The Tissue Plasminogen Activator (tPA)/Plasmin Extracellular Proteolytic System Regulates Seizure-induced Hippocampal Mossy Fiber Outgrowth through a Proteoglycan Substrate

Yan Ping Wu,* Chia-Jen Siao,** Weiquan Lu,*‡ Tsung-Chang Sung,† Michael A. Frohman,‡ Peter Milev,‡ Thomas H. Bugge,‡ Jay L. Degen,§ Joel M. Levine,¶ Richard U. Margolis,¶ and Stella E. Tsirka*‡

* Department of Psychiatry, ‡ Department of Pharmacological Sciences, and § Department of Neurobiology and Behavior, University Medical Center at Stony Brook, Stony Brook, New York 11794-8651; † Division of Developmental Biology, Children's Hospital Research Foundation, Cincinnati, Ohio 45229; and ¶ Department of Pharmacology, New York University Medical Center, New York, New York 10016

Abstract. Short seizure episodes are associated with remodeling of neuronal connections. One region where such reorganization occurs is the hippocampus, and in particular, the mossy fiber pathway. Using genetic and pharmacological approaches, we show here a critical role in vivo for tissue plasminogen activator (tPA), an extracellular protease that converts plasminogen to plasmin, to induce mossy fiber sprouting. We identify DSD-1-PG/phosphacan, an extracellular matrix component associated with neurite reorganization, as a physiological target of plasmin. Mice lacking tPA displayed decreased mossy fiber outgrowth and an aberrant band at the border of the supragranular region of the dentate gyrus that coincides with the deposition of unprocessed DSD-1-PG/phosphacan and excessive Timm-positive, mossy fiber termini. Plasminogen-deficient mice also exhibit the laminar band and DSD-1-PG/phosphacan deposition, but mossy fiber outgrowth through the supragranular region is normal. These results demonstrate that tPA functions acutely, both through and independently of plasmin, to mediate mossy fiber reorganization.

Key words: neurite • dentate • nonproteolytic • kainic acid • seizure

Introduction

Organization of neuronal pathways occurs during late prenatal and early postnatal development, and reorganization takes place continuously as neurons undergo stimulation. Short seizure episodes induce the development of new synapses and the reorganization of existing synapses along the mossy fiber pathway in the hippocampus. Such induced sprouting of mossy fibers (axons of dentate granule cells) can result in the formation of aberrant structures in the CA 3 infrapyramidal layer and the dentate supragranular layer (Ben-Ari and Represa, 1990). Synaptic organization and reorganization require remodeling of extracellular matrix (ECM) components through the action of extracellular proteases (Werb, 1997). Previously, we described a role for the extracellular proteases, tissue plasminogen activator (tPA) and plasminogen in mediating neuronal cell death after excitotoxic injury to the hippocampus (Tsirka et al., 1995, 1996, 1997). tPA is made by neurons and microglia, whereas plasminogen is expressed by neurons (Tsirka et al., 1997). In addition to its role after excitotoxic events, tPA expression is upregulated in the cerebellum during normal neuronal stimulation and changes in plasticity (Seeds et al., 1995). Furthermore, in hippocampal slices from tPA-deficient (tPA−/−) mice (Carmeliet et al., 1994), or wild-type mice in which tPA activity is inhibited, deficits in the late phase of long-term potentiation (LTP) are observed (Frey et al., 1996; Huang et al., 1996; Baranes et al., 1998). The mechanism(s) through which tPA mediates these changes is not understood, although the prevalent hypot...
esis has been that generation of plasmin by tPA leads to the degradation of ECM and cell surface components (Carroll et al., 1994; Werb, 1997). Supporting this model, plasmin-mediated degradation of laminin has been shown to be an early step in the pathological neurodegeneration process triggered by excitotoxins (Chen and Strickland, 1997).

