

# Spatiotemporal Expression Patterns of Metalloproteinases and Their Inhibitors in the Postnatal Developing Rat Cerebellum

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Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade the components of the extracellular matrix (ECM). The balance between MMPs and their inhibitors [tissue inhibitors of metalloproteinases (TIMPs)] in the pericellular environment determines the most significant proteolytic events in tissue remodeling. *In vitro* evidence is accumulating that these molecules may be crucial in the maturation of neural cells. Here, we investigated the *in vivo* expression of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 in the developing and adult rat cerebellum using immunohistochemistry and *in situ* hybridization. During postnatal development, all Purkinje (PK) cell somata expressed all the MMPs and TIMPs studied, whereas their growing dendritic trees expressed only MMP 3 and TIMP 3. In the adult, MMP 3 was confined to PK cell bodies, whereas TIMP 3 was expressed in PK cell somata and processes. Irrespective of the developmental stage, Bergmann glial processes contained only MMP 9, but their somata contained both TIMP 1 and MMP 9. In

granular cells, MMPs 3 and 9 and TIMPs 1, 2, and 3 were chiefly detected at a time when migration is known to be maximal; except for that of TIMP 1, their expression persisted in the internal granular layer in the adult. The functional relevance of MMP expression was verified by gelatin zymography. MMP 9 activity was maximal on postnatal day 10 (P10) and was detectable at a low level on P15 and in the adult, whereas MMP 2 activity remained similar throughout postnatal development. Regional and cell-specific expression of MMPs and TIMPs closely reflects the successive stages of cerebellar development, thereby suggesting a pivotal role for ECM proteolysis in brain development and plasticity.

*Key words:* metalloproteinase; tissue inhibitor of metalloproteinase; cerebellum postnatal development; neuronal migration; synaptogenesis; tyramide signal amplification immunohistochemistry; *in situ* hybridization; gelatin zymography

The matrix metalloproteinases (MMPs) constitute a family of zinc-binding endopeptidases characterized by their ability to degrade the components of the extracellular matrix (ECM), a structural support and physiological regulator of cell growth and differentiation (Bissell et al., 1982; Adams and Watt, 1993). MMPs play a pivotal role in modulating interactions between cells and their microenvironment, mainly through ECM receptors (Damsky and Werb, 1992). MMPs 2, 3, and 9 are involved in physiological tissue remodeling (Matrisian and Hogan, 1990), such as trophoblast implantation (Alexander et al., 1996) and mammary gland development (Talhouk et al., 1992; Thomasset et al., 1998), as well as in pathological events, such as tumor progression and metastatic invasion (Mignatti and Rifkin, 1993). MMP activity is tightly regulated at various levels, including gene transcription, activation of MMP proenzymes, and by endogenous tissue inhibitors of metalloproteinases (TIMPs). The balance between MMPs and TIMPs in the pericellular environment determines the most significant proteolytic events in tissue remodeling. Indeed, excessive cleavage of ECM caused by an im-

balance of the MMPs/TIMPs ratio confers oncogenicity on 3T3 cells (Khokha et al., 1989), and excessive proteolysis contributes to various brain pathologies (Rosenberg et al., 1992; Nakagawa et al., 1994; Mohanam et al., 1995). Although there is growing evidence for an important role of MMPs in inflammatory disorders of the CNS (Giraudon et al., 1996; Pagenstecher et al., 1998) and in brain tumor progression (Lampert et al., 1998), little is known about the cellular origin and physiological role of MMPs and TIMPs in the CNS. *In vitro* studies suggest that MMPs are required for neurite outgrowth (Pittman and Williams, 1989; Sheffield et al., 1994), and oligodendrocytes are known to use MMP 9 in migration and process extension (Uhm et al., 1998). MMPs and TIMPs may be expected to be involved in brain development, because extensive cellular migration and remodeling of the ECM are necessary for neural development (Reichardt and Tomaselli, 1991; Condic and Letourneau, 1997; Curran and D'Arcangelo, 1998). Indeed, serine proteinases (urokinase- and tissue-plasminogen activators) and their inhibitors are involved in the postnatal migration of granular cells (Moonen et al., 1982; Seeds et al., 1990), and plasminogen activator, strongly expressed in the adult cerebellum, is likely to play a role in the synaptic remodeling associated with learning and memory (Qian et al., 1993; Seeds et al., 1995; Frey et al., 1996). These previous studies clearly indicate that a precise knowledge of the relative distribution of the major MMPs and TIMPs is indispensable to delineate their possible role in brain development and plasticity.

Here, we used the cerebellum as a model to investigate the spatiotemporal expression patterns of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 using immunohistochemistry and *in situ*

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hybridization. MMPs 2 and 9 activity was confirmed by gelatin zymography. Depending on the stage of development, specific and differential expression of MMPs and TIMPs was seen, which may be related to granular cell migration, arborization of Purkinje (PK) cells, and synaptogenesis.

## MATERIALS AND METHODS

### Gelatin zymography

The gelatinase activity of MMP 2 (also known as 72 kDa type IV collagenase or gelatinase A) and MMP 9 (also known as 92 kDa type IV collagenase or gelatinase B) was determined using zymography (protease-substrate gel electrophoresis). This technique involves SDS-PAGE of nonreduced protein samples in gels containing the substrate gelatin (Herron et al., 1986).

