Deposition and Role of Thrombospondin in the Histogenesis of the Cerebellar Cortex

K. S. O'Shea,* J. S. T. Rheinheimer,* and V. M. Dixit‡

Departments of *Anatomy and Cell Biology and ‡Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109

Abstract. The patterns of deposition of thrombospondin (TSP), a trimeric extracellular matrix glycoprotein, were determined during the initial establishment of the external granule cell layer and the subsequent inward migration of granule cells forming the molecular and (internal) granule cell layers. The early homogeneous deposition of TSP became restricted to the rhombic lip in the region of granule cell exit from the neuroepithelium, and was present between migrating granule cells.

During the later inward migration of granule cells, little TSP was associated with dividing granule cells; it was enriched in premigratory granule cells. With the cessation of migration, TSP was lost except in association with fasciculating axons in the molecular layer where staining persisted briefly. At the EM level, TSP was associated with the leading process of granule cells as they associated with Bergmann glial cells and migrated through the molecular layer. TSP was present within granule cell axons; Purkinje cells and their dendrites, as well as Bergmann glial fibers and endfeet were negative for TSP.

When anti-TSP antibodies were added to explant cultures of cerebellar cortex during active granule cell migration, a dose-dependent inhibition of migration was observed. In control cultures, granule cells migrated into the (internal) granule cell layer, while granule cells exposed to anti-TSP antibodies were arrested within the external granule cell layer. These results suggest that TSP plays an important role in the histogenesis of the cerebellar cortex by influencing granule cell migration.

In the development of the nervous system, neurons migrate considerable distances from their site of origin to their adult locations. Although the pattern of the cellular migrations is relatively well delineated, the mechanisms underlying these rearrangements remain largely unknown. To date, research interest has emphasized the role of glial fiber guidance in directing neuronal cell migrations (cf., Rakic, 1971). More recently, interest has focused on the role of the extracellular matrix (ECM)[1] that surrounds and supports neurons and glia, and may provide guidance cues for neurons as they migrate.

Unlike the ECM of other developing tissues, which is known to consist of tissue-specific combinations of numerous components (e.g., Hay, 1981), relatively few ECM proteins have been identified in the developing central nervous system (cf., Sanes, 1989), although histochemical evidence for their presence existed for some time (Margolis et al., 1975; Kraynek, 1980). In addition to cell surface-associated cell adhesion molecules (Chuong and Edelman, 1984; Chuong et al., 1987; Persohn and Schachner, 1987), ECM proteins fibronectin (Hatten et al., 1982; Chun and Shatz, 1988; Stewart and Pearlman, 1987), laminin (Letourneau et al., 1989; McLoon et al., 1988), tenascin (Grumet et al., 1985; Erickson and Taylor, 1987), hyaluronectin (Delpech et al., 1982), hyaluronic acid (Ripellino et al., 1988), chondroitin sulfate proteoglycan (Aquino et al., 1984), and thrombospondin (O'Shea and Dixit, 1988) have recently been identified in the developing central nervous system.

Thrombospondin (TSP) is a large (420,000-M_r) adhesive glycoprotein densely deposited in the developing nervous system, among other locations (O'Shea and Dixit, 1988). It is associated with smooth muscle cell proliferation and cell migration (Majack et al., 1986), chemotaxis and haptotaxis of melanoma cells (Taraboletti et al., 1987), and serves as an attachment factor for a variety of cells (Varani et al., 1986; Lawler et al., 1988). TSP binds a number of ECM components including glycosaminoglycans (Dixit et al., 1984; Lawler et al., 1985), plasminogen and tissue plasminogen activator (Silverstein et al., 1984, 1986), laminin (Mumby et al., 1984), and collagens (Lahav et al., 1982, 1984; Mumby et al., 1984). Interestingly, binding of TSP to plasminogen and to tissue plasminogen activator greatly enhances plasminogen activation by tissue plasminogen activator (Silverstein et al., 1984, 1985, 1986), possibly creating a nidus of protease activity required for these cell migrations. Thus, the presence of TSP in regions of cell migration and process outgrowth, as well as its affinity for many other matrix components, suggests that TSP plays a role in determining the organization and characteristics of the ECM and thus directs developmental events.

1. *Abbreviations used in this paper*: ECM, extracellular matrix; TSP, thrombospondin.

In the current investigation we first determined the pattern of deposition of TSP in the establishment of the cerebellar cortex—a well-defined system characterized by precise geometrical organization and relatively few cell types (Palay and Chan-Palay, 1974). We then examined the role of TSP in the migration of granule cells from their site of proliferation near the pial surface to their adult location below the Purkinje cell bodies, forming the (internal) granule cell layer. Consistent with its dense deposition surrounding premigratory granule cells, addition of anti-TSP antibodies to cultures of day 10 cerebellar cortex explants arrested granule cell migration.