tPA protein and activity in the mouse hippocampus localize along the CA 3 pyramidal layer and the dentate gyrus (D G; Sappino et al., 1993). This expression pattern coincides with the mossy fiber pathway and the region in which sprouting and reorganization of mossy fibers occur (Ben-Ari and Represa, 1990; A maral and Witter, 1995). There are several genes known to be involved in the formation and structural reorganization of mossy fibers. Neuronal cell adhesion molecule (NCA M), a membrane glycoprotein expressed by astrocytes (Jucker et al., 1995), mediates adhesion between neuronal elements, induces neurite outgrowth (C remer et al., 1997), and may mediate the interaction of mossy fibers with other fibers and glial cells (Niquet et al., 1993). A mon NCA M's ligands are chondroitin sulfate proteoglycans. These proteoglycans are present in the brain E CM and are involved in cell-cell and cell-substrate interactions (Levine and Nishiyama, 1996). Two of these ligands are neurocan and D SD-1-PG/phosphacan. Neurocan is synthesized by neurons at high levels during late embryonic development (M argolis and M argolis, 1997). D SD-1-PG/phosphacan is expressed primarily by astrocytes, but only at low levels prenatally. The levels increase steadily in late embryogenesis, reach a plateau two weeks postnatally, and then remain stable (M argolis and M argolis, 1997). D SD-1-PG/phosphacan is the isolated, soluble extracellular domain of a receptor-type transmembrane protein tyrosine phosphatase (RP TP C/β). R PTP C/β induces cell adhesion and promotes neurite growth of primary tectal neurons (Peles et al., 1995; Saku rai et al., 1997), as well as neuronal migration (Maeda and N oda, 1998). D SD-1-PG/phosphacan and R PTP C/β both bind to NCA M and tenascin-C and R. A coagrinably, D SD-1-PG/phosphacan has been proposed to oppose R PTP C/β by competing for its binding sites. D SD-1-PG/phosphacan can affect neuronal adhesion and neurite outgrowth (M argolis and M argolis, 1997), although the precise effect may differ for hippocampal and spinal cord neurons (Garwood et al., 1999).

In this report, we demonstrate two roles for tPA in controlling mossy fiber sprouting, one of which involves regulated D SD-1-PG/phosphacan proteolysis.

Materials and Methods

Animals

We used wild-type C57B16, I PA/+, and placoginin-deficient (pg−/−) mice and their heterozygote littermates. The mice were genotyped as described previously (Bugge et al., 1996; Mcenas et al., 1997). The adult male mice, age-matched and more than 25 g, were injected intraperitoneally with atropine (0.6 mg/kg of body weight) and then deeply anesthetized with 2.5% avertin (0.02 mL/kg of body weight). They were placed in a stereotaxic apparatus, and injected unilaterally with 1.0 nmol of kainic acid (KA) in 2.5% avertin (0.02 mL/g of body weight). They were placed in a stereotaxic apparatus, and injected unilaterally with 1.0 nmol of kainic acid (KA) in 2.5% avertin (0.02 mL/g of body weight). The injection were: bregma, 2.16 mm; medial-lateral, 3.3 mm; and dorsoventral, 1.6 mm. The injection was directed at a concentration of 0.12 mg/mL in PBS, approximately in the midline at the level of the hippocampus (stereotaxic coordinates: bregma, −2.5 mm; medial-lateral, 0.5 mm; and dorsoventral, 1.6 mm) using an 1 Lza micro-osmotic pump (Model no. 2004) with a pumping rate of 0.25 μL/h. After the infusion, cannula was positioned and secured, and the mice were injected unilaterally with kainate into the amygdala at the coordinates cited above. 12 d after the infusion/injection, the mice were killed and their brains processed as above.

Histochemical Staining

Control and KA-treated animals were perfused with 0.2% sodium sulfide and then with 4% paraformaldehyde. Mousy fibers were stained according to a modified Timm silver staining method (Holm and Geneser, 1991).

