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Research Article

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Extracellular Proteolysis in the Adult Murine Brain

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Abstract

Plasminogen activators are important mediators of extracellular metabolism. In the nervous system, plasminogen activators are thought to be involved in the remodeling events required for cell migration during development and regeneration. We have now explored the expression of the plasminogen activator/plasmin system in the adult murine central nervous system. Tissue-type plasminogen activator is synthesized by neurons of most brain regions, while prominent tissue-type plasminogen activator–catalyzed proteolysis is restricted to discrete areas, in particular whithin the hippocampus and hypothalamus. Our observations indicate that tissue-type plasminogen activator–catalyzed proteolysis in neural tissues is not limited to ontogeny, but may also contribute to adult central nervous system physiology, for instance by influencing neuronal plasticity and synaptic reorganization. The identification of an extracellular proteolytic system active in the adult central nervous system may also help gain insights into the pathogeny of neurodegenerative disorders associated with extracellular protein deposition. (*J. Clin. Invest.* 1993. 92:679–685.) Key words: antiproteases • proteases • neurons • plasmin • tissue-type plasminogen activator

Introduction

The concerted action of extracellular proteases and antiproteases modulates interactions between cells and their environment through the controlled turnover of extracellular proteins (1, 2). Plasminogen activators (PAs)[1] are serine proteases that catalyze the conversion of plasminogen into plasmin, a protease capable of degrading directly or indirectly most extracellular proteins (2). Two PAs have been identified in mammals: urokinase (uPA) and tissue-type plasminogen activator (tPA). Both enzymes are secreted in the extracellular compartment where they can interact with different ligands, such as specific cell surface receptors, physiological inhibitors (PAIs), and extracellular matrix (ECM) components. Interactions between secreted PAs and their extracellular ligands are thought to focus plasmin-mediated proteolysis to defined regions of the pericellular milieu, providing PA-producing cells with efficient means to degrade ECM constituents in a highly controlled fashion. Since they are synthesized by a wide variety of cell types endowed with invasive and migrating properties, including trophoblasts, monocytes/macrophages, and regenerating keratinocytes, PAs are assumed to play a crucial role in controlling ECM degradation in the context of tissue remodeling. However, more recent in vivo investigations have shown that PAs are also produced by cell types that are not engaged in tissue remodeling, such as epithelial cells lining the vas deferens and the renal tubules, and endocrine cells, suggesting other possible functions for the PA/plasmin system (2).

Growing experimental evidence supports the participation of proteolytic cascades in the development and regeneration of nervous tissues (3, 4). For instance, glial and neuronal cells from peripheral and central nervous tissues can synthesize and release PAs (5–8), and the localized expression of a PA at the growth cone of cultured peripheral neurons (9) together with the production of tPA by migrating granule cells in the developing cerebellum (10) suggest that plasmin-mediated extracellular proteolysis may facilitate neurite outgrowth during ontogeny. By contrast, the identification of extracellular proteases in the adult mammalian brain has received until now only limited attention, and very little information is available regarding the possible production of PAs by adult neural tissues in vivo under physiological conditions.

By using techniques that allow the localization of sites of enzymatic activity and of enzyme synthesis, we have found that neuronal-derived tPA could be a major mediator of extracellular proteolysis in the adult normal murine brain. Our findings are in agreement with the concept that PA-catalyzed extracellular proteolysis is not restricted to tissue remodeling and/or fibrinolysis. They raise the possibility that tPA may be involved in more subtle modulations of cell/cell and cell/ECM interactions, such as required for neuronal and synaptic plasticity.

Methods

Tissue preparation. Brain tissues were obtained from 12–16-wk-old NMRI mice killed by cervical dislocation. Brains or brain dissected regions were frozen down in precooled methyl butane, and stored at −70°C until analyzed. For histological zymograms, 6–8-μm cryostat tissue sections were collected on microscope slides and stored at −20°C until examined. For in situ hybridizations, 6–8-μm cryostat tissues sections were mounted on poly-L-lysine-(Sigma Chemical Co., St. Louis, MO)–coated microscope slides, fixed in 4% paraformaldehyde in PBS for 5 min, rinsed in PBS, and stored in 70% ethanol at 4°C until analyzed.