Materials and Methods

Immunocytochemistry

Light Microscope Level. Embryos obtained from matings of CD-1 strain mice were dissected free of decidua and membranes and rapidly frozen in OCT, in hexane cooled over an acetone-dry ice slurry. Embryos were obtained on days 11-19 of gestation, and cerebella were isolated from male mice on postnatal days 1, 10, 20, and 60. 8-μm-thick sagittal sections were collected on polylysine-coated slides and stored at −80°C. For immunocytochemistry, sections were washed in PBS, exposed to normal goat serum (1:20), followed by anti-TSP IgG (1:20; O'Shea and Dixit, 1988) for 2 h at room temperature. Sections were rinsed, exposed to FITC-conjugated goat anti-rabbit IgG (1:50) for 30 min at room temperature, rinsed extensively, and then coverslipped with glycerol containing 0.1% phenylenediamine. For additional controls, the primary antibody was replaced by PBS, preimmune serum, or anti-TSP antibodies to which a 10-fold excess of TSP was added previously (preabsorption controls). The sections were examined and photographed on a photomicroscope (Aristoplan; E. Leitz, Inc., Rockleigh, NJ) using Tri-X Pan film.

Electron Microscope Level. Cerebella were rapidly dissected from postnatal day 10 male mice and fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer for 30 min at room temperature. They were then rinsed in buffer, surrounded with agar (13%), and sectioned on a vibratome at 50 μm. Slices were washed in buffer, preincubated in 5% normal goat serum with 2% BSA for 2 h at room temperature, and then rinsed in PBS with 1% BSA. Slices were then incubated in the primary antibody for 15 min at room temperature followed by overnight incubation at 4°C. Sections were rinsed, exposed to peroxidase-conjugated goat anti-rabbit Fab fragments for 2 h at room temperature, or to biotinylated goat anti-rabbit IgG (1 h, room temperature) followed by an avidin-biotinylated horseradish peroxidase complex (1 h, room temperature; Vectastain ABC; Vector Laboratories, Burlingame, CA). Both groups were rinsed, fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h, and rinsed in cacodylate buffer. Slices were then incubated in 0.1% diaminobenzadine with 0.01% hydrogen peroxide for 5 min. After an additional rinse, slices were stained in 1% osmium tetroxide and 1% potassium ferrocyanide for 45 min at 4°C. They were rinsed, dehydrated through alcohols, followed by propylene oxide, and embedded in Araldite resin. Thin sections were cut and viewed unstained in an electron microscope (model 400; Philips Electronic Instruments, Mahwah, NJ). In addition to exposure to anti-TSP antibodies (1:10), other sections were exposed to antibodies to the glial fibrillary acidic protein (1:50; Dakopatts, Santa Barbara, CA), or to PBS alone.

Explant Cultures

Male day 10 neonatal mice were decapitated, cerebella were isolated, and lateral portions of forming lobule VI (just caudal to the primary fissure) were dissected from remaining cerebellar tissue. Sagittal slices (0.5-0.75 mm) were cut using an irridectomy knife and transferred to polylysine-treated tissue culture filter inserts (Millicell-CM; Millipore Continental Water Systems, Bedford, MA). Polylysine treatment was carried out solely to augment flattening of the explant and had no effect on cell migration in these experiments. Explants were allowed to adhere briefly (<5 min); then inserts were transferred to wells of 24-well plates (Costar Data Packaging Corp., Cambridge, MA) containing two filter disks cut from type AP25 filter (Millipore Continental Water Systems) and saturated with 1 ml defined medium (N2; Bottenstein and Sato, 1979) containing 1 μCi/ml tritiated thymidine (ICN Biomedicals, Costa Mesa, CA). After a 1-h labeling period, filter inserts containing explants were transferred to similar wells containing media in which tritiated thymidine was replaced with cold thymidine (100 μM) to prevent reutilization. Additional wells contained anti-TSP IgG (50 or 100 μg/ml), or antibodies to fibronectin (50 μg/ml; Collaborative Research, Bedford, MA). Plates were placed in an incubator maintained at 37°C, 5% carbon dioxide. Medium was changed at 36 h of the 72-h culture period.

The antibodies used in the current investigation have been characterized previously, and are specific for TSP as determined by metabolic labeling, immunoprecipitation, and immunoblotting of protein extract from day 13 mouse embryos (O'Shea and Dixit, 1988).

Autoradiography and Quantification

For autoradiographic localization of thymidine-labeled cells, filter inserts were transferred to vials containing 1% glutaraldehyde in 0.1 M phosphate buffer and tissue was fixed for 30 min at room temperature. Fixative was replaced with phosphate buffer and inserts were stored at 4°C. After a graded alcohol dehydration, the explants were removed from the inserts and embedded in Araldite. 1-μm sections were cut, collected on glass slides, dipped in NTB2 emulsion (diluted 1:1; Eastman Kodak Co., Rochester, NY), and exposed for periods of 30 d at 4°C. Slides were developed in D-19 developer, fixed, stained with toluidine blue, and coverslipped; camera lucida drawings of cerebellar cortex were constructed at 25× magnification from at least eight explants per treatment group. A cell was counted as labeled if it contained at least six silver grains. Distance of each labeled cell from the pial surface was then determined using a digitizing tablet interfaced with a microcomputer. As sample sizes were considerably greater than 30, mean distances were evaluated using $\bar{z}$ scores (one-tailed test; Guilford and Fruchter, 1973).

