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Short communication

Embryonic form of N-CAM and development of the rat corticospinal tract; immuno-electron microscopical localization during spinal white matter ingrowth

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Abstract

The neural cell adhesion molecule N-CAM has been proposed to function in the guidance of outgrowing axons in the peripheral and central nervous system. Light microscopic observations have shown that the embryonic form of N-CAM (200–230 kDa) is present in the ventralmost part of the dorsal funiculus during corticospinal tract (CST) ingrowth in the first postnatal week (Joosten, *Dev. Brain Res.*, 78 (1994) 226–236). Here, the subcellular localization of the embryonic form of N-CAM (E-NCAM) is determined by pre-embedding staining on vibratome sections and by postembedding immunogold-labelling on Epon embedded spinal cord sections. The electron microscopical observations indicate that E-NCAM is present on the outer membrane of CST growth cones as well as other unmyelinated axons which are present in the ventralmost part of the dorsal funiculus. Furthermore, E-NCAM is localized in an irregular patchy way on the outer side of the axonal membrane of small unmyelinated, later arriving CST axons. From these results it may be deduced that E-NCAM is involved in CST tract formation through guidance of outgrowing pioneer CST growth cones along other unmyelinated axons and through mediation of axon fasciculation of later arriving CST axons.

Keywords: Neural cell adhesion molecule; Corticospinal tract; Growth cone; Guidance; Pyramidal tract; L1 ; Tract formation

The neural cell adhesion molecule N-CAM, present on cell surfaces and/or extracellular matrices, mediates adhesive interactions between neurons during development of the central nervous system [5,29]. N-CAM is highly concentrated in developing tissues, and in vitro studies show that it is localized on growth cones [31]. In the embryonic nervous system, N-CAM is present in a higher molecular weight form (MW = 200–230 kDa) that has more polysialic acid (PSA) residues and therefore less homophilic binding than the lower molecular weight NCAM forms present in the adult (MW = 120, 140, 180 kDa). The structurally distinct isoforms of N-CAM are expressed in a developmentally regulated and tissue specific manner [3,21].

In the developing mouse optic nerve the highly sialylated embryonic form of N-CAM (E-NCAM) is present on the growth cones of fasciculating axons [1]. As this highly sialylated form of N-CAM is less adhesive than the adult forms [3,26] it is thought to be involved in rather dynamic morphogenetic events [6,10]. The contact-mediated exploration of the environment by the growth cone is such a very dynamic morphogenetic event during which E-NCAM might be important.

Light microscopic immunocytochemical observations indicated that E-NCAM may be involved in corticospinal tract (CST) formation during spinal white matter ingrowth [11]. The rat CST, localized in the ventralmost part of the dorsal funiculus, is characterized by a staggered mode of outgrowth in spinal cord white matter [9,15,28,30]: the first CST pioneer fibers, which enter the spinal cord at postnatal day one (P1), are followed by a bulk of later arriving fasciculating axons between postnatal days 2 and 10.

Based on light microscopical data we suggested that E-NCAM may play an important role during both CST pioneer ingrowth and fasciculation of later arriving CST fibers [11]. With the use of the monoclonal antibodies 5A5

