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Research report

L1 and GAD65 are expressed on dorsal commissural axons in embryonic rat spinal cord

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Abstract

Using immunocytochemical methods, the cell adhesion molecule L1 was detected on axons crossing in the dorsal commissure of developing rat spinal cord. Immunoreactive axons were found in locations similar to fiber bundles illustrated by Ramón y Cajal and designated the anterior, middle and posterior bundles of the dorsal commissure. L1-immunoreactive dorsal commissural axons were first observed on embryonic day 17 (E17), appeared more numerous by E19, and remained detectable in early postnatal ages. The massive middle axon bundles extended bilaterally from the dorsolateral funiculi towards the midline and crossed in the central part of the commissure. In horizontal sections, bundles of L1-labeled middle axons were observed to traverse the dorsal commissure in a periodic pattern along the entire rostrocaudal extent of the spinal cord. Bundles of glutamic acid decarboxylase (GAD65)-positive axons were detected crossing in the middle and posterior regions of the dorsal commissure between E17 and E20. Results from double-labeling experiments demonstrated that GAD65-positive fibers were embedded in larger bundles of L1-labeled axons and that some dorsal commissural axons were double-labeled. To determine if there were axons crossing in the dorsal commissure that did not express L1, double-labeling experiments were conducted using neurofilament and L1 antibodies. Results indicated that bundles of axons identified with anti-neurofilament antibodies were also L1-positive, whereas individually coursing axons within the commissure were L1-negative. The predominance of L1 on fiber bundles traversing the dorsal commissure adds to the growing evidence that this molecule may play a role in axon outgrowth and fasciculation.

Theme: Development and regeneration

Topic: Axon guidance mechanisms and pathways

Keywords: Glutamic acid decarboxylase; GABA; Cell adhesion molecule; IgG superfamily; Axonal outgrowth

1. Introduction

In 1933, Ramón y Cajal first identified axon fibers that crossed in both the ventral and dorsal commissures of developing mammalian spinal cord. Axons that crossed in the ventral commissure appeared to originate from cells in the dorsal spinal cord, whereas those that traversed the dorsal commissure seemed to branch from white matter [34]. While the neurons forming the ventral commissure have been characterized extensively [15,16,43,55] and utilized in models of axon outgrowth, pathfinding, and chemotropism [19,39,48,51], relatively little is currently known about the development of axons that cross in the dorsal commissure. Considering the current interest in the cellular and molecular mechanisms of axon outgrowth and pathfinding, the axons of the dorsal commissure may provide a useful comparison with those of the ventral commissure.

Cell adhesion molecules (CAMs) are cell-surface molecules that are thought to play a significant role in axonal development by mediating cell–cell and cell–extracellular interactions. For example, CAMs have been associated with neurite outgrowth, fasciculation, and axonal guidance [17,38]. One such adhesion molecule is L1, an axonal glycoprotein and member of the immunoglobulin (Ig)

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superfamily [21,30]. L1 was first described in the mouse [36] and has been found to have an equivalent molecule in the rat (NILE) [4], and homologous molecules in chick (NgCAM and G4) [40,52], and *Drosophila* (neuroglian) [3]. L1-related glycoproteins have an extracellular domain that consists of six Ig-like domains followed by five fibronectin type III-like repeats, a single-pass transmembrane region, and a short, highly conserved cytoplasmic domain [30]. Studies have shown that L1 promotes axon outgrowth [1,20,21], mediates neuronal migration [24,29], fasciculates growing axons [5,13,37,48], and possibly plays a role in axon guidance [7]. L1 identifies specific subsets of axons and has been localized in many different regions of the central nervous system (CNS), including the spinal cord [41,48]. Although L1 has been found on the axons of ventral commissural neurons, the role of this CAM in mediating axonal growth across the midline is unclear, as reports from various species differ. Dodd et al. [9] suggested that rat ventral commissural neurons first express TAG-1 during their outgrowth to the floor plate and subsequently switch to express L1 once they cross the midline and extend longitudinally in the ventral marginal zone. However, in chick, L1 was observed on the somata and axons of ventral commissural neurons as they projected to the floor plate [40,41], and also is thought to be involved in the fasciculation of ventral commissural axons before they cross the midline [48].

Recent studies in rats [32] reported that a significant group of ventral commissural neurons express glutamic acid decarboxylase (GAD), the rate-limiting enzyme in the synthesis GABA, a major inhibitory neurotransmitter [2,27]. Early in development somata and axons projecting into the ventral commissure were detected with antibodies to GAD65, whereas several days later, ventral commissural neurons were identified with antibodies against the other isoform of GAD, GAD67 [18,32]. Ventral commissural neurons also were identified with antibodies against GABA, confirming that a significant subpopulation of these neurons were of the GABAergic phenotype. Furthermore, some of these GABAergic ventral commissural neurons were found to express L1 on both the ipsi- and contralateral surfaces of their axons [53]. We then asked if GABAergic neurons also projected axons across the dorsal commissure and subsequently, if those axons would express L1 CAM as they crossed the midline.

In this present study, we have found that two very different cellular markers, L1 and GAD65, both identify fiber bundles coursing through the dorsal commissure. We have described the temporal expression of L1 and GAD65 on dorsal commissural axons during late embryonic development and have compared these results with those obtained by neurofilament labeling of embryonic spinal cord. These data then are compared with similar developmental findings observed at earlier ages in the ventral commissure [53].

2. Materials and methods

2.1. Animals and tissue preparation

Adult male and female Sprague–Dawley rats (Charles River) were housed together for overnight breeding. The day a positive vaginal smear was obtained was labeled as embryonic day 0 (E0), and birth was designated as P0. Embryos utilized in this study ranged in age from E12 to E21, and postnatal animals were studied at P0.

Pregnant female rats were anesthetized with Nembutal (40 mg/kg), and embryos were delivered via Cesarean section. Individual embryos were immediately perfused through the heart with 4% paraformaldehyde for 10 min followed by postfixation in the same fixative for 1 h at room temperature or 4 h at 4°C. Following postfixation, spinal cords were dissected, and stored in 0.12 M Millonig's phosphate buffer containing 0.06% azide until processing.

Spinal cords were infiltrated with 30% sucrose in buffer, blocked for either transverse or horizontal sectioning, embedded in M1 (Shandon-Lipshaw) and frozen on dry ice. For horizontal sectioning the rostral ends of the spinal cords segments (~1.5 cm long) were marked before embedding the ventral surface down. Forty micron thick sections were cut with a cryostat, then rinsed and stored in Millonig's buffer with azide until immunocytochemical procedures were performed. Specimens were mounted on coated glass slides and allowed to dry for 20 min. A ring of silicon was placed around the sections to create a well for the reagents.

2.2. L1 and GAD65 immunocytochemical procedures

Axonal processes displaying the L1 surface antigen were identified by using either a monoclonal antibody (ASCS4 [49], obtained from the Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa, Iowa City, IA) or polyclonal antiserum (rabbit anti-rat L1 [21], a generous gift from Dr Vance Lemmon of Case Western Reserve University). For labeling with ASCS4, sections were rinsed in Tris-buffered saline (TBS; 0.1 M Tris buffer containing 1.4% NaCl, pH 7.4) and then incubated in 0.3% hydrogen peroxide (H_2O_2) and 0.1% sodium azide to quench endogenous erythrocyte peroxidase activity. Specimens were incubated in detergent (0.8% Triton X-100 in TBS) for 15 min, followed by 1 h in 0.1% Triton X-100 with 1.5% normal horse serum (NHS). Similar concentrations of Triton X-100 and NHS were added to all subsequent antisera steps. Specimens were incubated in primary monoclonal antibody (ASCS4 supernatant, 1:25) for 2 h at room temperature followed by overnight at 4°C. The following day, sections were washed and incubated in secondary antibody, rat adsorbed biotinylated horse anti-mouse IgG (diluted 1:200), for 1 h.

