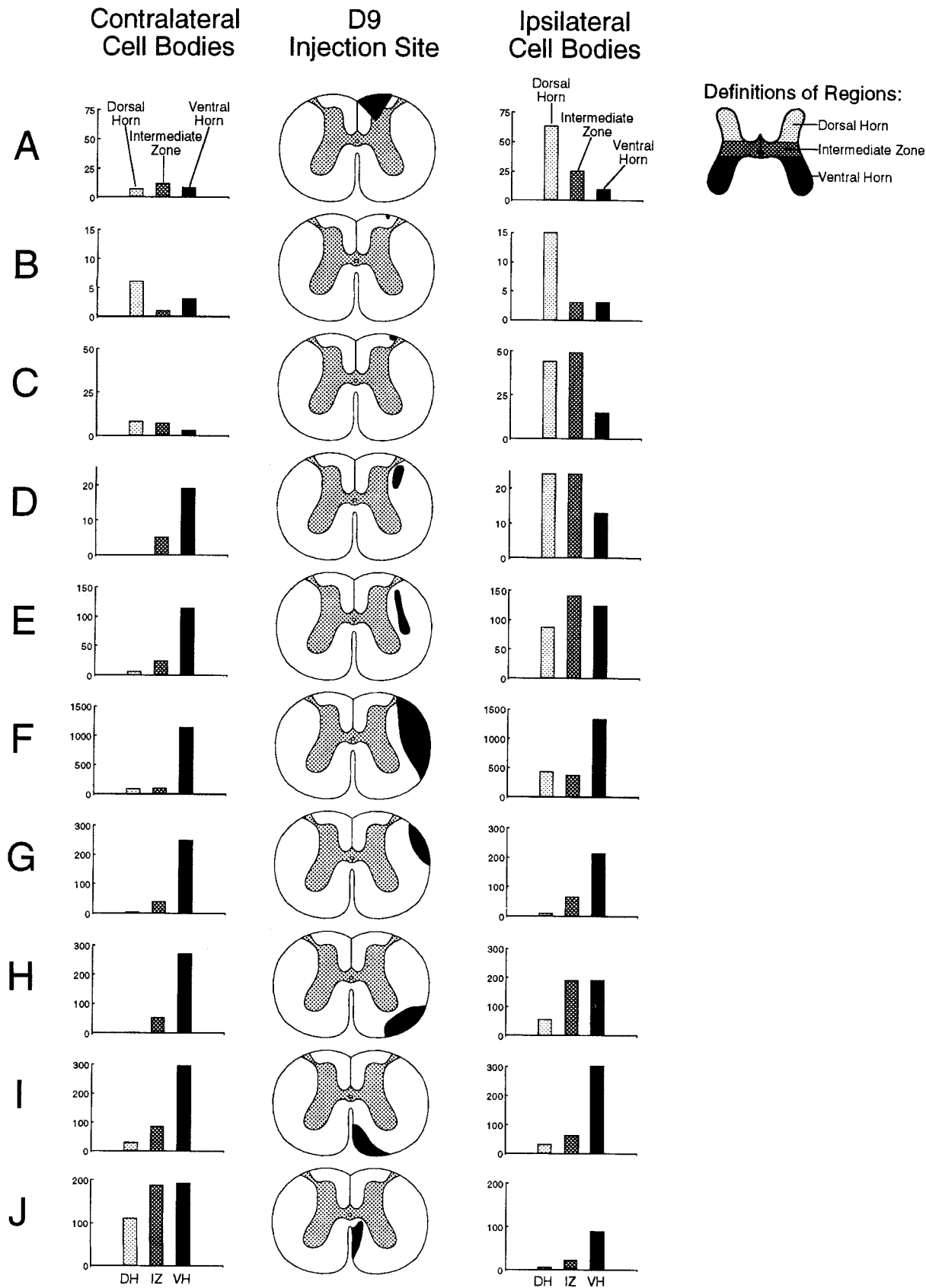


Figure 6

always granularly labeled (Figs. 2A, 3B,E). Diffusely labeled cell bodies were seen most clearly with brightfield illumination; granularly labeled cell bodies were seen most clearly with darkfield illumination.

Labeled cell bodies exhibited a very wide range of sizes and shapes, but most were ovoid, with a long diameter of 15–40 μm , and were bipolar or multipolar. The largest cell bodies tended to be multipolar. Many of these were found in the intermediate zone. However, in every region of gray matter, the sizes of labeled cell bodies encompassed a very wide range. No attempt was made to quantify the distributions of labeled cell body sizes.