Histological zymographies. Cryostat tissue sections were overlayed as described (11) with a mixture consisting of: 0.5 ml of an 8% commercial instant nonfat dry milk solution, 0.75 ml of PBS (with 0.9 mM Ca^{2+} and 1 mM Mg^{2+}), 0.7 ml of a 2.5% agar solution in water, and 20 μl of a 4 mg/ml solution of purified human plasminogen. After heating

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1. *Abbreviations used in this paper:* CNS, central nervous system; ECM, extracellular matrix components; PA, plasminogen activator; PAI, PA inhibitor; tPA, tissue-type PA; uPA, urokinase-type PA.

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at 50°C, 100 μl of this overlay mixture were applied to prewarmed cryostat tissue sections, and spread evenly under 24 × 32-mm glass coverslips. Slides were incubated at 37°C in humid chambers and zymograms were allowed to develop for 2–6 h. Identical experiments were carried out with overlay mixtures from which plasminogen was omitted or to which were added either 1 mM amiloride (12), 0.2 mg/ml of nonimmune goat immunoglobulins (Sigma Chemical Co. [E]), or 0.2 mg/ml of anti-human tPA goat immunoglobulins (American Diagnostica, Inc., Greenwich, CT). Photographs were taken using dark-ground illumination.

Plasmid constructions and in vitro transcriptions. The uPA antisense probe was prepared from pSP64-MU, containing the 658-bp PstI-HindIII fragment of the mouse uPA cDNA clone pDB29 (13). The tPA antisense probe was prepared from pSP64-MT3, containing the 726-bp PvuII-SpeI fragment of the mouse tPA cDNA clone pUC9-A33 (14); the tPA sense probe was prepared from a DraI fragment containing 455 bp corresponding to the 3′ terminal untranslated region of mouse tPA cDNA (15). The PAI-1 probe was prepared from pRPAI106, containing the 726-bp PstI-ApaI fragment of the rat PAI-1 cDNA (16). The PAI-2 probe was prepared from pSP65-MPAI-2 containing the 928-bp PstI-PvuII fragment of the mouse PAI-2 cDNA clone pDB5901 (17). The 977-bp murine PN-1 cDNA was obtained by using oligonucleotides corresponding to conserved regions between rat and human PN-1 cDNAs to amplify cDNA from murine seminal vesicle and was subcloned in pGEM-3Z.The 272-bp murine plasminogen cDNA (nucleotides 101–372) was obtained by PCR amplification of murine liver cDNA (18), and was subcloned in pBS-M13-KS. The probes were transcribed in vitro in the presence of 12.5 mM of α^{32}P-labeled UTP (400 Ci/mmol; Amersham International, Amersham, UK) or 30 mM ^{3}H-labeled UTP and 30 mM ^{3}H-labeled CTP (40 and 20 Ci/mmol, respectively; Amersham International). ^{3}H-labeled probes were reduced to an average size of 50–100 nucleotides by mild alkaline hydrolysis as previously described (19).

Northern blot analysis. Total RNAs were extracted as described elsewhere (20). RNA samples were denatured in glyoxal, electrophoresed in 1.2% agarose gels, transferred onto Hybond membranes (Amersham International), and probed with ^{32}P-labeled cRNAs (19). Membranes were exposed for 72–96 h at −80°C, between intensifying screens. Even loading and the integrity of RNAs were verified by staining membranes after transfer with methylene blue.

RNAse protection assays. They were performed as described (17).

In situ hybridization. Prehybridizations, hybridizations, and posthybridization washes were carried out as described elsewhere (19). 1–3 × 10^{6} cpm of ^{32}P-labeled RNAs or 0.4–1 × 10^{6} cpm of ^{3}H-labeled RNAs were applied to each section in 20–70 μl of hybridization mixture. After graded ethanol dehydration, sections hybridized to ^{32}P-labeled cRNAs were directly exposed to x-ray films (SB5; Kodak, Rochester, NY) between intensifying screens and the films developed after 5–10 d exposure at room temperature, while sections hybridized to ^{3}H-labeled cRNAs were immersed in NTB-2 emulsion (Eastman Kodak), diluted 1:1 in deionized water. After 12–16 wk exposure, they were developed in Kodak D-19 developer, fixed in 30% Na thiosulfate, and counterstained in methylene blue.