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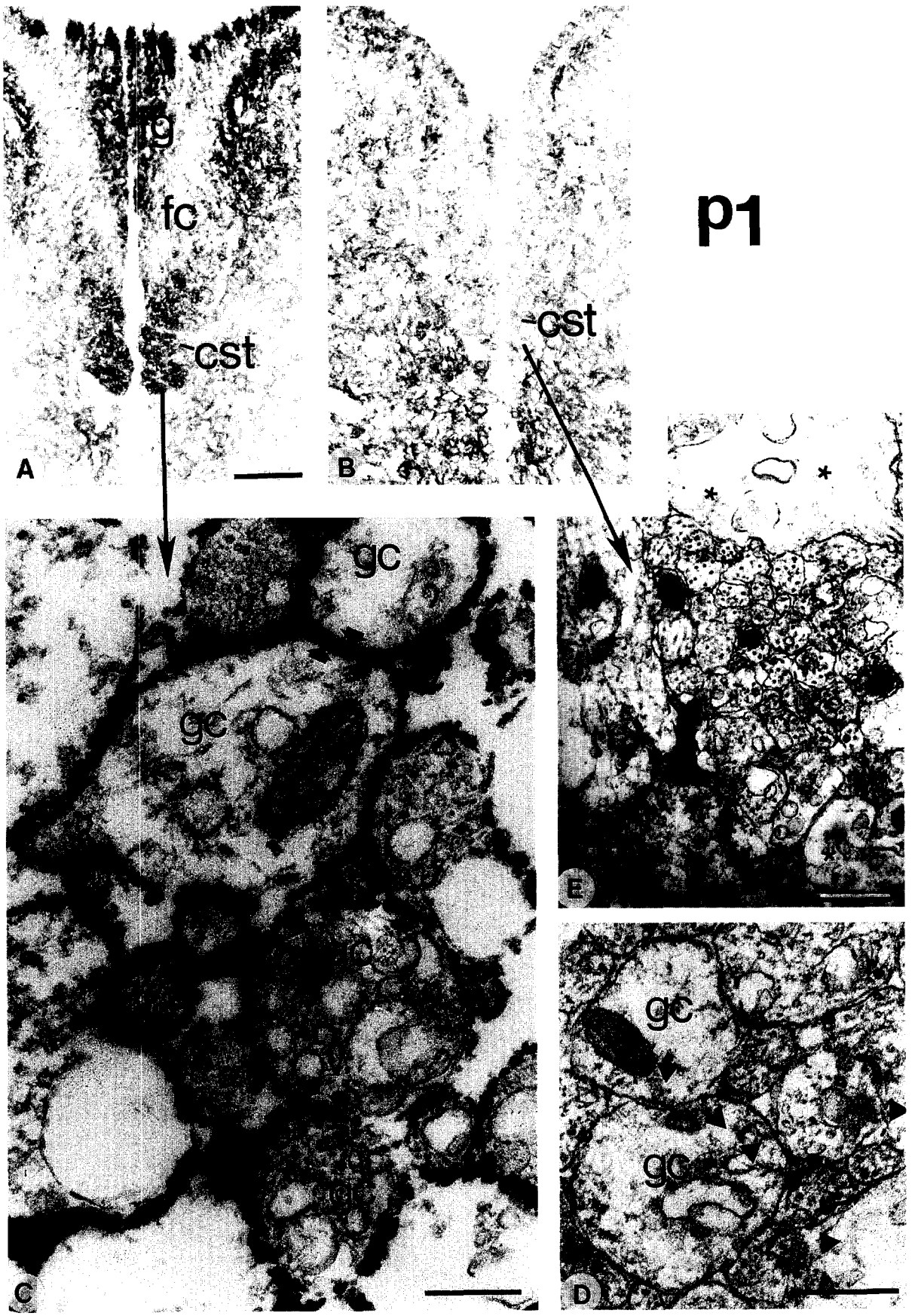
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[22] and 12F8 [2,18], which both recognize E-NCAM, an intense staining of the ventralmost part of the dorsal funiculus is observed. This immunostaining is restricted to the period of CST tract formation in spinal white matter, during the first postnatal week [11]. Therefore, the aim of the present study is to localize E-NCAM at the subcellular level during CST tract ingrowth into the spinal white matter. This may add to our understanding how CST fibers are guided to their spinal target and may be illustrative for processes underlying outgrowth and guidance in the central nervous system.

Wistar rats (UWU/CPB) pups varying in age between P1 and P4 were used in this study. The day of birth is considered P0. At least four animals of each age were used.

The monoclonal antibodies (MoAb) 5A5 (IgM) (gift from Dr. T. Jessell, New York, NY) and 12F8 (IgM) (gift from Dr. Carl Lagenauer, Pittsburgh, PA) were used in this study. The 5A5 MoAb was generated against mouse embryonic spinal cord membranes and recognizes an epitope associated with the polysialglycan chain of NCAM [22]. The MoAB 12F8, raised against affinity purified mouse N-CAM, recognizes polysialic acid on embryonic N-CAM [2,18].

