Ventral spinal cord inhibition of neurite outgrowth from embryonic rat dorsal root ganglia

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SUMMARY

Organotypic culture of embryonic rat lumbar spinal cord and dorsal root ganglia has been used to demonstrate an inhibitory effect of ventral spinal cord on neurite growth from dorsal root ganglion explants. When dorsal root ganglion explants from 14-15 day old embryos were cultured alone or in close proximity to a dorsal cord explant, the pattern of dorsal root ganglion neurite outgrowth was typically radial. However, when E14-15 dorsal root ganglion explants were cocultured for 22-24 hours in proximity to a ventral spinal cord explant from the same embryo, few, if any, dorsal root ganglion neurites grew in the direction of the ventral cord explant. This inhibitory effect appeared to be developmentally regulated; it was diminished or absent in cocultures prepared from 18 day old embryos. In con-

trast, in cocultures of dorsal cord and ventral cord explants from E14-15 embryos, dorsal cord neurites grew abundantly toward the ventral cord explant suggesting that the inhibition is not likely to be due to a nonspecific neurotoxic effect and that the activity responsible selectively inhibits dorsal root ganglion neurite outgrowth. We conclude that a diffusible, primary afferent inhibitory factor(s) produced by embryonic ventral horn may be responsible for the inhibition. Our results are discussed with respect to the possible involvement of inhibition in the normal development of primary afferent innervation of the spinal cord.

Key words: organotypic culture, primary afferents, growth factors, spinal cord

INTRODUCTION

Previous studies of the development of primary afferent innervation in the rat spinal cord have primarily focused on the time course of afferent innervation, the onset of reflex activity and the development of transmitter activity. However, the factors that contribute to the development of the dorsoventral termination pattern of morphologically and functionally distinct classes of primary afferent neurons are not known.

The dorsoventral termination pattern of dorsal root ganglion (DRG) primary afferents in the adult spinal cord is well known (Brown, 1981; Willis and Coggeshall, 1991). Primary afferents have been classified according to both their fibre diameter and their physiological response properties. Briefly, small diameter myelinated and unmyelinated afferents, many of which mediate noxious mechanical, thermal and chemical cutaneous sensory information, terminate predominantly in the most superficial regions of the dorsal horn, in laminae I-II (laminae designations according to Rexed, 1952). Large diameter low threshold cutaneous afferents terminate in deeper laminae III-V and group Ia afferents that mediate muscle monosynaptic reflexes terminate deeper still in the ventral horn.

In the rat spinal cord, the bundle of His, which contains

central projections of DRG cells, is well developed by embryonic (E) day 14, and collaterals first enter the spinal cord grey matter on E14-15 (Windle and Baxter, 1936; Vaughn and Grieshaber, 1973; Smith, 1983; Altman and Bayer, 1984; Fitzgerald et al., 1991). By birth at around E21.5, dense innervation is present in all areas of the grey matter. On the basis of electron microscopic and electrophysiological evidence, the earliest afferents appear to make functional connections during E15-17 in intermediate regions of the spinal grey matter. These afferents are likely to be collaterals of A cutaneous afferents and Group II muscle afferents that mediate polysynaptic spinal reflexes (Vaughn and Grieshaber, 1973; May and Biscoe, 1975a,b; Saito, 1979; Ziskind-Conhaim, 1990; Kudo and Yamada, 1987). Monosynaptic reflex activity does not occur until 1-2 days following the onset of polysynaptic activity (Saito, 1979; Kudo and Yamada, 1985, 1987; Ziskind-Conhaim, 1990), which is consistent with the fact that afferent axons do not innervate the lumbar ventral horn until about E17-18 (Smith, 1983; Kudo and Yamada, 1987; Ziskind-Conhaim, 1990). Afferent innervation of superficial regions of the dorsal horn by small diameter myelinated and unmyelinated fibres occurs relatively late in embryonic development during E19-21 (Smith, 1983, Fitzgerald, 1987).

Evidence from studies of axon guidance suggest that a

variety of adhesive, chemotropic and chemotrophic influences are likely to be involved in specifying the adult afferent innervation pattern (Lockerbie, 1987; Dodd and Jessell, 1988; Lumsden, 1988; Patterson, 1988; Davies and Lumsden, 1990; Tessier-Lavigne and Placzek, 1991). Whether a growth cone will advance, retract or change directions appears to depend on a delicate balance of adhesive forces and molecular interactions between growth cones, extracellular matrix and neuroepithelium. In addition to interactions involving contact, growth-promoting or growth-inhibiting influences may be caused by diffusible molecules released in the environment of growth cones (Ebendal, 1982; Lumsden, 1988; Patterson, 1988; Tessier-Lavigne and Placzek, 1991).

