Topographic specificity of corticospinal connections formed in explant coculture

Rong Zhen Kuang1, Michael Merline1,2 and Katherine Kalil1,2,*

1Department of Anatomy and 2Neuroscience Training Program, University of Wisconsin, Madison, Wisconsin 53706, USA

*Author for correspondence at address 1

SUMMARY

The corticospinal pathway connects layer V pyramidal neurons in discrete regions of the sensorimotor cortex to topographically matching targets in the spinal cord. In rodents initial pathway errors occur transiently during early postnatal development, such that visual cortical axons project inappropriately into the corticospinal tract. Nevertheless, only sensorimotor axons form corticospinal connections, which are topographically ordered in hamsters from the earliest stages of innervation. Previous work in vivo suggests that pathfinding is carried out by primary cortical axons whereas target innervation occurs by extension of axon collaterals at appropriate locations. In vitro studies have provided evidence that chemotropic factors may selectively attract extension of neurites into specific targets. To investigate the basis for corticospinal target selection during development, we have used an in vitro explant coculture system. Sensorimotor and visual cortical explants from newborn hamsters were presented with inappropriate targets from olfactory bulb and cerebellum and targets from the cervical (forelimb) and lumbar (hindlimb) enlargements of the early postnatal spinal cord. Under in vitro conditions, corticospinal target selection was highly specific and remarkably similar to corticospinal connectivity in vivo. Visual and sensorimotor cortical neurites extended nonselectively into the white matter of the spinal cord. However, only neurites from the sensorimotor cortex were able to extend into and arborize within the spinal gray. In the majority of cases, these connections were topographically appropriate, matching forelimb cortex to cervical cord and hindlimb cortex to lumbar cord. However, we found no evidence that chemotropic attraction was responsible for selection of appropriate targets by cortical neurites or that spinal target tissue promoted extension of cortical axon collaterals within the collagen matrix. These results suggest that the ability of cortical neurites to recognize correct spinal targets and form terminal arbors may require direct axon target interaction.

Key words: axon guidance, explant coculture, corticospinal development, target specificity

INTRODUCTION

The corticospinal pathway connects discrete regions of the sensorimotor cortex to topographically matching targets in the spinal cord. In rodents, corticospinal outgrowth and connectivity develop during the first several postnatal weeks (Donatelle, 1977; Reh and Kalil, 1981, 1982; Gribnau et al., 1986; Joosten et al., 1987; Schreyer and Jones, 1988; Schwab and Schnell 1991; reviewed in Stanfield, 1992; Kuang and Kalil, 1994). Efferent axons from pyramidal neurons in cortical layer V (Stanfield and O’Leary, 1985; Merline and Kalil, 1990) extend over long pathways through the brainstem and spinal cord and then select exact subsets of target neurons in the dorsal and ventral horn of the spinal cord. Recent studies of developing efferent cortical pathways suggest that axon pathfinding and target selection may be governed by separate mechanisms such that layer V cortical axons are guided into appropriate pathways in the brainstem and spinal cord by activities of the primary growth cones at the axon tips, whereas target selection occurs by extension of interstitial axon collaterals branching from the primary axon at appropriate locations (O’Leary and Terashima, 1988; O’Leary et al., 1990; 1991; O’Leary and Koester, 1993).

Studies of the developing corticospinal pathway in rodents suggest that initial pathfinding by cortical axons is relatively imprecise. Anatomical studies have shown that inappropriate axons from the visual cortex extend transiently into the corticospinal tract in the dorsal funiculus of the spinal cord (Stanfield et al., 1982; Adams et al., 1983; Stanfield and O’Leary, 1985; O’Leary and Stanfield, 1986; Joosten et al., 1987; Kuang and Kalil, 1994). Appropriate axons from sensorimotor cortex have also been shown to make initial projection errors by growing in the corticospinal tract past the level of their eventual targets in the spinal cord (Kuang and Kalil, 1994). However, despite these transient pathfinding errors in the corticospinal tract, corticospinal connections in the spinal gray are topographically appropriate from the earliest stages of innervation. Thus transient visual cortical axons in the corticospinal tract fail to innervate any level of the spinal cord and sensorimotor cortical axons form
terminal arbors only at topographically appropriate levels of the cord (Kuang and Kalil, 1994). Taken together, these results suggest that the corticospinal tract may be generally permissive for cortical axon growth whereas the spinal gray matter is permissive only for invasion of topographically appropriate corticospinal arbors. However, the mechanisms by which forelimb sensorimotor cortical axons select matching targets in the cervical cord and hindlimb sensorimotor cortical axons select targets in the lumbar cord are at present unknown.