After another set of rinses in TBS, specimens were incubated for 1 h in an avidin–biotin–peroxidase complex (1:100; Vectastain Elite Mouse IgG Kit), washed in buffer, and developed with 0.06% 3,3′-diaminobenzidine·4 HCL (DAB), intensified with either Ni-glucose oxidase [42] (purple-black product) or 0.02 M imidazole with 0.007% H_2O_2 (amber-brown product). Sections were subsequently washed, dehydrated, and coverslipped. Specimens incubated with the polyclonal antiserum [21] to L1 (1:100 000) were processed similarly to those labeled with the monoclonal antibody with the exception of normal goat serum in the blocking and antisera steps, and a secondary antibody consisting of biotinylated goat anti-rabbit IgG (Vectastain Elite Rabbit IgG Kit).

GAD6 [6] (obtained from the Developmental Studies Hybridoma Bank), a monoclonal antibody that preferentially recognizes GAD65 [18], was utilized to identify GABAergic axons. The methods used to localize GAD65 were identical to those previously published [32]. Sections were developed for approximately 8 min with 0.06% DAB diluted with a solution of Ni-glucose oxidase to produce a purple-black product. Specimens were then washed in sodium acetate buffer, dehydrated and coverslipped.

The goal of these double-labeling experiments was to determine if axons that contained GAD65 also expressed L1. The best double-labeling of axon bundles was obtained by processing GAD65 first using the DAB intensified Ni-glucose oxidase and temporarily coverslipping the sections with glycerin. After photographing the GAD65 staining, specimens were stored in Millonig's buffer at 4°C until proceeding with the second half of the experiment. The L1 immunocytochemistry was developed with DAB intensified with 0.02 M imidazole, yielding an amber-brown product that was easily distinguishable from the purple-black reaction product identifying GAD65. We also identified individual GAD65-positive fibers with the amber-brown DAB product and overlaid the L1 immunocytochemistry with purple-black DAB to determine if individual GAD65-labeled axons expressed L1 on their surfaces. For double-labeling controls, GAD65 experiments were followed by L1 localization in which NHS was substituted for the primary antibody. No purple-black DAB product was observed overlying any of the GAD65-labeled axons in these control experiments (data not shown).

2.3. Neurofilament immunocytochemical procedures

The monoclonal antibody 2H3 [9] (Developmental Studies Hybridoma Bank), was used to label neurofilaments within developing axons. Sections were rinsed in TBS buffer, incubated for 30 min in 0.3% H_2O_2 and 0.1% sodium azide and then transferred to 0.8% Triton X-100. After blocking with 1.5% NHS for 1 h, sections were incubated in primary antibody (2H3 supernatant, diluted 1:40) for 2 h at room temperature and then overnight at 4°C. Standard avidin–biotin immunolabeling techniques (Vectastain Elite Mouse IgG Kit) were employed with dilutions identical to the L1 procedures described above. Imidazole (0.02 M) was added to intensify the DAB step and to produce an amber-brown reaction product.

The goal of these double-labeling experiments was to determine if all axons contained within the dorsal commissure expressed the L1 antigen. The 2H3 immunocytochemistry first utilized the amber-brown chromogen, and sections were temporarily coverslipped with glycerin for color photomicroscopy of the single-labeling results. Following an overnight wash in Millonig's buffer, L1 immunocytochemistry was performed using DAB intensified with Ni-glucose oxidase, and the slides were dehydrated and coverslipped with Permount. Color photomicrographs were taken of the double-labeled sections and compared with those previously recorded of the single-labeled images.

3. Results

In his original drawing of the spinal cord, Ramón y Cajal [33] illustrates three bundles of fibers (anterior, middle, and posterior) coursing through the dorsal commissure (reproduced in Fig. 1). He describes these fibers as commissural collaterals of the sensory system, joining the ipsilateral dorsal and lateral funiculi with the contralateral gray matter of the dorsal horn. Fibers composing the

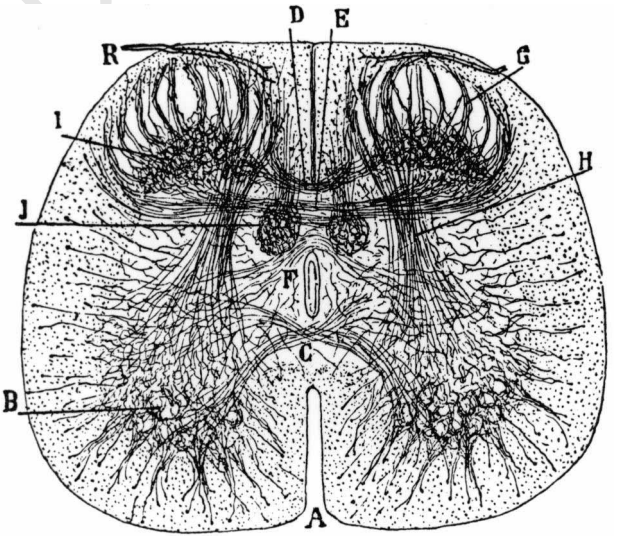


Fig. 1. Drawing of Golgi impregnated newborn mammalian spinal cord by Ramón y Cajal illustrating the spinal cord commissures and other collaterals in a transverse section. The drawing is oriented with the dorsal funiculi at the top and the lateral funiculi on each side. Ramón y Cajal's legend states, 'Plexus of collaterals of the anterior horn (B), ventral commissure (C), posterior (D), middle (E), and anterior bundles of the dorsal commissure (F), long or sensory-motor collaterals (H), plexus of collaterals in the column of Clarke (J).' Reproduced from Ref. [34].

anterior bundle arise from the anterior part of the lateral funiculus and cross the midline just dorsal to the central canal before scattering in many directions. Fibers forming the middle bundle emerge from the most posterior portion of the lateral funiculus and extend to the midline before coursing laterally to the base of the contralateral dorsal horn and lateral margin of the substantia gelatinosa. Fibers in the posterior bundle form a horseshoe-like pattern around the two cuneate fascicles and project from the innermost dorsal funiculus to the medial parts of the dorsal horn on the contralateral side [34].