Rat pups were anesthetized by intraperitoneal injection of an aqueous solution of Nembutal (18 mg/kg body weight) and perfused through the heart (via the left ventricle) either with 4% paraformaldeyhde in phosphate buffered saline (PBS) (pH 7.4) or with a combination of 2% paraformaldehyde and 0.12% glutaraldehyde PBS [8,14,16]. After perfusion the spinal cords were resected from the vertebral columns and then postfixed in the same fixative for about 1 h before being transferred into cold (4°C) PBS with 10% sucrose or 30% sucrose (until the tissue block sank in each solution).

For the electron microscopical studies only tissue from rats perfused with 2% paraformaldehyde and 0.12% glutaraldehyde were used. For pre-embedding imunocytochemistry, 50 μm transverse sections of the fifth cervical segment were cut on a BIO-RAD H1200 vibratome and collected in cold PBS. The vibratome sections were treated with 5% dimethyl sulfoxide (DMSO) in PBS and rinsed with PBS/BSA. To localize E-NCAM the vibratome sections were incubated overnight with the 5A5 antibodies (diluted 1:10 in 0.1 M PB). Control sections were incubated with 10% normal serum instead of primary antibodies. After rinsing 30 min in 0.1 M PBS/BSA, pH 7.4, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h (diluted 1:220 in PBS/BSA). After washing for 20 min (twice 10 min) with PBS/BSA the HRP was visualized using the chromogen diaminobenzidine hydrochloride (DAB; Sigma Chemicals) and cobalt and nickel as intensifying agents. Some sections were washed in PBS, dehydrated and embedded in Depex for light microscopical immunocytochemical localization. For electron microscopic examination sections were washed in PBS, postfixed in 1% OsO_4 in 0.1 M PBS (pH 7.4) for 1 h, dehydrated and embedded in Epon. Ultrathin sections were cut on a ultramicrotome and mounted on 75 mesh Fromvar-coated copper grids. All sections were counterstained with uranyl acetate (20 min) and lead citrate (10 min).

For postembedding immunocytochemistry 50 μm transverse vibratome sections (from tissue fixed with 2% paraformaldehyde and 0.12% glutaraldehyde) were washed in PBS, fixed in 1% OsO_4 for 1 h, dehydrated and embedded in Epon. Ultrathin sections were mounted on uncoated nickel grids and etched with 10% H_2O_2 for 5 min. To quench free aldehyde groups the sections were incubated in 0.02 M glycine solution in PBS, pH 7.4 (3 × 10 min). The immunostaining was carried out by sequentially floating the grids on drops of the following solutions: primary antibody (undiluted, overnight); PBS-glycine (3 × 10 min); secondary antibody rabbit anti-mouse (diluted 1:500 in 1% BSA for 1 h at room temperature); 0.1% BSA/PBS (3 × 10 min); protein A conjugated with 10 nm colloidal gold for 1 h; PBS (4 × 5 min); stabilization on 1% glutaraldehyde in PBS (5 min); distilled water (5 × 2 min). Control sections were incubated with 1% BSA in PBS instead of the primary antibodies.

In rat the main component of the CST is located in the ventralmost parts of the dorsal funiculus (Fig. 1A). Tissue treated with 2% paraformaldehyde and 0.12% glutaraldehyde showed immunostaining identical to that obtained with 4% paraformaldehyde fixed tissue. The 5A5 and the 12F8 antibodies, which both recognize polysialic acid on embryonic N-CAM, gave an indistinguishable staining pattern. Control sections invariably displayed no detectable immunoreaction.