Controls of specificity were performed as previously described (19), and included Northern blot hybridizations, the use of sense RNA probes and dot blot analysis under conditions of in situ hybridizations. Photographs were taken with a conventional camera (Canon), using PANF Ilford black and white film. Microphotographs were taken with a Zeiss photomicroscope (Zeiss, Oberkochen, FRG), equiped with an immersion dark-field condensor, using Kodak Ektachrome 50 color film.

SDS-PAGE zymographies. Protein extracts were prepared by homogeneizing 10 mg of tissue in 1 ml of 0.25% Triton X-100, 0.1 M Tris-HCl, pH 8.1, and aprotinin 200 Kallikrein inhibitor units/ml [Sigma Chemical Co.). Gel electrophoresis and zymography were performed as described elsewhere (21).

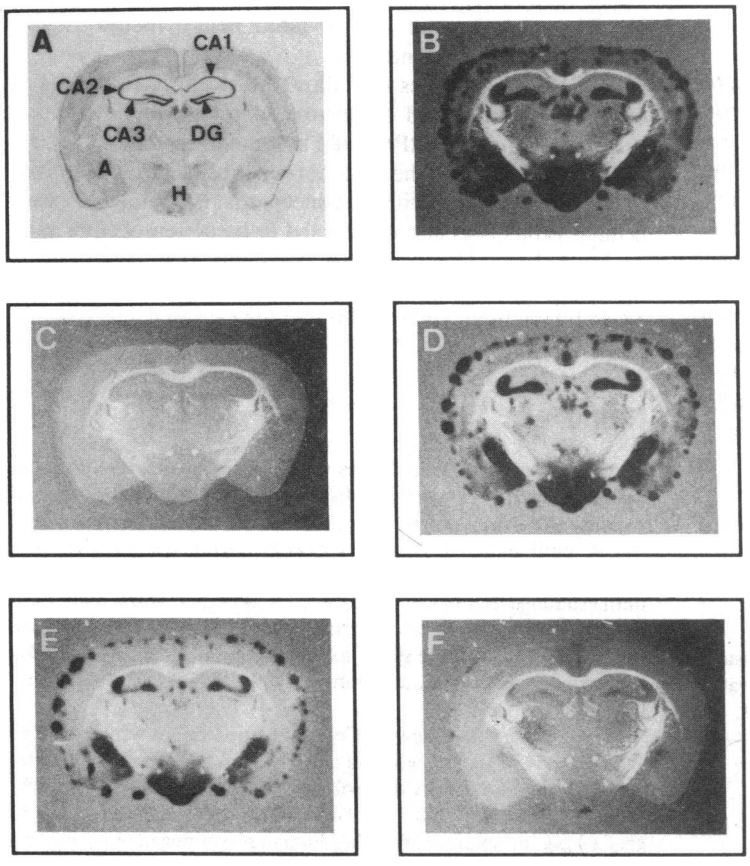


Figure 1. Localization of tPA-catalyzed proteolysis in the murine CNS. 6 μm consecutive coronal cryostat tissue sections were either stained with cresyl violet (*A*) or overlayed, as described in experimental procedures. Photographs were taken using dark-ground illumination after 3 h incubation at 37°C. Magnification, 3. (*A*) A, amygdala; H, hypothalamus; DG, dentate gyrus. (*B*) Caseinolytic activity appears as black zones of substrate lysis over the hippocampal complex, hypothalamus, amygdala and meningeal tissues; (*C*) no caseinolysis is observed in the absence of plasminogen; (*D*) caseinolytic activity is not prevented by amiloride; (*F*) the caseinolytic activity is abolished by anti-tPA antibodies, but not by irrelevant antibodies (*E*).

Results

Regional localization of tPA catalytic activity in the central nervous system (CNS). Whole brain cryostat tissue sections were analyzed by a histoenzymatic assay that reveals PA activity (11). In the forebrain, circumscribed zones of proteolysis were detected over discrete regions of the hippocampus, the hypothalamus, the amygdala, and, as expected (22), over the meningeal blood vessels (Fig. 1 *B*). To identify the enzymes responsible for substrate lysis, we performed histological zymographies in the absence of plasminogen, or in the presence of amiloride, which inhibits uPA (12), or of anti-tPA antibodies. These experiments demonstrated that the proteolytic activity was plasminogen dependent (Fig. 1 *C*), and that it was unaffected by amiloride (Fig. 1 *D*) but abolished by anti-tPA antibodies (Fig. 1 *F*), indicating that it resulted from tPA-catalyzed generation of plasmin.