3.1. L1-positive axon bundles cross in the dorsal commissure

L1 is known to be preferentially expressed on develop-

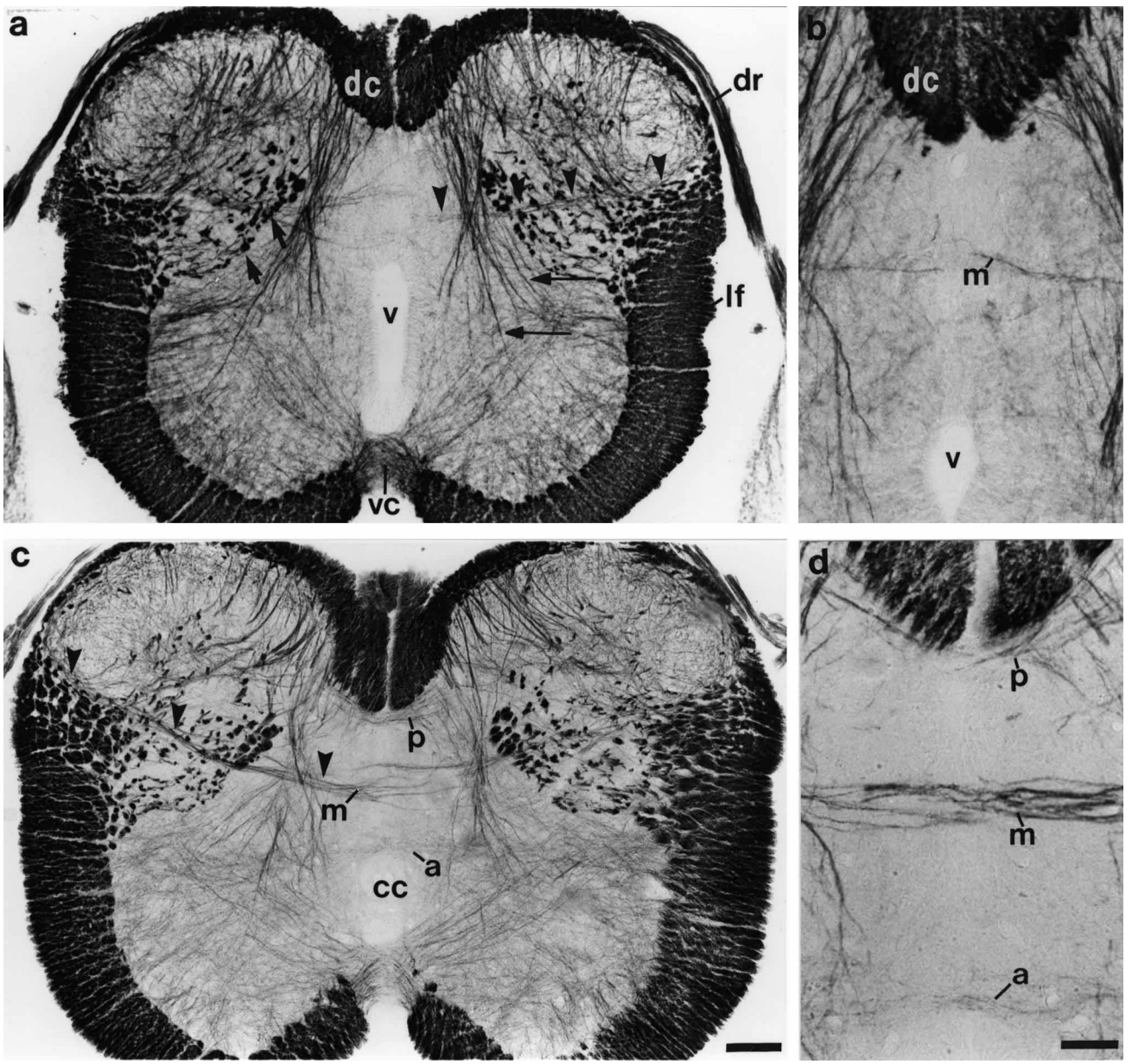


Fig. 2. L1 immunocytochemistry labels dorsal commissural fibers in cervical spinal cord at E17 (a, b) and E19 (c, d). (a): At E17, L1-labeled axons project medially (arrowheads) from the lateral funiculi (lf) to the midline. Other L1-labeled axons are found in the reticulated area (short arrows), ventral commissure (vc), dorsal columns (dc), dorsal root (dr), and sensory afferent fibers (long arrows). (b): Higher magnification photomicrograph of another E17 specimen illustrates L1-positive fibers of the middle (m) bundle coursing in the dorsal commissure between the ventricle (v) and the dorsal columns (dc). (c): By E19, the central canal (cc) is formed and three distinct bundles of L1-labeled axons course across the dorsal commissure: posterior (p), middle (m), and anterior (a). The middle bundle (arrowheads) can be followed laterally to the dorsal part of the lateral funiculus and medially to the midline, where it occasionally separates into component bundles. (d): Higher magnification photomicrograph of another E19 specimen illustrates the three L1-labeled fiber bundles (posterior, p; middle, m; anterior, a) crossing in the dorsal commissure. Scale (a,c)=100 μm; (b,d)=40 μm.

ing axons [47,54], and thus only fiber tracts and not cell bodies were found to be labeled at all ages studied. On both E16 and E17, axons within the ventral, lateral, and dorsal marginal zones were intensely labeled for L1 (Fig. 2a) similar to earlier observations of NILE expression by Stallcup et al. [47]. In addition, we observed L1-immunoreactive axons in the dorsal root and in primary sensory afferents that project ventrally from the dorsal marginal zone (Fig. 2a). Furthermore, bundles of L1-labeled axons were scattered within the reticulated area (laminae IV–VI; Fig. 2a). Numerous L1-labeled axons also coursed across the ventral commissure. On E17, immunoreactive axons were first detected coursing from the lateral part of the dorsal horn into and across the dorsal commissure in the position of the middle fiber bundle (Fig. 2a,b), as defined by Ramón y Cajal [34]. Axons in the anterior and posterior fiber bundles were not detectable at this age.

L1-immunoreactive fibers coursing through the dorsal commissure were more numerous by E19. At this age, three distinct bundles of immunoreactive fibers were observed traversing the dorsal commissure (Fig. 2c) in positions and relative numbers similar to the description by Ramón y Cajal (Fig. 1). The most distinctive L1-labeled fiber bundle in the dorsal commissure resembled the middle bundle. It extended laterally from the dorsolateral white matter to the midline, crossing the commissure approximately halfway between the central canal and the dorsal columns (Fig. 2c,d). Further dorsally, short L1-labeled axons were found wrapped around the dorsal columns in a pattern and location similar to those axons Ramón y Cajal called posterior fibers of the dorsal commissure (Fig. 1). The posterior fiber bundle crossed the midline just ventral to the dorsal columns and appeared to fan out into the adjacent dorsal gray matter (Fig. 2c,d). Further ventrally, several L1-labeled axons appeared adjacent to the central canal, in a position similar to that described by Ramón y Cajal as the anterior fiber bundle (Fig. 2d). A few L1-positive anterior fibers were detectable in the midline, but their lateral origins were difficult to delineate.

While coronally sectioned specimens allowed us to visualize three distinct bundles of fibers in the dorsal commissure, horizontally sectioned specimens were preferred to evaluate the overall developmental pattern of dorsal commissural axons along the rostrocaudal extent of the spinal cord. Horizontal sections illustrated that L1-labeled middle dorsal commissural fiber bundles formed with a periodic nature — that is, the bundles projected across the midline at regular intervals rather than continuously along the rostrocaudal extent (Fig. 3a). Occasionally, middle commissural fibers separated into several bundles near the midline and joined with other middle bundles. This periodic crossing pattern was similar at cervical, thoracic, and lumbar levels of E19 spinal cord. Horizontal sections through the reticulated area revealed numerous L1-positive fibers projecting rostrocaudally along a mean-