Previous studies on mechanisms of axon guidance and target recognition relating to primary afferent sensory neurons have focused more on peripheral than central target innervation. Organotypic culture of neural tissue in three-dimensional collagen matrices has been used to demonstrate developmental interactions between trigeminal ganglion neurite outgrowth and maxillary epithelium (Lumsden and Davies, 1983, 1986). We have now adapted this method to study interactions between lumbar DRG neurons and their central target tissue. In this paper, we describe the results of in vitro organotypic culture experiments, which demonstrate an inhibitory effect of ventral, but not dorsal spinal cord, on the neurite outgrowth from dorsal root ganglia. This inhibitory effect, which appears to be developmentally regulated, is likely to contribute to the development of the pattern of primary afferent innervation in the spinal cord.

MATERIALS AND METHODS

Surgery and embryo dissection

Embryos were obtained from timed pregnant Wistar rats. The day on which a vaginal plug was first detected was designated embryonic day (E) 0. Using sterile technique, embryos were removed by Caesarean section under pentobarbitone anaesthesia (40 mg/kg, i.p.). Embryos were immediately placed in minimum essential medium containing Hepes buffer (MEM/Hepes, Gibco) at 4°C, in which all dissections were carried out. Under sterile conditions embryos were decapitated, and the lumbar spinal cord and dorsal root ganglia were removed from the spinal canal. A length of spinal cord containing 2-3 segments was isolated and hemisected along the dorsoventral midline using etched tungsten needles. Individual ganglia were separated from the spinal cord and divided into 3-4 explants. Hemisected cord explants, or explants containing only dorsal or only ventral spinal cord were placed in a fresh droplet of MEM/Hepes in a sterile 35 mm culture dish. One or more DRG explants from the appropriate segment, or another cord explant, was then added to the droplet. Cultures from E14 and E15 embryos were treated as a single group; explants were cocultured in the following combinations: (a) DRG/hemisected spinal cord (n=6); (b) DRG/dorsal spinal cord (n=12); (c) DRG/ventral spinal cord (n=18); (d) dorsal spinal cord/ ventral spinal cord (n=8) and (e) DRG/ventral spinal cord/dorsal spinal cord (n=13). DRG/ hemisected cord (n=4) and DRG/ventral spinal cord (n=24) cocultures were prepared from E18 embryos. The distance between explants was 100-300 µm.

Culture conditions

The droplet of MEM/Hepes containing the explants was aspirated

and replaced by a freshly prepared 27 µl droplet of dialysed rat tail collagen solution (prepared according to Elsdale and Bard, 1972) which contained 2.7 µl of 10× Dulbecco's minimum essential medium (10× DMEM, Gibco) and 1.5 µl of a 7.5% solution of NaHCO$_3$ (Gibco). The collagen solution set within 15 minutes after which 2.5 ml of culture medium containing the following was added to each dish: 1.8 ml DMEM, 0.6 ml fetal calf serum (Gibco), penicillin/streptomycin antibiotic (final concentration 80 i.u./ml, Gibco), and 2.5S Nerve Growth Factor (final concentration 50 ng/ml, Sigma). The cultures were incubated for 22-24 hours at 36°C in humidified air containing 5% CO$_2$.
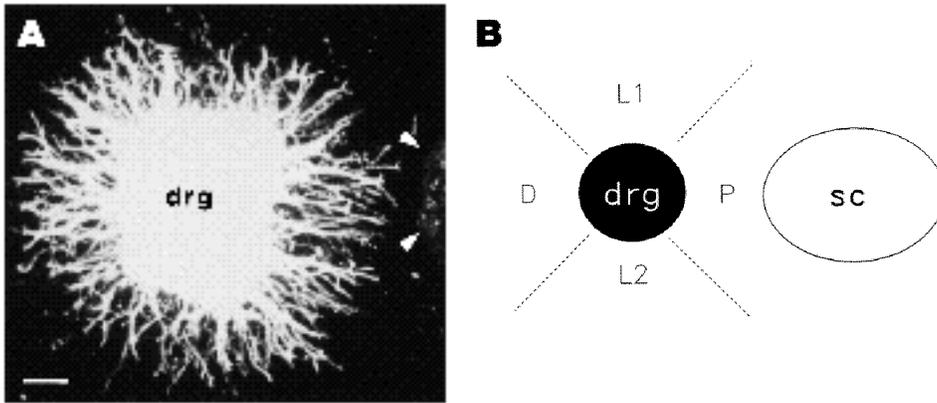
DiI labelling of neurite outgrowth

In cultures analysed quantitatively, neurite outgrowth was labelled with the lipid soluble fluorescent dye, DiI (1,1 -dioctadecyl-3,3,3 ,3 -tetramethyl-indocarbocyanine perchlorate, Molecular Probes). A 2.5% solution of DiI in ethyl alcohol was evaporated on the surface of a sterile culture dish which resulted in the formation of a thin layer of DiI crystals. A suspension of DiI crystals was then created in a droplet of MEM/Hepes using a tungsten needle. Explants were briefly immersed in this suspension and cultured as described above. Examination of labelled explants using phase-contrast microscopy and rhodamine epifluorescence indicated that this method produced reliable DiI labelling of all or nearly all neurite outgrowth after 22-24 hours in culture (see Fig. 2).