Five rats at each chosen stage of postnatal development [postnatal day 10 (P10), P15, and adult] were intraperitoneally anesthetized with sodium pentobarbital (12 mg/100 gm of body weight; Sanofi, Libourne, France), and then the cerebellum of each animal was dissected out. One hundred fifty milligrams of tissue was mechanically dispersed, placed in a culture dish without rinsing, and incubated for 12 hr with 500  $\mu$ l of DMEM medium (Life Technologies, Paisley, UK) without serum; this step allowed us to detect the activity of secreted MMPs 2 and 9 in the conditioned medium. Cell loss by lysis was quantified using Trypan blue and was minimal after tissue treatment (<10%). Briefly, equal volumes (20  $\mu$ l) of the conditioned medium, normalized for protein concentration, were mixed 1:4 with 4 $\times$  SDS sample buffer, and 25  $\mu$ l of the mixture was loaded into a well of a 10% zymography gel copolymerized with 0.1% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 30 min at room temperature and then incubated overnight at 37°C in 100 mM Tris buffer, pH 7.4, containing 15 mM CaCl<sub>2</sub>. They were then fixed with 30% methanol–10% acetic acid, stained with 0.25% Coomassie blue R-250 (Sigma, St. Louis, MO), and destained with 10% methanol–10% acetic acid. Enzyme activity, attributable to MMPs 2 and 9 on the basis of their respective molecular weights, was visualized in the gelatin-containing zymograms as clear bands on a blue background. For inhibition of metalloproteinase activity, the gels were incubated with 4 mM 1,10-phenanthroline as described previously (Adler et al., 1990).

### Immunohistochemistry

**Animals and tissue preparation.** Experiments were performed on five Sprague Dawley rats (Iffa Credo, L'Arbresle, France) at each stage of postnatal development studied (P10, P15, and the 200–250 gm adult). Animals were intraperitoneally anesthetized with sodium pentobarbital (12 mg/100 gm of body weight; Sanofi, Libourne, France) and intracardially perfused with 50 ml of 0.1 M phosphate buffer, pH 7.4, followed by 300 ml of fixative consisting of 4% paraformaldehyde and 5% sucrose in 0.1 M phosphate buffer, pH 7.4. The brains were removed, post-fixed for 24 hr at 4°C in the same fixative, and immersed for 5 d in 0.1 M phosphate buffer, pH 7.4, containing 20% sucrose, before being frozen in isopentane at –50°C using liquid nitrogen and embedded in O.C.T. compound (Tissue Tek, Elkhart, IN). Cryostat sections (10- $\mu$ m-thick) were cut and fixed to glass slides precoated with 2% aminopropyltriethoxysilane (Fluka, Buchs, Switzerland). All animals were treated according to the guidelines approved by the French Ethical Committee (decree 87–848).

**Immunohistochemical analysis.** Immunohistochemistry was performed on the slides using a tyramide signal amplification (TSA) kit (New England Nuclear, Boston, MA), which enhances fluorescent signals by a factor of up to 1000-fold (Van Heusden et al., 1997). The TSA technique uses fluorescein-labeled tyramide as the substrate for horse radish peroxidase, which catalyzes the formation of highly reactive tyramide radicals with a very short half-life, thus allowing the binding of fluorescein-conjugated tyramide to tyrosine residues only at the site of enzymatic reaction. This amplification was essential to detect the expression of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 in the cerebellar cortex of the postnatal and adult rats. The amplified immunohistochemical signals were highly specific, because no labeling was seen using nonimmune sera (data not shown).

The primary polyclonal rabbit antibodies, used at a 1:2500 dilution, were anti-MMP 2 (hinge region), anti-MMP 3 (hinge region), anti-MMP 9 (N-terminal region of the active form), anti-TIMP 1 (C-terminal region), anti-TIMP 2, and anti-TIMP 3 (Chemicon, Temecula, CA). The primary antibodies were raised against human MMPs and TIMPs but

recognize the rat proteins; their specificity was verified by Western blots using an extract of adult rat cerebellum (data not shown).

The secondary antibody, used at a 1:1000 dilution, was biotinylated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

Slices were permeabilized for 1 hr at room temperature in 0.1 M PBS, pH 7.4, containing 0.25% Triton X-100 and 1% bovine serum albumin (Sigma), and then fixed in methanol at –20°C for 15 min. Endogenous peroxidase activity was blocked by treating the slides for 15 min at room temperature with 3% hydrogen peroxide in methanol, and then the slides were washed at room temperature (three times) in TNT buffer (0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween 20). Non-specific binding sites were saturated by incubation for 15 min at 37°C with TNT buffer plus 5% normal goat serum, followed by incubation for 30 min with TNB blocking buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% of the blocking reagent supplied in the kit). The sections were incubated initially overnight at 4°C with primary antibody diluted in TNT buffer and then, after washing, for 2 hr at room temperature with biotinylated secondary antibody diluted in TNT buffer before being incubated with streptavidin-conjugated horse radish peroxidase for 30 min at room temperature, followed by washing with TNT. The fluorophore tyramide, diluted 1:50 with the diluent supplied in the kit, was added to the slides for 10 min at room temperature. After washing, the slides were mounted in PBS containing 50% glycerol for FITC-fluorescence microscopy (Axioskop FS; Zeiss, Jena, Germany). Controls consisted of the omission of the primary antibody or incubation with a nonreactive normal rabbit serum (Vector Laboratories, Burlingame, CA).

Bergmann glial cells were characterized using mouse monoclonal anti-vimentin antibody at a 1:200 dilution (Sigma) in PBS, and PK cells were stained using mouse monoclonal anti-calbindin-D antibody at a 1:200 dilution (Dako, Carpinteria, CA) in PBS. The secondary FITC-conjugated anti-mouse antibodies were used at a 1:1000 dilution in PBS.