One approach to addressing the mechanisms underlying the formation of specific corticospinal connections during development is to study target selection by cortical axons under in vitro conditions. Recent studies of axon target interactions in vitro have used explants of neural tissue cocultured in a threedimensional matrix. Results from some studies suggest that chemotropic factors from target tissues may act at a distance to attract extension of neurites into specific targets. For example, it has been shown that sensorimotor cortical neurites are selectively attracted toward specific pontine targets (Heffner et al., 1990), that visual cortical axons are attracted toward thalamic and tectal targets (Bolz et al., 1990; Novak and Bolz, 1993) and that spinal commissural axons are attracted toward the floor plate of the embryonic spinal cord (Tessier-Lavigne et al., 1988; Placzek et al., 1990). In addition to chemoattractant factors, long-range target-derived repulsive guidance cues have been postulated (Fitzgerald et al., 1993; Pini, 1993). In contrast, studies using similar coculturing strategies on thalamocortical systems have failed to find evidence for diffusible factors that act to regulate growth of afferent axons at a distance (Molnar and Blakemore, 1991; Bolz et al., 1992). Thus the formation of appropriate neural connectivity is probably governed in part by contact mediated mechanisms.

To investigate the basis for corticospinal target selection during normal development, we have used an in vitro explant coculture system. Sensorimotor and visual cortical explants from postnatal day 1 hamster were presented with targets of varying ages from cervical and lumbar enlargements of the spinal cord, which are innervated in vivo by forelimb and hindlimb sensorimotor cortex, respectively. Age-matched tissue from the olfactory bulbs and cerebellum, which cortical axons never innervate in vivo, were used as examples of inappropriate targets. We sought to determine whether cortical neurites in the absence of their normal pathways were able to express regional preferences for targets at different levels of the spinal cord. We also asked whether chemotropism played a role in the selection of appropriate targets by cortical neurites or their neuriters. We found that under in vitro conditions connectivity between discrete regions of sensorimotor cortex and the spinal cord was remarkably specific and mimicked the topography established by afferent sensorimotor cortical axons during normal corticospinal development. However, we found no evidence that chemotropic attraction at a distance was responsible for the selection of appropriate targets by cortical neurites or that target explants elicited the selective extension of cortical axon collaterals within the collagen matrix. Rather, the ability of cortical neurites to recognize and arborize within appropriate targets of the spinal cord appeared to require direct contact between primary neurites and target tissue.

**MATERIAL AND METHODS**

Infant Golden hamsters (*Mesocricetus auratus*) were obtained from timed pregnancies. Litters were usually born on the afternoon of the 16th day of gestation. The day of birth was considered to be postnatal day 0.

**Dissection of explants**

Cortical, olfactory bulb and cerebellar explants were obtained from 1 day postnatal littersmates. Animals were anesthetized by cooling on crushed ice and decapitated. To obtain cortical explants, forebrain was dissected from fresh brains and sectioned at 300 μm on a Vibratome into chilled sodium-free artificial cerebrospinal fluid (10 mM d-glucose, 2 mM MgSO₄, 2 mM CaCl₂, 1.25 mM NaH₂PO₄, 5 mM KCl, 25 mM HEPES, and 252 mM sucrose) after which pie-shaped explants (1-1.5 mm wide) spanning the full radial thickness of the cortex were cut from the sections using fine tungsten needles. Presumptive cervical and lumbar projection regions of sensorimotor cortex were roughly estimated based on previous studies of topography in neonatal corticospinal projections (Schreyer and Jones, 1988; Merline and Kalil, 1990; Kuang and Kalil, 1994). Visual cortex was dissected from the most caudal occipital region of the neocortex (Rhoades et al., 1991; Kuang and Kalil, 1994). Olfactory bulbs were similarly sectioned and dissected into 1-1.5 mm square explants. Cerebellar tissue was dissected free of meninges and 300 μm sections prepared using a McIlwain tissue chopper. Spinal cord explants were obtained from 1 or 5-8 day postnatal animals. The cervical or lumbar enlargement was dissected free of meninges and entire cross sections cut at 300 μm on a McIlwain tissue chopper. In some cases, spinal targets were hemisected. All explants were allowed to recover in sodium-free artificial cerebrospinal fluid at 4°C for 30 minutes.