Analysis of neurite growth

After 22-24 hours, cocultures were examined and photographed using phase-contrast microscopy, or, in cases where explants were labelled with DiI, rhodamine epifluorescence microscopy. Cultures in which either explant was phase dark or showed no evidence of neurite outgrowth were excluded from further analysis. Representative examples from different coculture combinations that contained DiI labelling were selected for quantitative analysis of neurite outgrowth.

Using confocal microscopy and image analysis (PC Image), neurite outgrowth in each of four quadrants surrounding an explant was quantified and recorded with respect to the position of another explant placed 100-300 µm away. Digitized images of optical sections at 20 µm increments through DiI-labelled explants were obtained using a Biorad confocal microscope. The optical sections were parallel to the bottom of the culture dish, and analysis was confined to the core region of growth approximately 50 µm above and below a plane through the centre of the explant. An image of the total growth in this region was reconstructed from the individual optical sections; an example is shown in Fig. 1A. Reconstructed images were then subdivided digitally into four separate images representing each of the four quadrants shown schematically in Fig. 1B. For each quadrant image, the total area of fluorescent label outside the explant core region was measured and expressed as a percentage of the total area in all four quadrants. Histograms were constructed from a number of cases, showing the relative area of DiI fluorescence (% neurite outgrowth) in each of the four quadrants for each category of coculture combination (Figs 2-5). The area of neurite growth was chosen as an index of growth because methods that involve counting individual neurites or neurite bundles are complicated by the difficulties of resolving individual neurites and the unknown numbers of neurites in fascicles. Other advantages of using confocal microscopy are that an image is obtained relatively free of the out-of-focus blurring common in conventional fluorescence microscopic images of neurite growth in three dimensions, and that a particular region of interest can be selected for analysis.

Fig. 1. (A) Confocal microscope image of DiI-labelled neurite outgrowth from an E14 dorsal root ganglion explant (drg) which was cocultured with a dorsal cord explant (arrowheads indicate the position of the dorsal cord explant which was not DiI labelled). (B) Schematic representation of coculture experiments in which DiI-labelled neurite outgrowth was quantified, relative to a spinal cord explant (sc), in four quadrants designated P (proximal), D (distal) and L1, L2 (lateral). Scale bar in A = 50 μm.

RESULTS

In 56% (32/57) and 50% (14/28) of the cocultures from E14-15 and E18 embryos, respectively, neurite outgrowth from DRG and/or spinal cord explants was considered sufficient to allow further analysis. In the remaining cocultures, one or more explants appeared dark and failed to extend neurites when viewed with phase-contrast microscopy. Reasons for failure of explants to survive and extend neurites were not obvious, but possibilities include trauma during dissection, prolonged time between removal of embryos from the uterus and culture preparation, and excessive amounts of DiI. There did not appear to be any correlation between failure of an explant to survive or extend neurites and the type of coculture; in fact, surviving and nonsurviving DRG explants were sometimes observed in the same coculture.

After 22-24 hours in culture, DRG neurites reached lengths exceeding 100 μm and typically exhibited a radial pattern of outgrowth when DRG explants were cultured alone. Neurites also consistently grew from spinal cord explants; however, there were fewer neurites compared to DRG explants and the pattern was typically nonradial. In most cultures, a small number of flat, multipolar nonneuronal cells migrated a short distance from explants. Under the conditions used in these experiments, the number of cells migrating from the explant increased with increased culture times but did not usually interfere with the analysis of neurite growth at culture times of 22-24 hours. Although DRG neurite fasciculation appeared to be more commonly associated with DRG explants cocultured in the presence of a ventral cord explant, it was, in fact, observed to a variable extent in all types of cocultures.

The pattern of neurite outgrowth from E14-15 DRG explants cocultured in proximity to dorsal cord explants or in proximity to the dorsal region of a hemisected cord explant was in all cases similar to the growth seen when DRG were cultured alone (*n*=9). There was no clear evidence of preferential growth towards or away from the dorsal cord explant. This is illustrated in Fig. 2A,B and shown in the histogram in Fig. 2C.