### In situ hybridization

**Preparation of tissue.** Three rats at each of the two chosen stages (P10 and adult) were intraperitoneally anesthetized with sodium pentobarbital (Sanofi), intracardially perfused with 50 ml of 0.1 M phosphate buffer, pH 7.4, followed by 300 ml of fixative containing 2% paraformaldehyde, and then decapitated. The brains were removed and post-fixed for 1 hr at 4°C in the same fixative before being immersed in 20% sucrose in 0.1 M phosphate buffer, pH 7.4, for 2 d. The samples were frozen in isopentane at –50°C using liquid nitrogen and embedded in O.C.T. compound (Tissue Tek). Cryostat sections (12- $\mu$ m-thick) were cut and fixed to glass slides precoated with 2% aminopropyltriethoxysilane (Fluka) and then dehydrated in a graded series of ethanol.

**Hybridization probes.** Three antisense oligodeoxynucleotide probes, specific for MMP 9, TIMP 2, or TIMP 3, were provided by Biognostik (Göttingen, Germany). The rat MMP 9, TIMP 2, and TIMP 3 hybrid probes were, respectively, 29, 29, and 30 bases long and targeted the region between bases 689 and 717, 309 and 337, and 422 and 451 of the respective total sequence. The probes were 3' labeled for 1 hr at 37°C using 5  $\mu$ M probe in a final volume of 10  $\mu$ l of 2.5 mM CoCl<sub>2</sub>, 17  $\mu$ M  $\alpha$ <sup>32</sup>S dATP (1250 Ci/mmol; New England Nuclear) and 22 U of terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany). The probes were separated from nonincorporated nucleotides by Micro Spin column chromatography through Bio-Gel polyacrylamide (Bio-Rad, Hercules, CA) and then diluted with 500  $\mu$ l of hybridization buffer (Hybribuffer; Biognostik). Eighty microlitres of the mixture was added to each of the sections, which were then incubated overnight at 39°C in a sealed humid container, and the slides were sequentially washed at room temperature for 1 hr once with 2 $\times$  SSC (1 $\times$ SSC: 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), once with 1 $\times$  SSC, and twice with 0.5 $\times$  SSC. After dehydration in a graded alcohol series, the slides were air-dried and exposed for 5 d to a Hyperfilm- $\beta$ max film (Amersham, Arlington Heights, IL); they were then dipped in Kodak NTB-2 emulsion (Eastman Kodak, Rochester, NY), dried, and stored in the dark for 4 weeks before being developed, counterstained with toluidine blue, and examined by dark- and bright-field microscopy.

When hybridization specificity was verified by hybridizing some sections in the presence of an excess (100-fold) of unlabeled probe, a substantially reduced signal was obtained (data not shown).

Cresyl violet was used to examine the general histology in cryostat sections adjacent to those used for immunohistochemistry. The neuronal classes were defined using the criteria of their position and the size of the nuclei, as described by Altman (1972a,b,c). PK cells were also character-

ized using anti-calbindin-D antibody (Enderlin et al., 1987). On P10, small round cells in the external granular layer (EGL) were identified as granular cells, whereas spindle-shaped cells descending along Bergmann glia fibers in the molecular layer (ML) were identified as migrating granular cells (Altman, 1972a; Komuro and Rakic, 1998). Medium and large cells in the ML were classified as ML cells and identified as either basket or stellate neurons on the basis of their morphology. Other cells were classified as ML neurons when located above the PK cell layer (PCL). Small cells encircling the PK cell bodies were classified as Bergmann glial cells. Small cells in the internal granular layer (IGL) were identified as granular neurons.

## RESULTS

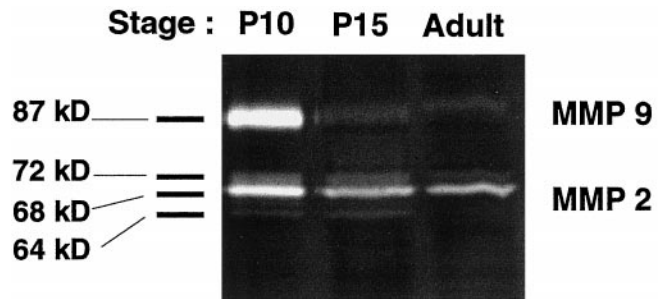
Three stages of postnatal development of the cerebellum, P10, P15, and the adult, were chosen for immunohistochemical study because of the major events occurring at these times during maturation. The P10 stage corresponds to the beginning of a sharp decline in the thickness of the EGL, caused by extensive and rapid granular cell precursor migration through the ML and PCL (Altman, 1972a). On P15, cerebellar development is marked by intensive outgrowth of PK cell dendritic branches in the ML. Growth occurs mainly in the secondary and tertiary branches and terminal branchlets bearing spines (Altman, 1972b). Synaptogenesis between parallel fibers and the dendritic PK tree ascends progressively from the PK cell somata toward the EGL. In the adult, granule cells from the EGL have migrated to give rise to the IGL. Various cellular and molecular mechanisms underlie this coordinated proliferation, migration, and synaptogenesis. Of the molecules involved, the functional domains of ECM components have been shown to be important in regulating cell attachment, migration, and process extension during development (Reichardt and Tomaselli, 1991). These functional domains of the ECM are generated by proteolysis involving several proteolytic enzymes because of their diverse macromolecular composition. Thus, multiple metalloproteinase family members could be involved. We therefore examined gelatinase activity in the developing and adult cerebellum.

### Gelatinase activity in the developing and adult rat cerebellum

MMPs are expressed and secreted as inactive precursors that are activated by the removal of an N-terminal propeptide (Okada et al., 1988). Important physiological activators of pro-MMPs are plasmin, a serine proteinase generated from plasminogen by the action of tissue or urokinase-plasminogen activator, or other proteinases (Basbaum and Werb, 1996). The latent and active forms of each enzyme can be differentiated on the basis of their molecular weights (MW). In the rat cerebellum, MMP 9 activity was high on P10 but barely detectable on P15 and in the adult (Fig. 1); MMP 9 was found to be in the active form (87 kDa), with the latent form (92 kDa) being undetectable. MMP 2 activity was constant throughout postnatal development and was higher than MMP 9 activity on P15 and in the adult. Furthermore, MMP 2 existed as both the latent and active forms (MW of the latent form, 72 kDa; MW of the active forms, 66 and 64 kDa) at all three stages. To determine their cellular origin and specific localization in the developing cerebellum, we determined their regional and cellular patterns of expression using immunohistochemistry and *in situ* hybridization.