Both 5A5 and 12F8 produce an intense staining of the CST at cervical levels (Fig. 1A), but not at lumbar spinal cord levels (Fig. 1B) at P1. The fasciculus gracilis (FG)

Fig. 1. Localization of 5A5/12F8 immunoreactivity in rat CST at postnatal day 1 (P1). The E-NCAM antigen is present in the ventralmost part of the dorsal funiculus at cervical (A) but not at lumbar spinal cord levels (B). Bar = 25 μm; cst: corticospinal tract; fc: fasciculus cuneatus; fg: fasciculus gracilis. Pre-embedding immunostaining on transverse vibratome sections from cervical spinal cord (C) reveal the presence of 5A5 immunoreactivity on the outer axonal membrane of CST growth cones (arrows) as well as on small unmyelinated axons. 5A5 immunoreactivity is present in the contact zone between growth cones. D: Postembedding immunogold staining of 5A5 antigen on CST growth cones (arrowheads) and unmyelinated axons. Bar = 0.2 μm. gc: growth cone. E: Pre-embedding 5A5 immunostaining on transverse section from lumbar spinal cord (prior to the arrival of CST fibers). No 5A5 immunoreactivity can be observed. Note the absence of growth cones. The pre-arrival area contains fascicles of unmyelinated fibers which are surrounded by watery profiles (asterisks) containing large vacuole-like structures. Bar = 0.5 μm.