Results

Immunocytochemistry

Prenatal Development (Days 11-19 of Gestation). From approximately days 9-11, the cerebellar anlagen is a pseudostratified epithelium located in the anterior portion of the roof of the fourth ventricle. Over the next several days, the thickness of the cerebellar plate gradually increases; as early as the 13th day three layers are present. Purkinje and Golgi type II cells are generated from the ependymal layer on days 13-15, they migrate radially to their adult locations in the cerebellar cortex, forming uniform layers by the 17th day. Granule cells are generated at the lateral margin of the fourth ventricle (rhombic lip) beginning on the 15th day, and migrate rostrally just below the pial surface to cover the cortical surface by day 19 (Miale and Sidman, 1961; Altman and Bayer, 1978). Fig. 1 illustrates the topographical changes of the cerebellum during its prenatal development.

As early as the 11th day of gestation, TSP was densely deposited around neuroepithelial cells and in the neuroepithelial basement membrane. By day 13, the deposition of TSP was beginning to become restricted. There was less TSP in the ependymal cell layer lining the future fourth ventricle. TSP was densely deposited throughout the middle portion (transitory zone) of the cerebellar plate at this stage, however (Fig. 2 *a*). By day 15, little staining was seen in the primitive neuroepithelium of the ependymal region. As neurons migrated radially from the lumen to their cortical positions, increased deposition of TSP was present in the mixed zone of deep neurons and migrating Purkinje cells. There was little TSP associated with the forming rhombic lip region, although considerable TSP was present in the developing choroid plexus (Fig. 2 *b*).

On the 17th day of development, staining was localized to the region of Purkinje and Golgi type II cells whose migration was ceasing. TSP was seen near the rhombic lip, where

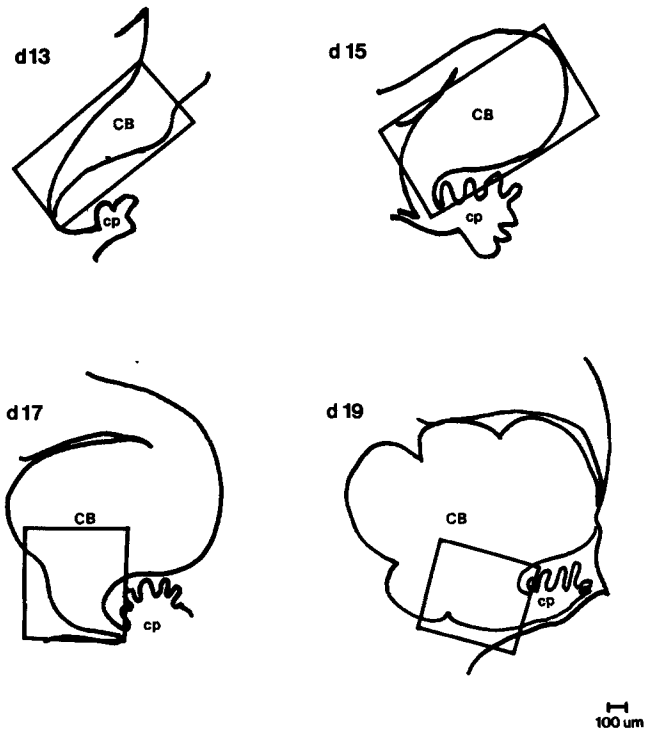


Figure 1. Camera lucida drawings of transverse sections through the developing cerebellum illustrate its prenatal development. As in the accompanying immunofluorescence micrographs (Fig. 2), the pial surface is located to the immediate left of the diagrams, rostral is toward the top of the figures. Section orientation in Fig. 2 is indicated by outlines. *CB*, cerebellar rudiment; *cp*, choroid plexus.

granule cells were migrating (Fig. 2 *c*). By day 19, granule cell migration had extended throughout the superficial cerebellar cortex. TSP was densely deposited at the interface of Purkinje cell bodies and granule cells; the region of deep nuclei neurons was also densely stained (Fig. 2 *d*) as was forming white matter. Little TSP was associated with the granule cells next to the pial surface.

Postnatal Development. During postnatal development, external granule cells divide rapidly near the pial surface; postmitotic granule cells move deep in this layer, then migrate through the molecular layer where their axons fasciculate with axons of earlier migrating granule cells to form the parallel fibers of the molecular layer. These axons synapse with inhibitory basket and stellate cells, and then with dendrites of the forming Purkinje cell dendritic tree; the granule cell bodies then migrate past Purkinje cell bodies to form the (internal) granule cell layer. Basket and stellate cells are also derived from the external granule cell layer, but are few in number compared with the numerous granule cells. The external granule cell layer attains its maximum thickness of 4–10 cells during the first postnatal week and is gradually reduced in thickness, disappearing during the third week (Miale and Sidman, 1961; Altman, 1972).

