ELSEVIER

Developmental Brain Research 79 (1994) 122–127

DEVELOPMENTAL BRAIN RESEARCH

Short Communication

Corticospinal axons and mechanism of target innervation in rat lumbar spinal cord

Elbert A.J. Joosten [a,b,*], Peter R. Bär [a], Willem-Hendrik Gispen [b]

[a] *Research Laboratory, Department of Neurology, Academic Hospital Utrecht, Room G02.314, P.O. Box 85500, 3508 GA Utrecht, The Netherlands*
[b] *Rudolf Magnus Institute, University of Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*

(Accepted 25 January 1994)

Abstract

The aim of the present study is to investigate the mechanism by which outgrowing rat corticospinal (CS) axons innnervate their spinal gray target areas. This study was carried out with the use of anterogradely transported horseradish peroxidase or 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) after application in the sensorimotor cortex of rat pups varying in age between 5 days postnatal (P5) and 10 days postnatal (P10). The CS axons of neurons situated in the sensorimotor cortex have reached the ventral most parts of the dorsal funiculus at mid-lumbar spinal cord levels at the fifth postnatal day (P5). After a waiting period of 2 days some CS fibers change their direction and directly enter the adjacent spinal gray target areas. One day later, i.e., P8, CS target innervation by the formation of collateral branches can be observed. Finally, the development of collaterals by interstitial budding from their parent axons appears to be the major, but not the exclusive, mechanism by which CS axons innnervate the lumbar spinal gray matter target area.

Key words: Corticospinal tract; Target finding; Collateral; Pathway formation; Horseradish peroxidase; 1,1-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI); Spinal cord

During the development of central nervous projections most studies on axon branching and target recognition have focused on the behavior of the growth cone of the primary axon [2,3,17,19]. Both in vertebrates and in invertebrates growth cone morphology dramatically changes as developing axons extend along their pathway and reach so-called 'decision points'. At a 'decision point' the axonal growth cone decides either to leave the tract and subsequently enter an appropriate target or to further extend via the tract [2,7,18,25,26]. However, during the target selection of rat cortical axons the primary growth cone does not play a role [20,21]. Instead, layer V neurons extend out of the cortex via the internal capsule and continue to grow caudally via peduncle and deccussation to the spinal cord, bypassing their targets in mid- and hindbrain. For example the rat corticopontine projection from layer V neurons develops by a delayed interstitial budding of collateral branches from corticospinal (CS) axons [20,21]. The 2-day delay between arrival of the primary growth cones and the ingrowth of the collaterals in the pons at predetermined points, may either be intrinsically or extrinsically regulated. In vitro experiments favor an extrinsic regulation of the corticopontine target selection [8,21]. Collagen co-culture experiments have shown that a diffusible chemotropic factor released by the pons induces the interstitial branching of cortical axons [8]. Also at the level of the spinal cord, ie. cervical as well as lumbar spinal gray matter, a diffusible factor is involved in target selection of corticospinal axons [13,16]. The latter in vitro observations indicate that the development of branches of axons originating from layer V neurons in sensorimotor cortex explants is specifically induced by the presence of a lumbar spinal target explant [13]. Nevertheless, the question remains if the CS projection to lumbar spinal cord gray matter in vivo develops by the back-branching of collaterals from parent axons. Anterograde tracer experiments have shown that the first CS axons reach cervical spinal cord levels at birth (P0), mid-thoracic spinal cord levels at postnatal day 2 (P2) and the

* Corresponding author. Fax: (31) (30) 542100.