Distributions of labeled cell bodies

Every injection into the white matter labeled descending propriospinal cell bodies, whether the injection was in the dorsal funiculus, lateral funiculus, or ventral funiculus, and whether it was near or far from the gray matter (Figs. 4–6). In addition, every injection gave rise to a very wide distribution of labeled cell bodies over the six spinal segments examined (Figs. 4–6). Every injection labeled cell bodies in the ipsilateral dorsal horn, intermediate zone, and ventral horn as well as the contralateral intermediate zone and ventral horn (Figs. 4–6). Labeled cell bodies were not placed in “laminae,” because laminar descriptions are unreliable in reptilian spinal cords (see Materials and Methods). In general, descending propriospinal cell bodies were concentrated in the ipsilateral deep dorsal horn, intermediate zone, and dorsal part of the ventral horn as well as the contralateral intermediate zone and ventral horn. Each white-matter region injected gave rise to a characteristic and reliable distribution of labeled cell bodies. Thus injections in the dorsal funiculus labeled cell bodies mostly in the ipsilateral dorsal horn and intermediate zone (Figs. 3A–C, 4A, 6A–C). Injections in the medial part of the lateral funiculus gave rise to a similar distribution of labeled cell bodies, except that many ventral horn cell bodies were also labeled bilaterally (Fig. 6D,E). In contrast, injections in the lateral part of the lateral funiculus gave rise to a more ventral distribution of cell bodies ipsilaterally. Labeled cell bodies were concentrated in the ipsilateral deep dorsal horn, intermediate zone, and ventral horn, as well as the contralateral ventral horn (Figs. 2, 4B, 6F,G). Finally, injections in the ventral funiculus labeled as many contralateral cell bodies as ipsilateral cell bodies, if not more (Figs. 3D–F, 4C, 6I,J). Cell bodies were concentrated in the ventral horn bilaterally, but could also include substantial numbers of contralateral intermediate zone and dorsal horn neurons.

The transversely sectioned case illustrated in Figure 4B strongly suggests that the ipsilateral cell bodies of descending propriospinal axons in the lateral part of the lateral funiculus are concentrated in the lateral part of the gray

matter. To test this suggestion more rigorously, mediolateral distributions of labeled cell bodies were quantified for four representative horizontally sectioned cases, one each with an injection in the lateral part of the lateral funiculus (case F in Figs. 5, 6), the medial part of the lateral funiculus (case E), the dorsal funiculus (case A), and the ventral funiculus (case I). The dorsal horn, the intermediate zone, and the ventral horn on each side were each divided into medial and lateral halves and the total number of labeled cell bodies in each division counted. Indeed, 81% of the ipsilateral cell bodies labeled in case F were in the lateral half of the gray matter. On the contralateral side, 67% were in the lateral half. One might expect, then, that injections in another region of spinal white matter would have labeled a mostly medial distribution of descending propriospinal cell bodies. However, this appears not to have happened. In case A, 60% of labeled cell bodies were in the lateral half ipsilaterally and 62% contralaterally. In case E, 84% were lateral ipsilaterally and 69% contralaterally. In case I, 76% were lateral ipsilaterally and 69% contralaterally. Thus cell bodies with descending propriospinal axons in each white-matter region tend to be concentrated in the lateral parts of the gray matter, especially ipsilaterally.

Any comparison of the absolute numbers of labeled cell bodies from different cases must be tentative, due to the variety of injection techniques used and possible variations in the robustness of HRP transport in different animals. Nonetheless, it does appear that injections into the lateral funiculus labeled greater numbers of cell bodies than injections into either the dorsal funiculus or ventral funiculus (Figs. 2–6). This was true for both medial and lateral injections into the lateral funiculus. In addition, injections into the ventral funiculus generally labeled more cell bodies than injections of similar size into the dorsal funiculus.

The number of labeled cell bodies was generally reduced in the more rostral spinal segments examined (Figs. 4, 5). This could be due to decreased transport of HRP over long distances, as the distance from the injection site to the D3 segment was 6–7 cm in these turtles. However, those cell bodies that were labeled in the D3 and D4 segments were generally just as heavily labeled as those in the D5 and D6 segments. Therefore, the decline in numbers of labeled cells rostrally may reflect a prevalence of short, descending propriospinal axons over long, descending propriospinal axons. The number of funicular descending propriospinal axons derived from cell bodies at a given rostrocaudal level may gradually decline caudally as axons terminate. Although every white-matter injection labeled cell bodies in every segment, some injections (cases A, C, and E) clearly labeled a peak number of ipsilateral cell bodies in the D8 segment, whereas others (cases F–I) clearly peaked in the D6 or D7 segment. Cases F–I all involved injections in the outer regions of the lateral funiculus or ventral funiculus. This observation provides evidence that descending propriospinal axons far from the gray matter tend to be derived from cell bodies further rostrally than are axons near the gray matter.

White-matter dendrites

Descending propriospinal cell bodies in the D7–D9 segments were occasionally labeled very heavily, along with substantial parts of their dendritic trees. Both ipsilateral (Fig. 7A) and contralateral (Fig. 7B) descending propriospinal neurons often had extensive dendritic trees in the white matter, especially in the lateral and ventral funiculi. In one

Fig. 6. Distributions of labeled cell bodies in the transverse plane, following white-matter HRP injections in ten animals (A–J). The center column depicts the caudal D9 injection sites on sketches of D9 cross sections, from dorsomedial (top) clockwise to ventromedial (bottom). The black regions show the effective injection sites. For each case, the total number of labeled cell bodies ipsilaterally (right column) and contralaterally (left column) is given for the dorsal horn, the intermediate zone, and the ventral horn. A sketch illustrating the definitions of these regions is shown in the top right (see Materials and Methods). All labeled cell bodies were counted in all 80 μm horizontal sections. A–J are the same cases illustrated in Figure 5A–J, respectively.