### Immunohistochemical distribution patterns of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 in the P10 rat cerebellum

In the EGL, subsets of cell bodies, described as granular cell precursors, were strongly labeled by antibodies specific for MMP



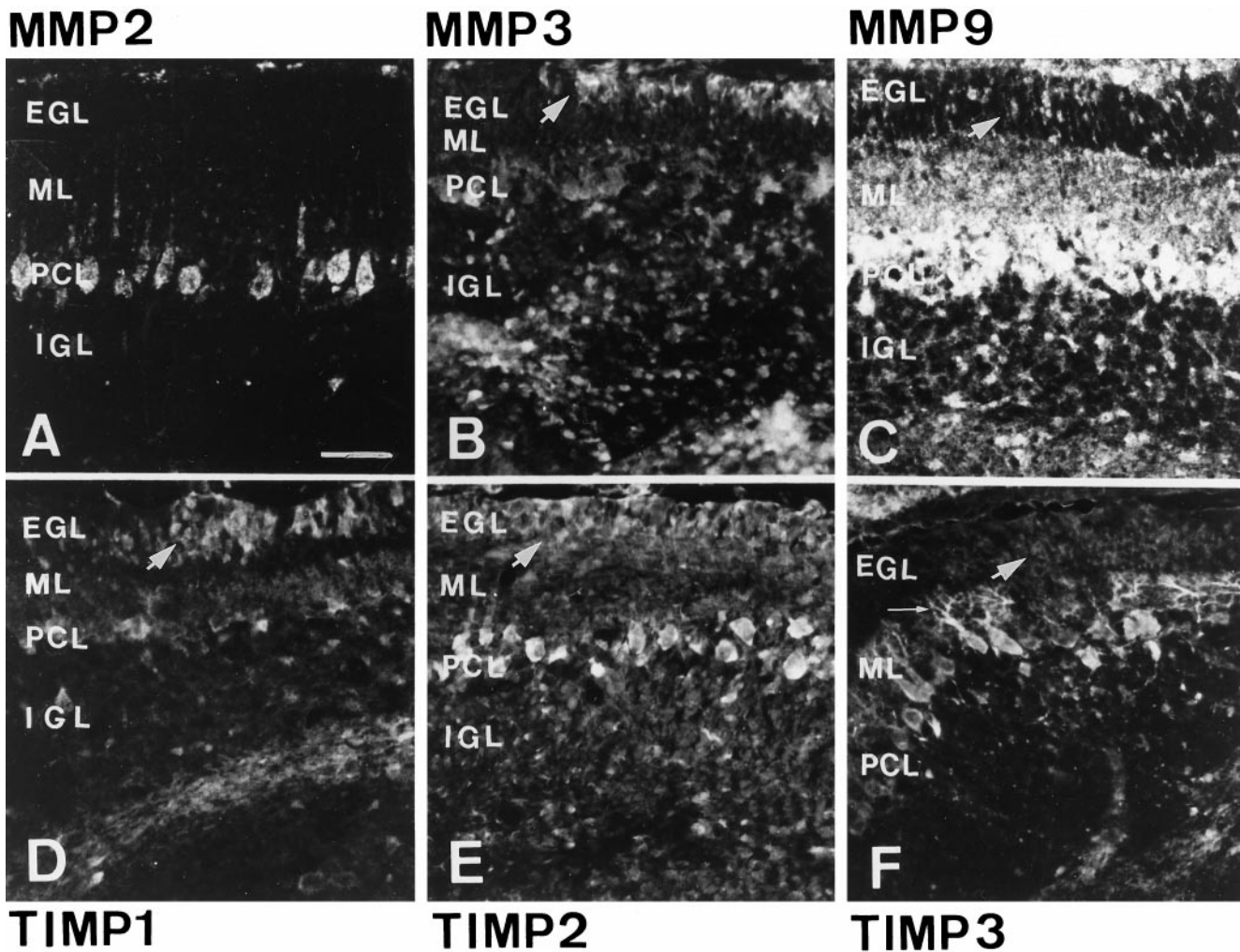
**Figure 1.** SDS-PAGE zymographic analysis of MMP activity in the rat cerebellum. Rat cerebellum extracts from P10, P15, or adult were examined. For each stage, 150 mg of tissue cerebellum was plated in tissue dish with 500  $\mu$ l of DMEM without serum. After 12 hr, 20  $\mu$ l of this conditioned medium at the same protein concentration was analyzed for MMP 2 and MMP 9 activity using SDS-PAGE zymography with gelatin as substrate. The active form of MMP 9 (87 kDa) and the latent form (72 kDa), as well as active forms (66 kDa, 64 kDa), of MMP 2 can be seen. The image was obtained by scanning the gel and using Adobe (San Jose, CA) Photoshop software to generate a digital image.