In contrast to DRG/dorsal cord cocultures, when DRG explants from E14-15 embryos were cocultured in proximity to ventral spinal cord, DRG neurites almost always appeared to avoid growing toward the cord explant (23/26

cocultures; Fig. 3). Few or no neurites were found growing from the DRG explant in the region nearest the ventral cord explant (Fig. 3A-C). DRG neurites in the lateral quadrants grew along more or less straight courses; however, neurites could occasionally be found to have altered their initial course in a direction further away from the ventral cord explant. A quantitative assessment of the amount of neurite growth in different quadrants surrounding DRG explants cocultured with ventral spinal cord explants demonstrated that the growth in the quadrant closest to the cord explant, i.e., the proximal quadrant, was inhibited compared with the growth in the distal and lateral quadrants; neurite growth in the distal and lateral quadrants was roughly equivalent (Fig. 3D). In two DRG/ventral cord cocultures monitored at 4, 10 and 18 hours of culture, DRG neurites were not observed to make contact with the ventral cord explant at any time. The inhibitory effect was found whether or not ventral cord neurites were present in the proximal quadrant. The orientation of the ventral cord explant relative to the DRG explant was not a strict requirement to demonstrate the inhibitory effect, since DRG explants positioned on opposite sides of a ventral cord explant both showed inhibition (Fig. 3B). The inhibitory effect of ventral cord appeared to be developmentally regulated; when DRG explants taken from E18 embryos were cocultured with ventral spinal cord explants from the same embryo, the inhibitory effect was absent, or greatly diminished (*n*=14; Fig. 4). The inhibitory effect of E14-15 ventral spinal cord on DRG neurite outgrowth was specific in that ventral cord did not inhibit outgrowth from dorsal cord explants (*n*=17; Fig. 5). The limited neurite outgrowth from dorsal cord explants relative to DRG explants makes direct comparison difficult, but it is clear from the photomicrograph in Fig. 5A that dorsal cord neurites readily grew towards ventral cord explants. In fact, quantitative analysis suggested a preference for dorsal cord neurite outgrowth in the proximal quadrant, nearest the ventral cord explant (Fig. 5B).

DISCUSSION

We have demonstrated a novel inhibitory effect of ventral spinal cord on neurite outgrowth from DRG in vitro. The inhibition appears to be developmentally regulated, present
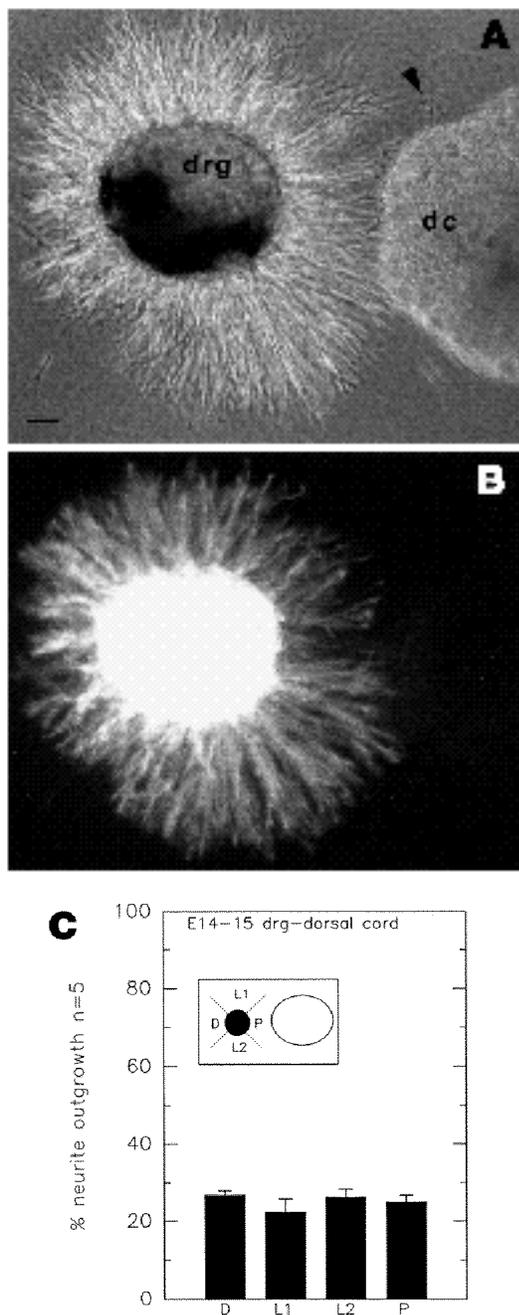
Fig. 2. E14-15 dorsal root ganglion (drg)/dorsal cord (dc) coculture. (A) Phase-contrast photomicrograph of a representative E14 drg/dc coculture in which the distribution of drg neurite outgrowth is essentially radial. The black area within the core of the drg explant is due to concentrated deposits of DiI crystals. The arrowhead points to a small neurite growing from the cord explant. Scale bar = 50 μm. (B) Same field as in A shown under epifluorescence illumination for DiI in which all or nearly all drg neurites have been labelled. The dorsal cord explant, which was not DiI labelled, is invisible. (C) Histogram showing the distribution (expressed as a percent of total outgrowth) of drg neurite outgrowth in four quadrants with respect to the position of the dorsal cord explant. Error bars indicate the standard error of the mean.