In vitro processing of purified 125I-neurocan and DSD-1-PG/phosphacan by tPA and plasmin purified 125I-neurocan (−2.5 ng/mL, 0.06 μCi/μL), and 125I-DSD-1-PG/phosphacan (−2.5 ng/mL, 0.09 μCi/μL), was pre-treated with protease-free chondroitinase ABC (Rettierz et al., 1996). 0.234 μCi of each proteoglycan was then incubated in 100 mM Tris-HCl, pH 7.5, 0.01% Triton X-100 in the absence or presence of various concentrations (100 ng/ml) of recombinant mouse tPA or human plasmin for 30 min at 37°C. To test for specificity of the proteolytic reactions, PA1-1 (a tPA inhibitor) or α2-antiplasmin (a plasmin inhibitor) were included in the reaction. At the end of the incubation period, SDS sample buffer was added and the samples were analyzed by 8% (for neurocan) and 7% (for DSD-1-PG/phosphacan) SDS-PAGE. The gels were then processed for autoradiography.

Immunoblotting

Cerebral tissue at the level of the hippocampal formation (~100 mg wet weight) was prepared for SDS-PAGE as described (Oohira et al., 1994). In brief, the tissue was homogenized in PBS containing protease inhibitors. A 1 aliquot was centrifuged at 6,000 g for 20 min. The supernate was mixed with 50 mM Tris-HCl, pH 7.5, containing protease inhibitors, and was boiled for 5 min. 3 vol of 95% ethanol containing 1.3% potassium acetate was added to precipitate the proteoglycans. The pellet was washed with 70% ethanol/1% potassium acetate, and was then subjected to digestion with protease-free chondroitinase ABC at 37°C for 60 min before performing Western blot analysis. The amounts of protein loaded were controlled, both before loading (Bradford protein assay), as well as through assessment using postblot staining with Pamseal S.

Scoring Method for Timm Histochemistry

The extent of mossy fiber sprouting was analyzed independently by three examiners who were unaware of the genotype of each brain section, using a previously published, standardized scoring procedure (Cavazos et al., 1991). This method gives a scoring scale from 0 to 5 of the distribution of Timm granules in the supragranular region of the DG. This scoring method assesses only the presence or absence of granules in the DG supragranular region or in the inner molecular layer in a wild-type animal. It does not, however, evaluate the morphology of the extending neurites.

Immunohistochemistry

Brain sections or hippocampal neurons of the mice, manipulated as described above, were incubated with antisera against neurocan, DSD-1-PG/phosphacan (polyclonal provided by Dr. M argolis, and monoclonal purchased from Developmental Studies Hybридoma Bank, University of Iowa, PSA-NCA M (a gift of Dr. T. Seki, Department of Anatomy, J untendo University, J apan), or dynorphin A (Peninsula Laboratories, Inc.), at the dilutions recommended by the suppliers. Biotinylated or rhodamine-conjugated (for dynorphin A) secondary antibodies were used (Vector Laboratories).
Substrate Preparation
To evaluate whether DSD-1-PG/phosphacan is inhibitory or repellent to elongating mossy fiber neurites, we used a previously described culture method (Dou and Levine, 1994). In brief, 60-mm petri dishes were coated with 750 μl nitrocellulose dissolved in methanol (5 cm² nitrocellulose in 12 ml of methanol) and air-dried in a tissue culture hood. To create a boundary between DSD-1-PG/phosphacan (proteolyzed or not) and laminin, a 2-μl drop of purified DSD-1-PG/phosphacan (20 μg/ml), or proteolyzed DSD-1-PG/phosphacan, was spotted onto the nitrocellulose; the drop was aspirated off after 10 min and then a larger droplet (8 μl) of laminin (0.7 μg/ml) was spotted over the DSD-1-PG/phosphacan spot, so that a circular border was formed with DSD-1-PG/phosphacan in the inner circle and laminin in the outer. When proteolyzed DSD-1-PG/phosphacan was used, the proteoglycan was incubated with plasmin (100 ng) for 4 h at 37°C. The reaction was terminated with the addition of 100 ng of α2-antiplasmin. The dish was washed with medium before adding the cells. Neurons from four tPA2/2 hippocampi were plated on each 60-mm dish, as described (Rogove and Tsirka, 1998). The cultures were photographed after 5 d of growth. The images of the cultures were captured, and the number of neurites (pyramidal vs. granule) that were in the proximity of the border crossing into the phosphacan substrate was quantitated. This quantitation was done in four individual experiments by two reviewers blinded to the experimental conditions.