**Preparation of explant cocultures**

Explants were cultured by the Roller-tube method (Gahwiler et al., 1983) or in three-dimensional collagen matrix gels (Lumsden and Davies, 1983; Heffner et al., 1990). Both methods gave identical results.

For the Roller-tube method, explants were embedded in approximately 20 μl of collagen (9 parts rat tail collagen (Lumsden and Davis, 1983), 0.9 parts 10× MEM (Gibco), and 0.1 parts 7.5% NaHCO₃) on a 9×22 mm glass coverslip precoated with non-polymerized rat tail collagen. The explants were positioned 300-500 μm apart and the collagen spread using a fine paintbrush. The coverslips were then placed in a humidified 5% CO₂ incubator at 36.5°C for 30 minutes in order to polymerize the collagen. Coverslips were then inserted into roller tubes containing 1 ml of culture medium (25% fetal bovine serum (Hyclone), 25% Hanks balanced salt solution (Gibco), 50% Hepes-buffered MEM (Gibco) supplemented to 10 mM NaHCO₃, 0.5% glucose and 0.5 mM L-glutamine). Beginning on the second day, the tubes were rotated in a roller drum kept within the incubator under conditions described above.

For the cultures in the three-dimensional collagen matrix, explants were embedded in a 20 μl drop of unpolymerized collagen as described above and placed on the bottom of plastic culture dishes (Corning). After polymerization, the collagen gels were covered with culture medium and maintained in the incubator but without rotation. Culture medium was changed every other day and after 2-4 days the cultures were fixed with fresh 4% paraformaldehyde in 0.1 M phosphate-buffered saline.

**DiI labeling of explant cultures and data analysis**

Cultures were stored in fixative solution and after 3 days were removed for labeling with the lipophilic tracer DiI ([1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchlorate) (Honing and Hume, 1989; Godement et al., 1987)]. For anterograde labeling of cortical axons, small crystals of DiI were inserted into the upper layers of the cortical explant using a fine glass micropipette. To retrogradely
label cortical cells extending neurites out of the explant. DiI crystals were inserted into the collagen gel in the region containing neurite growth from the cortical explant. To label cortical cells extending neurites into target tissue, the DiI crystals were inserted into the target explant. The cultures were then returned to fixation solution and stored in the dark at 37°C for 5-7 days to allow diffusion of the DiI.

Labeled cultures were observed using the rhodamine optics of a Zeiss inverted microscope (Axiovert 35M) and images of fluorescently labeled explants and neurites were captured with a silicon-intensified-target (SIT) camera, enhanced using an image processing system (Image 1, Universal Imaging), and stored on laser discs with an optical memory disc recorder (Panasonic TQ-2028F). Photographs of selected images were taken from the video monitor. Borders of the target tissue and the spinal gray matter were determined from matching phase images projected on the video monitor.

The DiI-labeled cortical explants were examined and scored on the basis of neurite growth into the target tissue. Each case was scored as either having neurites that entered the target tissue, that were stopped or deflected from the edge of the tissue or that showed a mixed result. Neurites that stopped at the border between the white matter and the gray matter of the spinal cord targets were counted as stopped by the tissue. Mixed cases most often involved only small percentages of anomalous fibers. In all cases explants were scored only when it was clear that the labeled fibers were in the same focal plane as the target tissue. However, some mixed cases may have resulted from occasional errors in determining the exact focal plane of the fibers. Since many of the cocultures contained two different target explants (Fig. 1A), the results for each of these targets were counted as a separate case in the data analysis. Similarly, other cocultures contained sensorimotor and visual cortex with a single spinal target (Fig. 1B). Results for each cortical explant were counted as separate cases.

