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Development of specific muscle and cutaneous sensory projections in cultured segments of spinal cord

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SUMMARY

Development of sensory projections was studied in cultured spinal segments with attached dorsal root ganglia. In spinal segments from stage 30 (E6.5) and older chicken embryos, prelabeled muscle and cutaneous afferents established appropriate projections. Cutaneous afferents terminated solely within the dorsolateral laminae, whereas some muscle afferents (presumably Ia afferents) projected ventrally towards motoneurons. Development of appropriate projections suggests that sufficient cues are preserved in spinal segments to support the formation of modality-specific sensory projections. Further, because these projections developed in the absence of muscle or skin, these results show that the continued presence of peripheral targets is not required for the formation of specific central projections after stage 29 (E6.0).

Development of the dorsal horn in cultured spinal segments was assessed using the dorsal midline as a marker. In ovo, this midline structure appears at stage 29. Lack of midline formation in stage 28 and 29 cultured spinal segments suggests that the development of the dorsal horn is arrested in this preparation. This is consistent with earlier reports suggesting that dorsal horn development may be dependent on factors outside the spinal cord.

Because dorsal horn development is blocked in cultured spinal segments, this preparation makes it possible to study the consequences of premature ingrowth of sensory axons into the spinal cord. In chicken embryos sensory afferents reach the spinal cord at stage 25 (E4.5) but do not arborize within the gray matter until stage 30. During this period dorsal horn cells are still being generated. In spinal segments, only those segments that have developed a midline at the time of culture support the formation of specific sensory projections. The end of the waiting period therefore coincides with the formation of a dorsal midline and, interestingly, also with the development of cues in the dorsal horn that are required for the formation of specific sensory projections. Based on these results, we propose that one important function of the waiting period is to delay the ingrowth of sensory fibers until appropriate guidance cues have developed within the dorsal horn.

Key words: guidance cues, sensory afferents, organotypic cultures, waiting period

INTRODUCTION

A critical feature of neuronal development is the establishment of precise, modality-specific synaptic connections between different sets of neurons. During embryonic development both axonal trajectories and choices of presynaptic and postsynaptic targets are modulated by the environment surrounding the neuronal somata or encountered by their axons (Dodd and Jessell, 1988; Goodman and Shatz, 1993). Spinal sensory neurons, with bipolar axons that project both peripherally and centrally, are capable of a remarkable coordination between the functional modalities of their peripheral and central targets. Relatively little is known about the underlying mechanisms that enable neurons to project axons to appropriately matched target areas. To this end, it will be useful to establish preparations where this phenomenon can be studied in culture.

Over the last several years, significant progress has been made in establishing preparations for studying the development of axonal projections (Gahwiler, 1981; Stewart et al., 1991). Organotypic co-cultures of thalamus and neocortex have been developed in which thalamic sensory afferents project to the appropriate cortical layers (Caeser et al., 1989; Yamamoto et al., 1989, 1992; Bolz et al., 1990, 1992). However, it has not been possible to demonstrate modality-specific projections in these preparations (Molnar and Blakemore, 1991). Spinal sensory neurons are particularly interesting in this regard because different functional classes of sensory neurons can be identified on the basis of both their peripheral and central projections. For example, cutaneous afferents are confined to the dorsal laminae of the cord (Davis et al., 1989; Woodbury and Scott, 1991; Woodbury, 1992), while sensory neurons supplying muscle spindles project down through the dorsal layers to arborize more ventrally (Jhaveri and Frank, 1983; Mendelson et al., 1992). This projection pattern determines the choice of postsynaptic target within the spinal cord; only muscle spindle afferents make direct synaptic contacts with motoneurons (Brown, 1981). Moreover, one can distinguish among different functional classes of sensory

neurons in vitro because the peripheral axons of different neuronal types project to their targets (skin versus muscle) in separate peripheral nerves. We have exploited these advantages to develop a preparation of spinal segments with attached sensory ganglia in which modality-specific sensory connections are established in culture.

A well-documented aspect of axon growth in several systems is the delay between arrival of axons at the boundary of their target and their innervation of it. For example, thalamic axons grow to the appropriate region of developing cortex and then pause within the subcortical zone before they project to the appropriate cortical layers (Lund and Mustari, 1977; Rakic, 1977). Motoneuronal axons also pause within the plexus region before they enter the developing limb bud (Swanson and Lewis, 1982). Similarly, axons of spinal sensory neurons grow rapidly to the dorsolateral margin of the spinal cord but project into the gray matter of the dorsal horn only after a significant delay (Lee et al., 1988; Mendelson et al., 1992). During this period these axons establish their longitudinal projections rostrally and caudally within the developing dorsal columns. Although the existence of this waiting period has been determined in several different systems, its functional significance is unclear. Our new preparation gave us an opportunity to explore this significance. When spinal segments were placed in culture before the end of the normal waiting period, further development of the dorsal horn did not occur, yet sensory axons projected into the gray matter. By placing the segments into culture at various times during the waiting period, we were able to assess the development of cues within the spinal cord that are required for the establishment of correct patterns of sensory input.

Finally, peripheral targets of sensory neurons have been found to have an important influence on the development of the central projections of these neurons within the spinal cord (Frank and Westerfield, 1982; Smith and Frank, 1987). However, the time period during which this influence is manifest has not been studied. Our new preparation helps to define a time window for this influence after which the peripheral target is not necessary for the development of appropriate central projections.