Regional localization of tPA mRNA in the CNS. Northern blot analysis of total RNAs extracted from dissected brain regions showed the presence of tPA mRNA, but not of uPA mRNA (Fig. 2 *A*); tPA mRNA was detected in the hypothalamus, the hippocampus, the cerebellum, and, to a lesser degree, in the cerebral cortex, confirming that the tPA gene is transcribed in the adult brain. When hybridized to a ^{32}P-labeled tPA cRNA probe, brain tissue sections exhibited maximal labeling in regions of high neuron density, including the hippocampal layers, the dentate gyrus (Fig. 2 *B*) and the cerebellar cortex (Fig. 2 *D*). A weaker signal was observed over the meninges, the hypothalamus, the amygdala, the olfactory bulb, and the entire cortical mantle, while the white matter remained unlabeled. No detectable labeling was obtained by hybridizing adjacent tissue sections to uPA cRNA (Fig. 2 *C*) and to sense t-PA (Fig. 2 *E*) probes.

tPA mRNA is present in many CNS neurons. To identify cellular sites of tPA mRNA accumulation, we hybridized corresponding cryostat tissue sections to a ^{3}H-labeled cRNA probe. As expected from macroscopic localization, tPA mRNA was predominantly localized in neurons; quantification of autoradiographic grains demonstrated that the strongest signal was observed in hippocampal pyramidal neurons, while comparable amounts of tPA mRNA were found in dentate granule, hypothalamic, thalamic, as well as neocortical neurons (Fig. 3); in contrast, the grain content of glial cells was similar to background level.