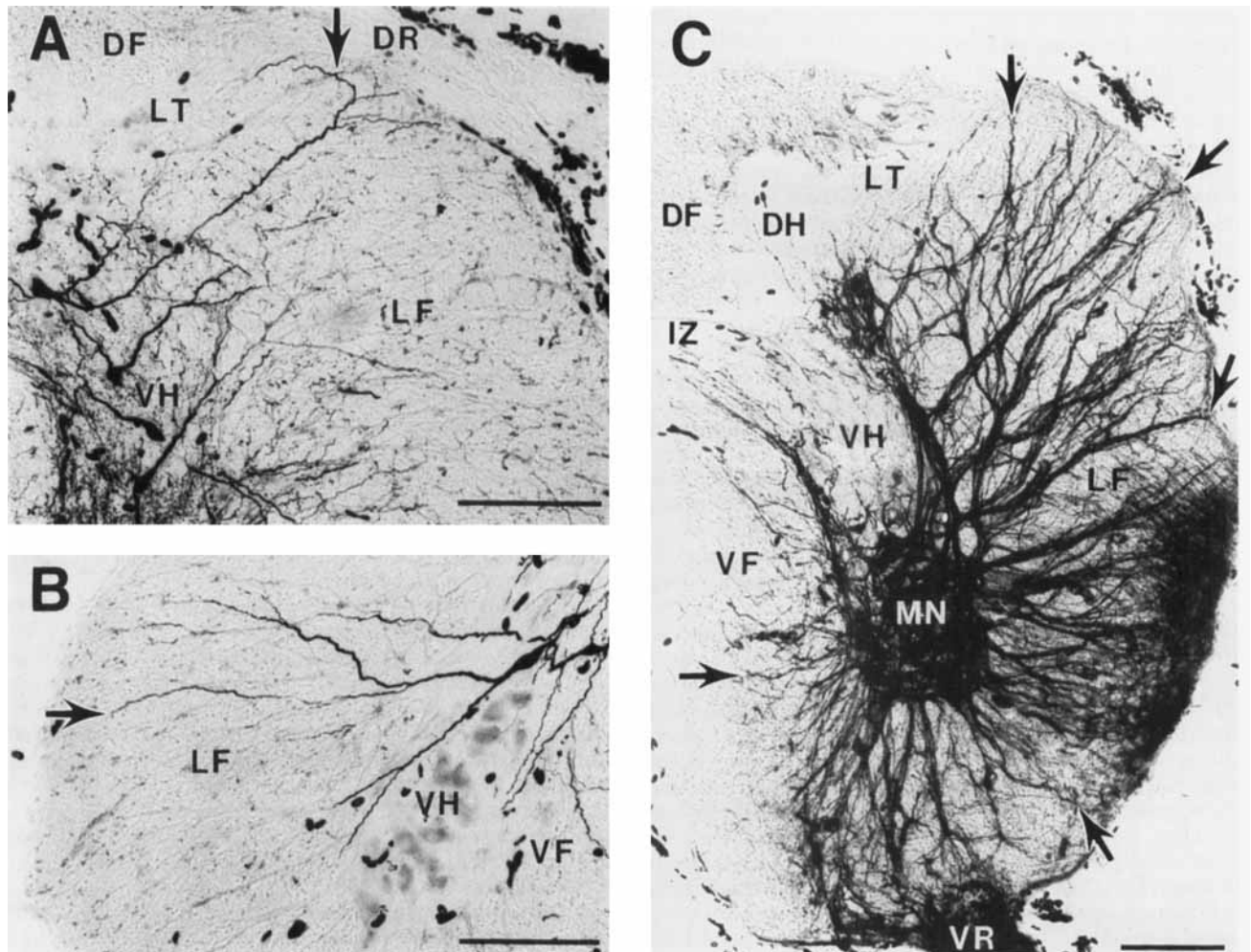


Fig. 7. Examples of white-matter dendrites of spinal neurons. **A,B:** Darkly labeled descending propriospinal neurons in (counterstained) transverse sections through the right (A) and left (B) D9 spinal cord, rostral to an HRP injection in the right ventral funiculus. Arrows indicate some of the white-matter dendrites that reach deeply into the (A) dorsal part and (B) ventral part of the lateral funiculus. **C:** Labeled D9 motoneurons in a transverse section (not counterstained), following

HRP labeling of the right D9 ventral roots. Arrows indicate some of the white-matter dendrites that reach deeply into the lateral funiculus and ventral funiculus. Dorsal is upward; right is right. DH, dorsal horn; IZ, intermediate zone; VH, ventral horn; DF, dorsal funiculus; LT, Lissauer's tract; LF, lateral funiculus; VF, ventral funiculus; DR, dorsal root; VR, ventral root; MN, motoneuron cell bodies. Scale bars = 200 μ m.

experiment, D9 segmental motoneurons were unilaterally labeled with HRP by injection of the D9 ventral rootlets (see Materials and Methods). The motoneuron cell bodies and dendrites were heavily labeled. The dendritic field of this set of motoneurons (Fig. 7C) clearly included most of the ipsilateral lateral funiculus and some of the ipsilateral ventral funiculus.