Intrahippocampal Delivery of rtPA and S478A tPA and Injection of Kainate in the Amygdala
Adult tPA2/2 male mice were subjected unilaterally into the hippocampus to infusion of either buffer (PBS; n = 4) or 200 μl of wild-type rtPA (n = 4), or S478A tPA (0.12 mg/ml; n = 8, a gift of Genentech, Inc.), as described (Rogove et al., 1999). Then, kainate (1.5 nmol of KA in 300 nl of PBS) was injected unilaterally into the amygdala, as above. The brains were processed and evaluated for mossy fiber sprouting and the presence of tPA protein (by activity and immunohistochemistry; Tsirka et al., 1995). The recombinant catalytically inactive tPA, S478A, has a serine to alanine mutation at the active site of tPA.

Results
tPA2/2 and plg2/2 Mice Display Differing Degrees of Aberrant Mossy Fiber Sprouting after Kainate Stimulation
Since tPA is active as an extracellular protease in the CA3 and DG, we sought to investigate whether it has a role along the mossy fiber pathway (Carroll et al., 1994; Werb, 1997). We used a modification of a previously published protocol for stimulation-induced mossy fiber sprouting in the rat (Niquet et al., 1993). In brief, KA was injected into the amygdala of wild-type and tPA2/2 mice, which were then monitored for development of seizures as evidence of adequate neural stimulation. Only mice that exhibited seizures soon after recovery from anesthesia were used subsequently to assess the pattern of sprouting using Timm staining. Marked differences were obvious in two respects between the injected and uninjected sides of the wild-type and tPA2/2 mice (Fig. 1). First, extensive stimulation-dependent sprouting was evident in wild-type mice across the width of the supragranular region of the DG (Timm score 2.17 ± 0.39, see Table I), whereas only limited sprouting occurred in tPA2/2 mice. The few sprouts observed in tPA2/2 mice were not as well-defined or dense as the wild-type sprouts. This phenotype is comparable to the pattern of mossy fiber sprouting observed for mice treated with endo-Neuraminidase, which removes the polysialic acid associated with NCAM (Seki and Rutishauser, 1998), in that after endo-Neuraminidase treatment, the bundles were less compact and the fascicles were much smaller.
Mossy fiber synaptic reorganization was evaluated using a previously established scoring scale, which used the following criteria: 0, no granules between the crest and tip of the DG; 1, sparse granules in the supragranular region in a patchy distribution between the tips and crest of the DG; 2, more numerous granules in the supragranular region in a continuous pattern between tips and crest with occasional patches of confluent granules between the tips and crest of the DG; 3, prominent granules in the supragranular region that form a confluent dense laminar band between tips and crest; 4, confluent dense laminar band of granules in the supragranular region that extends into the inner molecular layer (Cavazos et al., 1991). Table I.

| Genotype                  | Average Timm score* ± SD | Mice used |
|---------------------------|--------------------------|-----------|
| Wild-type                 | 2.17 ± 0.39              | 12        |
| tPA−/−                    | 4.44 ± 0.51              | 16        |
| plg−/−                    | 3.00 ± 0.00              | 6         |
| Wild-type/tPA Stop       | 4.50 ± 0.71              | 6         |

*According to Cavazos et al. (1991).

In addition to the histological Timm stain, we evaluated neurite outgrowth along the mossy fiber pathway using immunohistochemistry for polysialylated NCAM (PSA-NCAM), which has been used extensively as a reliable marker for mossy fiber sprouting (Seki and Rutishauser, 1998). PSA-NCAM is not normally expressed in the adult (Seki and Arai, 1999), but its expression is required for activity-dependent morphological plasticity (Theodosia et al., 1999). As seen in Fig. 2 B, PSA-NCAM is expressed by the compact and dense extending neurites in wild-type DG (Fig. 2 B, wt). In tPA−/− mice, PSA-NCAM+ neurites are stubby and disorganized (Fig. 2 B, tPA−/−, arrows), and the laminar band is observed (Fig. 2 B, tPA−/−, arrowheads), in agreement with the Timm histological stain. plg−/− neurites are dense and compact through the granule cell layer (Fig. 2 B, plg−/−, arrows), but still form the laminar band (Fig. 2 B, plg−/−, arrowheads). Taken together, these results suggest that tPA+, but not plasminogen, promotes mossy fiber outgrowth across the supragranular layer, whereas formation of the aberrant laminar band occurs when plasmin (or tPA+, which activates plg) is absent.