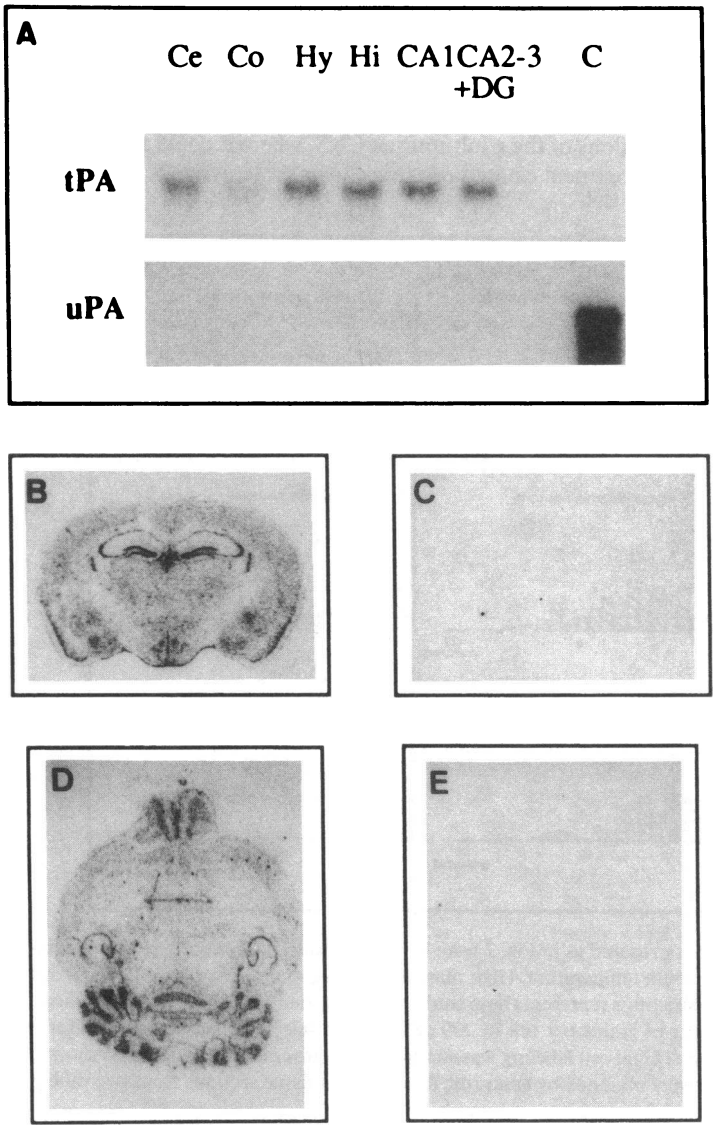


Figure 2. Localization of tPA mRNA in the murine CNS. (*A*) 5 μg of total RNA from dissected cerebellum (Ce), neocortex (Co), hypothalamus (Hy), hippocampus (Hi), isolated CA1, and CA2-3 and dentate gyrus regions (CA-2-3 + DG) was analyzed using ^{32}P-labeled tPA and uPA cRNAs. C, control, total RNA from murine kidney. A single-size tPA transcript and no uPA mRNA are detected in each specimen. (*B–E*) 5–8 μm coronal (*B* and *C*) or horizontal (*D* and *E*) brain cryostat tissue sections were hybridized to tPA cRNA (*B* and *D*), uPA cRNA (*C*), or tPA sense (*E*) ^{32}P-labeled probes as described in experimental procedures. (*D*) The rostral part (olfactive bulb) is at the top of the figure and the caudal part (cerebellum) at the bottom. Film autoradiograms were developed after 10 d exposure at room temperature. Magnification, 3. (*B* and *D*) tPAmRNA is detected over all hippocampal subdivisions including the CA1 region.

Plasminogen mRNA is present in CNS tissues. Plasminogen is the main substrate for PAs; it is synthesized and secreted by the liver, and is present at high concentrations in plasma and many other extracellular fluids. Though it can be detected in cerebrospinal fluid during pathologic conditions, circulating plasminogen would be expected to remain, through the blood-brain barrier, inaccessible to neuronal tPA under physiological conditions. We therefore searched for the presence of plasminogen mRNA by RNAse protection analysis of total RNAs from dissected brain regions. Plasminogen mRNA was detected in RNA preparations from all brain regions analyzed, with an abundance approximately three orders of magnitude lower than in liver RNA (not shown). Due to its low abundance, we could not localize the cellular sites of accumulation of plasminogen mRNA by in situ hybridizations of brain tissues.

Uncoupling of tPA mRNA accumulation and of tPA catalytic activity. Striking discrepancies were noticed between regions of tPA mRNA accumulation and zones displaying enzymatic activity. In the hippocampus, all regions had a similar tPA mRNA content (Fig. 4 *A*), while tPA-catalyzed proteolysis was localized exclusively over the CA2 and CA3 regions and the dentate gyrus, but not over the CA1 region (Fig. 4 *C*); in the cerebellum, a strong tPA mRNA signal was detected in the granule cell layer (Fig. 4 *B*) whereas no proteolysis was noticed over that layer (Fig. 4 *D*); similarly, neocortical neurons contained tPA mRNA (Fig. 2 *B* and Fig. 3), while no proteolytic activity was observed over the cerebral cortex (Fig. 1 *B*). Paired analyses by in situ hybridization and histological zymography performed on consecutive tissue sections confirmed these discrepancies and excluded potential microanatomical differences within these regions. The uncoupling of tPA mRNA and tPA-catalyzed proteolysis could result from translational control, as shown in murine oocytes (15) or from accumulation of tPA protein at sites distinct from those of its synthesis. These two possibilities can be ruled out: protein extracts from cerebral cortex, cerebellum, dissected regions of hippocampus, and hypothalamus were analyzed by SDS-PAGE and zymography, and tPA was found in equivalent amounts in all samples analyzed (Fig. 4 *E*). Since electrophoresis separates PAs and their known inhibitors (unless they have interacted to form stable complexes) before the assessment of enzymatic activity and therefore allows the detection of free active enzyme in tissue extracts even if inhibitors are present, this observation suggests that the differences observed in tPA-catalyzed proteolysis in tissue sections might be due to the differential presence of antiproteases. To search for the presence of known PAIs in regions devoid of detectable tPA enzymatic activity, we probed Northern blots of total RNAs from dissected brain regions for three characterized PAIs, namely, PAI-1, PAI-2, and protease-nexin-1 (PN-1) (2). No PAI-1 or PAI-2 mRNAs could be detected; in contrast, PN-1 mRNA was found in each specimen, at comparable levels (Fig. 4 *F*). Biochemical analysis of protein extracts confirmed the presence of active PN-1 in the different CNS regions (data not shown).