Labeled axons

Only labeled cell bodies were analyzed in detail in this study because a spinal cell body retrogradely labeled by a more caudal HRP injection must be a descending propriospinal neuron, as it has both a cell body and an axon contained within the spinal cord. (It may also have an ascending axon.) However, a spinal axon labeled rostral to a white-matter HRP injection could be either propriospinal or supraspinal and either descending or ascending. Thus, we could not selectively follow labeled descending propriospinal axons within the white matter. However, our data

provide suggestive evidence about courses taken by spinal axons in general. The bundle of heavily labeled axons seen just rostral to each injection gradually spread out rostrally, until labeled axons could be seen in all three funiculi on both sides (data not shown), before axonal labeling became too weak to observe, in the D7 or D6 segment. These data suggest that some spinal axons do not maintain a particular position within the white matter throughout their course. More evidence for this comes from examples of individual labeled axons that, in opportune sections, could be followed for some rostrocaudal distance without being confounded with other labeled axons. In these cases, axons occasionally changed their cross-sectional location within a funiculus (Fig. 8A) or, less frequently, switched from one funiculus to another (Fig. 8B).

Funicular axons may synapse with the extensive white-matter dendrites (Fig. 7) of spinal neurons. In fact, axonal swellings, possibly en passant synaptic boutons, were observed on labeled axons in both the gray matter and the

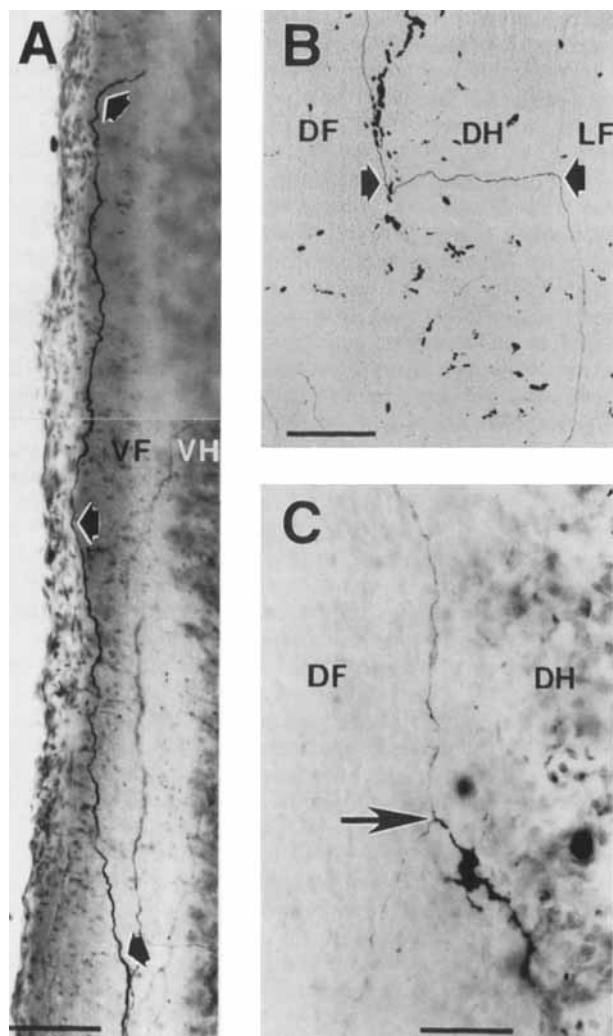


Fig. 8. Examples of labeled axons. **A:** Photomontage of a (counterstained) horizontal section at two focal planes, showing a labeled axon that changes its mediolateral position along its rostrocaudal course through the D9 ventral funiculus. Arrows indicate this axon. **B:** Horizontal section (not counterstained) showing a labeled axon switching funiculi along its rostrocaudal course in the D9 segment. Arrows indicate this axon. **C:** Horizontal section (counterstained) through a labeled D9 dorsal horn neuron with an axon that bifurcates into ascending and descending branches in the dorsal funiculus. The arrow indicates the bifurcation point. In A–C, rostral is upward; lateral is right. DF, dorsal funiculus; LF, lateral funiculus; VF, ventral funiculus; DH, dorsal horn; VH, ventral horn. Scale bars = 100 μ m in A, 200 μ m in B, 50 μ m in C.

white matter, including the lateral part of the lateral funiculus (Fig. 9). These swellings typically occurred along thin collaterals of the main axon (Fig. 9B,C); sometimes, they occurred along the main axon as well (Fig. 9B).