RESULTS

Most explants of sensorimotor and visual cortex showed robust neurite outgrowth after 48 hours in culture. Neurites often tipped with growth cones could grow to lengths of 1-2 mm if they did not encounter target tissue. Cultures were discarded if neurite outgrowth was sparse or if the explants were poorly positioned. Spinal cord explants maintained their normal structure, such that white matter and gray matter retained distinct borders (Fig. 3A). At the times observed little or no cellular migration occurred from any of the target explants. Spinal cord and cerebellar explants showed little neurite outgrowth whereas long neurites extended from olfactory bulb or cerebellar targets were positioned on opposite sides of spinal target (Fig. 3A,B). Similarly, when spinal, olfactory bulb or cerebellar targets were positioned on opposite sides of the same sensorimotor cortical explant, cortical neurites did not show enhanced growth toward the appropriate spinal target in comparison with growth toward the inappropriate cerebellar target. Observation of cortical neurites with phase microscopy after DiI labeling also showed that neurites did not reorient their trajectories toward spinal targets. Instead, as shown in Fig. 3, neurites growing in straight trajectories continued to grow in this fashion without altering their pathways to contact targets in close proximity. Cortical explants were also able to extend neurites from the pial surface, although this outgrowth was relatively sparse. Placement of appropriate spinal tissue facing the pial side of cortical explants (n=33, data not shown) did not stimulate neurite outgrowth in this direction. In these cases retrograde DiI labeling from the spinal tissue revealed only a few fluorescently labeled cortical neurones. Cortical neurites exhibited occasional branches within the collagen gel, but there was no evidence that target tissue elicited branching activity at a distance. These results suggest that the direction of neurite outgrowth from cortical explants was non-selective.
and that the presence of appropriate target tissue did not enhance neurite outgrowth, attract cortical neurites at a distance or increase the extension of collateral branches within the collagen.

**Specificity of interactions between cortical neurites and target explants**

Visual and sensorimotor cortical neurites encountering inappropriate targets failed to enter them in the overwhelming majority of the cocultures. Tissue from olfactory bulb and cerebellar cortex were positioned in the cocultures so that the ventrolaterally directed cortical neurites would encounter them (Fig. 1). Results for olfactory bulb and cerebellar targets were similar. In 91% of the cocultures with targets from olfactory bulb \(n=74\) and 88% of the cocultures with cerebellum \(n=192\) cortical neurites grew to the target explants but stalled at the edges and failed to enter the tissue. As shown in Fig. 4, Dil-labeled neurites from sensorimotor cortex did not extend into olfactory and cerebellar targets. Neurites approaching the targets in straight trajectories typically stopped abruptly at the

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**Fig. 2.** Sensorimotor cortical explants maintain organotypic organization in culture, as shown in photomicrographs in phase microscopy of cortical explants at 0 hours in culture (A) and after 30 hours in culture (B). Laminar organization is maintained in the explants in which the cortical plate (cp) and layer VI are visible. Neurite outgrowth occurs from the subcortical fiber layer (*). Sensorimotor cortical neurons in the cortical plate retrogradely labeled with Dil (C and D) extend axons ventrally to the subcortical fiber layer and then turn to exit the cortex. Retrogradely labeled cortical neurons at higher magnification (E and F) have typical pyramidal morphologies. Scale bar in A-D, 250 μm; in E, 150 μm and in F, 50 μm.
Corticospinal specificity in coculture

Neurites from sensorimotor cortex grew into appropriate spinal targets and were able to discriminate between targets from different segmental levels of the spinal cord. Coculture experiments in which explants of forelimb regions of sensorimotor cortex were presented with topographically appropriate spinal targets from the cervical cord or inappropriate targets from the lumbar cord showed that cortical neurites were highly selective in their choices of spinal targets. As shown in Fig. 5, many neurites from forelimb sensorimotor cortex entered target explants of the cervical spinal cord, remained within the tissue and in some cases formed terminal arbors. Arborization of cortical neurites was never observed within the collagen but occurred only within the gray matter of the spinal cord explants. The arbors were often tipped with growth cones (Fig. 5) and resembled typical widely ramifying corticospinal arbors that form in vivo (Kuang and Kalil, 1990). Forelimb cortical neurites invaded targets from the cervical spinal cord in the overwhelming number of cocultures examined, regardless of whether spinal explants were obtained from 5- to 8-day-old cord (87%, n=161) or 1-day-old cord (89%, n=428. See Fig. 9 and Table 1). In contrast, when explants of forelimb sensorimotor cortex were presented with topographically mismatching targets from 5-8 day lumbar spinal cord, the forelimb cortical neurites extended into the gray matter in only 22% of the cultures. Most of the forelimb cortical neurites grew for short distances into the lumbar cord explant but stopped abruptly at the border between the white matter and the spinal gray (Fig. 6A,B). This border was easily visible in phase microscopy (Fig. 3A). Failure of ingrowth of forelimb cortical neurites occurred more often with target explants from 5-8 day lumbar cord than for 1 day lumbar targets (see Table 1 and Fig.