Discussion

We have shown here that tPA is produced in many different regions of the adult murine CNS, where it could function as a prominent catalyst of extracellular proteolysis. By demonstrat-

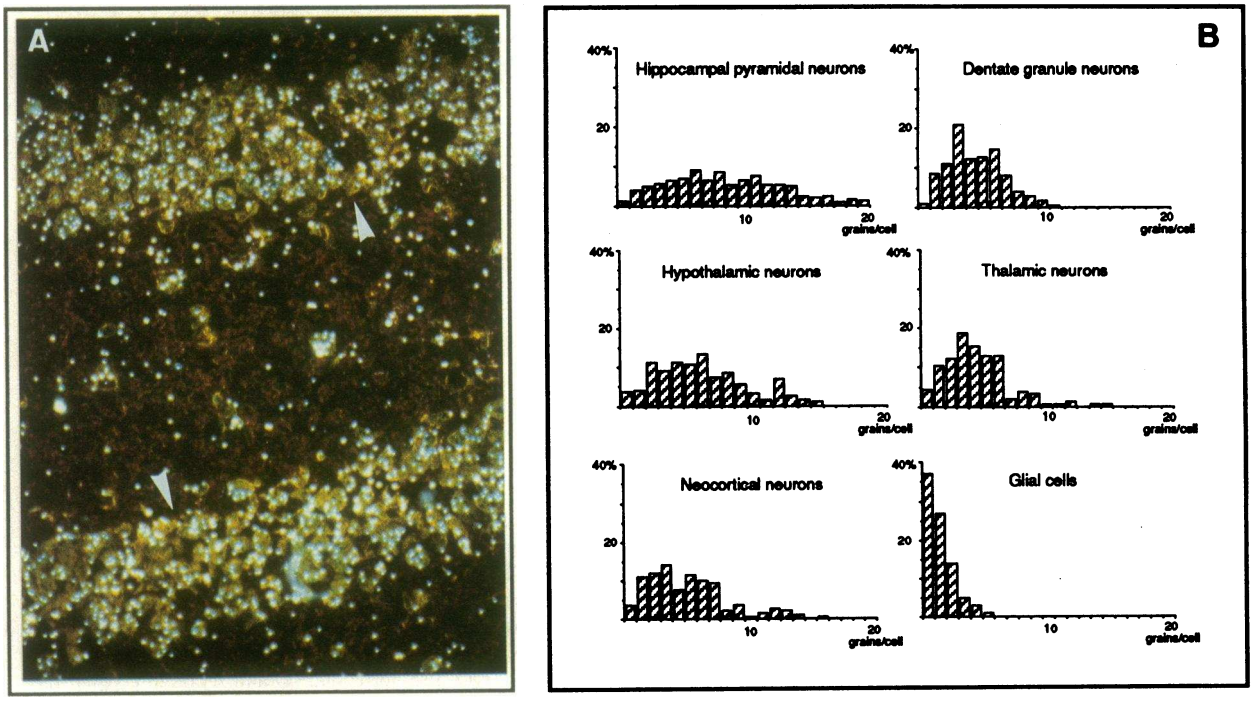
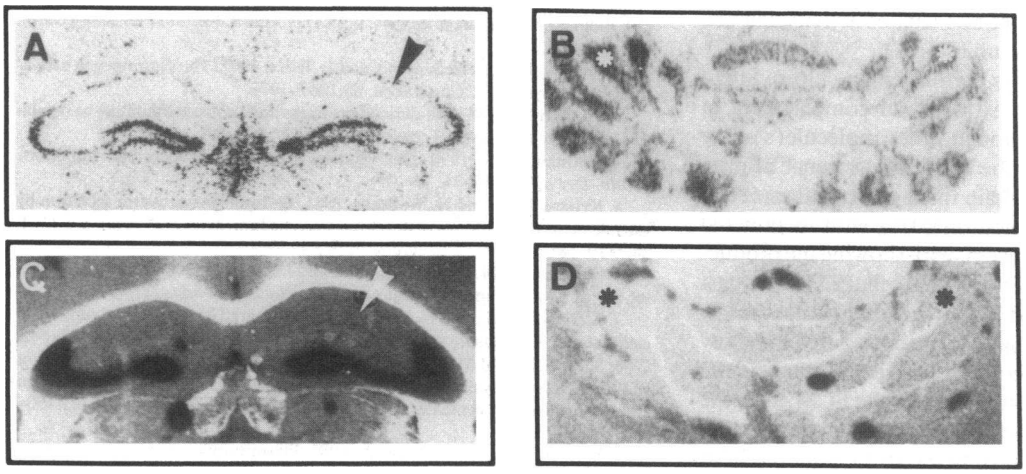
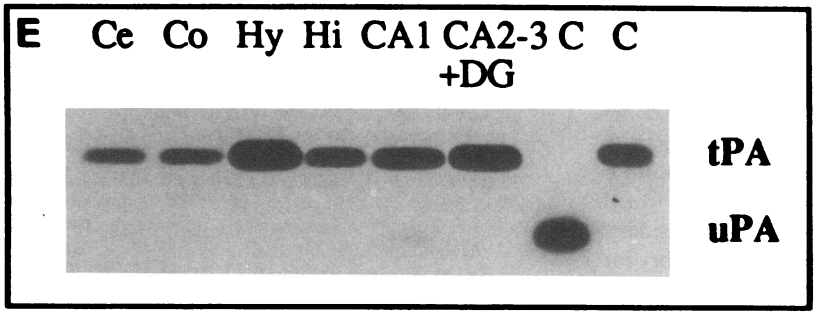