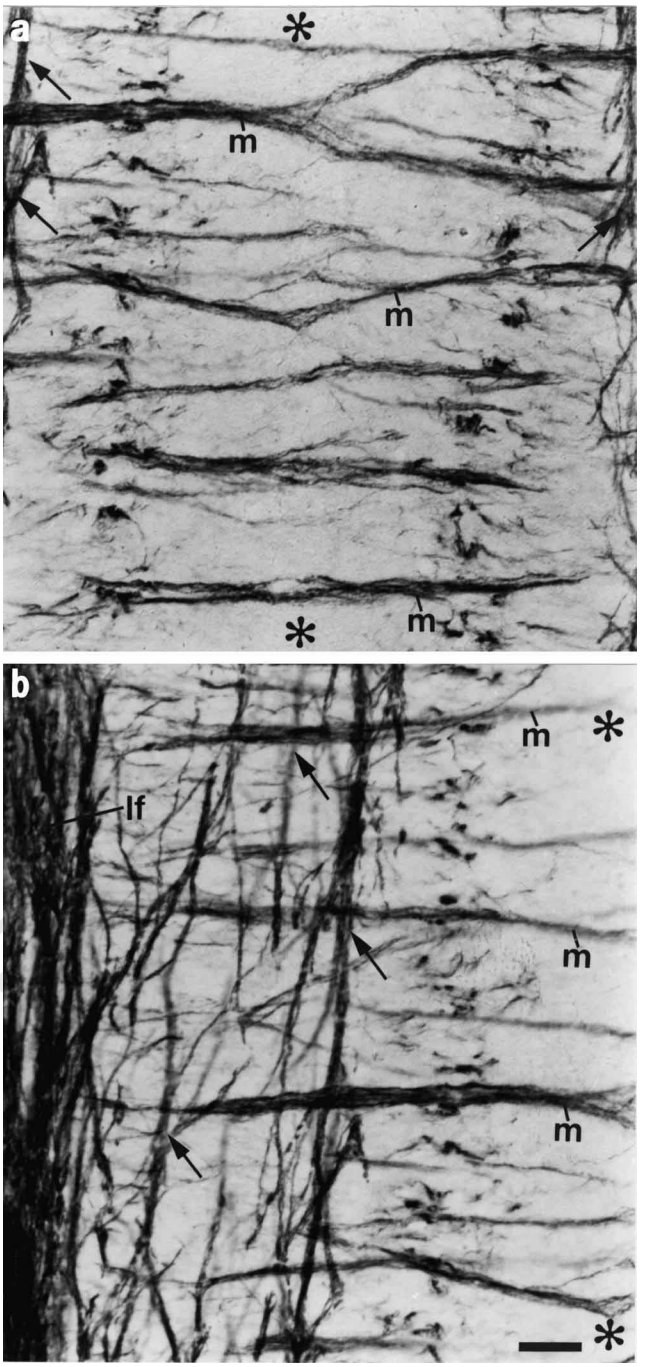


Fig. 3. Immunoreactive middle fiber bundles cross periodically in the dorsal commissure in a L1-labeled horizontal section of E19 cervical spinal cord. The rostral spinal cord is oriented toward the upper border of the photomicrographs. (a): Bundles of L1-positive axons course through the midline (between asterisks) of the dorsal commissure in a periodic pattern. Some axons within the middle bundles (m) separate into smaller bundles and then appear to join other L1-labeled middle bundles. Immunoreactive reticulated fibers (long arrows) are seen on the lateral edges of this photomicrograph. (b): Middle dorsal commissural bundles extend perpendicularly from the lateral funiculi (lf), crossing the reticulated fibers (long arrows) as they course to the midline (between asterisks). Scale (a,b)=40 μm.

dering course (Fig. 3b). L1-labeled middle commissural axons did not appear to emerge from the rostrocaudally-oriented fibers in the reticulated area but rather extended across these fibers to reach the dorsolateral marginal zone (Fig. 3b).

While the middle fiber bundles could be visualized easily along several segments of horizontally sectioned spinal cord, the posterior fiber bundles were thinner, shorter, and more difficult to detect because of their horseshoe-like shape. However, the posterior fiber bundles were observed to cross the midline periodically at all axial levels. Compared to the posterior and middle fiber bundles, those crossing in the anterior part of the dorsal commissure on E19 were very thin, lightly immunoreactive, and appeared to be arranged more diffusely in the midline of the commissure (data not shown).

Throughout the remainder of embryonic development, L1-labeled axons in the dorsal commissure and reticulated