DISCUSSION

Every HRP injection into the spinal white matter labeled a substantial number of neuronal cell bodies in spinal cord segments rostral to the injection. These data demonstrate that all major regions of the white matter in the hindlimb enlargement of the red-eared turtle contain substantial

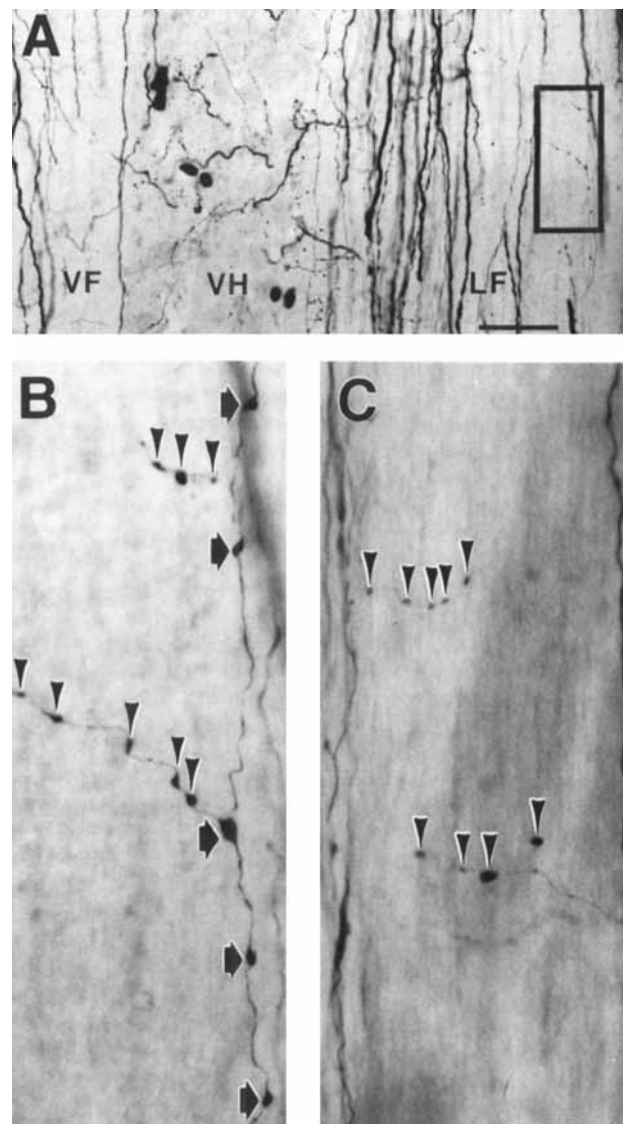


Fig. 9. Putative synaptic boutons in the white matter. **A:** Horizontal section (counterstained) through the right side of the D8 spinal cord, showing axonal swellings, perhaps synaptic boutons, in both the gray matter and the white matter. The axons were labeled by an HRP injection into the medial part of the right lateral funiculus in the caudal D9 segment. Rostral is upward; the lateral edge of the spinal cord is at right. **B:** The boxed area in A, at higher magnification, showing labeled axonal swellings in the lateral part of the lateral funiculus. Arrowheads indicate swellings along collaterals; arrows indicate swellings along the main axon. **C:** Horizontal section (counterstained) showing labeled axonal swellings in the lateral part of the right D8 lateral funiculus of another case, in which HRP was injected into the middle of the right lateral funiculus in the caudal D9 segment. VF, ventral funiculus; VH, ventral horn; LF, lateral funiculus. Scale bars = 100 μ m in A, 20 μ m in B and C.

numbers of descending propriospinal axons. This result is consistent with the results of successive anterograde degeneration studies in dogs (Sherrington and Laslett, 1903) and turtles (Kusuma and ten Donkelaar, 1980). In those experiments, the rostral-most spinal cord was completely transected (Sherrington and Laslett, 1903) or hemisected (Kusuma and ten Donkelaar, 1980); after a period of at least 9

months, during which all supraspinal fibers degenerated, a second, more caudal lesion was made in the spinal cord. The anterograde courses of degenerating descending propriospinal axons were examined following the second spinal lesion. Degenerating fibers were seen throughout the lateral and ventral funiculi and in parts of the dorsal funiculus. Our results are also consistent with a recent study (Alstermark et al., 1990) in which wheat germ agglutinin conjugated to HRP was injected into the lateral parts of lamina VI–VII in the cat cervical spinal cord. Labeled descending axons were found both near and far from the gray matter in the lateral and ventral funiculi (Fig. 6C in Alstermark et al., 1990). In addition, physiological experiments have confirmed that descending propriospinal axons are found both near and far from the gray matter. Two physiological studies in cats used white-matter electrical stimulation to measure the distribution of descending propriospinal axons in the lateral funiculus (Jankowska et al., 1974; Hoskin et al., 1988). Both studies indicated that descending propriospinal axons are found both near and far from the gray matter in the lateral funiculus. Finally, single descending propriospinal axons were recently recorded both near and far from the gray matter in the dorsal and lateral funiculi of the red-eared turtle (Berkowitz, 1993; Fig. 10 in Berkowitz and Stein, 1994a). Many of these descending propriospinal axons could be excited by stimulation of the contralateral body surface (Berkowitz, 1993; Berkowitz and Stein, 1994a); this fits well with the current finding that many descending propriospinal projections are crossed. Crossed propriospinal projections may also be involved in bilateral interactions underlying scratching, locomotion, and withdrawal of all four limbs (Berkowitz and Stein, 1994b).