During this period, TSP was densely deposited within the cerebellar cortex during the first ~20 postnatal days, the period of division and migration of granule cells, after which it was rapidly lost from the cortex. On postnatal day 1, the situation was very similar to that seen on the 19th day of gestation. TSP was present surrounding granule cells, and was particularly densely deposited at the interface of granule cells and Purkinje cells (Fig. 2 *e*). On the 10th day, during the active migration of external granule cells, TSP was present in the pia, the external granule cell layer, the forming molecular layer, and the forming white matter.

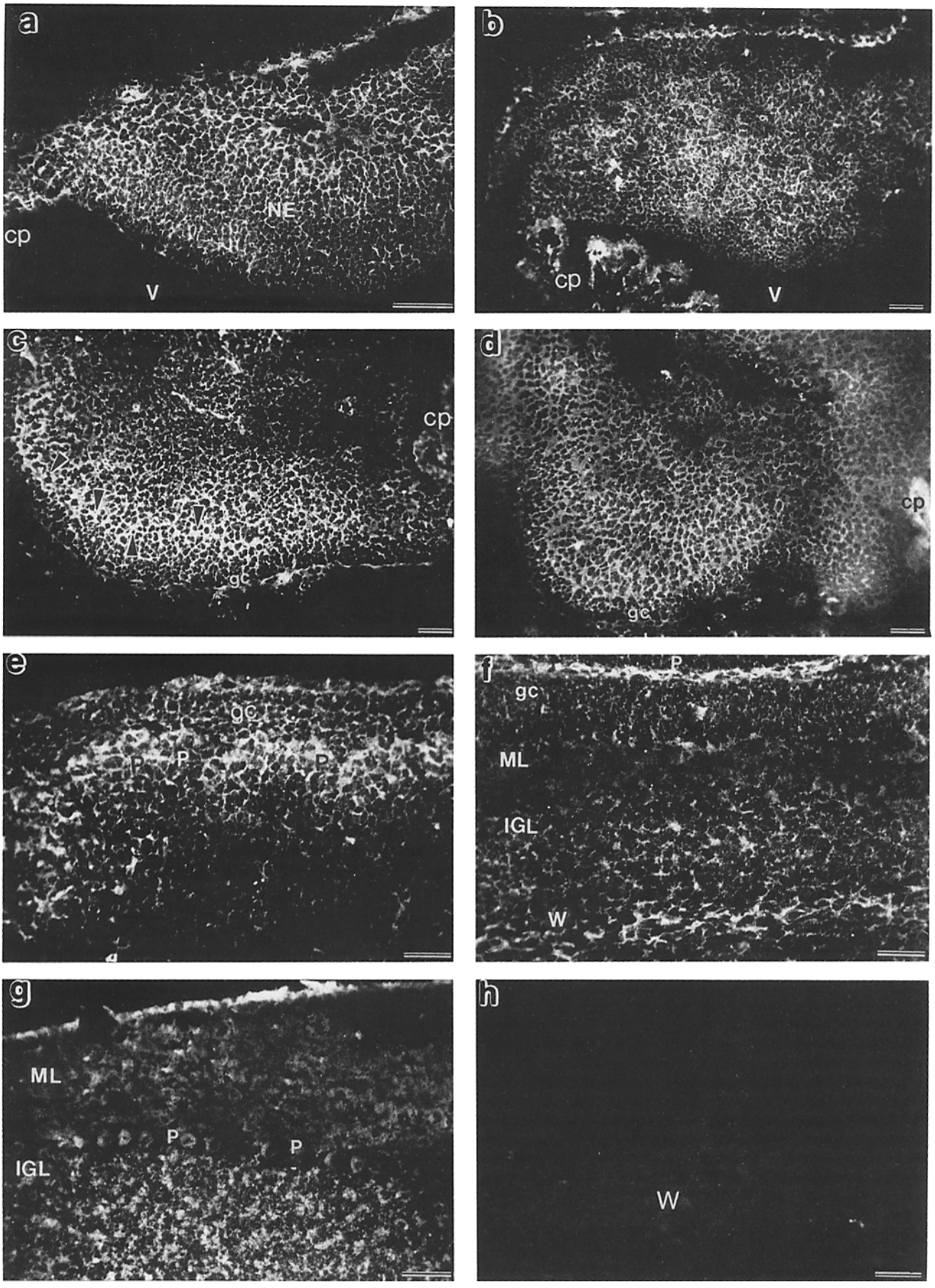
In the external granule cell layer, dividing cells near the pial surface contained less TSP, while stacks of granule cells about to begin migration were intensely stained, particularly at their leading (deep) surface. TSP was deposited in the forming molecular layer and in association with granule cells present deep to the Purkinje cells (internal) granule cells. There was slight autofluorescence of Purkinje cells at this stage. EM analysis confirmed the absence of TSP staining from the Purkinje cells. Cells deep to the Purkinje cell layer with the morphological appearance of Golgi epithelial cells were also stained. Astrocytes present in the white matter were densely stained, as were blood vessels and the pial surface (Fig. 2 *f*).

By postnatal day 20, staining was restricted to the parallel fibers of the molecular layer and astrocytes present in the white matter. Endothelia and pial surface were also densely stained (Fig. 2 *g*).