Identification of DSD-1-PG/Phosphacan as a Substrate for Plasmin in the Mossy Fiber Pathway

NCAM promotes mossy fiber sprouting by facilitating neuronal cell interactions (Cremer et al., 1997) and is essential for accurate neurite outgrowth, synaptogenesis, and long-term changes in synaptic strength (Cremer et al., 1998). In addition, it has been reported that tPA+ and plasmin can proteolyze the highly polysialylated NCAM that is associated with mossy fibers (Endo et al., 1998). Therefore, we investigated NCAM as a potential substrate for plasmin in the mossy fiber pathway.
levels of NCAM in wild-type, tPA$^{-/-}$, and plg$^{-/-}$ mice injected with buffer or kainate appeared similar, and no differences in degradation or cleavage of recombinant NCAM were observed after incubation with brain homogenates prepared from similarly treated animals (data not shown). Similarly, aberrant accumulation of DSD-1-PG/phosphacan has been reported before in lesioned rat hippocampi (Deller et al., 1997).

To determine whether the accumulated DSD-1-PG/phosphacan had in fact failed to be processed correctly, we prepared protein extracts from hippocampi of stimulated and control wild-type, tPA$^{-/-}$, and plg$^{-/-}$ mice. The proteoglycans were partially purified (Retzler et al., 1996) and analyzed by gel electrophoresis. Extracts from all three genotypes contained similar basal levels of DSD-1-PG/phosphacan (Fig. 4, uninjected lanes). However, extensive DSD-1-PG/phosphacan proteolytic cleavage, primarily of the 180-kD band (the protein core), was observed in the extracts from stimulated wild-type mice, but not in the extracts from tPA$^{-/-}$ or plg$^{-/-}$ mice (Fig. 4, injected lanes). These data indicate that significant proteolytic processing of DSD-1-PG/phosphacan occurs via the tPA/plasmin system.

Figure 3. In vitro cleavage of neurocan and DSD-1-PG/phosphacan by plasmin, but not tPA. Radiolabeled with $^{125}$I, purified neurocan (~9.75 ng) and DSD-1-PG/phosphacan (~6.5 ng) were incubated with recombinant mouse tPA or human plasmin in the absence or presence of PAI-1 or $\alpha_2$-antiplasmin. The inhibitors were preincubated with the recombinant enzymes for 5 min at 37°C. The samples were analyzed by SDS-PAGE and autoradiography. Lane 1, tPA 1 ng; lane 2, tPA 10 ng; lane 3, tPA 100 ng; lane 4, tPA 100 ng and PAI1 100 ng; lane 5, proteoglycan incubated with buffer alone; lane 6, proteoglycan by itself; lane 7, plasmin 1 ng; lane 8, plasmin 10 ng; lane 9, plasmin 100 ng; lane 10, plasmin 10 ng and $\alpha_2$-antiplasmin 50 ng; lane 11, plasmin 10 ng and $\alpha_2$-antiplasmin 100 ng. The arrows point to bands that disappear after incubation with plasmin.

Figure 4. Proteolytic processing of DSD-1-PG/phosphacan in wild-type mice after KA injection. Partially purified protein extracts from KA-injected (inj) or PBS-injected (uninj) wild-type (wt), tPA$^{-/-}$ and plg$^{-/-}$ hippocampi, as described in Materials and Methods, were analyzed by SDS-PAGE and immunoblotting, using an anti-DSD-1-PG/phosphacan antibody. Note the processing that has occurred in the wild-type injected lane, and the absence of such processing in the corresponding tPA$^{-/-}$ and plg$^{-/-}$ lanes. The arrows point to the 400-kD DSD-1-PG/phosphacan protein band (upper arrow), and the DSD-1-PG/phosphacan protein core of 180 kD (lower arrow), which appears more susceptible to plasmin cleavage. Top, Coomassie-stained gel showing comparable loading among the different lanes; bottom, Western blot of the Coomassie-stained gel (top). This experiment has been repeated five times.
ystem during mossy fiber sprouting in wild-type mice, that endogenous plasmin is produced in enough quantities locally to mediate phosphacan’s cleavage, and suggest that this processing may be physiologically important.