Our results are inconsistent with interpretations (Nathan and Smith, 1959; Brodal, 1981) of experiments in which the lumbosacral spinal cord of a dog (Tower, 1937) or monkey (Tower et al., 1941) was isolated by spinal cord and dorsal root transections and examined 1–2 weeks (Tower et al., 1941) or 5–6 months (Tower, 1937) later to determine the original courses of propriospinal fibers. Tower et al. (1941) assumed that supraspinal fibers would degenerate and propriospinal fibers would remain. Their illustration actually showed “healthy appearing fibers” spread throughout the white matter but concentrated near the gray matter (Fig. 6 in Tower et al., 1941). In later reviews and textbooks (Nathan and Smith, 1959; Crosby et al., 1962; Brodal, 1981; Carpenter and Sutin, 1983; Carpenter, 1991; see also Chung and Coggeshall, 1983, for a discussion of this literature), propriospinal fibers were depicted as a tract immediately adjacent to the gray matter. Experiments that isolate a few mammalian spinal cord segments (Tower, 1937; Tower et al., 1941; Chung and Coggeshall, 1983, 1988) are very hard to interpret because 1) mammalian supraspinal fibers take about 9 months to degenerate completely (Sherrington and Laslett, 1903); 2) there are many long propriospinal fibers (Burton and Loewy, 1976; Molenaar and Kuypers, 1978; Matsushita et al., 1979; Skinner et al., 1979; Kusuma and ten Donkelaar, 1980; Yezierski et al., 1980; Menetrey et al., 1985; Cassidy and Cabana, 1993; Martin et al., 1993), which may also degenerate; 3) there are several classes of spinal neurons with both spinal and supraspinal axon collaterals (Verburch and Kuypers, 1987; Bras et al., 1988; Skinner et al., 1989), which may or may not degenerate; and 4) much rearrangement of surviving axons may occur. Therefore, such experi-

ments may not provide an accurate indication of the normal distribution of descending propriospinal axons.

In combination with previous studies in turtles (Kusuma and ten Donkelaar, 1980; Berkowitz and Stein, 1990, 1991, 1994a; Berkowitz, 1993), cats (Jankowska et al., 1974; Hoskins et al., 1988; Alstermark et al., 1990), and dogs (Sherrington and Laslett, 1903), the current results suggest that descending propriospinal axons may be widely distributed throughout all three funiculi in vertebrates generally. This suggestion conflicts with the contemporary view (Brodal, 1981; Carpenter and Sutin, 1983; Carpenter, 1991) that descending propriospinal axons are concentrated in or limited to fasciculi proprii close to the gray matter. However, there is little experimental evidence that specifically reveals the funicular courses of descending propriospinal fibers in mammals, especially in primates. Experiments involving small injections of a neuronal tracer into distinct regions of the spinal cord in various mammals would be of help in resolving this issue. For example, a retrograde tracer could be injected into a small region of the spinal white matter, as in the current study. Alternatively, an exclusively anterograde tracer could be injected into a small region of the spinal gray matter and the courses of labeled descending propriospinal axons viewed directly.

It is not clear why the results of the Sherrington and Laslett (1903) experiments were not more influential in shaping the contemporary view. It may be that the experiments were done so long ago that they are rarely read today or that current researchers have little confidence in the technique of anterograde degeneration. Another possibility is that the Sherrington and Laslett work is not taken seriously because the authors mistakenly concluded that long, descending propriospinal projections are solely ipsilateral. This conclusion has been refuted based on several studies using unilateral injections of a retrograde neuronal tracer (Burton and Loewy, 1976; Molenaar, 1978; Molenaar and Kuypers, 1978; Matsushita et al., 1979; Kusuma and ten Donkelaar, 1980; Menetrey et al., 1985; Hongo et al., 1989; Cassidy and Cabana, 1993; Martin et al., 1993; current study). However, it may be that this conclusion by Sherrington and Laslett is not a reflection of the limitations of their particular technique or carelessness in its application but rather is the necessary outcome of any technique that involves anterograde tract tracing of descending propriospinal fibers (see also Fig. 4B in Kusuma and ten Donkelaar, 1980). In combination with the experiments demonstrating crossed propriospinal projections, this suggests that most descending propriospinal axons that cross do so near their cell bodies. This hypothesis would explain why Sherrington and Laslett saw few degenerating fibers contralateral to the lesion: The vast majority of crossed descending propriospinal fibers had crossed rostral to the lesion site and were, in fact, derived from cell bodies on the opposite side. This would indicate a similarity in the decussation patterns of descending propriospinal axons and those of the spinothalamic and rubrospinal tracts, which also cross near their cell bodies (Brodal, 1981).