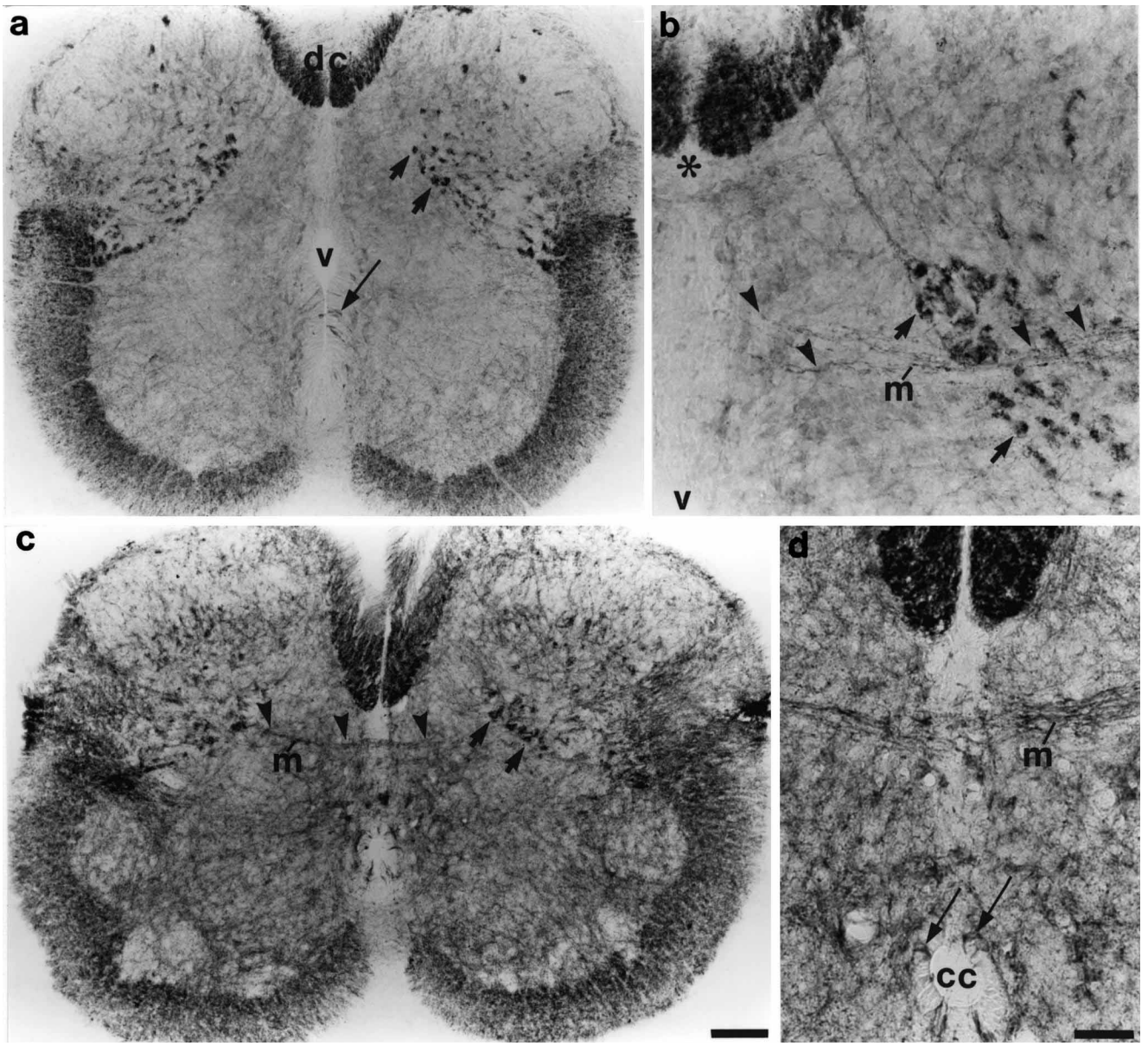


Fig. 4. GAD65 is present in dorsal commissural fibers in cervical spinal cord on E17 (a,b) and E19 (c,d). (a): GAD65-immunoreactive axons are present in the ventral and lateral funiculi and dorsal columns (dc) on E17, as well as within the reticulated area (short arrows). Some of the cells lining the ventricle (v) contain GAD65 immunoreactivity (long arrow). (b): An enlargement of a different E17 section illustrates the dorsal commissure (on left side of the photomicrograph, between asterisk and ventricle, v) and reticulated portion of the dorsal horn. A few GAD65 immunoreactive axons (arrowheads) course among the GAD-positive fibers of the reticulated area (short arrows) in the position of the middle fiber bundle (m), and extend toward the midline. (c): On E19, GAD65 immunocytochemistry labels the middle bundle (m, arrowheads) coursing through the dorsal commissure, as well as axons in the reticulated area (short arrows). (d): Enlargement of c illustrates a number of GAD65-labeled fibers that course together in the middle bundle (m). Note GAD65-positive somata (long arrows) within the ependymal layer of the central canal (cc). Scale (a,c)=100 μm; (b,d)=40 μm.

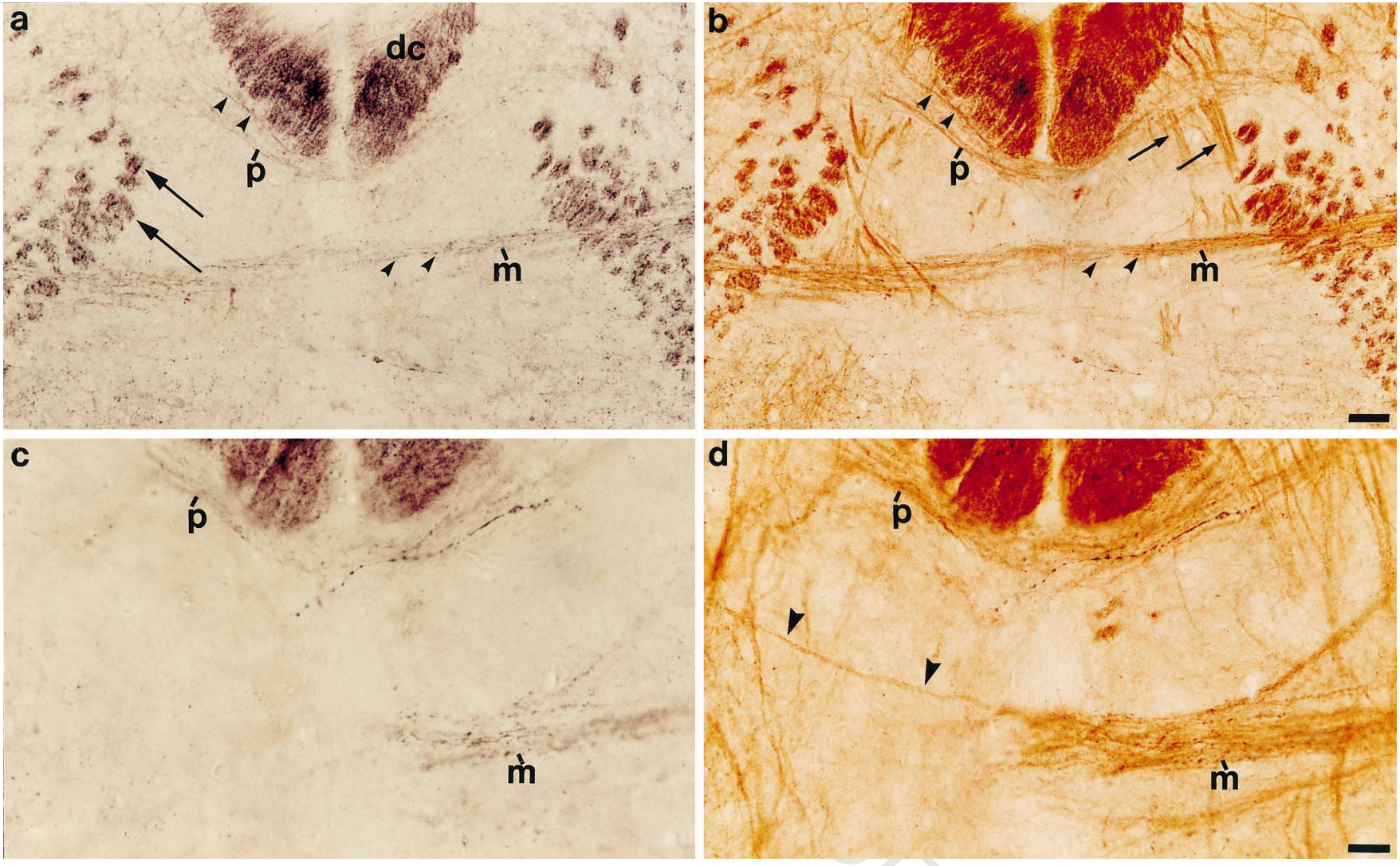


Fig. 5. Double-labeling of GAD65 (a,c) and L1 (b,d) in coronal sections of the dorsal commissure on E19. (a): GAD65 immunocytochemistry reveals purple-black axons in both the middle (m) and posterior (p) fiber bundles (small arrowheads), dorsal columns (dc) and reticulated area (long arrows). (b): Subsequent L1 labeling of the section illustrated in a. The middle (m) and posterior (p) bundles contain fibers that are both GAD65- (purple-black chromogen; small arrowheads) and L1-positive (amber-brown chromogen). Color specificity of the double-labeling procedure is demonstrated in the primary sensory afferent fibers (short arrows) that are L1-positive but GAD65-negative. (c): GAD65 immunocytochemistry identifies axons in both the middle (m) and posterior (p) fiber bundles. (d): After the double-labeling procedure, occasional fibers in the middle (m) bundle that were not labeled for GAD65 could be observed to express L1 on their surfaces (arrowheads). Scale (a,b)=40 μm; (c,d)=20 μm.

area appeared fundamentally unchanged from the observations described on E19. At P0, the pattern of L1-labeling was similar to that observed in late embryonic specimens, but the intensity of immunoreactivity expressed on dorsal commissural axons was consistently less (data not shown).

3.2. GAD65-positive axon bundles cross in the dorsal commissure

GAD65-positive axons were present in both the middle and posterior fiber bundles of the dorsal commissure but were not obvious in the anterior fiber bundle. On E17, the ventral, lateral, and dorsal marginal zones and the reticulated area were immunoreactive for GAD65 (Fig. 4a). Occasionally GAD65-positive axons were detected extending from the dorsolateral marginal zone toward the dorsal commissure at the level of the middle fiber group (Fig. 4b). By E18, GAD65-immunoreactive fibers had crossed the midline of the dorsal commissure, and by E19, labeled GAD65 axons were identified in both middle and posterior fiber groups (Figs. 4c,d and 5a,c). Horizontal sections through the level of the dorsal commissure showed that the GAD65-labeled middle bundles had a periodic pattern of crossing (Fig. 6a,c) similar to those observed in L1-labeled sections (Fig. 3a,b). In addition, the GAD65-labeled posterior bundles also showed an intermittent pattern of crossing the dorsal commissure but were less robust than the middle bundles.