On the 60th postnatal day all staining from the cerebellar cortex was gone; some residual, very slight immunoreactivity was present in the white matter. In addition, endothelial cell basal lamina and the pial surface remained very slightly stained (Fig. 2 *h*).

EM Level. To correlate with studies of explant cultures, and also because all phases of postnatal granule cell development are present concurrently, EM immunocytochemistry focused on 10th postnatal day cerebella, although adult cerebella have also been examined. As at the light level, PBS controls showed no reaction product (Fig. 3 *a*), while incubation in the presence of anti-glial fibrillary acidic protein antibodies produced linear patterns of reaction product restricted to glial fibers. Staining contrast was considerably improved using the Avidin-Biotin method compared with peroxidase-labeled secondary antibody, and was therefore used to illustrate these results. In contrast, TSP was densely deposited throughout the developing cerebellar cortex, with staining present intracellularly as well as on cell surfaces. There was slight staining of TSP on the surface and within the cytoplasm of granule cells near the pial surface; as granule cells became postmitotic, approached, and entered the forming molecular layer, TSP was present in lateral and lower surfaces (Fig. 3 *b*). There was considerable TSP within and on the surface of fasciculating axons in the molecular layer, although the Purkinje dendritic tree was negative, as were the Purkinje cell bodies (Fig. 3 *c*).

Granule cells preparing to emigrate from the external granule cell layer (Fig. 3 *d*) as well as those moving through the lower portion of the molecular layer (Fig. 3 *e*) exhibited a striking enrichment of TSP associated with the granule cell leading process. Associated Bergmann fibers did not exhibit any staining except at sites of contact with granule cells (Fig. 3 *f*) where TSP was present on the granule cell surface. Their endfeet were similarly negative for TSP, although the perikaryl cytoplasm contained some reaction product. TSP was also present in regions of contact between granule cells and remained on the surface and within the granule cell cytoplasm as they reached their adult locations in the (internal) granule cell layer. Although granule cells and their axons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within the molecular layer contained considerable TSP reaction product, stellate cells and their forming axons were negative for TSP. TSP was also deposited in endothelia throughout the cortex, in the pia arachnoid, as well as in association with astrocytes present in the white matter.

Explant Culture Migration Assay

When the distance each labeled granule cell had migrated from the pial surface was measured, granule cells in cultures grown without antibodies migrated an average of 54.0 ± 5.8 μm, 88.5% of the distance from the pial surface. Those grown in the presence of anti-fibronectin antibodies had migrated a mean distance of 55.4 ± 6.1 μm, 90.8% of the distance to the pial surface. As these data are not statistically different ($\bar{z} = 0.4$, $P = 0.35$), they have been combined for clarity in Fig. 4. Granule cells from these preparations migrated an average of 54.4 ± 4.3 μm, or 88% of the distance from the pial surface to the Purkinje cell layer (Fig. 4). In sections of this material, the normal histotypic arrangement of thinning external granule cell, molecular, and Purkinje and forming (internal) granule cell layers was observed. The molecular layer in particular, increased in thickness at the expense of the external granule cell layer during the 72-h culture period (Fig. 5). The pial surface remained smooth and unbroken; migrating granule cells were slightly elongate in shape and were tightly applied to Bergmann glia processes.

Labeled granule cells in cultures grown in the presence of 50 or 100 μg/ml anti-TSP antibodies had migrated significantly less, 28.0 ± 2.6 μm and 12.8 ± 4.5 μm, respectively, 45.2% and 36.8% of the distance from the pial surface to the Purkinje cell layer (Fig. 4). This inhibition of cellular migration resulted in abnormalities of cortical architecture. The external granule cell layer remained thickened with resulting thin molecular and (internal) granule cell layers (Fig. 5). Granule cells were also found outside the pial surface in a number of cases. In the premigratory zone near the forming molecular layer, granule cell bodies were clumped and occasional cells were pyknotic. In addition, the leading edge of granule cells present in the molecular layer was often directed away from the underlying Bergmann glial fiber. Potential dendritic alterations or ultrastructural abnormalities were not assessed in the current investigation.

Discussion

Both the polarized deposition of TSP and the inhibition of granule cell migration produced by anti-TSP antibodies observed in the current investigation are consistent with a role for this important ECM protein in cellular migrations. In fact, there is considerable evidence from other systems that TSP plays a role in cell movement: of melanoma cells (Taraboletti et al., 1987), smooth muscle cells (Majack et al., 1986), as well as squamous carcinoma cells (Varani et al., 1986). This is, however, the first report of a correlation between the deposition of TSP and cell migrations in situ.

The molecular mechanisms by which TSP affects cellular behavior have not been elucidated, and are likely to be multiple. TSP is deposited in cell attachment sites and in "tracks" formed by migrating cells (Vischer et al., 1988), suggesting that TSP may play a role in the sequential adhesions and deadhesions involved in cellular migrations. Increased deposition of TSP immediately preceding and during migration may reflect its role in destabilizing cell–cell and cell–matrix contacts before migration, as in cultured bovine aortic endothelial cells (Murphy-Ullrich and Hook, 1989). Loss of TSP from the cortex after the period of granule cell migration may therefore indicate stabilization of cell–cell contacts and establishment of the adult histotypic pattern.