MATERIALS AND METHODS

Spinal segment cultures

Spinal cords with attached dorsal root ganglia (DRG) and peripheral nerves were dissected in oxygenated Tyrode's solution (Landmesser, 1978) from stage 28-30 (E5.5-6.5) chicken embryos (White Leghorn, obtained from Penn State University Farms). Thick spinal slices (500-800 μm) were cut from the lumbosacral 1 (LS1) to LS3 levels. Spinal segments consisted of spinal cord, two DRGs attached through dorsal and ventral roots, and, for embryos older than stage 28, peripheral nerves (obturator, a muscle nerve and cutaneous femoralis medialis (CFM), a cutaneous nerve). Spinal segments were embedded in 2% low melting point agarose (SeaPrep, FMC Laboratory, Rockland, ME) in defined medium (MEM, progesterone 20 nM, insulin 40 μg/ml, putrescine 1 μg/ml, sodium selenite 20 nM and transferrin 40 μg/ml; all materials from GIBCO) supplemented with chick muscle extract (1 μg prot./ml, Davies, 1986) and nerve growth factor (15 ng/ml, Sigma). The agarose was allowed to gel at 4°C for 6 minutes and embedded spinal segments were then cultured in the same medium saturated with 95%/5% O_2/CO_2 at 37°C.

Spinal slice cultures

Spinal cords with attached DRG were dissected from stage 31 (E7) embryos. Slices (200-250 μm) were cut on a Vibratome; those slices with intact dorsal and ventral roots and attached DRG were used for culture. Slice cultures were grown on collagen-coated dishes (rat tail collagen, 1 mg/ml, 50 μl per 35 mm dish) in serum-free defined medium as described above.

Labeling of sensory afferents

Sensory projections were labeled with a saturated solution of DiI (1,1′-diocta-decyl-3,3,3′,3′-tetramethylindocarbocyanin perchlorate; Molecular Probes) in ethanol. For spinal segments with peripheral nerves, DiI was applied to the cut end of the nerve after embedding the segment in agarose and allowed to diffuse throughout the culture period. In spinal segments without the peripheral nerve and in spinal slices, DiI was injected into the DRG after the culture period just prior to fixation and allowed to diffuse overnight at room temperature. All cultures were fixed in phosphate-buffered 4% paraformaldehyde. Labeled axons were visualized using a rhodamine filter set with either a confocal or conventional epifluorescence microscope in 50 μm Vibratome sections (for spinal segments) or intact cultures (for spinal slices).

RESULTS

Development of sensory projections

To provide a framework for the analysis of sensory projections that develop in culture, we first describe the pattern of these projections in normal chicken embryos shortly after they begin to form. Although sensory axons begin to arrive at the edge of the spinal cord (the dorsal root entry zone, or DREZ) by stage 25 (E4.5), there is no appreciable growth into the future gray matter until after stage 30 (E6.5, Fig. 1A). By stage 34 (E8), however, a characteristic projection pattern is apparent (Fig. 1C). The basic characteristics of the sensory projections that develop during this 1.5 day period in ovo include: (i) restriction of all the sensory projections to the ipsilateral dorsal horn and (ii) segregation of the projections into two distinct regions of the spinal gray matter - a dorsolateral region just medial to the DREZ and a more medial region where afferents projected through the dorsal laminae to more ventral layers.

Separate labeling of muscle and cutaneous nerves with DiI reveals that the two regions of projections correspond to two different functional classes of sensory afferents, confirming earlier observations (Lee et al., 1988; Lee and O'Donovan, 1991; Mendelson et al., 1992). The projections of cutaneous afferents are restricted to the dorsolateral laminae (Fig. 1C, right), while one class of muscle afferents (known from earlier studies to be muscle spindle afferents) makes the more medial projections towards the ventral layers (Fig. 1C, left).

A similar pattern of sensory projections developed in cultured spinal segments. When spinal segments from embryos at stage 30-32 (n=14) were placed in culture for 2 days, sensory axons grew into the spinal gray matter but did not cross over the midline (Fig. 2A,B), just as in normal development. Prelabeling of muscle and cutaneous sensory axons just before the segments were placed in culture, as in Fig. 2A, showed that each class of afferent made its normal projections. Cutaneous afferents were restricted to the dorsolateral laminae, while some muscle afferents projected medially and then ventrally. When muscle and cutaneous afferents were labeled together,