SSDI 0165-3806(94)00011-N

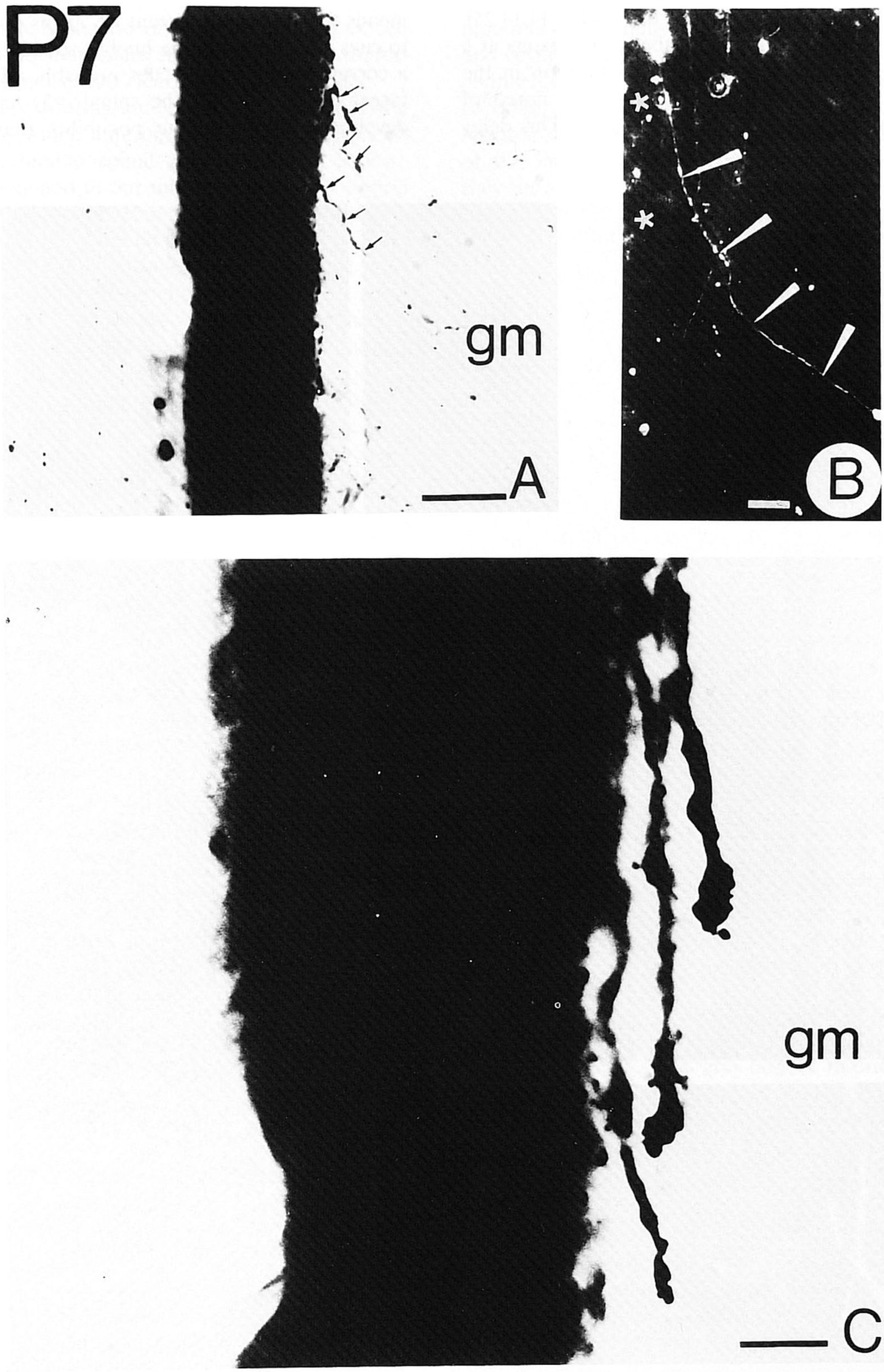

Fig. 1. Initial ingrowth of corticospinal axons into the lumbar spinal gray matter at P7. A: horizontally sectioned spinal cord. The CST (asterisks) is heavily HRP-labeled because of the bulk of later arriving axons. The first HRP-labeled CST axons enter lumbar spinal gray at an angle varying between 130 and 170 degrees (arrows), gm: gray matter, Bar = 100 μm. B: detail of a DiI-labeled CST axons (arrowheads) directly deflecting and entering the lumbar spinal gray matter. The corticospinal tract area is indicated by asterisks Bar = 20 μm. C: detail of HRP-labeled CST axons entering the lumbar spinal gray matter. gm, gray matter, bar = 50 μm.

lumbar enlargement at postnatal day 5 (P5) [4,14,24]. Between the arrival of the first labelled CS axons at a given spinal cord segment and their extension in the respective spinal gray matter, a delay was noted of about 2 days [4,14,24] (for review see [24]). This delay means that either the parent CS axons do not respond to cues which identify the lumbar spinal gray matter as a correct target or that cues probably released by the target interneurons in the spinal gray have yet to be developed. However, the possibility that during the