at E14-15 but greatly diminished or absent by E18. Importantly, dorsal spinal cord did not inhibit DRG neurite outgrowth nor did ventral cord inhibit neurite outgrowth from dorsal cord.

Our finding that dorsal cord neurite outgrowth was not inhibited by ventral cord at E14-15 is in agreement with the results of Tessier-Lavigne et al. (1988) who demonstrated that E14 floor plate explants, and to a lesser extent ventral cord explants, have a chemoattractant effect on neurite outgrowth from commissural cells of dorsal cord explants. Although the sample size for quantitative analysis was not large, our results are sufficient to suggest that ventral cord explants have an attractive influence on dorsal explant neurites. The fact that dorsal cord neurites grew readily toward ventral cord explants rules out the possibility of a nonspecific neurotoxic effect of ventral cord on the DRG neurites as well as the possibility that ventral cord renders the collagen substratum nonpermissive for any neurite growth.

In vivo, inhibitory mechanisms affecting axon growth in the spinal cord would have to be selective, because the ventral horn is clearly not devoid of other developing axons at E14. For example, dorsally situated commissural neurons project axons through the ventral horn toward the floor plate at E11-14 (Altman and Bayer, 1984; Tessier-Lavigne et al., 1988). Electron microscopic studies demonstrate the presence of growth cones and synapse formation within the ventral horn during E14-18 (Vaughn and Grieshaber, 1973; May and Biscoe, 1975a,b). The abundance of immunoreactivity for the growth-associated protein, GAP-43, further suggests the presence of active axonal growth in the ventral horn at E14-15 (Fitzgerald et al., 1991).

Our results are consistent with a diffusible factor(s), derived from ventral spinal cord, which acts at a distance to inhibit DRG neurite outgrowth. Collagen gel matrices can support gradients of diffusible molecules (Ebendal and Jacobson, 1977; Tessier-Lavigne and Placzek, 1991), and such gels have been used to study the effects of diffusible chemoattractant factors on neurite growth (Lumsden and Davies 1983, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990). In cocultures where inhibition was present, DRG neurites were never found contacting ventral cord explants. Likewise, observations of neurite growth throughout our culture period indicated that at no time did DRG neurites make contact with the ventral cord explant or appear to be in the process of retraction. Our results are <u>not</u> consistent with previously described characteristics of contact inhibition (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987). For example, when retinal neuron growth cones come into contact with sympathetic axons in culture, they collapse, retract, then recover and make repeated attempts to advance. A portion of the collapsed growth cone usually remained in contact with the sympathetic axon and in some cases, failed to retract at all for observation periods exceeding 1 hour. If a similar mechanism requiring contact were involved in DRG neurite inhibition by ventral cord, we would expect to see a considerable number of DRG neurites contacting or at least growing in the direction of the ventral cord explant, especially in light of the relatively slow growth rate of neurites in three-dimensional collagen substrata. Rather, we invariably observed a clear
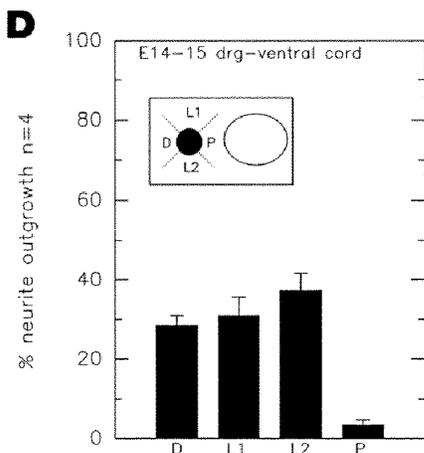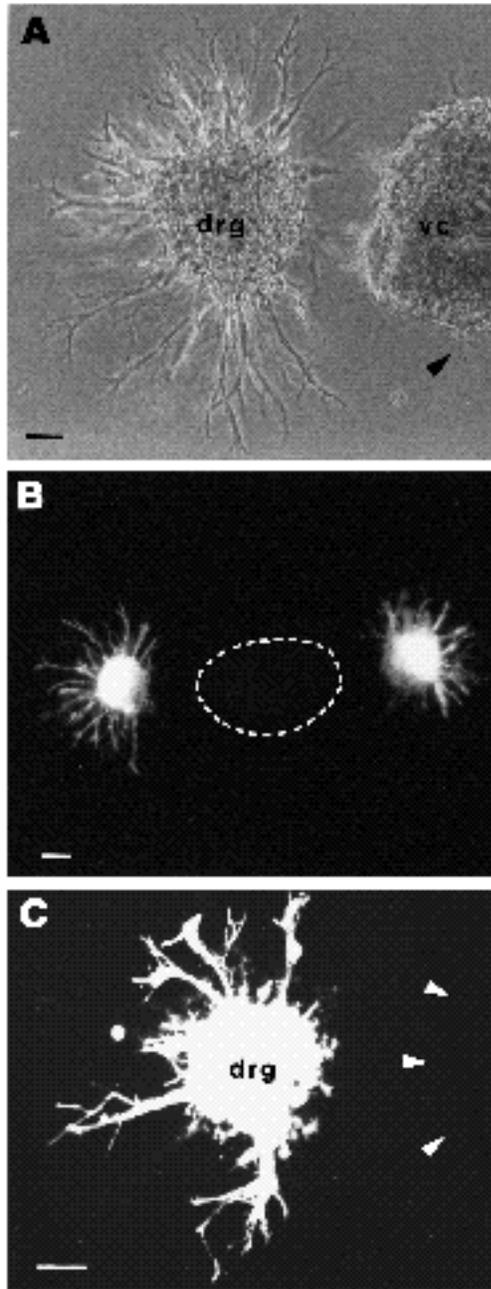
absence of DRG neurites in the vicinity of the ventral cord explant, and no obvious differences were apparent in the lengths of DRG neurites that were present. The inhibitory effect of ventral cord on DRG neurite outgrowth is therefore more likely to involve a diffusible molecule(s). The fact that cultures were not continuously monitored with video microscopy throughout the culture period, however, precludes completely ruling out a contact-mediated mechanism.