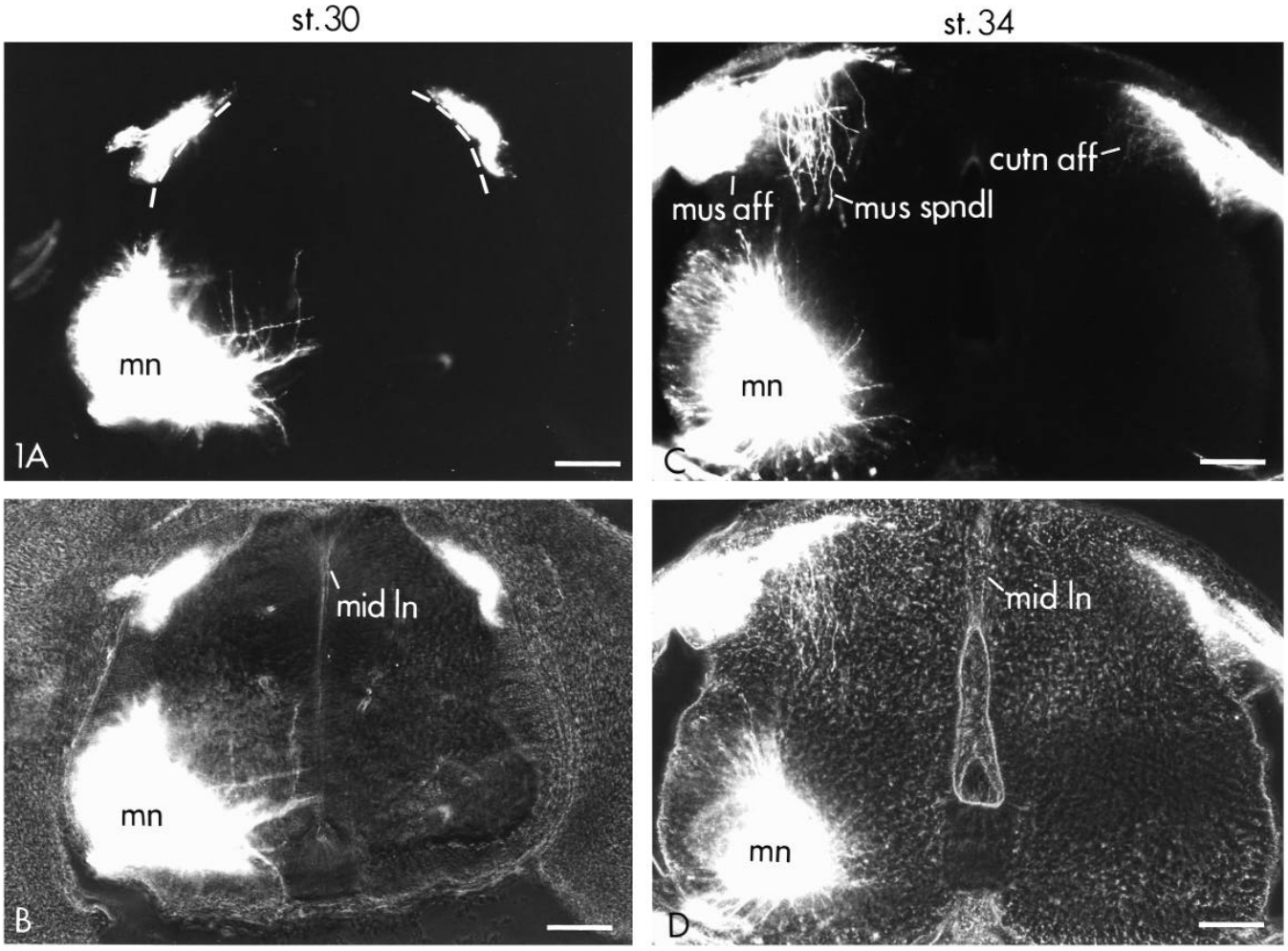

Fig. 1. Development of sensory afferent projections in chicken embryos in ovo, at stage 30 (A,B) and at stage 34 (C,D). Muscle and cutaneous afferents (left and right sides, respectively, in each panel) were labeled with DiI placed on a peripheral nerve. At stage 30, muscle and cutaneous sensory afferents at LS 1-3 levels remain at the dorsolateral margin of the spinal cord and have not projected through the boundary between gray and white matter (broken line in A). By stage 34 sensory afferents have grown into the spinal gray matter and characteristic muscle and cutaneous projections have developed. Muscle afferents make two distinct projections: muscle spindle afferents (mus spndl) enter the dorsal horn dorsally and project first medially and then ventrally towards dendritic arbors of motoneurons (mn); other muscle afferents (musl aff) remain confined to dorsolateral laminae. Cutaneous afferents (cutn aff) enter dorsolaterally and are restricted to superficial dorsal laminae. Sensory afferents neither cross the midline (mid ln) nor project into the ventral horn at these stages. Scale bar, 100 μm.

this distinctive projection pattern was still apparent (Fig. 2B). Afferent projections could be easily identified in specific areas of the dorsal horn: a dorsolateral region adjacent to the DREZ, and a more medial group of afferents projecting ventrally towards the lateral motor column. This result was useful because at earlier stages of development (see below), muscle and cutaneous nerves could not be labeled separately; instead DiI was injected directly into the DRGs.

These results show that, in this culture system, sufficient cues are preserved both on sensory neuronal axons and within the spinal cord to permit the establishment of modality-specific sensory projections. Moreover, sensory neurons maintain their distinctive phenotype, as evidenced by their central projections, despite the absence of their peripheral target tissue. Continued contact with peripheral target after stage 29 (E6.0) is therefore not required for the development of correct central projections.

Development of dorsal horn

The presence of appropriate guidance cues within the dorsal horn is required for the development of specific sensory projections. For example, the roof plate and the sparsely cellular spinal dorsal midline have been suggested to restrict sensory afferent projections to the ipsilateral dorsal horn (Snow et al., 1990). We have used the dorsal midline as a structural landmark to assess the development of dorsal horn architecture and its preservation in cultures of spinal segments and spinal slices as it is easily visible both during and after the culture period.