Fig. 3. Forelimb sensorimotor cortical explant (right) and visual cortical explant (left) cocultured with cervical spinal cord (center) at low (A) and higher (B) magnification. In phase microscopy, the lack of directional selectivity of neurite outgrowth from both cortical explants is apparent. In (A) the spinal gray can also be distinguished from the white matter. Higher magnification photomicrograph (B) of cortical neurites from the same coculture shows that neurites extending from the forelimb cortex do not orient selectively toward their appropriate spinal target. Note that some neurites (near arrow) even turn away from the spinal explant (Arrow indicates corresponding points in A and B). Scale bar, 250 µm.

Fig. 4. Exclusion of cortical neurites from inappropriate targets. DiI-labeled neurites from sensorimotor cortex are stopped or deflected at the edges of olfactory (A) and cerebellar (B) explants. Arrows indicate neurites deflected from the edge of the inappropriate targets. Arrowheads indicate edges of the explants. Scale bar, 250 µm.
9). These results show that forelimb sensorimotor fibers extended selectively into appropriate cervical spinal targets. In contrast, they were often excluded from growth into inappropriate targets from the lumbar cord, which they do not innervate in vivo at any stage of development.

Results from experiments to show target selectivity for neurites growing from hindlimb sensorimotor cortical explants were more variable. In all likelihood this was due to the small size of the hindlimb cortical representation and hence the difficulty of obtaining explants limited to this region in 1-day-old cortex. Thus many of the cortical explants probably contained forelimb as well as hindlimb sensorimotor areas. Nevertheless, neurites from 'hindlimb' cortical explants showed some selectivity in their choice of spinal targets. They failed to extend into the gray matter of a mismatched explant from the cervical cord in 56% of such cocultures (n=34). As shown in Fig. 7A

Fig. 5. Growth of forelimb sensorimotor cortical neurites into appropriate cervical targets from hemisected 1-day-old spinal cord. Cortical explants were positioned at upper left. A, C and E are low power photomicrographs from three different cocultures. Note that cortical neurites remain within the gray matter rather than extending to the edge of the spinal explant. B, D and F are corresponding higher power images showing branching corticospinal arbors and growth cone tips. Arrowheads indicate the edge of the spinal explant. Arrows show corresponding points in low and high power photomicrographs. Scale bar in A, C and E, 250 μm; in B, D and F, 50 μm.
1943Corticospinal specificity in coculture

the same cervical spinal target that excluded hindlimb cortical neurites was able to admit appropriate neurites from an explant of forelimb cortex positioned in the culture dish on the opposite side of the cord. When neurites extending from explants of hindlimb cortex were presented with appropriate 6 day lumbar cord targets they grew readily into the gray matter in 72% of the cases (n=61) (Fig. 7B).

A final set of experiments showed that visual cortical neurites, in contrast to sensorimotor fibers, were unable to invade spinal gray targets. When explants of occipital cortex were cocultured with spinal explants from different levels of the cord, we found that visual cortical neurites were overwhelmingly excluded from both cervical and lumbar spinal targets. Visual cortical neurites typically stalled at the border between white matter and gray matter. In experiments in which sensorimotor and visual cortical explants were positioned on opposite sides of a spinal cord explant in the same coculture, one side of the cord excluded visual cortical neurites but the other side was permissive for growth of sensorimotor neurites (Fig. 8). Visual cortical neurites failed to grow into 81% of the cervical spinal explants obtained from 5-8 day cord (n=43), and 81% of explants (n=17) from 1 day cervical cord. The ability of the lumbar spinal cord to exclude growth of visual cortical axons was more pronounced with explants from 5-8 day cord (68%, n=102) than with 1 day old cord (39%, n=46). These results on the growth of visual cortical neurites mimic the in vivo situation in which visual cortical axons can extend transiently into the corticospinal white matter but are prevented from invading targets in the spinal gray.