With regard to the specific mechanism by which DRG neurites are inhibited, our results do not provide conclusive evidence as to whether the inhibitory factor acts in a soluble form, or as a component of extracellular matrix which moves by bulk flow. In both cases inhibition could result from a direct action on the growth cone or from an effect on the collagen matrix to produce a nonpermissive substratum for neurite growth. Although instances of axon trajectories deviating away from the ventral cord explant were observed, inhibition was more commonly characterised by an almost complete absence of neurites emerging from the DRG explant face nearest the ventral cord explant. The latter observation would suggest that the inhibitory activity has a direct effect on the rate of growth or the ability of DRG cells in this region to extend neurites; however, these cells may have extended neurites which grew back through the DRG explant. The response of DRG cells and growth cones is likely to be dependent on the concentration of inhibitory factor. The molecular identity of the inhibitory factor remains to be determined. In chicks, a chondroitin sulfate proteoglycan has been implicated in the inhibition of DRG neurites by epidermal explants in vitro; whether it acts in soluble or substratum-bound form is not yet clear (Verna, 1985; Fichard et al., 1991).

An inhibition of chick sympathetic ganglia neurite outgrowth when cocultured with spinal cord explants, similar to the inhibition reported here, has been described by Ebendal (1982). Ebendal observed that chick DRG neurite outgrowth was similarly inhibited by spinal cord explants, however, no data was presented and effects due to different cord regions were not addressed. Davies et al. (1990) demonstrated that chick DRG neurite growth is also inhib-
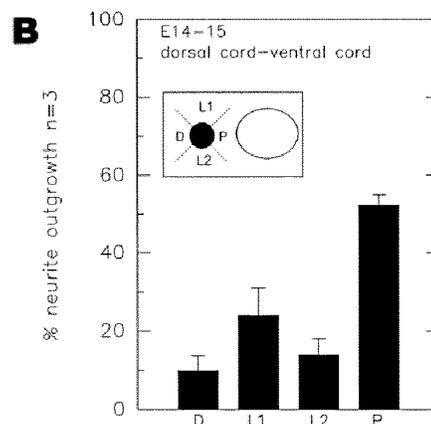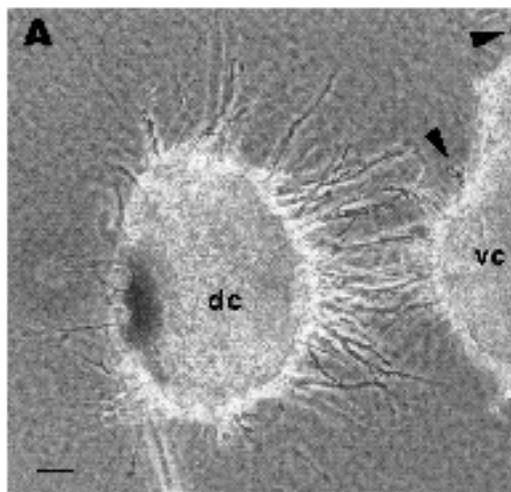
Fig. 3. E14-15 dorsal root ganglion (drg)/ventral cord (vc) coculture. (A) Phase-contrast photomicrograph showing inhibition of drg neurite outgrowth when a ventral cord explant is placed in close proximity. Neurites can be seen growing in all directions other than toward the ventral cord explant. The arrowhead points to a small bundle of neurites growing from the cord explant. Nonneuronal cells out of the plane of focus can also be seen in the region between explants. (B) Low magnification photomicrograph of two DiI-labelled drg explants on opposite sides of a ventral cord explant (position indicated by dashed outline) which was not DiI labelled. Neurites from both explants appear to avoid growing toward the ventral cord explant. C. Confocal microscope image of a DiI-labelled