These results link tPA/plasmin activity to the regulation of a cell adhesion molecule known to inhibit neural adhesion and neurite outgrowth (in this case, mossy fiber sprouting) through interaction with NCAM.

Unprocessed DSD-1-PG/Phosphacan Directly Repels Mossy Fiber Outgrowth in Culture

To determine whether DSD-1-PG/phosphacan specifically inhibits mossy fiber sprouting, we plated hippocampal neurons onto nitrocellulose/laminin-coated plates that additionally contained a region in which DSD-1-PG/phosphacan had been applied before the laminin coating (Dou and Levine, 1994). The pattern of neurite outgrowth was evaluated at the border between the laminin and the phosphacan/laminin regions. To differentiate between pyramidal neurites and genuine mossy fiber axons, immunohistochemistry was used (Fig. 5 E) to detect dynorphin A, which is a marker that visualizes mossy fiber axons from DG granule cells, but not those from CA1-CA3 cells (Baranes et al., 1996).

Neurons attached and extended neurites over the laminin-coated region. Pyramidal neurons could extend neurites into the phosphacan/laminin-coated region (98.71 ± 0.24% of all pyramidal neurites crossed the boundary between laminin and phosphacan). However, the phosphacan/laminin coated area was potently inhibitory to the axons of dentate granule cells (Fig. 5; only 7.55 ± 1.78% of granule cell neurites crossed the boundary). In fact, direct avoidance of DSD-1-PG/phosphacan by the outgrowing neurites was readily observed at the boundary (Fig. 5 B, red arrows, and D–F). This avoidance was not due to a border created by the two components, since when the border was generated by two different layers of laminin (Fig. 5 A, blue arrows), neurites could readily cross over. Furthermore, when phosphacan was incubated with plasmin before plating, no neurite outgrowth inhibition was observed (Fig. 5 C).

These results demonstrated that mossy fiber axons avoided regions that contained full-length DSD-1-PG/phosphacan, but otherwise continued to extend, supporting the in vivo data (Figs. 1 and 2).

**tPA Promotes Mossy Fiber Outgrowth Acutely through a Proteolysis-independent Mechanism**

To evaluate whether the involvement of tPA in neuritic pathfinding and outgrowth is due to its active participation...
in such processes (rather than to a developmental abnormality of the tPA−/− or plg−/− mice), we delivered back into tPA−/− mice recombinant active tPA or the catalytically inactive mutant, S478A tPA, and then stimulated amygdala with the injection of kainate. As seen in Fig. 6, the delivery of tPA into the tPA−/− animals just before kainate stimulation results in reversal (Fig. 6, tPA−/−/tPA) of the tPA−/− mossy fiber sprouting phenotype (Fig. 6, wt), making the animals comparable to wild-type animals. The effectiveness of the tPA infusion was assessed by visualizing tPA activity by in situ zymography (Sappino et al., 1993) or visualizing tPA protein by immunohistochemistry.

The more extensive phenotype observed for mossy fiber outgrowth in tPA−/− mice, as opposed to plg−/− mice, suggested either that tPA cleaves other effector proteins in addition to plg, or that it also acts through a proteolysis-independent pathway. To address this issue, we infused S478A tPA.