The dorsal midline first appears while the sensory afferents remain within the DREZ (from stage 25 to stage 30). In stage 28 and early stage 29 embryos, the midline has not yet developed and it fails to develop in our cultures even after 2 days (Fig. 3A,B). Development of the midline in ovo proceeds

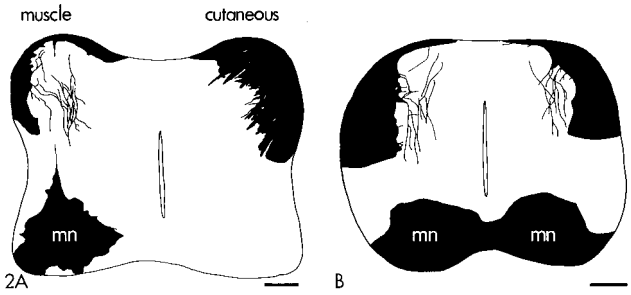

Fig. 2. Development of sensory projections in stage 30 spinal segments after 2 days in culture. Afferent projections that developed in vitro and in the absence of peripheral targets were similar to sensory projections that develop in ovo (compare Fig. 2 with Fig. 1 C,D). In A, muscle (obturator, left side) and cutaneous (cutaneous femoralis medialis, right side) afferents were labeled separately with DiI placed on their peripheral axons at the time the segment was placed in culture, before central projections had developed. Motoneurons (mn) are labeled only on the left side. In B, DiI was placed in DRGs so both types of sensory afferents were labeled on each side of the segment. Dye also spread to ventral roots, resulting in labeled motoneurons (mn) bilaterally. Drawings were made from a digitally reconstructed confocal image using ten 5 μm optical sections in A and from conventional phase-contrast and fluorescence micrographs in B. Scale bar, 100 μm.

from the roof plate ventrally to the central canal. By late stage 29, a clearly defined midline structure extends through the entire dorsal half of the spinal cord (Fig. 3C). From this stage onwards, the midline remains a distinctive feature in cultured spinal segments (Fig. 3D, stage 30). To the extent that midline development is a marker for the development of the dorsal horn in general, these results show that spinal segments in culture preserve the target area of the sensory afferents that existed at the time that they were placed in culture. Further development apparently does not occur.

The midline is not preserved, however, in slices of spinal cord cultured on collagen-coated dishes (Fig. 5). When spinal slices are cultured on a two-dimensional substratum, many cells can be seen migrating on the collagen matrix around the spinal cord and the DRG. In contrast, the agarose gel used in spinal segment cultures provides a matrix that is not conducive to cell migration and it therefore helps to maintain the morphological inter-relationship among cells.

Dependence of projection specificity on target development

During development in ovo, sensory afferents begin to project into the spinal gray matter after stage 30 (E6.5). In cultured spinal segments from stage 30 and older embryos, growing sensory afferents therefore encounter dorsal horn that is at the same stage of development as in ovo. By arresting the development of the dorsal horn in spinal segments from younger embryos, we could challenge the sensory afferents to grow in an underdeveloped target.

In spinal segments from stage 28 and early stage 29 embryos, sensory projections failed to develop specifically. In these cultures from younger embryos sensory afferents did not segregate into one group that terminated dorsolaterally and another group that projected medially and then ventrally.

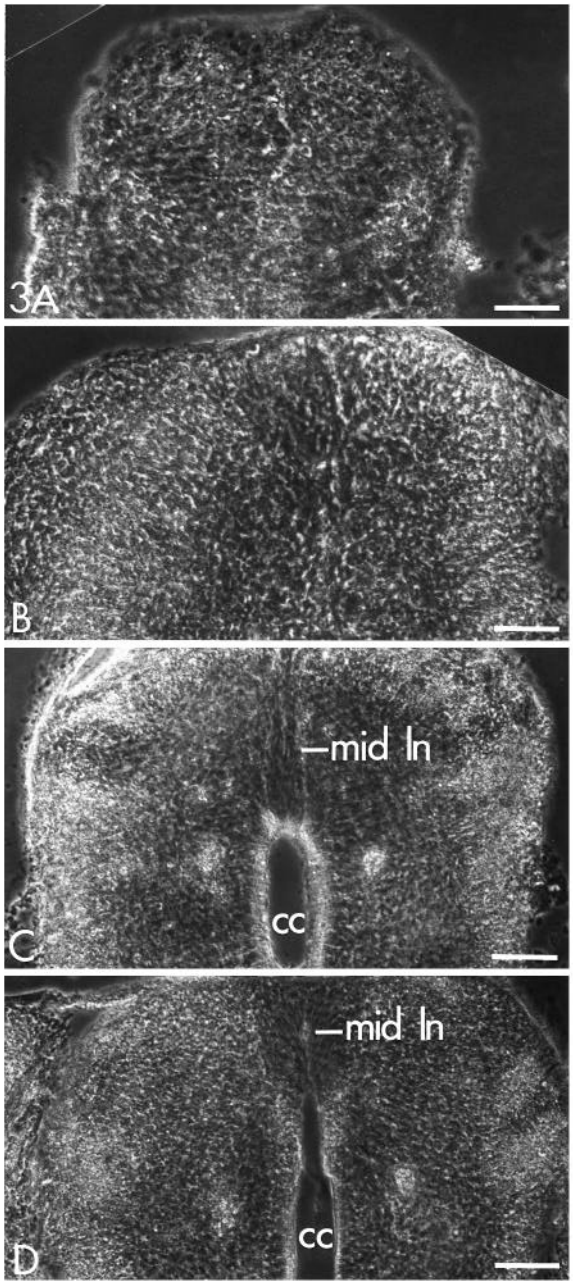

Fig. 3. Development of the dorsal horn, as assessed by the presence of a dorsal midline structure, is arrested in cultured spinal segments. In stage 28 (A) and early stage 29 (B) spinal segments, the dorsal midline has not developed and fails to develop even after 2 days in culture. At late stage 29 (C) and stage 30 (D), the dorsal midline (mid ln) has appeared and is distinct all the way to the central canal (cc). Scale bar, 100 μm.