Although the highly localized deposition of TSP in granule cell leading processes may be involved in recognition, adhesion, or guidance of the neuron along the Bergmann fiber, it seems more likely that this restricted deposition reflects a focus of protease activity required for penetration and movement through a cell- and matrix-filled environment. TSP has a high affinity for both plasminogen and tissue plasminogen activator, and binding of TSP to these ligands greatly enhances plasminogen activation by tissue plasminogen activator (Silverstein et al., 1986). Production of tissue plasminogen activator by cerebellar granule cells (Krystosek and Seeds, 1981; Verrall and Seeds, 1988, 1989) and neural crest cells (Valinsky and LeDouarin, 1985; Menoud et al., 1989) appears to play a critical role in migration per se, as movement of both neural crest (Erickson, 1988) and granule cells (Moonen et al., 1982) was arrested by inhibitors of the tissue plasminogen activator - plasminogen - plasmin system. Interestingly, the pattern of cell migration inhibition observed

Figure 2. Sagittal sections illustrating the pattern of immunofluorescence localization of TSP during prenatal (*a–d*) and postnatal (*e–h*) histogenesis of the cerebellar cortex. The pial surface is oriented toward the left in *a–d* and toward the top of *e–h*. Orientation of sections in *a–d* is indicated in Fig. 1. (*a*) Sagittal section through the cerebellar plate on day 13 of gestation. TSP is uniformly distributed throughout the stratifying neuroepithelium (*NE*). *cp*, the position of the nascent choroid plexus; *V*, forming fourth ventricle. (*b*) Sagittal section on day 15 of gestation illustrating the dense deposition of TSP in the transitory zone (mantle layer), and in the choroid plexus. There is little TSP associated with dividing cells near the fourth ventricle. (*c*) Cerebellum from an embryo on day 17 of gestation cerebellum illustrating the dense deposition of TSP in the transitory zone (*arrows*) particularly associated with radially migrating neurons attaining their final positions in the cortex. Note the considerable rotation of the cerebellum compared with the embryo at day 15 of gestation above. *gc*, Forming granule cell layer. (*d*) Section near the rhombic lip area from an embryo on day 19 of gestation illustrating the very dense deposition of TSP in this region surrounding and at the base of stratifying granule cells. Deep nuclear neurons are also stained. (*e*) Section through the cerebellar cortex on postnatal day 1 illustrating the dense deposition of thrombospondin between granule cells, and particularly at their base where they border Purkinje cells. *P*, Purkinje cell zone. (*f*) Section from postnatal day 10 cortex. TSP was present in pia (*p*), between granule cells, in the forming molecular layer (*ML*) and internal granule cell layers (*IGL*), as well as in association with astrocytes in the deeper white matter (*W*). (*g*) Postnatal day 20 cortex illustrating pial staining, and residual staining of the molecular layer and internal granule cell layers. Purkinje cell bodies (*p*) exhibit a slight autofluorescence. (*h*) Postnatal day 60 cortex illustrating the lack of staining except in association with astrocytes present in the white matter. Bars, 50 μm.

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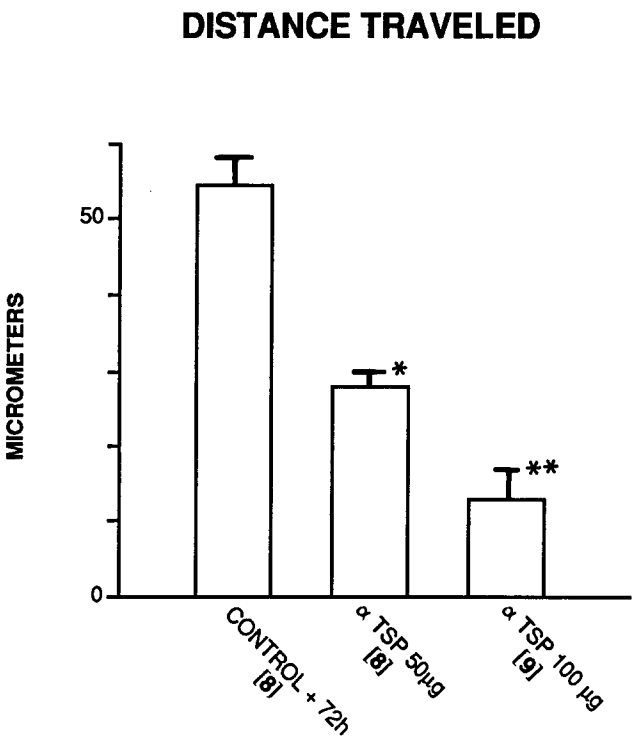

Figure 4. Histogram illustrating the mean distance labeled granule cells migrated from the pial surface. Number of replicate experiments carried out are indicated in brackets. Number of cells counted were (control) 442; (50 μg/ml anti-TSP) 349; and (100 μg/ml anti-TSP) 526. (*) Control > anti-TSP 50 μg/ml ($\bar{z}$ = 10.03, $P < 0.001$). (**) Control > anti-TSP 100 μg/ml ($\bar{z}$ = 14.01, $P < 0.001$).

in that study (Moonen et al., 1982) was very similar to that seen in the current investigation; failure of granule cells to enter the molecular layer, rather than a piling up of granule cells within the molecular layer.