drg explant which was cocultured in close proximity to a ventral cord explant (position of vc explant indicated by arrowheads). D. Averaged distribution of drg neurite outgrowth (expressed as a percentage of total growth) with respect to a nearby vc explant showing that the neurite growth in the proximal quadrant was considerably less compared to the growth in the other three quadrants. Scale bars in A and C = 50 µm; scale bar in B = 100 µm.
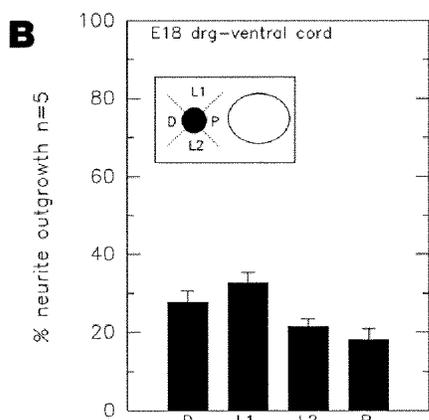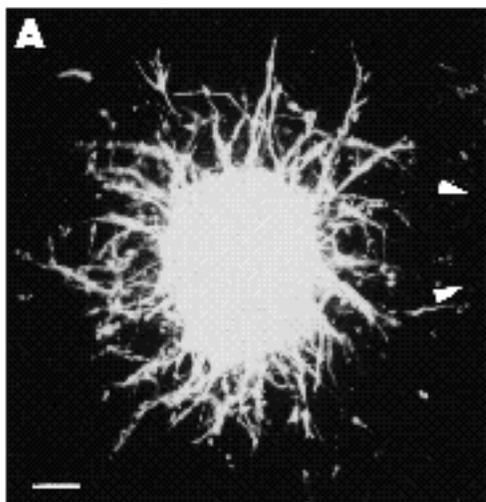
Fig. 4. E18 drg/ventral cord coculture. (A) Confocal microscope image of DiI-labelled neurite outgrowth from an E18 drg explant cocultured in close proximity to a ventral cord explant (position indicated by arrowheads). The inhibition of outgrowth appears to be greatly diminished or absent at this stage. Scale bar = 50 μm. (B) Averaged distribution of E18 drg neurite outgrowth (expressed as percentage of total growth) with respect to a nearby ventral cord explant.

Fig. 5. E14-15 dorsal cord (dc)/ventral cord (vc) coculture. (A) Phase-contrast photomicrograph showing numerous dorsal cord explant neurites growing toward a ventral cord explant. Neurites can also be seen growing from the vc explant (arrowheads). Scale bar = 50 μm. (B) Averaged distribution of dc neurite outgrowth (expressed as a percentage of total growth) with respect to the position of a nearby vc explant.

ited by glycoprotein fractions derived specifically from proximal half somites, a result that suggested this inhibitory activity may be involved in spinal cord segmentation patterning.

Other studies suggest that regulation of axon growth via diffusible inhibitory molecules may be more common than previously thought. The neurotransmitters, serotonin and dopamine, have been shown to have selective inhibitory effects on axon growth in dissociated cultures of *Helisoma* neurons (Haydon et al., 1984, 1987; McCobb et al., 1988). More recently, in collagen matrix coculture experiments, a diffusible inhibitory factor has been implicated in the inhibition of embryonic olfactory tract axons by septal explants (Pini, unpublished data). It remains to be determined whether this septal inhibitory factor is identical to, or has properties in common with, the factor responsible for the ventral cord inhibitory effect described here. Although there is as yet no direct evidence that the serine protease, thrombin, acts via a diffusible mechanism, it has been shown to

have inhibitory effects on neurite outgrowth, and thrombin mRNA has been localised in cells of the central nervous system (Hawkins and Seeds, 1986, 1989; Dihanich et al., 1991; Suidan et al., 1992).