Instead, most sensory fibers grew across the dorsal horn, from the dorsal root entry zone towards the midline. Many fibers continued their growth across the midline and projected into the contralateral dorsal horn. This non-specific projection pattern was seen consistently in all the 10 stage 28 (Fig. 4A,B) and 6 early stage 29 (Fig. 4C,D) segments examined. The specificity of sensory projections developing in culture was tightly correlated with the presence of the dorsal midline (refer

to Fig. 3 showing phase-contrast micrographs of the same segments). In stage 28 segments, in which the midline is not visible, sensory fibers made contralateral projections all along the dorsoventral extent of the dorsal horn. In early stage 29 segments, contralateral projections were seen only in the ventral half of the dorsal horn. By late stage 29, however, when the dorsal midline has developed, sensory projections were made specifically in all 8 segments examined (Fig. 4E,F).

This non-specific pattern of sensory afferent projections was correlated with an absence of the dorsal midline in two other situations as well. When slices of spinal cord from stage 31 embryos were cultured on a collagen matrix, the midline disappeared, as described earlier. Sensory afferents growing into these slices failed to segregate into their characteristic spinal regions and projected into the contralateral dorsal horn (Fig. 5). A further example comes from observations of axon growth at the rostral and caudal cut edges of later stage spinal segments. In the interior of these segments, sensory projections, which had already developed in ovo at the time of culture (refer to Fig. 1C) were specific (not shown). Within the first 50 μm of the cut edge of the spinal cord, however, the morphology of the spinal cord (evaluated in terms of the midline, compare Fig. 6A with Fig. 1D) was disrupted and sensory afferents failed to project specifically (Fig. 6B,C).

DISCUSSION

Formation of specific synaptic connections by sensory neurons is likely to be accomplished by a series of interrelated mechanisms. Some of these mechanisms relate to how the phenotype of sensory neurons is determined, while others must provide the molecular cues within the spinal cord that permit sensory neurons to project to their appropriate central targets. The fact that these neurons establish modality-specific projections within the spinal cord in the spinal segment preparation described in this report implies that at least several of these mechanisms survive in culture. One class of muscle sensory afferents, presumably those that supply muscle spindles, retain their phenotypic identity in this preparation sufficiently well that they make central projections indistinguishable from those that they form in vivo. The pattern of these projections is quite distinct from those made by cutaneous afferents, suggesting that these neurons, too, maintain their distinctive phenotype in culture. Moreover, for these neurons to make their characteristic central projections, the appropriate molecular cues within the spinal