3 (Fig. 2B), MMP 9 (Fig. 2C), TIMP 1 (Fig. 2D), or TIMP 2 (Fig. 2E) and weakly labeled by anti-TIMP 3 antibodies (Fig. 2F); MMP 2 expression was undetectable (Fig. 2A). In addition, MMP 9 (Fig. 2C) was expressed in thin parallel processes reaching the pial surface, which were identified as Bergmann glial fibers (Altman, 1972a) by double labeling with anti-vimentin antibodies (data not shown). In the ML, PK cell dendrites, characterized by their positive calbindin-D immunoreactivity (data not shown), were intensely stained with antibodies specific for MMP 9 (Fig. 2C) or TIMP 3 (Fig. 2F) and weakly stained with antibodies specific for MMP 2 (Fig. 2A), MMP 3 (Fig. 2B), or TIMP 1 (Fig. 2D). MMP 3 (Fig. 2B), MMP 9 (Fig. 2C), TIMP 1 (Fig. 2D), and TIMP 2 (Fig. 2E) were present in both the cell body and the extracellular space, whereas TIMP 3 (Fig. 2F) was strictly restricted to the PK cell arborization. In the PCL, PK cell somata stained positive for all the markers but to a varying extent. MMP 9 (Fig. 2C) and TIMP 1 (Fig. 2D) were also found in small cell bodies corresponding to Bergmann glial somata surrounding labeled and unlabeled PK cell somata. In the IGL, expression of MMPs 3 (Fig. 2B) and 9 (Fig. 2C) and all TIMPs tested was detectable in some granular cells, sparsely distributed throughout the layer. The heterogeneous labeling observed may represent modulation of MMP expression by the local environment, which can cause arrest of granular cells migration (Komuro and Rakic, 1998).

### Immunohistochemical distribution patterns of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 in the P15 rat cerebellum

On P15, when the thickness of the EGL is restricted to three or four layers of cells, MMP 2 (Fig. 3A), MMP 9 (Fig. 3C), TIMP 1 (Fig. 3D), TIMP 2 (Fig. 3E), and TIMP 3 (Fig. 3F) were undetectable in granular cell precursors; a few residual precursors exhibited MMP 3 reactivity (Fig. 3B). In the ML, numerous fibers, arising from PK cell somata on some section planes and corresponding to the enlarged PK cell dendrites arborization, and other fibers, possibly corresponding to portions of Bergmann glia fibers, were labeled by anti-MMP 3 antibodies (Fig. 3B). This staining was intense but diffuse, suggesting secretion into the extracellular environment, whereas TIMP 3 expression (Fig. 3F)





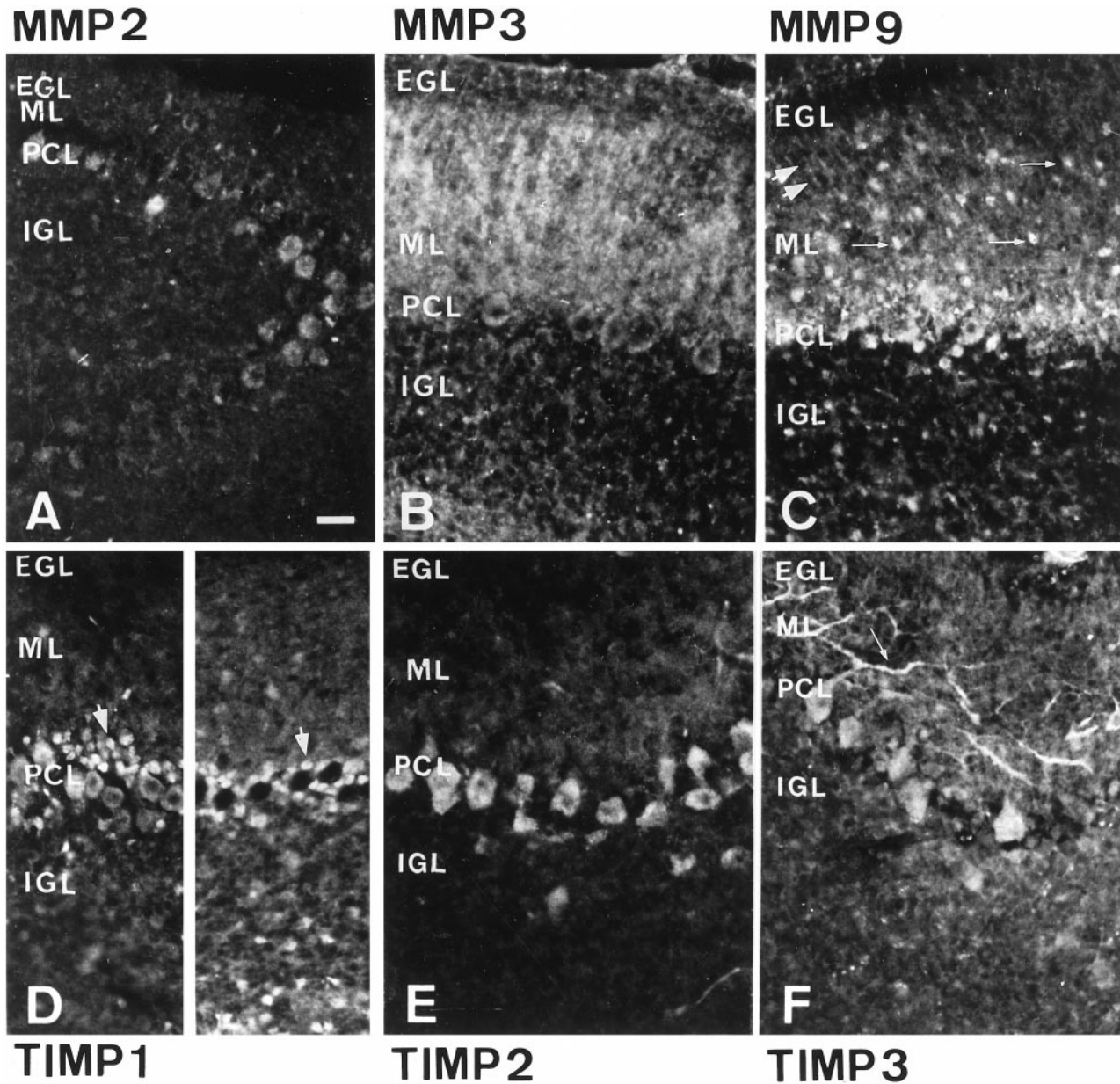
**Figure 2.** Immunohistochemical distribution pattern of MMP 2 (*A*), MMP 3 (*B*), MMP 9 (*C*), TIMP 1 (*D*), TIMP 2 (*E*), and TIMP 3 (*F*) in the cerebellar cortex of 10-d-old rats. Immunohistochemical labeling was performed on 10- $\mu$ m-thick frontal rat cerebellar sections showing the EGL, the ML, the PCL, and the IGL. The labeling with polyclonal antibodies was enhanced using the fluorescein tyramide signal amplification system (New England Nuclear). In the EGL, MMP 3 (*B*), MMP 9 (*C*), TIMP 1 (*D*), TIMP 2 (*E*), and to less extents for TIMP 3 (*F*), were seen in granular precursor cells. In addition, MMP 9 was detected along the Bergmann glial fibers in this layer (*C*). Arrows point to strips of stained granular precursor cells. In the ML, MMP 3, MMP 9, and TIMP 1 were diffusely present over PK cells dendrites, whereas TIMP 3 was restricted to the cytoplasm of the PK cell dendrites (the *small arrow* in *F* points to the labeled PK cell dendritic arborization). In the PCL, the PK cell somata showed labeling for each marker, and MMP 9 expression was seen in the Bergmann glial cell somata. Some granular cells in the IGL were immunopositive for MMPs 3 and 9 and TIMPs 1, 2, and 3. Scale bar, 50  $\mu$ m.