Numerous axons coursing rostrocaudally in the reticulated area were GAD65-immunoreactive (Fig. 6a). GAD65-positive axons that crossed in the middle fiber group appeared to be a separate set of axons unrelated to those coursing rostrocaudally in the reticulated area. Rather, the middle group of dorsal commissural fibers coursed from the edge of the dorsolateral marginal zone into the midline and then occasionally bifurcated into several bundles. By E20, there were remarkably few GAD65-labeled dorsal commissural fibers or fiber bundles in the reticulated area, however, punctate staining in the gray matter had increased substantially (data not shown).

3.3. L1- and GAD65-labeled axon bundles have an overlapping distribution in the dorsal commissure

Individual L1 and GAD65 immunocytochemical experiments revealed that both antigens were found on middle and posterior axon bundles and appeared in a similar pattern. In order to determine the relationship between the L1- and GAD65-labeled axons, double-labeling experiments were performed. Several single-labeled structures served as internal controls for the double-labeling procedure. The dorsal roots and primary sensory afferents only expressed the L1 antigen (Figs. 2a,c and 5b), while cell bodies found near or within the ependymal layer [2] only expressed GAD65 (Fig. 4a,d). As double-labeled specimens contained both single-labeled GAD65 and L1 structures, we were confident that the specificity of labeling was maintained throughout the procedure.

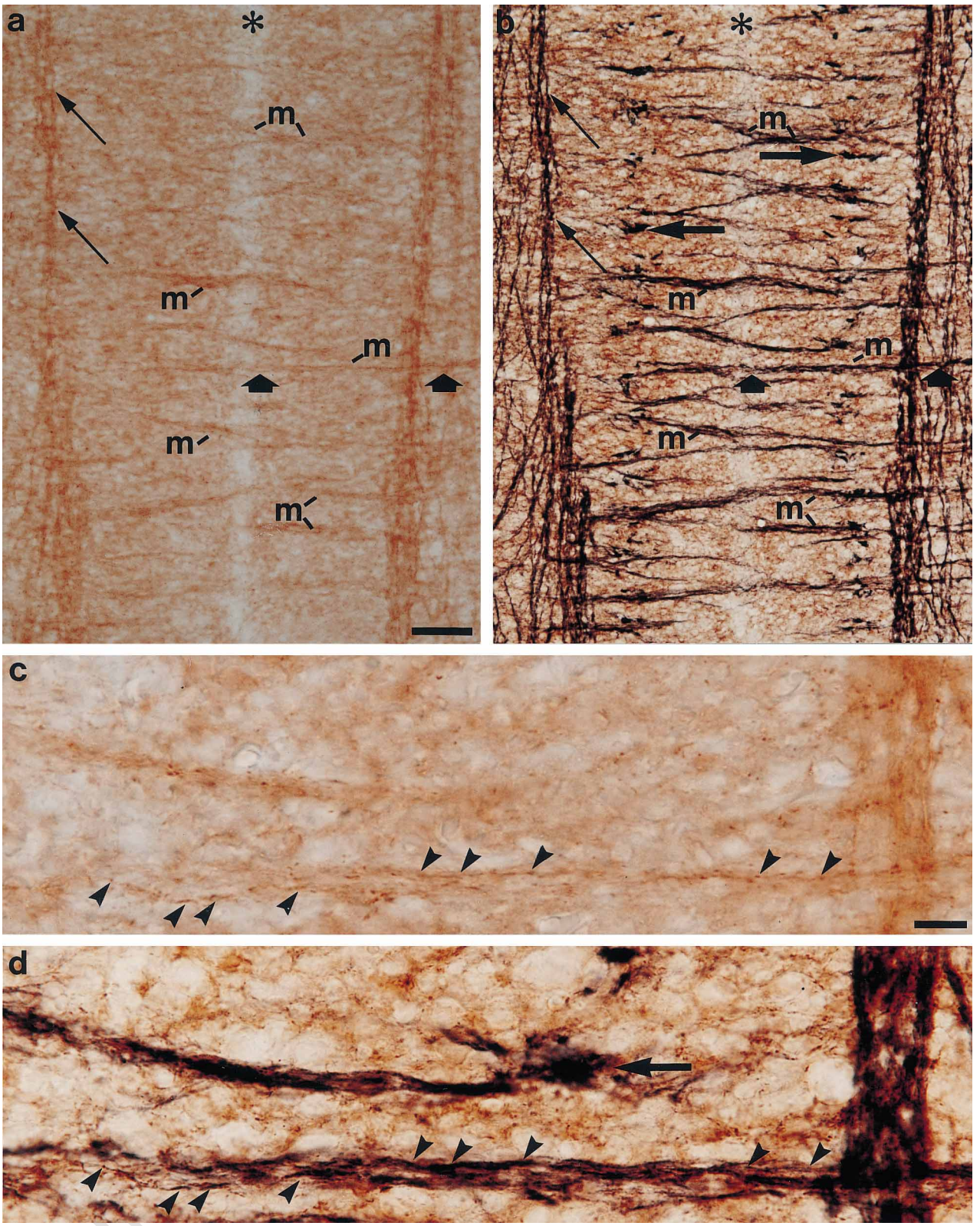
Embryonic day 19 specimens were first labeled for GAD65 using a black (Fig. 5a,c) or amber-brown (Fig. 6a,c) chromogen. Then they were photographed before a second immunocytochemical procedure was performed in which L1 was localized with the alternate chromogen (Figs. 5b,d and 6b,d). Data from these experiments showed that both the middle and posterior dorsal commissural fiber groups contained GAD65 and L1. In horizontal sections, GAD65 immunocytochemistry revealed numerous fibers crossing periodically in the dorsal commissure (Fig. 6a,c). When these sections were subsequently labeled for L1, the same axon bundles and even individual axons within these bundles appeared to have L1 on their surfaces (Fig. 6b,d). In the rostrocaudally coursing axons of the reticulated area, there was a similar overlapping pattern of GAD65- and L1-positive fibers (Fig. 6a,b).

L1-labeled fibers in the dorsal commissure appeared to be much more numerous than fibers containing GAD65. Occasionally, an L1-positive dorsal commissural fiber was observed in a double-labeled specimen that was not initially labeled by GAD65 immunocytochemistry (Fig. 5d). Observations such as these suggested that there were more L1- than GAD65-labeled axons within the dorsal commissural bundles. The overlapping expression pattern indicated that GAD65- and L1-positive axons follow the same pathways into and through the dorsal commissure.

3.4. L1 labels axon bundles but not all axons in the dorsal commissure

Anti-neurofilament antibodies (2H3) were utilized to determine if there were additional axons present in the

Fig. 6. Horizontal section demonstrating an overlapping pattern of GAD65-(amber-brown chromogen; a,c) and L1-labeled (black chromogen; b,d) fibers crossing in the dorsal commissure on E19. Asterisks designate the midline of the sections that are oriented rostral towards the top and lateral to each side. (a): GAD65 immunocytochemistry detects numerous middle fiber bundles (m), as well as rostrocaudally-coursing lateral reticular bundles (long arrows). The middle fiber bundle marked with two block style arrows is enlarged in c and double-labeled in b and d. (b): After subsequent L1 immunocytochemistry on the section illustrated in a, axon bundles in both the dorsal commissure (m) and reticulated area (long arrows) were double-labeled. L1-positive sensory afferents (thick arrows) were immunolabeled in b only. (c): Higher magnification of the GAD65-immunoreactive middle fiber bundle that is marked by two block style arrows in the specimen depicted in (a). Amber-brown GAD65-labeled fibers (arrowheads) can be followed within the bundle that courses from the midline into the lateral reticular fibers. (d): Higher magnification of the GAD65-L1 double-labeled specimen illustrated in (b) (between the two block style arrows). The GAD65-immunoreactive middle axon fibers (arrowheads) visualized in (c) are now double-labeled with L1 on their surfaces as they course within the L1-labeled bundles. Note that cross-cut L1-labeled sensory afferents (thick arrow) are not found in (c). Scale (a,b)=100 μm; (c,d)=20 μm.