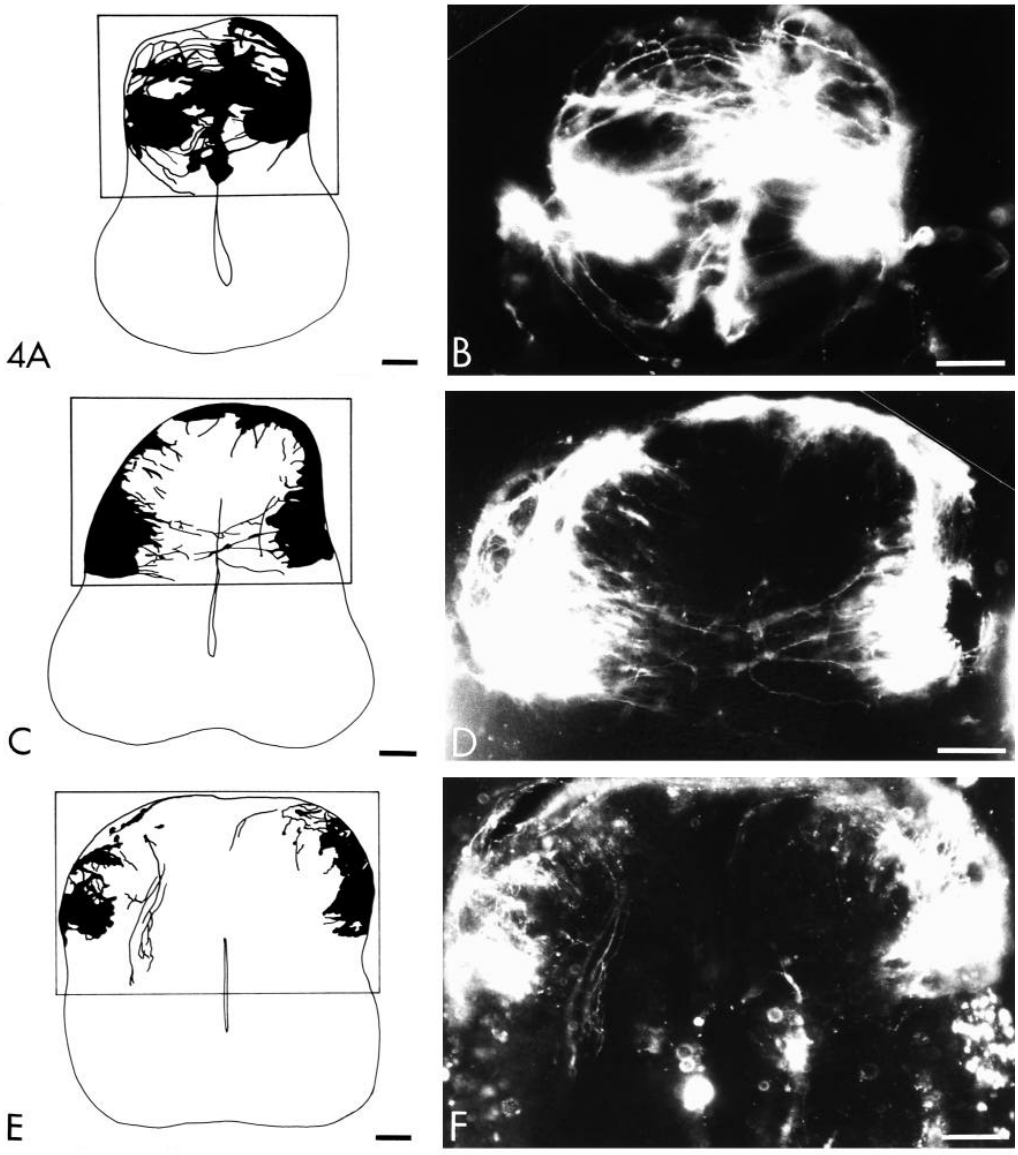

Fig. 4. Development of sensory projections in cultured segments at different developmental stages. In segments placed in culture at stage 28 (A,B) or early stage 29 (C,D), sensory projections do not develop appropriately. Muscle and cutaneous afferents fail to segregate as in ovo. Many axons project medially and cross into the contralateral dorsal horn. At later stages of development, muscle and cutaneous afferents project appropriately, as shown for the late stage 29 segment in panels E and F. At stage 28 and early stage 29, DiI was placed on the dorsal roots. For the late stage 29 segment, muscle and cutaneous afferents were labeled separately on the left and right sides. Phase-contrast micrographs of these same segments are shown in Fig. 3A-C. Drawings were made from montages of phase and fluorescence micrographs. The fluorescence image (whose position is indicated by the boxes in the drawings) is shown to the right of each drawing. Scale bar, 100 μm in each panel.

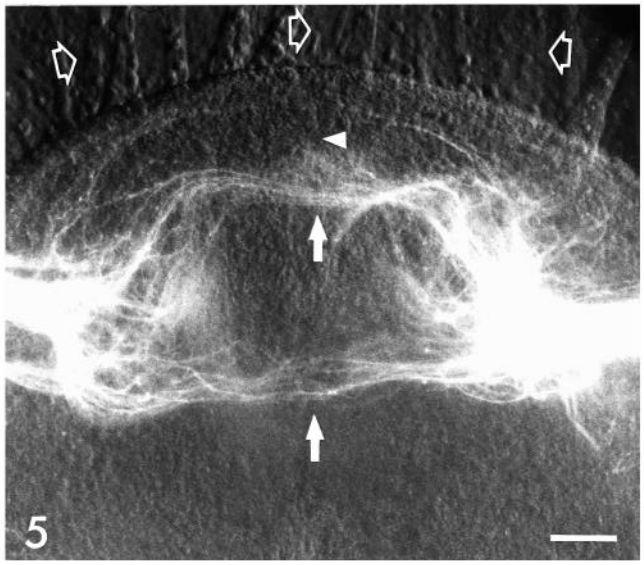

Fig. 5. Spinal slices cultured on a collagen matrix do not retain a dorsal midline structure, despite the fact that this structure had already developed at the stage (31) they were placed in culture (solid arrowhead marks the site at which the midline structure was located). Numerous spinal cells can be seen migrating on the collagen matrix (open arrows), away from the spinal explant. In these spinal slice cultures, sensory afferents grow into the spinal gray matter but project aberrantly within the ipsilateral dorsal horn. Many cross over into the contralateral dorsal horn (filled arrows). They are still excluded from the ventral horn, however. Scale bar, 100 μm.

cord itself must also be maintained in these cultures. Using this preparation, it should therefore be possible to study some of these mechanisms in greater detail than has been possible in vivo.

Specification of sensory neuron phenotype

An important aspect of this problem is how, during development, sensory neurons establish their distinctive phenotypes. What factors control, for example, whether a sensory neuron differentiates into a cutaneous mechanoreceptor or a muscle spindle afferent? The present results with cultured spinal segments show that neurons projecting in cutaneous and muscle nerves already have distinctive phenotypes shortly after these nerves are formed, arguing against the idea that the specificity of peripheral innervation is achieved by selective cell death. The earliest projections to skin form at stage 27 in chickens (Honig, 1982), and most naturally occurring cell death of sensory neurons occurs after stage 29-30 (Hamburger et al., 1981) in brachial ganglia, and presumably somewhat later in lumbosacral ganglia (Hamburger and Levi-Montalcini, 1949). Yet already by stage 29 (the earliest stage at which it was possible to label muscle and cutaneous nerves separately with DiI), neurons with axons in cutaneous nerves developed central projections characteristic of cutaneous, but not muscle spindle, afferents. It is unlikely that muscle afferents in cutaneous nerves would have died during the time in culture, because sensory neurons projecting in muscle nerves did develop the appropriate central projections for muscle afferents.