Ventral cord inhibition of DRG neurites occurs at a developmental stage consistent with an involvement in early afferent terminal arborizations. Two distinct groups of afferent fibres can be seen in the dorsal horn of the rat lumbar spinal cord at E15-16, a medial bundle, which appears to be directed toward the ventral horn, and a more laterally directed bundle. The laterally directed group are the earliest afferents to form terminal-like arborizations, at E15-17, in intermediate and deep dorsal horn regions (Smith, 1983; Kudo and Yamada, 1987). An inhibitory factor originating in the ventral horn may divert axons of this lateral group towards target fields in the dorsal and intermediate regions of the grey matter and prevent them from pursuing a ventral, and functionally inappropriate, course. Inhibition may also delay penetration of the medially situated bundle into the ventral horn until E17-18 when

monosynaptic connections between Group Ia afferents and motorneurons begin to form (Vaughn and Grieshaber, 1973; Kudo and Yamada, 1987). It is conceivable that delay of afferent growth into the ventral horn assures that peripheral target innervation occurs first and directs the development of central connections. Comparison of the times growing cutaneous or muscle afferent axons reach peripheral targets with the times they reach central targets suggests that peripheral target innervation precedes central innervation (Smith and Frank, 1988; Davis et al., 1989; Fitzgerald et al., 1991; Reynolds et al., 1991). That peripheral targets specify central connections is also supported by the result in frogs that thoracic DRG, which normally does not contain muscle afferents, will innervate muscle and form appropriate central connections when transplanted to brachial levels (Smith and Frank, 1987). On the other hand, central factors also seem to contribute to the formation of central target connections. Fitzgerald et al. (1990), using intra-axonal HRP labelling, demonstrated that the terminal morphology of hair follicle afferents, which sprouted into denervated regions of the spinal cord, was dependent on the nerve territory invaded by the sprouting terminals.

The presumed delay of afferent innervation into ventral cord would not be expected to be related to immaturity of ventral horn target cells. Developing lumbar spinal cord motorneurons are nearly all postmitotic by E13 and undergo considerable cytological and physiological maturation well before connections with group Ia afferents are first made (Altman and Bayer, 1984; Ziskind-Conhaim, 1988). Early ventral cord maturation and release of inhibitory molecules might be involved in assuring that peripheral axons of primary afferents do not inappropriately enter the spinal cord during early outgrowth.

The inhibitory effect of ventral spinal cord on DRG neurite outgrowth is greatly diminished or absent by E18 when, in vivo, afferents have clearly penetrated the ventral horn and have begun to form functional connections. The fact that some residual inhibition may be present in our experiments may reflect slight variations in the actual ages of the embryos used in our experiments. Or, the inhibitory effect may not have completely disappeared at E18 but has decreased to the extent that some afferents begin to grow into the ventral cord. The inhibitory effect described here would not be expected to influence innervation of superficial dorsal horn regions, which does not occur until E19-21.5 (Smith, 1983; Fitzgerald, 1987).

The possibility that different populations of DRG cells have different trophic requirements raises the question of whether our culture conditions favour the survival of certain types of primary afferents and the related question of whether ventral cord selectively inhibits a subset of primary afferent neurons. Recent in vitro studies suggest that group Ia afferents do not survive well in culture (Spenger et al., 1991) and that different DRG neurons may require trophic support from neurotrophic factors other than NGF (Davies et al., 1986a,b; Ernfors and Persson, 1991; Carroll et al., 1992). Studies on the effects of different members of the neurotrophin family of neurotrophic factors on the inhibition produced by ventral cord explants will help resolve these questions.

The inhibition of afferent axons by a diffusible factor emanating from the ventral horn would almost certainly act in conjunction with other mechanisms, attractive or repulsive, to guide afferent axons towards their targets. Other studies have focused on mechanisms involving direct interactions between axons and other axons, neuroepithelium, glia or components of the extracellular matrix (Lockerbie, 1987; Dodd and Jessell, 1988; Reichardt and Tomaselli, 1991). Chemoattractant mechanisms that involve freely diffusible substances might also be involved in central afferent innervation (Lumsden and Davies, 1983, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990). Potential cellular sources of the inhibitory factor include motorneuronal, neuroepithelial and glial cell populations.

In conclusion, we have used organotypic cultures of rat spinal cord and DRG explants in a three-dimensional collagen matrix to demonstrate an inhibition by ventral cord, but not dorsal cord, on the neurite growth from DRG. The effect is consistent with inhibition by a diffusible inhibitory molecule(s), the identity of which remains unknown. These results suggest that this factor may be involved in directing primary afferents to appropriate targets in intermediate and deep dorsal horn, and could possibly act to delay afferent innervation of ventral horn.

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