was confined to PK dendritic branches. MMP 9 (Fig. 3*C*) and TIMP 1 (Fig. 3*D*) dendritic staining was very weak compared with that seen on P10. MMP 9 (Fig. 3*C*) was also detected in small cells, probably corresponding to basket and stellate interneurons, and also along Bergmann glial cell processes. In the PCL, Bergmann glial somata were positive for MMP 9 (Fig. 3*C*) and TIMP 1 (Fig. 3*D*). MMP 2 (Fig. 3*A*), MMP 3 (Fig. 3*B*), MMP 9 (Fig. 3*C*), and TIMP 3 (Fig. 3*F*) could all be detected in all PK cell somata to varying extents. Only TIMP 2 (Fig. 3*E*) was homogeneously and strongly expressed in all PK somata. The TIMP 1 expression pattern (Fig. 3*D*) in PK somata made it possible to distinguish two types of PK populations, consisting either of patches of TIMP 1-positive PK cell somata, restricted to parts of the cerebellar hemispheres, or clusters of unlabeled PK cell somata. A few granular cells in the IGL were positive for all markers except MMP 2 and TIMP 2.

### Immunohistochemical distribution patterns of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 in the adult rat cerebellum

In contrast with the expression patterns seen on P10 and P15, that of a more diffuse distribution in the extracellular space around the cells, expression of MMPs and TIMPs in the adult was mainly confined to cell bodies.

In the ML, the PK cell dendritic trees showed no labeling for MMP 2 (Fig. 4*A*), MMP 3 (Fig. 4*B*), MMP 9 (Fig. 4*C*), TIMP 1 (Fig. 4*D*), or TIMP 2 (Fig. 4*E*). Only TIMP 3 (Fig. 4*F*) was detected in the dendritic elongations of all PK cells. The immunostained processes showed varicosities, which may correspond to PK cell synaptic glomeruli (Altman, 1972b). MMP 3 (Fig. 4*B*), MMP 9 (Fig. 4*C*), TIMP 1 (Fig. 4*D*), and TIMP 2 (Fig. 4*E*) were strongly expressed in numerous cell bodies described as interneurons. MMP 9 expression (Fig. 4*C*) was maintained along the



**Figure 3.** Immunohistochemical distribution pattern of MMP 2 (*A*), MMP 3 (*B*), MMP 9 (*C*), TIMP 1 (*D*), TIMP 2 (*E*), and TIMP 3 (*F*) in the cerebellar cortex of 15-d-old rats. Ten-micrometer-thick frontal sections of rat cerebellum showed the EGL, the ML, the PCL, and the IGL. Immunohistochemical staining was performed using polyclonal antibodies and amplified by tyramide signal amplification (New England Nuclear). EGL immunostaining for MMPs 3 and 9 and TIMPs 1, 2, and 3 was lost. The PK cell dendrites were still stained for MMP 3 and TIMP 3. Note the diffuse extracellular distribution of MMP 3 as opposed to the cytoplasmic distribution of TIMP 3. ML labeling for MMP 9 and TIMP 1 in the PK cell dendrites decreased, but MMP 9 labeling of Bergmann glial fibers was maintained. MMP 9 labeling in ML cells, probably corresponding to stellate and basket cells, was detected. The *large double arrows* point to MMP 9-positive Bergmann glial fibers, and the *small arrows* point to MMP 9-positive ML cells (*C*). In the PCL, expression of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 was noted in the PK cell somata. TIMP 1 was differentially expressed in clusters of PK cell somata (stained and unstained PK cell somata are respectively indicated by *large arrows* at the *left* and *right* of *D*). TIMP 1 was also present in Bergmann glial somata around labeled and unlabeled PK cell bodies (*D*). Granular cells in the IGL were positive for MMPs 3 and 9 and TIMPs 1 and 3. Scale bar, 25  $\mu$ m.