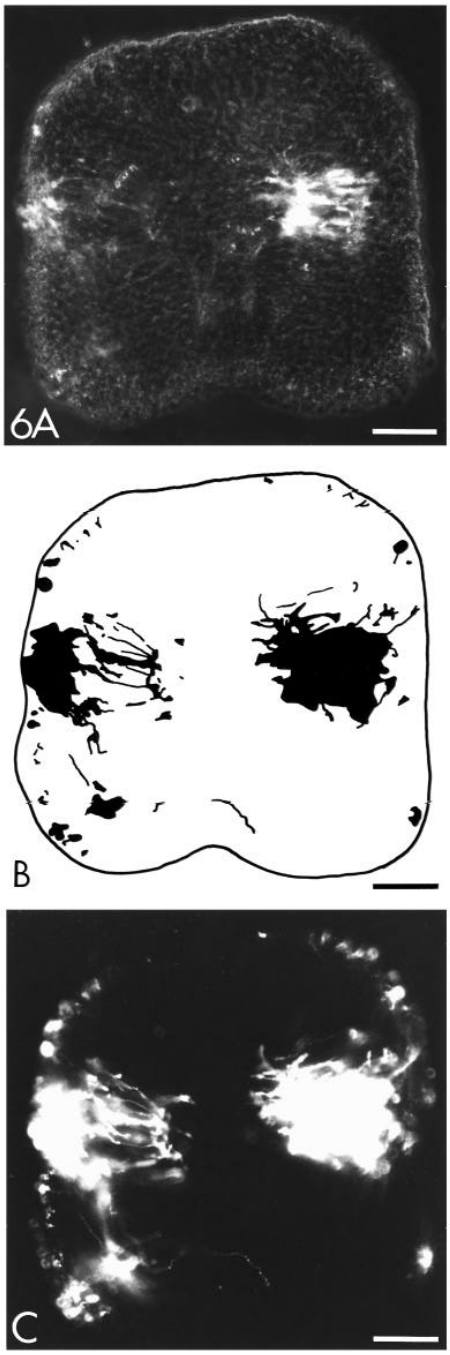

Fig. 6. The disruption of a dorsal midline structure is correlated with the loss of specificity of sensory projections. Near the cut surface of a stage 34 spinal segment, the midline structure has disappeared (compare panel A with Fig. 1D). Within this region, sensory afferents are no longer segregated into distinct regions within the dorsal horn (compare panels B and C with Fig. 1C). Micrographs and drawing were prepared as for Figs 3 and 4. Scale bar, 100 μm.

These experiments therefore place important restrictions on plausible mechanisms underlying the differentiation of sensory neurons supplying muscle spindles. If their phenotype is specified by the target muscle itself, then this specification must occur shortly after they contact their target, within less

than a day, because specific central projections are formed despite the absence of peripheral targets in cultured segments. In any case, it must occur before the majority of naturally occurring cell deaths. The absence of muscle spindle afferents in cutaneous nerves even at early stages argues either that they are already specified to project in muscle, but not cutaneous, nerves from the outset, or that some factor present in muscle, but not cutaneous, nerves is responsible for their differentiation. Future experiments in which sensory neurons from embryos before stage 29 are heterochronically transplanted into older host spinal segments will help to determine precisely when sensory neurons develop their distinctive phenotypes and when they acquire the ability to make appropriate central projections.

Development of projection cues within the spinal cord

For modality-specific sensory projections to develop within the spinal cord, not only must different classes of sensory afferents have distinct molecular phenotypes, but the target in which these projections form must also provide the appropriate cues to guide this specific growth. These cues are maintained in the cultured segments of spinal cord taken from later stage embryos, because specific sensory projections develop in such preparations. The normal development of these cues can therefore be studied by taking spinal segments from embryos at various developmental stages and determining whether sensory neurons establish specific projections within them.

We found that appropriate projections developed only in preparations from late stage 29 or older embryos. Before this stage, sensory fibers made extensive projections across the midline into the contralateral dorsal horn. Furthermore, sensory axons appeared to project throughout the entire dorsal horn. After stage 29, however, contralateral projections did not develop and ipsilateral projections were restricted into one group terminating solely within the superficial dorsolateral laminae and another (the spindle afferents) growing more medially before turning ventrally to project towards the lateral motor column. The stage at which sensory projections began to be made specifically was closely matched to the time when sensory afferents normally begin to grow into the gray matter of the spinal cord in ovo (see below).

The stages during which these guidance cues develop (approximately stage 27-30) are a period of rapid development of the dorsal spinal cord. At these stages, neuroepithelial cells are still dividing in the dorsal half of the ventricular zone and then migrating to their final locations within the dorsal horn (Langman and Haden, 1970, and unpublished observations of K. S., Z. K. and E. F.). Dorsal horn development has not been studied in detail and the appearance of a dorsal midline structure provided a convenient marker to chart its development in the cultured spinal segments. Based on the presence or absence of this structure, we found that further development of the dorsal cord did not occur in our cultures, although the structures present at the time that the segments were placed in culture did persist throughout the culture period. Interestingly, sensory projections grew specifically only in those spinal segments in which the midline had developed. This tight temporal correlation between development of the dorsal horn and correct patterns of projections suggests that the cues for specificity of sensory projections may be derived from cellular organization within the dorsal horn.