Figure 3. tPA mRNA in CNS neurons. (*A*) 6 μm cryostat tissue sections prepared as in Fig. 2 were hybridized to ^{3}H-labeled tPA cRNA and revealed by emulsion autoradiography. Slides were exposed for 4 mo at room temperature. High magnification view showing tPA mRNA in dentate granule neurons (*arrowheads*) (×1,200). The two layers of labeled neurons represent the granule cells of the dentate gyrus. (*B*) Quantification of autoradiographic grains was performed by counting the number of grains per cell in 200 cells of each selected CNS areas. Data for six different regions were used to generate frequency distribution histograms. Glial cell labeling was similar to background. The histograms shown represent values obtained from a single tissue section; identical results were obtained by analyzing four different tissue sections from two different brains.





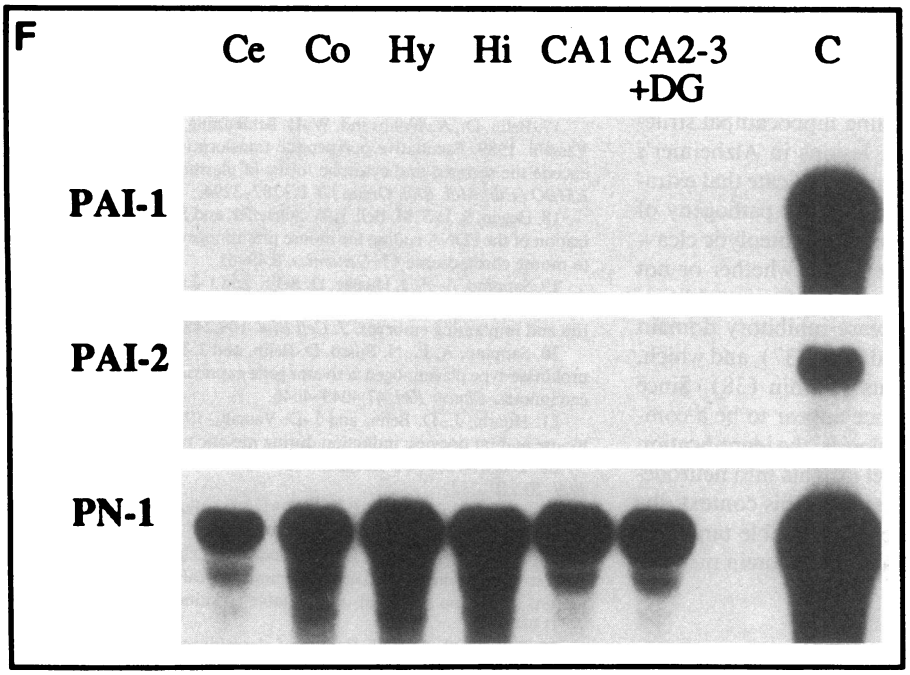


Figure 4. (*A–D*) Discrepancies between sites of tPA mRNA accumulation and tPA-catalyzed proteolysis. 6–8 μm consecutive coronal (*A* and *C*) or horizontal (*B* and *D*) cryostat tissue sections were either hybridized to a ^{32}P-labeled tPA cRNA probe (*A* and *B*), as described in Fig. 2, or overlayed with a casein solution containing plasminogen (*C* and *D*) as described in Fig. 1. Magnification, 6. (*A*) tPA mRNA is present in all hippocampal regions, including CA1 (*arrowhead*), while (*C*) no caseinolysis is observed over the CA1 region (*arrowhead*). (*B*) In the cerebellum, tPA mRNA is expressed by granule cell layer (*asterisks*), while (*D*) no proteolytic activity is observed over this layer (*asterisks*). (*E*) Zymographic analysis of PAs in the murine CNS. 15 μl of protein extracts of dissected brain regions (see legend to Fig. 2) were analyzed by SDS-PAGE and zymography as described in experimental procedures. Photograph was taken after 18 h incubation at 37°C. C, murine uPA and tPA standards as described (11). tPA-catalyzed proteolytic activity is observed in each protein extract; no detectable high mol wt PA/PAI complexes were observed, even after prolonged incubations of the zymograms. (*F*) Northern blot analysis of PAI-1, PAI-2, and PN-1 mRNAs in the murine CNS. Northern blots prepared as described in Fig. 2 were probed with rat PAI-1, murine PAI-2, and murine PN-1 ^{32}P-labeled cRNAs. Membranes were exposed for 96 h at −80°C, between intensifying screens. C, control RNAs; PAI-1, total RNA from murine placenta; PAI-2, total RNA from resident murine macrophages; PN-1, total RNA from murine seminal vesicle.

ing that neurons are the major sites of tPA synthesis in vivo, and that enzymatic activity is restricted to defined areas, our results extend previous studies which had revealed PA activity and tPA mRNA in adult rodent brain (23, 24). They also suggest that the net proteolytic activity in discrete CNS regions cannot be directly inferred from the sites of protease synthesis, and that it is likely to result from a protease-antiprotease balance. Differential expression of the three main PAIs is unlikely