In the present study, each unilateral injection into the white matter labeled a very wide distribution of cell bodies, including the ipsilateral dorsal horn, intermediate zone, and ventral horn as well as the contralateral intermediate zone and ventral horn. Labeled cell bodies were found in all six spinal segments examined. In general, descending propriospinal cell bodies were concentrated in the lateral halves of the ipsilateral deep dorsal horn, intermediate

zone, and dorsal part of the ventral horn as well as in the contralateral intermediate zone and ventral horn. This appears to correspond to areas V–VI, VII–VIII, and X of Kusuma et al. (1979) bilaterally as well as area IV ipsilaterally. This distribution agrees well with previous studies that used unilateral spinal injections of a retrograde tracer in lizards (Kusuma and ten Donkelaar, 1980), turtles (Kusuma and ten Donkelaar, 1980), opossums (Cassidy and Cabana, 1993; Martin et al., 1993), rats (Menetrey et al., 1985), cats (Molenaar and Kuypers, 1978; Matsushita et al., 1979; Hongo et al., 1989), and monkeys (Burton and Loewy, 1976; Molenaar, 1978; Molenaar and Kuypers, 1978). One of the examples illustrated by Kusuma and ten Donkelaar (1980; their Fig. 7A, segment 14), however, showed that most descending propriospinal neurons with cell bodies in the dorsal horn (areas III and IV) and intermediate zone (areas V and VI) had crossed descending projections; in our experiments, most had uncrossed projections (Fig. 6). The previous studies all involved large HRP injections into both gray matter and white matter (Burton and Loewy, 1976; Molenaar and Kuypers, 1978; Matsushita et al., 1979; Kusuma and ten Donkelaar, 1980; Cassidy and Cabana, 1993; Martin et al., 1993) or injections into gray matter alone (Molenaar, 1978; Menetrey et al., 1985; Hongo et al., 1989). None of the injections was restricted to a particular region of white matter. Thus these experiments did not indicate the funicular courses of propriospinal axons arising from cell bodies in different regions of the gray matter. However, the current study does allow one to discern the distribution of cell bodies that project descending propriospinal axons into each region of the white matter. At least four distinct distributions could be recognized, one each for the dorsal funiculus, the medial part of the lateral funiculus, the lateral part of the lateral funiculus, and the ventral funiculus. The distributions are detailed in Results and are summarized in Figure 10.

These distributions are consistent with the partial evidence provided by a study (Molenaar et al., 1974) of retrograde degeneration of cat spinal neurons following spinal lesions. In certain cases, the lesion was restricted to the white matter (Figs. 4, 6, 8 in Molenaar et al., 1974). The data from these cases suggest that descending propriospinal axons in the dorsal part of the lateral funiculus derived from cell bodies mainly ipsilaterally, in the lateral parts of the deep dorsal horn, intermediate zone, and dorsal portion of the ventral horn; descending propriospinal axons in the ventral funiculus derived mainly from the ipsilateral ventral horn. However, chromatolytic cell bodies were only found relatively close to the lesion site.

Sherrington and Laslett (1903) and others (Nathan and Smith, 1959) have suggested that long propriospinal fibers are found farther from the gray matter than short propriospinal fibers, the axons shifting outward as they grow further from their cell bodies. This would mark a similarity in the organizations of propriospinal axons and ascending afferents of the mammalian medial lemniscus pathway (Brodal, 1981). This suggestion receives modest support from the current data. HRP injections into the outer regions of the lateral funiculus or ventral funiculus (cases F–I in Figs. 5) labeled more ipsilateral cell bodies two or three segments away than in the adjacent segment, in contrast to certain other injections (cases A, C, and E). However, it must be emphasized that even the most superficial white-matter injections labeled many nearby cell bodies and the deepest injections labeled many distant cell

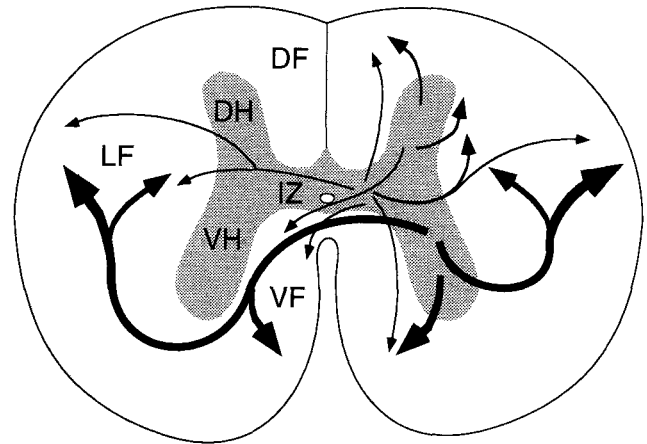


Fig. 10. Schematic summary of major funicular, descending propriospinal projections from the D3–D8 segments of the spinal cord in the red-eared turtle. Tails of arrows indicate regions of the gray matter on the right side of the spinal cord where descending propriospinal cell bodies were found. Arrowheads indicate the regions of the white matter through which these neurons most often projected their descending axons. Thicker arrows represent larger projections. Smallest projections are omitted for clarity. Regions of the gray matter and white matter are labeled only on the left side for clarity. This summary was inferred from the distributions of retrogradely labeled cell bodies presented in this study. The courses that axons take from the cell body into the white matter are not known and are sketched arbitrarily. DH, dorsal horn; IZ, intermediate zone; VH, ventral horn; DF, dorsal funiculus; LF, lateral funiculus; VF, ventral funiculus.