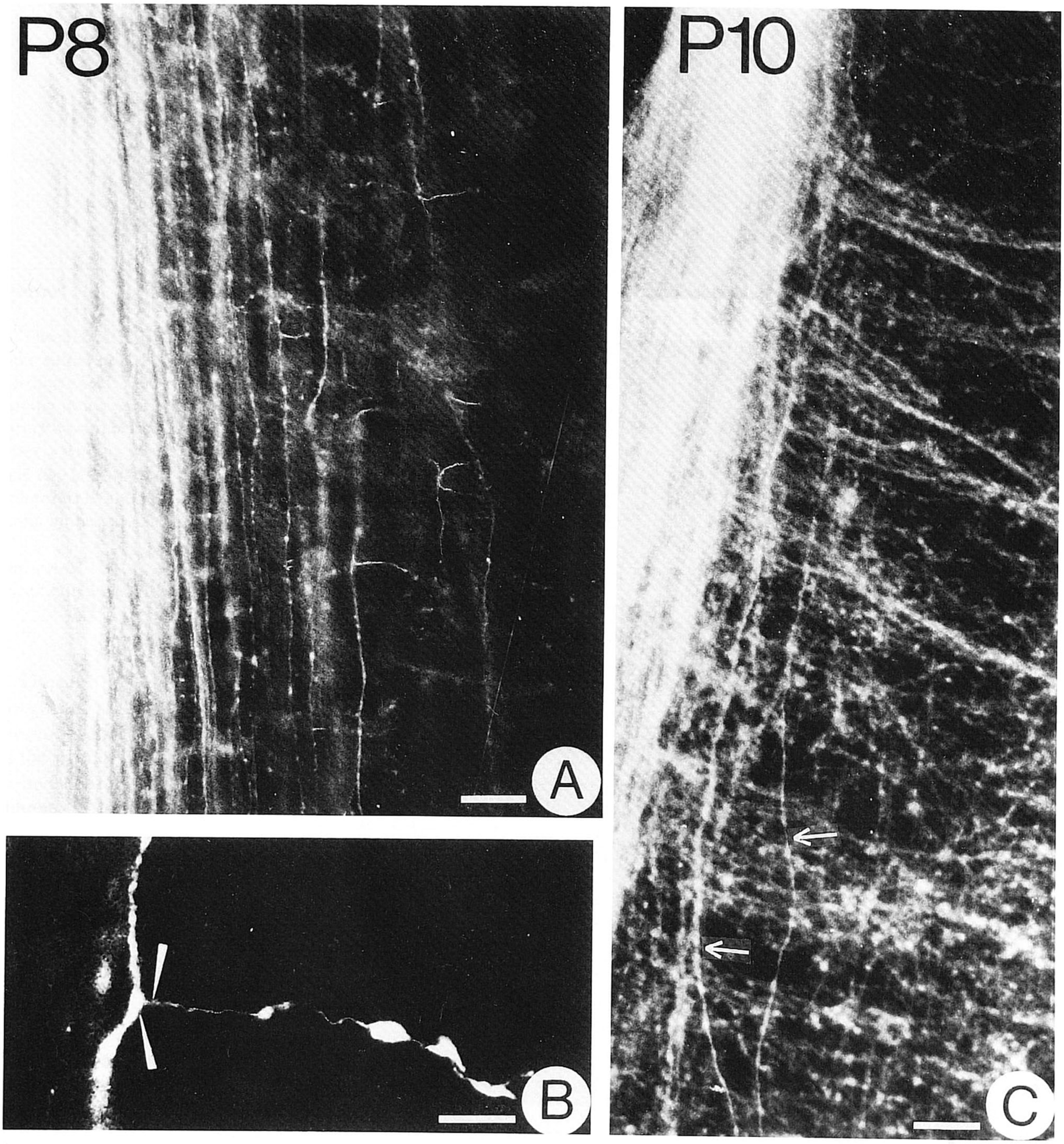

Fig. 2. Horizontally sectioned spinal cord and DiI labeled CST axons. A: target innervation by the formation of collaterals at P8. Collaterals enter spinal gray at sharp turning angles nearly perpendicular to the parent axons in the tract. Bar = 50 μm. B: detail of a DiI-labeled CST axon-collateral at P8 (arrowheads indicate branching point parent axon and its collateral). Bar = 10 μm. C: massive ingrowth of DiI labeled CST axon-collaterals into lumbar spinal gray at P10. Some directly deflecting CST axons can still be observed (arrows). Bar = 50 μm.

delay period the axons are responding to the cues, but that there is no immediate morphological manifestation of their reponse can clearly not be ruled out.