to account for the observed absence of tPA-catalyzed proteolysis in the CA1 pyramidal layers, the cerebellum, and the neocortex, raising the possibility that this lack of activity may be due to other(s) tPA or plasmin inhibitor(s).

It can be inferred from studies performed on cultured neurons that tPA is secreted in the extracellular compartment of the CNS (5, 6, 9, 25, 26). Although other molecule(s) may serve as substrate(s) for tPA in the brain, the presence of plasminogen mRNA indicates that brain tissues can synthesize this zymogen and directly provide neuronal tPA with its prefered substrate; this is in accord with recent work showing the production of plasminogen by cultured microglial cells (27). Amongst the cohort of enzymes involved in extracellular proteolysis in essentially all tissues, PAs and plasmin are considered to be key participants (2): they can, directly or indirectly, catalyze the degradation of most protein components of the ECM, alter the structure of surface proteins, and activate latent growth factors (28–30). Our observations suggesting the participation of tPA to adult CNS physiology raise the possibility that, in neural tissues, the enzyme may fulfill other functions than those that have been so far ascribed to it: the presence of PAs in the developing nervous system, as well as in cultures of CNS neurons, has been mainly related to cell migration and tissue remodeling events during ontogeny (6, 7, 9, 10). tPA and/or plasmin-mediated extracellular proteolysis in the adult CNS could participate in the processing of neuro-peptides and survival-promoting factors (31), or contribute to remodeling events, for instance in the context of neuronal plasticity, which is considered to be an essential determinant of adaptation and memory formation (32). In accord with the proposal that the enzyme may play a role in the structural changes that are associated with activity-dependent plasticity, electrical and pharmacological stimulations of neuronal activity have recently been shown to induce the accumulation of tPA mRNA in the rat hippocampus (33).

tPA-catalyzed extracellular proteolysis is prominent in discrete regions of the adult CNS, including hippocampal structures which are sites of characteristic lesions in Alzheimer's disease. There is now abundant evidence to indicate that extracellular proteolysis plays a decisive role in the pathogeny of Alzheimer's disease: the precise extracellular proteolytic cleavage of amyloid precursor protein determines whether or not amyloid will accumulate in the brain (34, 35); in addition amyloid precursor protein has a protease inhibitory domain (36), which appears important for the disease (37), and which, in addition to other enzymes, inhibits plasmin (38). Since dysregulations of the proteolytic balance appear to be a common denominator in a variety of pathologies, the identification of tPA in the adult CNS provides novel insights into neurodegenerative disorders and ageing processes. In this context, the neuronal PA/plasmin system represents a possible target for pharmacological modulations of extracellular protein metabolism in the CNS.

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