bodies. In addition, this tendency was not apparent for contralaterally labeled cell bodies or generally for dorsal funiculus injections. It is possible that this type of organization is more pronounced in mammals than in turtles.

Descending propriospinal cell bodies were concentrated in the lateral halves of the gray matter, especially ipsilaterally. In Nissl-stained sections, there does not appear to be a greater density of neuronal cell bodies in the lateral gray matter (data not shown). It may be that ascending propriospinal or supraspinal neurons are concentrated in the medial halves of the spinal gray matter. Previous experiments involving large HRP injections into the spinal cord in turtles suggested that the cell bodies of ascending neurons with crossed axons may be concentrated in the medial half of the spinal gray matter (Figs. 7, 8 in Kusuma and ten Donkelaar, 1980). Qualitative observations of cell body labeling caudal to the HRP injections of this study (data not shown) also suggest that contralateral ascending neurons may be concentrated medially.

Differences in funicular axon distributions may be associated with differences in functional connectivity of descending propriospinal neurons. Spinal motoneurons in fish (Schnitzlein and Brown, 1975), frogs (Szekely, 1976; Bregman and Cruce, 1980; Antal et al., 1986; Birinyi et al., 1992), lizards (Cruce, 1975), and turtles (Fig. 7C; see also Banchi, 1903; Ruigrok et al., 1984) have extensive dendritic trees in the white matter, often reaching the subpial surface. White-matter dendrites of spinal motoneurons have also been found in mammals (Light and Metz, 1978; Rose, 1981; Rose and Richmond, 1981; Ulfhake and Kellerth, 1981, 1983; Egger and Egger, 1982; Cameron et al., 1983; Ulfhake et al., 1988), including humans (Schoenen, 1982). This is especially clear in early development in both

chicks and mammals (Ramon y Cajal, 1909; Snider et al., 1992). Spinal interneurons (i.e., spinal neurons with neither dorsal root nor ventral root axons) with white-matter dendrites have been shown in turtles (Fig. 7A,B; see also Banchi, 1903), chicks (Ramon y Cajal, 1909), and mammals (see Rose and Richmond, 1981). Synapses onto white-matter dendrites of spinal motoneurons have been demonstrated in cats (Rose and Richmond, 1981) and frogs (Antal et al., 1986) via electron microscopy. In the current study, axonal swellings were often seen along thin collaterals of labeled funicular axons, both near and far from the gray matter (Fig. 9). These swellings had an appearance similar to that of synaptic boutons in the spinal gray matter of turtles (Fig. 9A; Ruigrok et al., 1985) and mammals (Brown, 1981); that these are actually synapses remains to be confirmed using electron microscopy. Skydsgaard and Hounsgaard (1992) have recently demonstrated that distal white-matter dendrites of spinal motoneurons in turtles can be selectively depolarized by focal iontophoresis of glutamate. Thus it is likely that synaptic contacts occur in the spinal white matter of turtles. The different funicular distributions of descending propriospinal axons arising from cell bodies in different regions of the gray matter may enable these neurons to select the distributions of motoneuron and propriospinal neuron dendrites with which they synapse. Further study is needed to determine the relationship between propriospinal axon distributions and the spinal circuitry controlling various behaviors.

Differential distributions of propriospinal axons may also have implications for axonal pathfinding in development, although it is not known whether the organization of descending propriospinal axons seen here in adults is similar to the organization in early development. Propriospinal axons are among the first spinal axons to develop (Nordlander, 1984; Oppenheim et al., 1988; Kuwada et al., 1990; Yaginuma et al., 1990) and may be involved in pioneering pathways for later-arising spinal axons (Kuwada et al., 1990; Yaginuma et al., 1990). The courses taken by propriospinal axons in early development may influence their functional connectivity with white-matter dendrites as well as the functional connectivity of later-growing spinal axons. The rostrocaudal trajectories of axons labeled in this study (which may include all types of spinal axons) may offer additional clues to the factors that influence the trajectories of early axons. Labeled axons did not always maintain a particular position within the white matter (see Fig. 8A,B), occasionally even switching from one funiculus to another (Fig. 8B). If this relative freedom from constraints of pathway choice reflects the situation seen in early development, it may indicate either that mistakes in axon trajectory occur and can be corrected by course changes or that an axon's position in the white matter is not crucial for determining its functional connectivity.

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