The purpose of this study was to investigate the mechanism by which anterogradely labelled CS axons innervate the lumbar spinal gray target area in vivo. The results may add to our understanding how central nervous system tracts develop and the way its axons are guided towards their targets.

Thirty-five newborn Wistar rats aged between postnatal days 5 (P5) and postnatal day 10 (P10) were used. The day of birth was accounted as P0, whereas the age of the animals given in the present paper were always the ages at their respective days of tracer-injection.

Rat pups were anaesthetized with sodium-pentobarbital (18 mg per kg body weight). They were then held in a stereotactic apparatus, the skin incised and with the use of a fine needle small holes were drilled into the skull and the underlying cerebral cortex. The entire sensorimotor and frontal cortex of the left hemisphere was labelled either by implantation of three horseradish peroxidase (HRP) gels [11] or the injection of the lipophylic fluorescent tracer 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) [9]. The pups were allowed to survive for 24 h.

After injecting a second lethal amount of sodiumpentobarbital the animals were perfused transcardially with 30 ml sucrose in 0.1 M phosphate buffer (PB), pH 7.2, followed by the fixative. For HRP-histochemistry 40 ml 1% paraformaldehyde and 2% glutaraldehyde in the same buffer. For an optimal DiI-visualization rats were perfused with 4% paraformaldehyde solution in 0.1 M PB (pH 7.2). After perfusion the brains and spinal cords were removed. Thereafter, transverse pieces were hand-cut through the second and third lumbar spinal cord segment (L2–L3). Then 50 μm thick transverse or horizontal vibratome sections were cut on an Oxford Vibratome.

A very intense HRP staining was obtained after execution of the following procedure [11]: sections were first incubated with tetramethylbenzidine (TMB) as a chromogen and ammoniumheptamolybdate (AHM) as a stabilizing agent [22]. After rinsing twice in 0.1 M PB, pH 7.4, the HRP-TMB-(AHM) crystals were prevented from being washed out by an additional diaminobenzidine (DAB) nickel intensified incubation [1]. The latter reaction was terminated by rinsing the sections twice in PB. In order to obtain optimal lightmicroscopic visualization of anterogradely HRP-labelled axons the sections were subsequently incubated in a DAB-cobalt glucosidase mixture as described by Itoh et al. [10]. Finally, the sections were rinsed in 0.1 M PB twice, mounted on albumin-coated slides, dehydrated in alcohol, cleared in xylene and coverslipped with Depex.

For DiI-visualization, vibratome sections were mounted on albumin-coated slides, washed in PB-buffer and coverslipped in gelvatol.

In order to visualize outgrowing CS axons, the cells of origin in layer V of the sensorimotor cortex were labelled either with HRP or DiI. Careful examination of the injection- or implantation-areas revealed that they invariably encompassed both the sensorimotor and motor areas of the cerebral cortex as well as adjacent cortical regions, at all ages studied.

The first CS axons entering the lumbar gray matter can be observed at P7 (Fig. 1). These CS axons enter the lumbar target area at an angle varying between 130 and 170 degrees (Fig. 1). The tract area, located in the ventral most part of the dorsal funiculus, is very intensely HRP-stained (Fig. 1A). Due to this accumulation of HRP-labeling in the tract area it is very difficult to determine if the labeled CS axons change their direction and directly grow out into the spinal gray matter. However, with the use of the anterograde label DiI directly deflecting CS axons can be visualized at P7 (Fig. 1B).

One day later, i.e., P8, initial budding of collaterals from the parent CS axons occurs (Fig. 2A). This development of collaterals by the mechanism of budding mainly occurs at sharp turning angles of 90 degrees perpendicular to the parent axons (Fig. 2A,B). This process of budding does not seem to occur at predetermined points (Fig. 2A).

A massive outgrowth of back-branches from CS parent axons into the spinal gray target area can be observed at P10 (Fig. 2C). The mechanism of interstitial budding of collaterals of parent axons seems to be the major mechanism by which labeled CST axons innervate lumbar spinal gray matter. There are obviously no predetermined points along the rostocaudal axis of the CS tract where back-branches develop.