dorsal commissure that were not detected by L1-labeling. Embryonic day 19 horizontal spinal cord sections contained bundles of 2H3-labeled fibers (Fig. 7a), and at high magnification additional axons that were not part of a bundle were observed coursing within the dorsal commissure. Results from the double-label experiments demonstrated that the prominent middle axon bundles identified with neurofilament antibodies also were labeled with L1, as shown respectively in Fig. 7a,b. Upon closer inspection of the double-labeled sections, individual single-labeled,

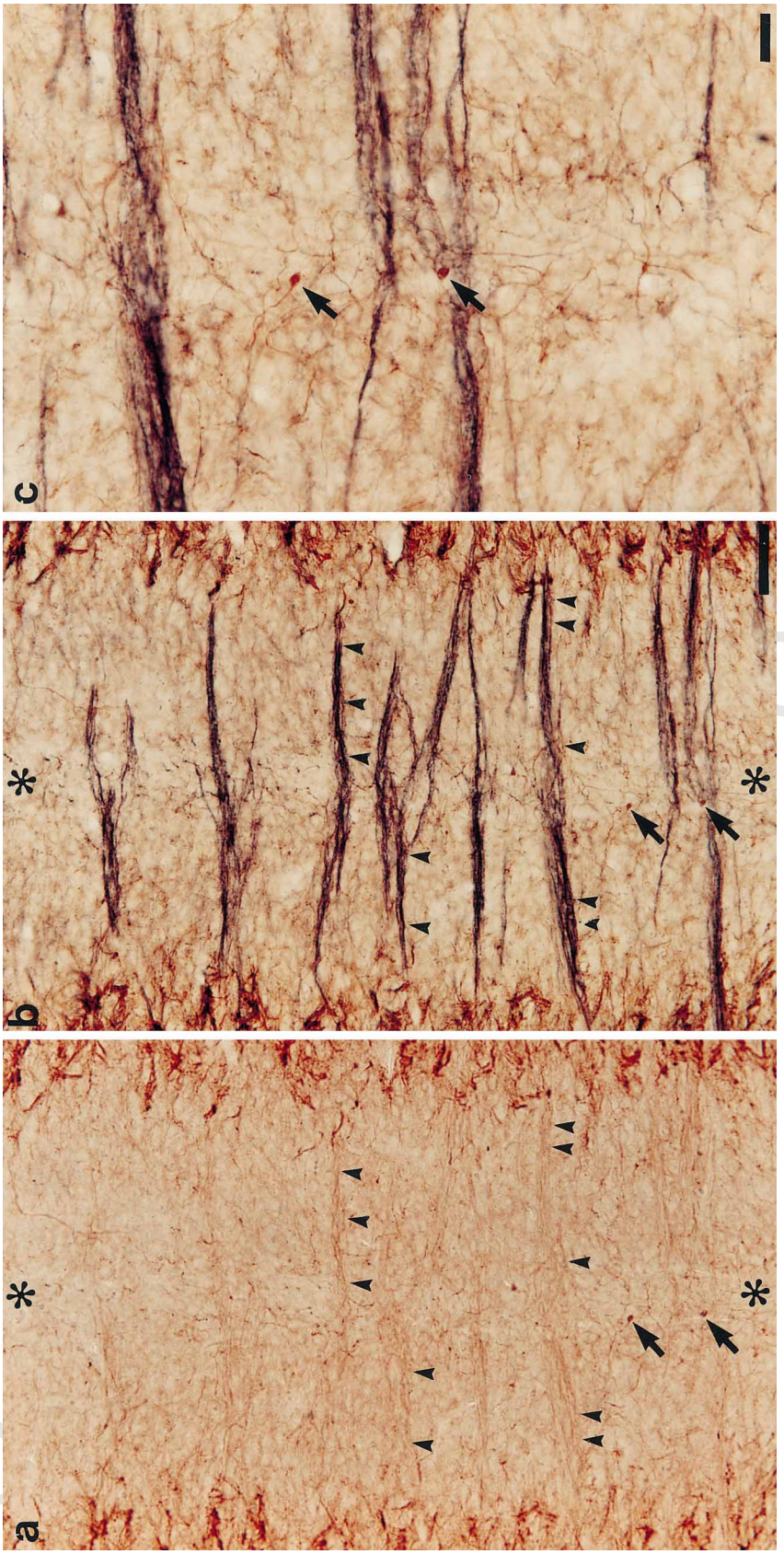


Fig. 7. Double-labeling of neurofilament- (a) and L1-labeled axons (b,c) in a horizontal section of E19 cervical spinal cord. Rostral spinal cord is oriented at the top of each photomicrograph and asterisks delineate the midline of the dorsal commissure. Neurofilament-positive primary sensory afferents cut in cross section are seen along the sides of panels a and b. (a): A section stained with anti-neurofilament antibody (amber-brown chromogen) to identify the middle axon bundles (arrowheads) was temporarily coverslipped with glycerin and photographed. Several prominent varicosities (arrows) are seen along individual axons labeled with the 2H3 antibody and are enlarged in (c). (b): Double-labeling of the section illustrated in a with L1 antibody (black chromogen). Middle axon bundles (arrowheads) are labeled with both antibodies, but axons in between the bundles and the prominent varicosities (arrows) appear to be labeled only with the neurofilament antibody. (c): Higher magnification of the lower part of panel b illustrates the same neurofilament-labeled varicosities (arrows) seen in a and b. L1-positive middle fiber bundles appear to be stained with both neurofilament and L1 and consequently are amber brown and overlaid by black reaction product. Many single-labeled neurofilament-positive fibers course within the dorsal commissure. Scale (a,b)=60 μm; (c)=20 μm.

neurofilament-positive fibers coursed within the dorsal commissure and were oriented in rostrocaudal or mediolateral directions (Fig. 7c). In addition, prominent varicosities were observed along some of the neurofilament-only labeled axons found within the dorsal commissure (Fig. 7c).

4. Discussion

In this study, we have described the development of several groups of commissural axons that are strikingly similar to bundles of fibers illustrated in Ramón y Cajal's original drawings [33]. We have presented evidence that between E17 and E19, a large number of dorsal commissural fibers express the cell surface glycoprotein L1, and many contain the cytoplasmic neurotransmitter synthesizing enzyme GAD65. Embryonic day 17 was the first time during development that L1-, GAD65- or neurofilament-labeled axons were detected crossing the dorsal commissure. To our knowledge, the axon bundles of the dorsal commissure have not been explicitly examined since the original Golgi studies by Ramón y Cajal.

4.1. Cellular origin of dorsal commissural axons

It is likely that the location of the cell bodies of origin for each of the three dorsal commissural bundles differs and that each bundle contains axons from more than one source. Ramón y Cajal suggested that axons forming the middle fiber bundle were collaterals from axons coursing rostrocaudally within the dorsal-most part of the lateral funiculus. Initially, we presumed that prominent L1- and GAD65-positive fibers coursing rostrocaudally in the reticulated area might be a source of the middle axon bundles. However, fibers in the reticulated area were never seen giving rise to axons turning medially into the dorsal commissure. Instead, they resembled those described by Ramón y Cajal [35] as displaced fascicles of white matter that coursed towards their terminal fields within the spinal gray matter. According to Ramón y Cajal, collaterals from the displaced fascicles arborized in the sensory horn of the ipsilateral spinal cord, a description consistent with our observations that L1- and GAD65-immunoreactive fibers within the reticulated area appear to be a separate system of fibers from those crossing in the dorsal commissure.