Interestingly, TSP was present on the surfaces of granule cell axons, with little TSP on Purkinje cell dendrites or Bergmann glial fibers. After the period of granule cell migration, TSP was largely lost from the cortex except from fasciculating axons of the parallel fibers. After fasciculation was completed, TSP was similarly lost from the molecular layer. The temporal and spatial correlation of TSP deposition with initial axon outgrowth (O'Shea and Dixit, 1988), and its presence on axons of parallel fibers during fasciculation, are consistent with a role for TSP in the fasciculation process. Similarly, the absence of TSP from axons of stellate cells which pass perpendicular to those of the parallel fibers, may facilitate their extension through this compact layer (see also Persohn and Schachner, 1987). Additional studies must be carried out to examine this possibility.

It has been assumed, based on histochemical studies as well as various estimates of the extracellular space and water content of the cerebellum (Margolis et al., 1975), that there is considerable ECM in the cerebellar cortex during the period of active granule cell migration. Since that time, a number of specific ECM components have been identified in the region. Among them, the distribution of hyaluronic acid (Ripellino et al., 1988), chondroitin sulfate proteoglycan (Aquino et al., 1984), the hyaluronic acid–binding region, and link protein epitopes (Ripellino et al., 1989) share a number of similarities with TSP. All were developmentally regulated, with major alterations in depositional pattern occurring with cell migration. Like TSP, little hyaluronic acid and chondroitin sulfate proteoglycan was associated with granule cells until migration, when they became enriched at the base of the external granule cell layer. All were associated with Golgi epithelial cells but not their Bergmann glial fibers, and were absent from Purkinje cells and dendrites. Like TSP, their depositional pattern was greatly altered with cessation of migration, in this case moving from an extracellular to intracellular position. They did not show the deposition associated with the leading process of granule cells, suggesting that, as in other developing systems, these matrix proteins may play a role in producing and maintaining extracellular space required for (granule cell) migration.

The distribution of the ECM protein, cytotactin, has been extensively examined in the chick embryo during all phases of granule cell migration (Chuong et al., 1987). It was associated with premigratory granule cells, and found later in the molecular layer particularly on the surface of Bergmann glial fibers. Unlike TSP, antibodies to cytotactin arrested granule cell migration in the molecular layer, presumably by altering interactions with the Bergmann fibers.

Cell adhesion molecules, N-CAM, L1, Ng-CAM, which, like cytotactin, share a carbohydrate epitope containing a sulfated glucuronic acid residue, have been extensively studied in the developing cerebellar cortex. Briefly, during layering of the cortex, N-CAM was deposited in all layers during all developmental periods (Chuong et al., 1987; Persohn and Schachner, 1987); antibodies to N-CAM had little effect on the pattern of development of the cortex (Chuong et al., 1987). Ng-CAM was found on premigratory granule cells and in the molecular layer (Chuong et al., 1987), while L1 was present, like TSP, on premigratory granule cells, and

Figure 3. EM immunocytochemical localization of TSP in sagittal sections of day 10 cerebellar cortex. Pial surface is oriented to the top of each micrograph. (*a*) Section through the cerebellar cortex during active granule cell migration, in which the primary antibody was replaced by PBS. Nuclei belong to granule cells in various stages of division and migration. Arrowheads indicate paths of Bergmann glial fibers. (*b*) Similar section illustrating the gradient of TSP in the cortex. All nuclei belong to granule cells. TSP was largely absent from dividing cells (*M*); and was enriched in granule cells leaving the external granule cell layer, particularly at their leading edge (*arrows*). Bergmann glial fibers (*arrowheads*) have no reaction product. *ml*, forming molecular layer. (*c*) Higher magnification view of a portion of a Purkinje cell (*P*) and its dendrite (*D*) which are negative for TSP. Reaction product is densely deposited within axons (*arrowheads*) present in the forming molecular layer. (*d*) Section taken from a region near the limit of antibody penetration of vibratome slices, illustrating the very dense deposition of TSP in the leading process and on the surface (*arrowhead*) of a granule cell beginning to associate with a Bergmann glial fiber to initiate its migration. (*e*) Enrichment of TSP at the leading edge (*arrowheads*) of a granule cell migrating near the base of the molecular layer. (*f*) Higher magnification view of two granule cells migrating along glial fibers (*arrowheads*). Note the absence of reaction product from the Bergmann glia, despite its abundance in the granule cells. Bars, 1 μm.