**DISCUSSION**

The present study has examined specificity of corticospinal connections in an explant coculture system. The results show that sensorimotor cortical neurites arose from appropriate pyramidal neurons, chose spinal targets from topographically appropriate levels of the spinal cord and were able to form highly branching terminal arbors within the spinal gray. Visual cortical neurites in contrast invaded the white matter but not the gray matter of the spinal cord. However, there was no evidence that the presence of target tissue influenced at a distance the growth or branching of cortical neurites. Rather, target recognition by cortical neurites and formation of

![Fig. 6](image_url)

Fig. 6. Examples of forelimb sensorimotor cortical neurites excluded from inappropriate lumbar spinal targets. DiI-labeled neurites from two different forelimb sensorimotor cortical explants stop at the border between the white and gray matter of 6 day lumbar spinal cord (A,B). Solid outline indicates the border of the spinal gray matter, which were drawn from a matching phase image of the spinal explant. Arrowheads indicate the edge of the spinal explant. Scale bar, 250 µm.
terminal arbors appeared to require direct interactions between cortical neurites and their potential targets. Our results, summarized in Table 1 and in the graphs in Fig. 9, show that the responses of cortical neurites to a given type of target tissue were highly consistent across large numbers of cocultures. The variability that we did observe may have several explanations. First, particularly for hindlimb cortex, our dissections may have included both forelimb and hindlimb regions of sensorimotor cortex. In addition a forelimb or hindlimb cortical explant could contain some neurons topographically unrelated to the spinal segment used in the coculture. Such neurites would not enter the spinal target and would thus account for a variable result. In addition, neurites and targets in different focal planes could have contributed to ‘mixed’ results. Finally, differences related to the age of the spinal cord targets most likely contributed to the more variable results obtained for the lumbar cord.

Origin of cortical neurite outgrowth
In previous studies of cortical connectivity in vitro, the laminar position and morphology of cortical neurons that innervated target explants were found to be specific and target dependent.

Fig. 7. Selective growth of cortical neurites into spinal targets. In A most of the neurites from hindlimb sensorimotor cortex (at left) stop at the edge of the gray matter of an explant of 1 day hemisected cervical spinal cord but neurites from forelimb sensorimotor cortex (at right) enter the gray matter of the same spinal explant. In B neurites from hindlimb sensorimotor cortex enter and remain within an explant of 6 day lumbar cord. Arrowheads indicate edge of the spinal explant. Arrows indicate midline of the spinal cord explant. Scale bar, 250 µm.

Fig. 8. Comparison of neurite growth from visual (left) and forelimb sensorimotor cortex (right) into 1 day cervical spinal cord. Visual cortical neurites stall at the white/gray matter border but sensorimotor neurites extend into the spinal gray. Arrows indicates midline of the cord. Solid outline indicates the border of the spinal gray matter on the left side as drawn from a matching phase image. Corresponding border on the right side of the cord is out of the frame of the photograph. Scale bar, 250 µm.
Corticospinal neurons exclusively. A class of efferent cortical projection cells, as opposed to cortical neurons, innervating pontine targets in vitro were found to reside in layer V, the normal origin of corticopontine axons (Heffner et al., 1990). Similarly, studies of neurite outgrowth from visual cortical explants in vitro have shown that neurons innervating thalamic, tectal and cortical targets were usually located in their appropriate cortical layers (Bolz et al., 1990; Yamamoto et al., 1992; Novak and Bolz, 1993) and were of the appropriate cell type.

In the present study, retrograde labeling with DiI showed that cortical neurites growing from the explants originated from pyramidal neurons in the cortical plate. In vivo, corticospinal axons arise exclusively from layer V pyramidal neurons in sensorimotor cortex (Wise and Jones, 1977; Killackey et al., 1989; Merline and Kalil, 1990). Transient visual cortical axons projecting in the corticospinal pathway also arise from layer V pyramidal neurons (Stanfield and O'Leary, 1985; O'Leary and Stanfield, 1986). Results from the present study, however, suggest that spinal targets did not affect neurite outgrowth from specific cortical neurons because retrogradely labeled neurons had pyramidal morphologies and similar laminar positions within the cortical plate regardless of the presence or appropriateness of target tissue. These results suggest that labeled pyramidal neurons belong to a general class of efferent cortical projection cells, as opposed to corticospinal neurons exclusively.