To investigate whether the above observation was reversible in wild-type mice, we employed tPA Stop, an inhibitor of tPA’s proteolytic activity, to selectively block proteolysis-dependent pathways. tPA Stop (or buffer) was infused into the hippocampus of wild-type mice, which were subsequently injected unilaterally with kainate in the amygdala, and analyzed 12 d later. tPA activity was detected in the CA3/dentate hippocampal region of the buffer-infused mice, but not in the tPA Stop-infused mice (data not shown). Mossy fiber sprouting was evaluated by Timm staining. Extensive and dense sprouting was observed by in situ zymography (Sappino et al., 1993) or visualizing tPA protein by immunohistochemistry. The more extensive phenotype observed for mossy fiber outgrowth in tPA−/− mice, as opposed to plg−/− mice, suggested either that tPA cleaves other effector proteins in addition to plg, or that it also acts through a proteolysis-independent pathway. To address this issue, we infused S478A tPA.

These results indicate that the tPA Stop infusion was effective, since DSD-1-PG/phosphacan processing, the step determined to be plasmin-dependent (and hence, tPA-dependent), was blocked and a laminar band was observed, yielding a phenotype similar to that observed for plg−/− mice (Fig. 2). However, since mossy fiber outgrowth was unperturbed, as opposed to the stunted outgrowth observed in tPA−/− mice (Fig. 1), mossy fiber outgrowth...
would appear to be mediated by a nonproteolytic tPA downstream effector pathway.

To demonstrate directly the physiological pathways mediated by tPA through this noncatalytic mechanism, we infused a recombinant, catalytically inactive tPA mutant, S478A, into tPA−/− mice. The inactivity of this enzyme was confirmed by zymography and its presence by tPA immunohistochemistry (Rogove et al., 1999; and data not shown). When S478A tPA was infused, the neurite out-growth and pathfinding profile through the granule cell layer (Fig. 6, tPA/S478A tPA, arrowheads) was comparable to that of wild-type mice (Fig. 6, wt). A new apparent laminar band, however, was present (Fig. 6, tPA−/−/S478A tPA, arrows), as had been observed in stimulated tPA−/− and plg−/− mice (Figs. 1, 2, and 6).

Discussion

NCAM promotes establishment of synaptic connections during development and synaptic plasticity in adults (Cremer et al., 1997) by regulating stimulation-dependent sprouting, growth, and synaptogenesis of mossy fibers (Niquet et al., 1993). NCAM-deficient mice exhibit learning and behavioral deficits ensuing from aberrant mossy fiber growth and pathfinding that leads to innervation of CA3 pyramidal cells at ectopic sites (Cremer et al., 1998). DSD-1-PG/phosphacan binds to many cell adhesion and ECM proteins, presumably to regulate cell–cell and cell–matrix interactions (Margolis and Margolis, 1997). DSD-1-PG/phosphacan has been demonstrated to potentiate neurite outgrowth in culture by opposing Ng-CAM and NCAM’s promotion of continued neurite extension. We do not presently know whether the termination of neurite extension is mediated directly by the processed DSD-1-PG/phosphacan or whether processing makes accessible a distinct termination signal otherwise hidden by the unprocessed DSD-1-PG/phosphacan. Our finding that unprocessed DSD-1-PG/phosphacan inhibits mossy fiber axon outgrowth (but not lateral growth) in culture is consistent with previous findings which demonstrated that...
both DSD-1-PG/phosphacan, as well as other brain chondroitin sulfate proteoglycans, can repel neurite outgrowth (Dou and Levine, 1994; M argolis and M argolis, 1997). Recently, it was reported that DSD-1-PG/phosphacan could promote or inhibit neurite outgrowth dependent on the lineage of neuronal cells (Garwood et al., 1999). In that context, pyramidal neurons transverse the phosphacan/laminin border (data not shown), indicating that the inhibitory effect is specific for the axons of dentate granule cells (Fig. 6 E).

The nature of the nonproteolytic mechanism exhibited by tPA remains to be defined. Previously, we reported that tPA-mediated activation of microglia does not require plasmin or catalytically active tPA (Tsirka et al., 1997). A corollary, mossy fiber pathfinding and outgrowth might represent another such biological phenomenon. Alternatively, it is possible that microglia activated by secreted tPA may effect the mossy fiber pathfinding and outgrowth. In this context, other proteases released by macrophages or microglia have been shown to promote neurite growth (Petanceska et al., 1996; Bednarski et al., 1997).

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