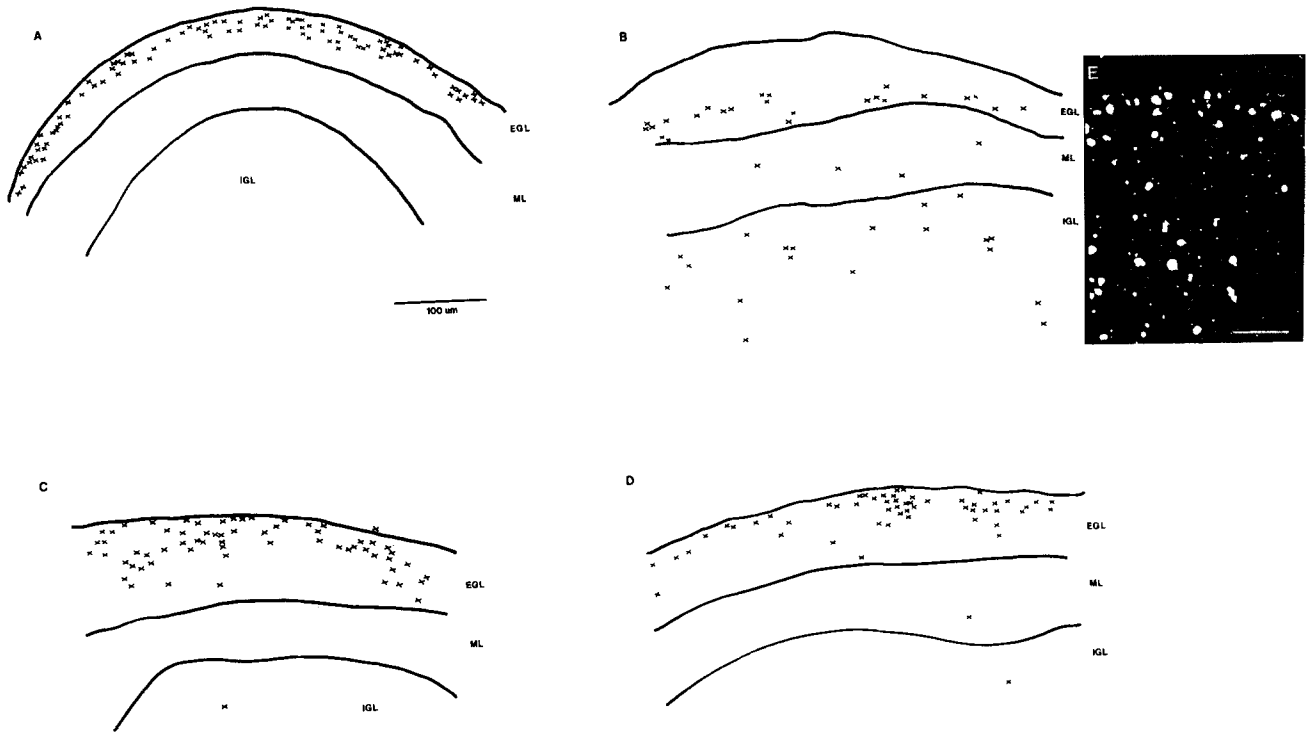

Figure 5. Camera lucida drawings illustrating the topology and positioning of labeled granule cells from representative sections after 1 h in vitro (*A*), 72 h in vitro (*B–D*), under control conditions (*B*), or after exposure to 50 μg/ml anti-TSP antibodies (*C*) or 100 μg/ml anti-TSP antibodies (*D*). (*E*) Dark-field autoradiograph of the cortex of the cerebellum in a control explant (no antibodies) grown for 72 h, illustrating an average preparation. *X*, labeled granule cells; *EGL*, external granule cell layer; *ML*, molecular layer; *IGL*, internal granule cell layer; and *P*, pial surface.

was absent from mitotic cells, basket and stellate cells, as well as from Bergmann glia. It was present on axons of parallel fibers, but not on dendrites or Purkinje cell bodies. Like TSP, L1 was largely lost after granule cell migration was complete. Similarly, there appeared to be a preferential localization of L1 to the leading process of migrating neurons (Persohn and Schachner, 1987). Antibodies to L1 inhibited granule cell migration largely in the external granule cell layer (Lindner et al., 1983), possibly by inhibiting clustering of granule cells before migration. Anti-Ng-CAM antibodies, like TSP, arrested granule cells at the interface of external granule cells with the molecular layer, and had little effect on granule cell migration within the molecular layer (Chuong et al., 1987).

Despite the extensive migrations and considerable remodeling of the cerebellum during its prenatal development, few studies have examined matrix deposition during these early stages. Hatten et al. (1982) reported that fibronectin was deposited near the rhombic lip just preceding the initial wave of granule cell emigration from the neuroepithelial layer, and that it disappeared as granule cells spread over the cortex and became mitotic. During migration of granule cells from the rhombic lip of the chick embryo, both N-CAM and cytotactin were present in the forming external granule cell layer; as mitotic and premigratory granule cells segregated, cytotactin was restricted to the premigratory group (Chuong et al., 1987). In the current investigation, TSP was associated with migrating cells, with the radial movement of neurons, and emigration and migration of granule cells over the cortical surface to form the external granule cell layer.

It appears that TSP may be an important component of the complex of cell surface and ECM components that play a role in producing the ordering of cells and their processes in CNS histogenesis. Its precise role in specifying these cellular behaviors remains to be determined.

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