**Selection of appropriate targets by cortical neurites**

Previous studies in explant cocultures have demonstrated varying degrees of axon target specificity. For example, sensorimotor cortical neurites were shown to discriminate between appropriate pontine targets and inappropriate targets such as hypothalamus and olfactory bulb (Heffner et al., 1990). Thalamocortical axons in vitro showed laminar specificity in their terminations (Yamamoto et al., 1989; 1992; Molnar and Blakemore, 1991; Gotz et al., 1992; Bolz et al., 1992), but showed no regional preferences (Molnar and Blakemore, 1991) or modality specificity (Yamamoto et al., 1992).

The present study has also shown that cortical neurites in vitro were able to discriminate generally between appropriate targets such as those from the spinal cord and inappropriate targets such as those from the olfactory bulb and cerebellum. More importantly, however, we have shown that cortical neurites in explant cocultures expressed regional preferences for targets at different segmental levels of the cord and discriminated between white matter tracts and gray matter targets of the spinal cord. These findings are the first to demonstrate topographic organization of efferent cortical connections formed in explant cocultures and are consistent with results from studies of corticospinal development in vivo. In vivo studies have shown that both visual and sensorimotor axons project non-selectively into the rodent corticospinal tract early in development (Stanfield and O'Leary, 1985; O'Leary and Stanfield, 1986; Joosten et al., 1987; Kuang and Kalil, 1994), but that visual cortical axons fail to extend into the gray matter of the cord. The present study is consistent with these results since visual cortical neurites in vitro also stopped at the border between the white matter and the gray matter of the spinal cord. Forelimb cortical neurites grew into the white matter but not the gray matter of lumbar spinal explants. Taken together these results suggest that spinal cord tracts are generally permissive for growth of visual and sensorimotor cortical neurites. In contrast gray matter targets in the spinal cord permit growth of only those axons from topographically appropriate cortical areas. The selectivity of corticospinal connections in vivo and in vitro suggests that targets in the spinal gray matter contain molecules recognized by specific corticospinal axons. Although we have not systematically varied the developmental ages of the spinal targets, we did note that 1-day-old lumbar spinal cord was more permissive for growth of inappropriate visual and forelimb sensorimotor neurites. In vivo, no corticospinal axons are present at lumbar levels until 7 days postnatal. Thus the signals regulating growth of cortical axons into lumbar targets may not be fully developed until later in development when cortical axons normally arrive in the lumbar cord.

Once cortical neurites invaded topographically appropriate spinal targets in explant cocultures they sometimes formed terminal arbors. These arbors had widespread branches similar to corticospinal arbors in vivo (Kuang and Kalil, 1990; 1994). Cortical neurites rarely branched in the collagen and never formed terminal arbors in the collagen or the white matter of the spinal cord explants. Thus contact with cells in appropriate spinal targets may signal cortical axons to begin forming characteristic arbors. In vitro studies of thalamic arbors formed in visual cortex (Yamamoto et al., 1989; 1992; Bolz et al., 1992) and arbors formed in CNS targets by axons of trigeminal ganglion neurons (Erzurumlu et al., 1993) have suggested
that axon arborization is dictated by the target tissue. Moreover, the development of trigeminal arbors in postnatal but not embryonic barrel field cortex (Erzurumlu et al., 1993) has provided evidence that the target-derived signals stimulating arbor formation may be developmentally regulated. In the present study, however, we observed well-developed arbors in the cervical spinal cord prior to postnatal day 4 (Fig. 5) when forelimb cortical axons in vivo begin to arborize in the cervical cord (Kuang and Kalil, 1994).