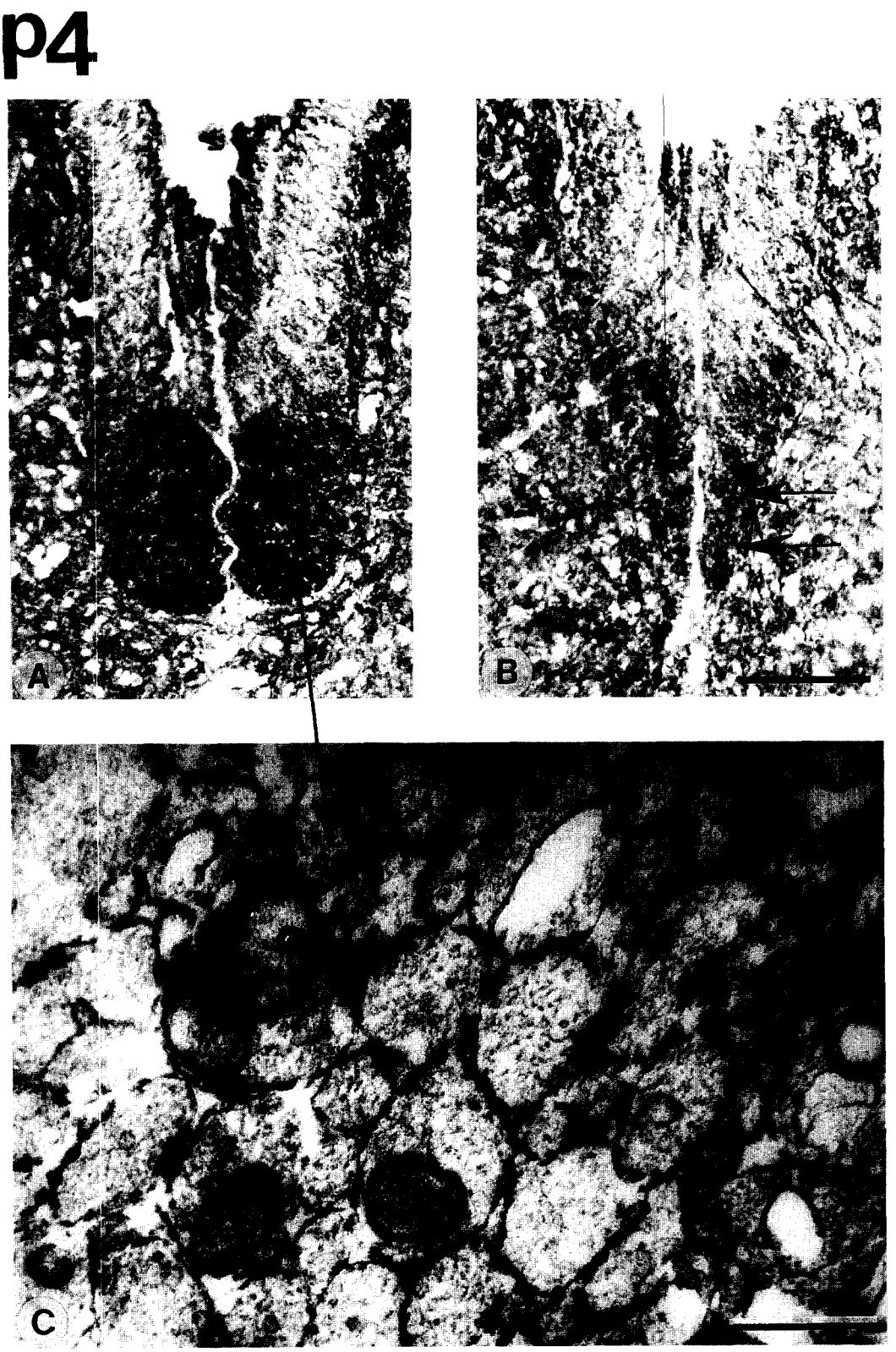

Fig. 2. Localization of 5A5/12F8 immunoreactivity in rat CST at postnatal day 4 (P4). The E-NCAM antigen is present in the ventralmost part of the dorsal funiculus during fasiculation of later arriving CST fibers at cervical levels (A) and during the ingrowth of the first CST fibers at lumbar spinal cord levels (B). Bar = 25 μm. C: 5A5 immunolabelling performed by pre-embedding staining procedures on transversally sectioned CST from cervical spinal cord levels at P4. Note the patchy distribution of the DAB reaction product on the outer axonal membrane of later arriving fasciculating CST axons. Bar = 0.2 μm.

was stained with 5A5 and 12F8 (Fig. 1A), whereas the fasciculus cuneatus (FC) was devoid of staining both at cervical and at lumbar spinal cord levels at P1 (Fig. 1A,B). The spinal gray adjacent to the dorsal funiculus is characterized by a very low, rather diffuse staining pattern with 5A5 (Fig. 1A,B). At P4 the ventralmost part of the dorsal funiculus at both cervical and lumbar levels showed staining with 5A5 (Fig. 2A,B) and 12F8 (not shown). Anterograde tracer studies have shown that the first CST axons reach lumbar spinal cord levels at P4, whereas CST formation at cervical levels is at the same time characterized by the addition of large amounts of later arriving fasciculating fibers [9,15].

For electron microscopical studies only rats perfused with 2% paraformaldehyde and 0.12% glutaraldehyde were used. The presence of 0.12% glutaraldehyde did not affect the 5A5/12F8 immunoreactivity of the spinal cord tissue and resulted in a considerably improved ultrastructural morphology as compared to 4% paraformaldehyde fixed tissue.

In general identical results were obtained after pre-embedding–DAB staining as compared to the postembedding–immunogold technique. The postembedding–immunogold staining technique on vibratome sectioned tissue did not result in high amounts of labelling as compared to the pre-embedding technique (compare Fig. 1C and Fig. 1D). Nevertheless the complete absence of immunogold labelling in the control sections indicated a relatively high specificity. We therefore used both the pre- and postembedding technique and conclude that misinterpretations due to for instance diffusion of the peroxidase product and/or incorrect localization of the immunogoldlabelling (within a range of 10–15 nm of the antigen) can be excluded.

During the entrance of CST pioneer fibers into the ventralmost part of the dorsal funiculus at cervical levels (at P1) and at lumbar levels (at P4) a clear 5A5 immunoreactivity was noted on the outer membrane of growth cones, at the interface between growth cones and also on small, unmyelinated axons (Fig. 1C,D). These unmyelinated axons in the CST ingrowth area are not of cortical origin [15,28] and probably belong to ascending systems located in the dorsal funiculus. Astroglial cells and its processes invariably displayed no 5A5 immunoreactivity (not shown).

Prior to the entrance of CST fibers (i.e. lumbar spinal cord at P1) the ventralmost part of the dorsal funiculus or pre-arrival zone is characterized by the absence of 5A5 immunoreactivity (Fig. 1E). The pre-arrival zone exhibits fascicles of unmyelinated fibers surrounded by lucent amorphous structures (Fig. 1E).