The reasons that further development of the dorsal spinal cord is arrested in these cultures are unknown. Unlike the development of ventral spinal cord, dorsal horn development is not dependent on signals from the notochord or floor plate (Placzek et al., 1991; Yamada et al., 1991; Ericson et al., 1992). Recently it has been shown that dorsalin-1, a member of the TGF-β gene family, is selectively expressed by cells in the dorsal neural tube (Basler et al., 1993). Signals from notochord and floor plate restrict the expression of dorsalin-1. However, dorsalin-1 can influence differentiation of neural tube cells independent of ventral signals (Basler et al., 1993). The lack of further differentiation of the dorsal horn in our cultured spinal segments is consistent with the idea that the notochord and ventral spinal cord are not sufficient to induce this differentiation. Further, our results suggest that dorsal horn development might depend on structures outside the spinal cord, which were not included in our cultures. A developmentally relevant, although reverse, interaction between the neural tube and the overlying mesenchyme has been reported (Takahashi et al., 1992). The expression of the *Quox-8* gene, a member of the family of homeobox-containing genes, in the mesenchyme can be induced by dorsal but not ventral spinal cord (Takahashi et al., 1992). The possibility that surrounding tissues are important in dorsal horn development will be tested in future experiments by including mesenchymal tissue present around the spinal cord at these stages.

What features of the spinal cord that develop during this period might serve as cues to direct the growth of sensory fibers? The prominent dorsal midline structure might ensure the restriction of sensory fibers to the ipsilateral dorsal horn, as has been suggested previously (Snow et al., 1990). If so, more than a direct barrier function is likely to be involved, because sensory afferents do not project up to the midline itself. The ventral half of the spinal cord may also restrict growth of sensory fibers to the dorsal horn, at least during their initial growth (up through stage 34) before the collaterals of muscle spindle afferents project more ventrally directly into the lateral motor column. Consistent with this idea, sensory axons grow into explants of dorsal, but not ventral, spinal cord, suggesting the presence of some inhibitory factor within or released by the ventral cord (Fitzgerald et al., 1993). In our cultures, growth of sensory axons within the ventral cord did not occur even when projections within the dorsal horn were non-specific, both in cultured spinal segments before stage 29 and in slice cultures. These observations suggest that the inhibitory influence of the ventral cord is distinct from cues within the dorsal horn responsible for the specific segregation of afferents subserving different sensory modalities. These cues are apparently contained within the dorsal horn itself, because it is within the dorsal laminae that distinct types of sensory projection are first apparent. Moreover, these cues develop relatively late, while sensory afferents are already waiting at the dorsolateral margin of the spinal cord.

Functional implications of the waiting period

A characteristic feature of the development of sensory axons is a distinct pause in their growth when they initially reach their target field. Peripherally, sensory (and motor) axons wait within the plexus region of the spinal nerves for about one day

before growing into the developing limb bud (Swanson and Lewis, 1982). Centrally, they wait within the DREZ at the dorsolateral margin of the spinal cord from stage 25 to stage 30, after which they project specifically within the dorsal horn. Similarly, thalamic afferents wait in the cortical subplate and innervate cortex only when their postsynaptic target neurons have been generated in layer 4 (Lund and Mustari, 1977). Although the existence of such waiting periods is well documented, neither their underlying mechanisms nor their functional significance is well understood.

In certain cases, the target tissue itself may be refractory to neurite growth during the waiting period. For example, thalamic afferents in culture do not grow into explants of developmentally immature neocortex, although they do project to explants of older cortex (Bolz et al., 1993). In spinal segments, however, immature dorsal horn is not refractory to the ingrowth of sensory fibers, suggesting that the 'wait signal' is not located within the dorsal horn itself. Another possibility is that the waiting period is mediated by mechanisms located at the edge of the target. Such a mechanism has been suggested for the development of thalamo-cortical projections. Waiting thalamic afferents make transient synapses with subplate neurons (Ghosh et al., 1990) and ablation of these neurons leads to failure of thalamic afferents to halt their growth at the proper cortical area (Ghosh and Shatz, 1993). Transient synapses have also been reported at the lateral margin of embryonic primate spinal cord (Knyihar et al., 1978), so it is possible that sensory axons form synaptic contacts with cells in the DREZ during the waiting period. Whether the formation of transient synapses serves as a mechanism to prevent sensory axons from growing into the dorsal horn has not been tested.

Although the mechanisms responsible for the blockade of sensory axon growth into the dorsal horn are unknown, our results suggest an important functional consequence of this waiting period. Sensory neurons are born over a protracted time period during development and their central axons begin to arrive at the edge of the neural tube by stage 25. At this time, the dorsal horn is still undergoing extensive development, as evidenced by the appearance of the dorsal midline structure. The tight temporal correlation between the appearance of this structure and the ability of sensory afferents to establish specific central projections argues that one function of the waiting period is to delay the ingrowth of sensory fibers until appropriate guidance cues have been established in the dorsal horn.

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