The present study demonstrates that the development of collaterals by interstitial budding of the parent axons forms the major mecchanism CST axons innervate their lumbar spinal gray targets. In order to visualize outgrowing CST axons during early postnatal development we used two anterograde tracers. First of all, we used HRP in combination with a modified staining technique [11]. This allowed us to study labeled axons as well as their leading tips or growth cones. As previously verified at the electron microscopic level, the HRP-staining procedure reveals a complete and intense staining of labeled profiles of outgrowing CS axons [11]. In the present study a complication with this technique is the massive labeling of CST axons in the tract. The intense and massive labeling often masks the presence of directly deflecting CST axons. As previously demonstrated, the development of collaterals by the mechanism of budding mainly occurs at sharp turning angles nearly perpendicular to the parent axons [21,23]. However, the HRP-labeled CST axons

enter the lumar spinal gray at an angle varying between 130 and 170 degrees (Fig. 1A,B): a result which is clearly not in favor of the budding-hypothesis. Nevertheless, we used a second anterograde tracer, DiI. With the use of this tracer we could clearly demonstrate the presence of directly deflecting CST axons in lumbar spinal cord levels at P7 (Fig. 1B). Although initially some directly deflecting axons are noted (at P7) this mechanism is clearly of minor importance during CS target innervation. Nevertheless, an intriguing question is whether the directly deflecting CS axons belong either to a population of (later arriving) axons which immediately upon arrival at a certain spinal cord segment enter their target or that they belong to the first labelled axons entering spinal white matter (at P5) subsequently wait for 2 days and then change their direction and enter the spinal gray. It is our opinion that only a carefull in vivo-time lapse study [6] of anterogradely labeled CST axons might give an answer to this question. In any event, the growth cones of these directly deflecting CS axons probably are able to recognize the lumbar spinal gray matter as a correct target. Preliminary (unpublished) observations reveal striking variations among CST growth cones related to their position in the lumbar spinal cord: CST growth cones in the tract are characterized by their long and rather simple configuration [5,11], whereas the tips of the directly deflecting CST axons invading the lumbar spinal gray are more complex (see Fig. 1A,C). Furthermore, this change in growth cone morphology from tract to target area not only reflects the varying interactions with the microenvironment but also indicates a change of the predominant guidance mechanism [2,19, 23,27]. It has been described that in the CS tract area various cell adhesion molecules are involved in the guidance of outgrowing CST axons [12,15] whereas in the spinal target areas diffusible,tropic factors are released [13,16]. In vitro collagen co-culture experiments have shown that the lumbar spinal gray matter specifically initiates and directs the branching of axons with their cells of origin located in layer V of the cortex explant [13]. Our observations indicate that also in vivo the formation of collaterals from parent axons by interstitial budding is of major importance in the way CST fibers innervate the lumbar spinal gray. In conclusion, both in vivo and in vitro observations strongly suggest that the diffusible factor released by the lumbar spinal gray is involved in the target innervation through the formation of interstitial collaterals of CST axons.