During the period in which the bulk of later arriving unmyelinated CST axons enter the ventralmost part of the dorsal funiculus, i.e. at cervical spinal cord levels between P2 and P7, 5A5 immunoreactivity was noted on small, unmyelinated faciculating CST axons (Fig. 2C). The local accumulation of DAB reaction product strongly suggests an irregular patchy distribution of E-CAM on the outer side of the axonal membrane.

In the rat spinal cord the outgrowth of the major component of the pyramidal tract (PT), the CST, primarily occurs during the first 10 postnatal days [9,15,28]. Our results demonstrate that N-CAM (E-NCAM) is present on CST growth cones and unmyelinated fibers in the ventralmost part of the dorsal funiculus. Tracer studies indicated that at that time these unmyelinated fibers are not of cortical origin [15], but probably belong to ascending systems located in the dorsal funiculus [20]. The localization of E-NCAM during ingrowth of the first CST axons in spinal white matter on the outer side of the plasma membrane of CST growth cones as well as on the surrounding unmyelinated axons indicates that guidance of the pioneer CST axons probably occurs by homophilic interactions between these structures. Adhesion mediated by N-CAM is thought to involve homophilic binding of N-CAM molecules on both adhesion membranes [7,25]. The highly sialylated embryonic form of N-CAM is known to be less adhesive than the adult form(s) [3], and therefore thought to be involved in more dynamic morphogenetic events such as the development of the retinal projections [27] or the cerebellar cortex differentiation [6,10]. The CST growth cone contact mediated inventarization of the spinal white matter environment is a very dynamic event during which the embryonic, highly sialylated form of N-CAM is important. Although homophilic binding of N-CAM molecules is important during CST pioneer ingrowth, N-CAM (E-NCAM) also binds to heparan sulfate proteoglycan, suggesting a role in extracellular matrix adhesion [4]. Based on our observations, interactions between E-NCAM and extracellular matrix components during CST pioneer ingrowth into spinal white matter cannot be excluded.

In addition to a role in the guidance of CST pioneer fibers our findings provide evidence for the implication of E-NCAM in the fasciculation of the later arriving bulk of CST fibers. In vitro antibody perturbation experiments [23] and in vivo observations [8,19] have shown that N-CAM is involved in the fasciculation of neurites.

Immunoelectron microscopical studies have shown that the cell adhesion molecule L1 [24] is also involved in the fasciculation of the later arriving CST axons [14,16]. The presence of E-NCAM as well as L1 on fasciculating later arriving CST axons during spinal white matter favors previous in vitro observations that interactions between these two different glycoproteins (of the same immunoglobulin superfamily [23]) result in the potentiation of neurite outgrowth [17]. An assisted homophilic interaction between E-NCAM and L1 may result in potentiation of the neurite outgrowth of later arriving CST axons in rat spinal white matter.

Besides the presence of E-NCAM on later arriving CST axons, light microscopic observations strongly suggested the presence of adult forms of N-CAM on later arriving

CST axons [11]. In this respect it should be noted that the presence of E-NCAM considerably decreased between P7 and P10, whereas the presence of adult N-CAM forms increased during the end stages of CST white matter ingrowth [11]. This shift from E-NCAM (with relatively low affinity) to adult N-CAM (with relatively high affinity) during CST fasciculation may indicate that once the CST projection is established, plasticity is no longer desirable and therefore only the higher affinity N-CAM is expressed. Because of its role during CST development we currently use the immunocytochemical visualization of N-CAM, and especially E-NCAM, as a marker for regrowth or sprouting of CST fibers after transection in the adult rat spinal cord [12,13].

In summary, our electron microscopic observations demonstrate that E-NCAM is involved in CST tract formation in various ways. First, through guidance of outgrowing pioneer CST growth cones along other unmyelinated axons in the ventral most part of the dorsal funiculus. Second, through mediation of the fasciculation of later arriving CST axons. The presence and role of E-NCAM during rat CST development makes it a potentially valuable marker for stuying CST regrowth in the injured adult rat spinal cord.

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