A second potential source of axons contributing to the middle fiber bundle are primary sensory afferents. Studies utilizing horseradish peroxidase or carbocyanine dye to identify primary afferents at several different spinal cord levels have reported axons crossing in the dorsal commissure [22,23,26,28,44]. Studies identifying visceral primary afferents have described numerous fibers entering the dorsal commissure through the lateral collateral pathway in sacral spinal segments [31]. Central processes of the primary afferents express L1 as they enter the spinal cord, and thus likely contribute to the L1-labeled middle fiber bundle. In contrast, however, there is little evidence to date that any of the subsets of primary afferents are GABAergic, but rather they have been suggested to utilize glutamate, substance P, and calcitonin gene-related peptide (CGRP) as neurotransmitters and/or neuromodulators [10,46,56]. Therefore, it is likely that the GAD65-positive component of the middle bundle originates from sources other than the dorsal root ganglion. However, dorsal commissural axons identified only with L1, as illustrated in Fig. 5d, may represent the lateral collateral pathway.

A third possible source of the middle fiber bundle would be spinal interneurons located near the dorsolateral funiculi. GABAergic neurons have been identified in all lamina of the dorsal horn [2,25,27] and may project across the dorsal commissure in the middle bundle at the same or adjacent spinal segments.

The posterior and anterior fiber bundles may also have cell bodies of origin that are more rostral or caudal to the spinal cord segment where their axons cross the midline. GABAergic interneurons appear to project axons across the dorsal commissure in the posterior bundle. In addition, primary afferents have been described traversing a pathway similar to that of the posterior bundle [28,44] and thus probably contribute L1-labeled fibers to the posterior bundle. Primary afferents also have been observed to branch and terminate with large varicosities within the dorsal commissure [26] and appear similar to the individual neurofilament-labeled axons with prominent varicosities observed in this study.

4.2. Comparison of axons crossing in the ventral and dorsal commissures

Axons traversing the ventral and dorsal commissures have distinct patterns of origin and of spatial and temporal development. Axons forming the ventral commissure have been observed to originate from cell bodies within the dorsal and ventral spinal cord and to extend their axons towards the floor plate at the same level where the fibers cross the midline [15,16,32,43,55]. In the dorsal commissure, many axons are likely to extend from cell bodies that are not within the same spinal cord level (i.e. supraspinal or propriospinal in origin). Axons crossing in the ventral and dorsal commissures also differ in their projection patterns along the length of the spinal cord. In the ventral commissure, axons traverse the midline continuously along the rostrocaudal extent ([53] Tran and Phelps, unpublished observations), while in the dorsal commissure, bundles of axons cross the midline periodically. After crossing the midline, these axons also differ in their turning behavior. Ventral commissural axons turn orthogonally before extending rostrally in the ventral funiculus [9,15,43] whereas dorsal commissural axons extend directly toward the contralateral dorsal horn ([44], present results).

Ventral and dorsal commissural axons also vary in their

temporal development. In rats, fibers projecting to the ventral commissure do so early in development, beginning around E12, while axons extending across the dorsal commissure do so later, between E16 and E17. Ventral commissural axons are known to be attracted to the midline by the secretion of netrin-1, a long range chemoattractant expressed by floor plate cells [19,39,50], whereas currently little is known about the guidance of dorsal commissural axons. Snow et al. hypothesized that axons may not be able to cross the dorsal midline until the expression of inhibitory molecules, such as the glycosaminoglycan keratan sulfate, were no longer present in the roof plate [45]. Thus, dorsal commissural axons may have to wait to cross the midline until inhibitory molecules are removed from the roof plate [45]. It will be interesting to determine if the roof plate also plays a chemoattractant role in guiding axon populations across the dorsal midline as has been shown with the floor plate and axons crossing the ventral midline [50,51].

Ventral and dorsal commissural axons also have features in common such as their neurotransmitter phenotype. Numerous GABAergic neurons extend their axons across the midline to the contralateral side of the spinal cord in both the ventral [32,53] and dorsal commissures (present results). In addition, bundles of axons crossing in both commissures express the L1 glycoprotein on their surfaces both before and after they traverse the midline of the spinal cord [53].

4.3. Possible function of L1 expression in commissural axons

L1-like molecules are present during early axon guidance and pathfinding in both the CNS [41,47] and peripheral nervous system (PNS) [8]. The precise role of L1 during the development of axonal fiber tracts, however, remains an active area of investigation. Rathjen et al. [37] reported that the fasciculation of retinal axons could be strongly inhibited by antibodies to L1-related antigens. Similar defasciculation results have been observed by Stoeckli and Landmesser [48] in antibody perturbations of the L1 homologue (NgCAM) in chick ventral commissural axons. Their results suggest that the L1 cell adhesion molecule may be involved specifically in the bundling of developing axons but not in their guidance. The presence of L1 on both ventral and dorsal commissural axons supports a possible role for this molecule in axons that bundle together before crossing the midline. Furthermore, the finding that L1-negative axons course between the labeled bundles is consistent with the role of L1 in axon fasciculation. Cohen et al. [7] reported that mice lacking L1 have defects in axonal guidance of the corticospinal tract as many of these axons fail to cross the midline at the decussation of the pyramids. Our results indicate that L1 is present at the appropriate time to play a role in axon outgrowth and fasciculation of commissural fibers across the midline.

4.4. GAD65 in developing spinal cord commissures

GAD65 has been described in elongating ventral commissural neurons as early as E12 [32], and in this study we report that GAD65 identifies axons as they extend across the midline in the dorsal commissure between E17 and E19. The decrease in GAD65 within dorsal commissural axons and increase in GAD65 terminal staining within the adjacent neuropil is reminiscent of findings in the embryonic ventral commissure and hippocampal formation and has been interpreted as a shift in the intracellular distribution of GAD65 within GABAergic neurons [11,12,32]. Thus, the current results suggest that a number of GABAergic neurons extend their axons across the neural axis through the dorsal commissure.

While GAD65 and L1 have overlapping patterns of expression, the coincidence of these two antigens within the spinal commissures is not exact. In the dorsal commissure, GAD65 and L1 are found in both the posterior and middle fiber bundles, however, GAD65-immunoreactive axons were less numerous than those labeled with L1-immunoreactivity. Additionally, while L1-labeled axons were present in the anterior fiber bundle, GAD65 was not detectable in these fibers, suggesting that there are few or no GABAergic axons traversing this portion of the dorsal commissure. In the ventral commissure, both GAD65 and L1 have been identified in some commissural axons and demonstrate an overlapping pattern of expression [53].

Although the ventral and dorsal commissures develop at different times and have different spatial distributions, they both possess a significant number of GABAergic and L1-immunoreactive axons. These groups of fibers provide useful models for further studies into the cellular and molecular mechanisms involved in axonal outgrowth and guidance of axons that cross the midline. The expression of L1 on several populations of commissural axons during axonal development supports a previously proposed role for this molecule in axonal outgrowth and fasciculation [1,13,20,21,48]. With the recent availability of L1 knockout mice [7,14], it will be important to examine the different populations of commissural neurons to determine if defects exist in their midline crossing patterns.

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