Mechanisms of corticospinal target selection

Results from recent studies of neurite outgrowth in various explant cocultures have led to different conclusions about whether axons are selectively attracted toward specific targets by diffusible chemoattractant signals. Heffner et al. (1990) for example showed that when cortical explants were cocultured in the presence of pontine and non-target tissue, cortical neurites and their collaterals grew selectively in the direction of the pons. Chemoattractant activity has also been demonstrated in the peripheral nervous system in cocultures of embryonic trigeminal ganglia and maxillary arch targets (Lumsden and Davies, 1983; 1986). In vitro studies on projections of spinal commissural neurons have provided further evidence for guidance of CNS axons by chemoattractants from intermediate floor plate targets (Tessier-Lavigne et al., 1992; Placzek et al., 1990; Tessier-Lavigne and Placzek, 1991). Similar mechanisms based on axon guidance by diffusible factors have been proposed to inhibit neurites from growth into inappropriate targets. In explant cocultures olfactory bulb axons were found to turn away from septal tissue (Pini, 1993), and dorsal root axons were shown to be inhibited from growth into the ventral horn of the spinal cord (Fitzgerald et al., 1993). These studies suggest that developmentally regulated chemoattractant and chemorepulsive factors acting in concert could guide axons toward their targets. In other in vitro studies of axon-target interactions, however, no selective chemotropism was apparent. For example, Molnar and Blakemore (1991) found that thalamic axons innervated cerebral cortex from appropriate and inappropriate areas, suggesting that thalamocortical connectivity is achieved not by selective chemotropism but by contact guidance mechanisms in axon pathways. Similar studies by Bolz et al. (1992) showed that, although thalamic axons in vitro terminated in appropriate cortical layers, there was no evidence for directed growth of thalamic axons toward cortical targets.

Results of the present study have shown no effects of targets on the amount or direction of cortical neurite outgrowth and are consistent with the view that target explants neither attract nor repel afferent neurites at a distance by chemotropic or chemorepulsive factors. The ventrolateral trajectories of efferent cortical neurites from the explants resembled that of cortical efferents in vivo. When we attempted to enhance cortical neurite outgrowth from the pial surface by placement of spinal targets close to the pia, we found that cortical efferents did not reorient their preferred ventrolateral direction of growth from the explant. This stands in contrast to results from corticotectal and corticothalamic explant cocultures in which target tissue ectopically positioned did promote neurite outgrowth from the pial surface of the cortex (Bolz et al., 1990; Novak and Bolz, 1993).

The lack of chemotropic effects on cortical neurite outgrowth by spinal targets reported in the present in vitro experiments may be relevant to the development of corticospinal connections in vivo. Studies of descending cortical pathways during development have led to the hypothesis that the growth cones of layer V neurons are responsible for axon guidance over long pathways but do not themselves invade targets to form connections in the brainstem and spinal cord (O’Leary et al., 1990, 1991; O’Leary and Koester, 1993). Innervation of targets in the pontine nuclei (O’Leary and Terashima, 1988) and spinal cord (Kuang and Kalil, 1994) has been shown to occur not by the directed growth of primary axons but by the extension of interstitial axon collaterals. Thus it is possible that primary cortical neurites in our experiments in vitro did not respond to diffusible molecules emanating from spinal targets since they are not guided by such cues in vivo. However, we have shown that in vivo once the growth cones of primary neurites make direct contact with target tissue they are able to recognize and invade appropriate spinal targets. The present experiments have shown no long-range influences of target tissues on the growth of interstitial branches from cortical neurites within the collagen such as were reported in corticopontine explant cocultures (Heffner et al., 1990). It may be that in vivo spinal targets do exert a localized tropic effect on cortical axons to extend interstitial collaterals but that these influences require an intact corticospinal pathway which is lacking in our explant coculture. Alternatively, in vivo, local cues within the corticospinal tract itself may elicit collateral branching from specific axons at appropriate segmental locations along the spinal cord. Such cues would be distinct from those that guide primary cortical axons through the corticospinal pathway.

The present study has shown that cortical neurites in vitro are not attracted selectively toward spinal targets at a distance, but discriminate between appropriate and inappropriate targets by direct contact. Video microscopic observation of behaviors of cortical growth cones in explant cocultures (Merline et al., 1993) supports this view. Cortical growth cones contacting inappropriate targets such as the cerebellum collapsed and withdrew whereas those approaching appropriate spinal cord targets grew readily into them. An increasing body of evidence suggests that cues regulating interactions between afferent axons and target neurons may be membrane bound (Walter et al., 1990; Baier and Bonhoeffer, 1992; Simon and O’Leary, 1992; Gotz et al., 1992; Boxberg et al., 1993; Wizenmann et al., 1993). The present results are consistent with the existence of membrane-bound cues that either fail to support or inhibit growth of cortical axons into inappropriate olfactory and cerebellar targets but permit or promote their extension into appropriate spinal explants. Differential expression of membrane-bound cues may also underlie the ability of cortical growth cones to discriminate between targets from different segmental levels of the cord. The corticospinal system, because of its topographic specificity in vivo and in vitro will be useful in studies of target cues that regulate growth of afferent axons into correct targets at appropriate stages in development.

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