[1] Adams, J.C., Heavy metal intensification of DAB-based HRP reaction product, *J. Histochem. Cytochem.*, 29 (1981) 775.
[2] Bovolenta, P. and Mason, C.A., Growth cone morphology varies with position in the developing mouse visual pathway from retina to first targets, *J. Neurosci.*, 7 (1987) 1447–1460.
[3] Dodd, J. and Jessell, T.M., Axon guidance and the patterning of neuronal projections in vertebrates, *Science*, 242 (1988) 105–116.
[4] Donatelle, J.M., Growth of the corticospinal tract and the development of placing reactions in the postnatal rat, *J. Comp. Neurol.*, 212 (1977) 207–232.
[5] Gorgels, T.G.M.F., Outgrowth of the pyramidal tract in the rat cervical spinal cord: growth cone ultrastrcuture and guidance, *J. Comp. Neurol.*, 306 (1991) 95–110.
[6] Harris, W.A., Holt, C.E. and Bonhoeffer, F., Retinal axons with and without their somata, growing to and arborizing in the tectum of Xenopus embryos: a time-lapse video study of single fibers in vivo, *Development*, 101 (1987) 123–133.
[7] Harris, W.A., Holt, C.E., Smith, T.A. and Gallenson, N., Growth cones of developing retinal cells in vivo, on culture surfaces and in collagen matrices, *J. Neurosci. Res.*, 13 (1985) 101–122.
[8] Heffner, C.D., Lumsden, A.G.S. and O'Leary, D.D.M., Target control of collateral extension and directional axon growth in the mammalian brain, *Science*, 247 (1990) 217–220.
[9] Honig, M.G. and Hume, R.I., Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures, *J. Cell Biol.*, 103 (1986) 171–187.
[10] Itoh, K., Konishi, A., Mizuno, N., Nakamura, Y. and Sugimoto, T., Application of coupled oxidation reaction to electronmicroscopic demonstration of horseradish peroxidase: cobalt glucose oxidase method, *Brain Res.*, 175 (1979) 341–346.
[11] Joosten, E.A.J., Light and electron microscopical visualization of anterogradely labelled corticospinal growth cones using a new combination of HRP staining techniques, *J. Neurosci. Methods*, 37 (191) 199–207.
[12] Joosten, E.A.J., Developmental expression of N-CAM epitopes in the rat spinal cord during corticospinal tract axon outgrowth and target-innervation, *Dev. Brain Res.*, in press.
[13] Joosten, E.A.J., Bär, P.R. and Gispen W.H., Tropism and corticospinal target selection in the rat, *Neuroscience*, in press.
[14] Joosten, E.A.J., Gribnau, A.A.M. and Dederen, P.J.W.C., An anterograde tracer study of the developing corticospinal tract in the rat: three components, *Dev. Brain Res.*, 36 (1987) 121–130
[15] Joosten, E.A.J., Gribnau, A.A.M. and Gorgels, T.G.M.F., Immuno-electronmicroscopical localization of cell adhesion molecule L1 in developing rat pyramidal tract, *Neuroscience*, 38 (1990) 675–686.
[16] Joosten, E.A.J., Van der Ven, P.F.M., Hooiveld, M.H.W., and Ten Donkelaar, H.J., Induction of corticospinal target finding by release of a diffusible, chemotropic factor in cervical spinal grey matter, *Neurosci. Lett.*, 128 (1991) 25–28.
[17] Landmesser., L., The development of specific motor pathways in the chick embryo, *Trends Neurosci.*, 7 (1984) 336–340.
[18] Mason, C.A., Growing tips of embryonic cerebellar axons in vivo, *J. Neurosci. Res.*, 13 (1985) 55–73.
[19] Nordlander, R., Axonal growth cones in the developing amphibian spinal cord, *J. Comp. Neurol.*, 263 (1987) 485–496.
[20] O'Leary, D.D.M., Bricknese, A.R., De Carlos, J.A., Heffner, C.D., Koester, S.E., Kutka, L.J. and Terashima, T., Target selection by cortical axons: alternative mechanisms to establish axonal connections in the developing brain, *Cold Spring Harbor Symp. Quant. Biol.*, 15 (1990) 453–468.
[21] O'Leary, D.D.M. and Terashima, T., Cortical axons branch to multiple subcortical targets by interstitial axon budding: implications for target recognition and "waiting periods", *Neuron*, 1 (1988) 901–910.
[22] Olucha, F., Martinez-Garcia, F. and Lopez-Garcia, C., A new stabilizing agent for the tetramethylbenzidine (TMB) reaction product in the histochemical detection of horseradish peroxidase (HRP), *J. Neurosci. Meth.*, 13 (1985) 131–138.
[23] Reh, T.A. and Constatine-Patton, M., Growth cone target interactions in the frog retinotectal pathway, *J. Neurosci. Res.*, 13 (1985) 89–100.

[24] Schreyer, D.J. and Jones, E.G., Growth and target finding by axons of the corticospinal tract in prenatal and postnatal rats, *Neuroscience*, 7 (1982) 1837–1853.
[25] Stanfield, B.B., The development of the corticospinal projection, *Prog. Neurobiol.*, 38 (1992) 169–202.
[26] Yagimuna, H., Homma, S., Hunzi, R. and Oppenheim, R.W., Pathfinding by growth cones of commisural interneurons in the chick embryo spinal cord: a light and electron microscopic study, *J. Comp. Neurol.*, 304 (1991) 78–102.
[27] Williams, R.W., Bastiani, M.J., Lia, B. and Chalupa, L.M., Growth cones, dying axons and developmental fluctuations in the fiber population of the cat's optic nerve, *J. Comp. Neurol.*, 246 (1986) 32–69.