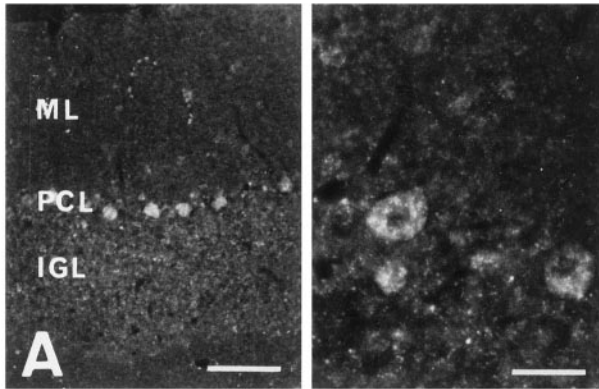
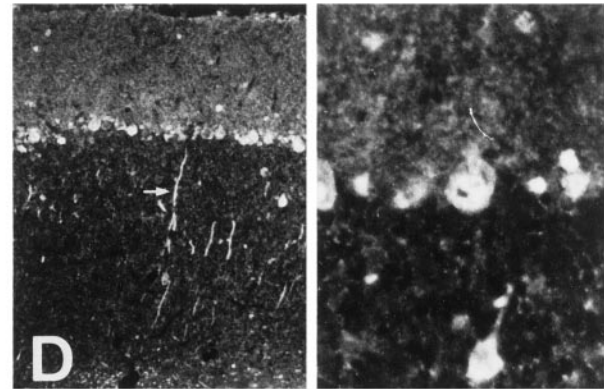
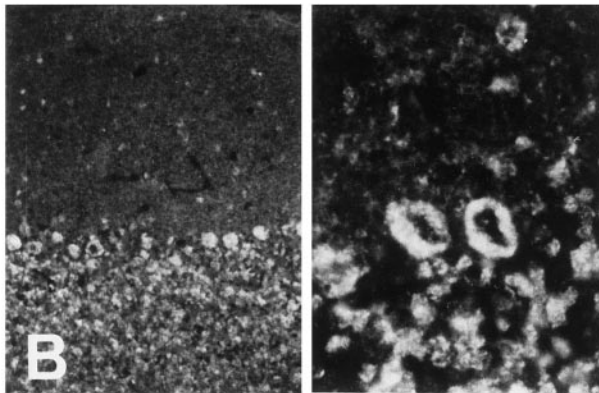
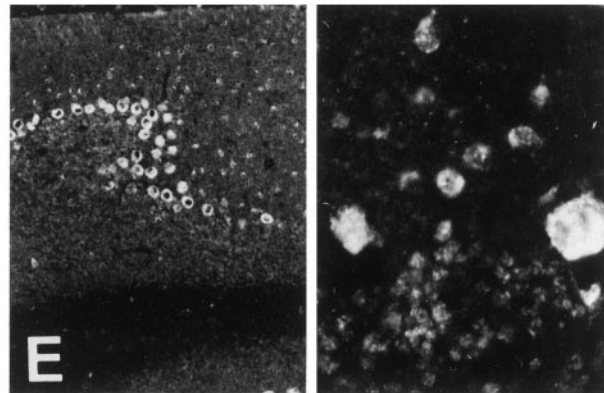
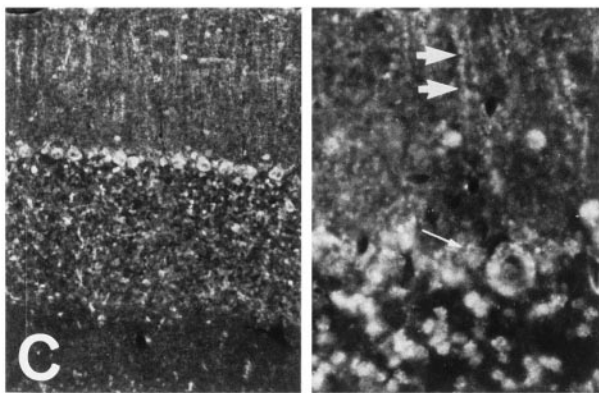
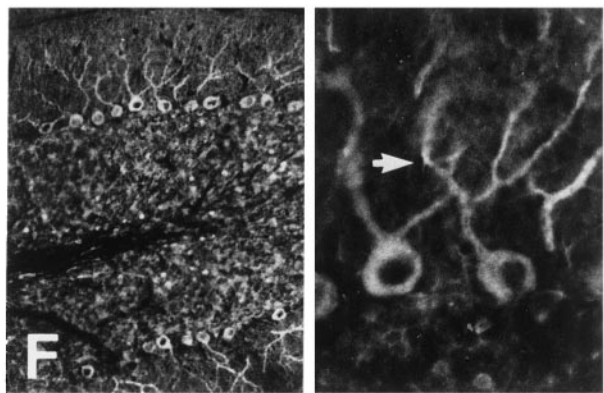
Bergmann glial processes. In the PCL, MMP 2 (Fig. 4*A*), MMP 3 (Fig. 4*B*), MMP 9 (Fig. 4*C*), TIMP 1 (Fig. 4*D*), TIMP 2 (Fig. 4*E*), and TIMP 3 (Fig. 4*F*) were expressed in PK cell somata, and MMP 9 (Fig. 4*C*) and TIMP 1 (Fig. 4*D*) were still detectable in the Bergmann glial somata. In the IGL, MMP 3 (Fig. 4*B*), MMP 9 (Fig. 4*C*), TIMP 2 (Fig. 4*D*), and TIMP 3 (Fig. 4*F*) were strongly expressed in many granular cells, whereas TIMP 1 (Fig. 4*D*) was poorly expressed, except along large unidentified fibers

in the IGL and in the white matter, probably corresponding to PK cell axons.

#### Spatiotemporal distribution of MMP 9, TIMP 2, and TIMP 3 mRNA expression in the P10 and adult rat cerebellum

MMP and TIMP transcriptional expression was investigated to determine whether protein expression corresponded to cellular



**MMP 2****TIMP 1****MMP 3****TIMP 2****MMP 9****TIMP 3**

**Figure 4.** Immunohistochemical distribution pattern of MMP 2 (*A*), MMP 3 (*B*), MMP 9 (*C*), TIMP 1 (*D*), TIMP 2 (*E*), and TIMP 3 (*F*) in adult rat cerebellar cortex. Ten micrometer frontal sections of adult rat cerebellum showed the EGL, the ML, the PCL, and the IGL. Labeling with polyclonal antibodies was amplified by using tyramide signal amplification (New England Nuclear). Note, for each marker, the cytoplasmic staining at the adult age compared with the diffuse staining seen on the developmental stages P10 and P15. In the ML, MMPs 3 and 9 and TIMPs 1, 2, and 3 protein expression was lost in PK cell arborization. However, MMP 9 expression persisted in the Bergmann glial fibers. *Double arrows* point to one fiber in *C*. Only the anti-TIMP 3 antibodies labeled PK cells dendrites. *Arrow* indicates TIMP 3 dendritic immunolabeling in *F*. Interneurons of the ML were labeled for MMPs 3 and 9 and TIMPs 1 and 2. In the PCL, all markers were detected in PK cell somata. In addition, MMP 9 and TIMP 1 were seen in the Bergmann glial cell bodies surrounding the PK cell somata. In the IGL, MMPs 3 and 9 and TIMPs 2 and 3 were strongly expressed in many granular cells, whereas TIMP 1 was poorly expressed. Magnification of the *left* of each panel, 100 $\times$ . Magnification of the *right* of each panel, 400 $\times$ . Scale bars, 100  $\mu$ m.

synthesis. Expression of MMP 9, TIMP 2, and TIMP 3 mRNAs was studied by *in situ* hybridization in the P10 and adult rat cerebellum (Fig. 5). Regional mRNA distribution in the cerebellar layers was analyzed using autoradiographic film, and the cellular transcriptional distribution was determined using the same sections coated with emulsion. The regional and cellular differences between MMP 9, TIMP 2, and TIMP 3 mRNA patterns were in general agreement with the immunohistochemical patterns. However, TIMP 3 mRNA was absent from the dendrites of PK cells, and thus, did not colocalize with its encoded protein in this area.

On P10, the EGL was significantly labeled for MMP 9 mRNA over the whole cerebellar cortex on autoradiographic film. MMP 9 mRNA was faintly detectable in the ML, whereas the PCL and IGL were strongly stained (Fig. 5A). In the adult, labeling was detected in the PCL and IGL (Fig. 5B). MMP 9 transcripts were confined to ML interneurons and PK somata (Fig. 5C).

On P10, TIMP 2 mRNA was detected in the whole EGL and in the PCL on autoradiographic film (Fig. 5D). In the adult, strong labeling was seen in the PCL and IGL (Fig. 5E). At high magnification, ML interneurons and the whole PK cell layer were shown to be labeled. Some clusters of granular cells were also stained in the IGL (Fig. 5F).

On P10, strong expression of TIMP 3 transcripts was noticed in the EGL, whereas the ML was unstained and appeared as a black line (Fig. 5G). The PCL and IGL were strongly labeled. In the adult, labeling was localized to the PCL and IGL (Fig. 5H). At high magnification, staining of ML interneurons and PK cell somata was seen (Fig. 5I).

## DISCUSSION

Cerebellar ontogenesis is characterized by postnatal directional migration, differentiation, and synaptogenesis with precise spatiotemporal order of positioning, leading to three highly organized cellular layers. In the adult, the cerebellar cortex requires synaptic remodeling of PK cell inputs for motor tasks and the learning of complex motor skills. MMPs, by focalized and controlled proteolysis, may be crucial in determining the hierarchy of processes involved in brain development and plasticity. This hypothesis was supported by the differential and spatiotemporal expression of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 during cerebellar development. These regional and cellular expression patterns are evidence for tightly regulated proteolysis-mediated alteration of ECM components, which might be related to the migration of granular precursors and PK cell arborization.

### Differential expressions of MMPs 2, 3, and 9 in granular precursors and PK cells in the rat developing cerebellum

Between birth and P14, the EGL of the cerebellum contains rapidly dividing precursor cells that migrate inward and cross the expanding ML to reach their final position in the IGL (Rakic, 1971; Komuro and Rakic, 1998), the peak of this migratory activity occurring between P7 and P10 (Altman, 1972a). The expression of MMPs 3 and 9 on P10 in EGL cells may be related to the migration of granular cell precursors. In fact, it has been demonstrated that these molecules are required for cell migration in the morphogenesis of the mandibular arch (Chin and Werb, 1997). MMPs act by degrading or activating cell surface proteins and ECM proteins, revealing cryptic domains that signal in a different manner to the intact protein. Interestingly, MMP 3 is known to cleave tenascin into different domains (Imai et al.,

1994). These different domains in the fibronectin type III repeats of tenascin promote the migration of granular cells in the EGL (Chuong et al., 1987; Bartsch et al., 1992) and neurite outgrowth (Husmann et al., 1992). In addition, MMP 3 and MMP 9 are able to cleave laminin, another ECM molecule, which contains a domain on its B2 chain that inhibits the migration of granular cells (Liesi et al., 1992) and which has been localized to the PK/Bergmann cell area of the developing cerebellum (Liesi et al., 1995). Thus, MMPs 3 and 9 could be involved in the migration of cerebellar cell precursors by causing local proteolysis and thus modifying cell signaling by the presence or absence of ECM molecules in different parts of the cerebellum. This hypothesis is corroborated by the dramatic decrease in MMP 9 activity on P15, when the rate of granular precursors migration declines (Altman, 1972a).

In the developing ML, granular cell migration is guided by surface-mediated interactions with Bergmann glial processes (Rakic, 1971, 1990; Edmondson and Hatten, 1987); MMP 9, specifically expressed in Bergmann glial fibers, could contribute to this migration.

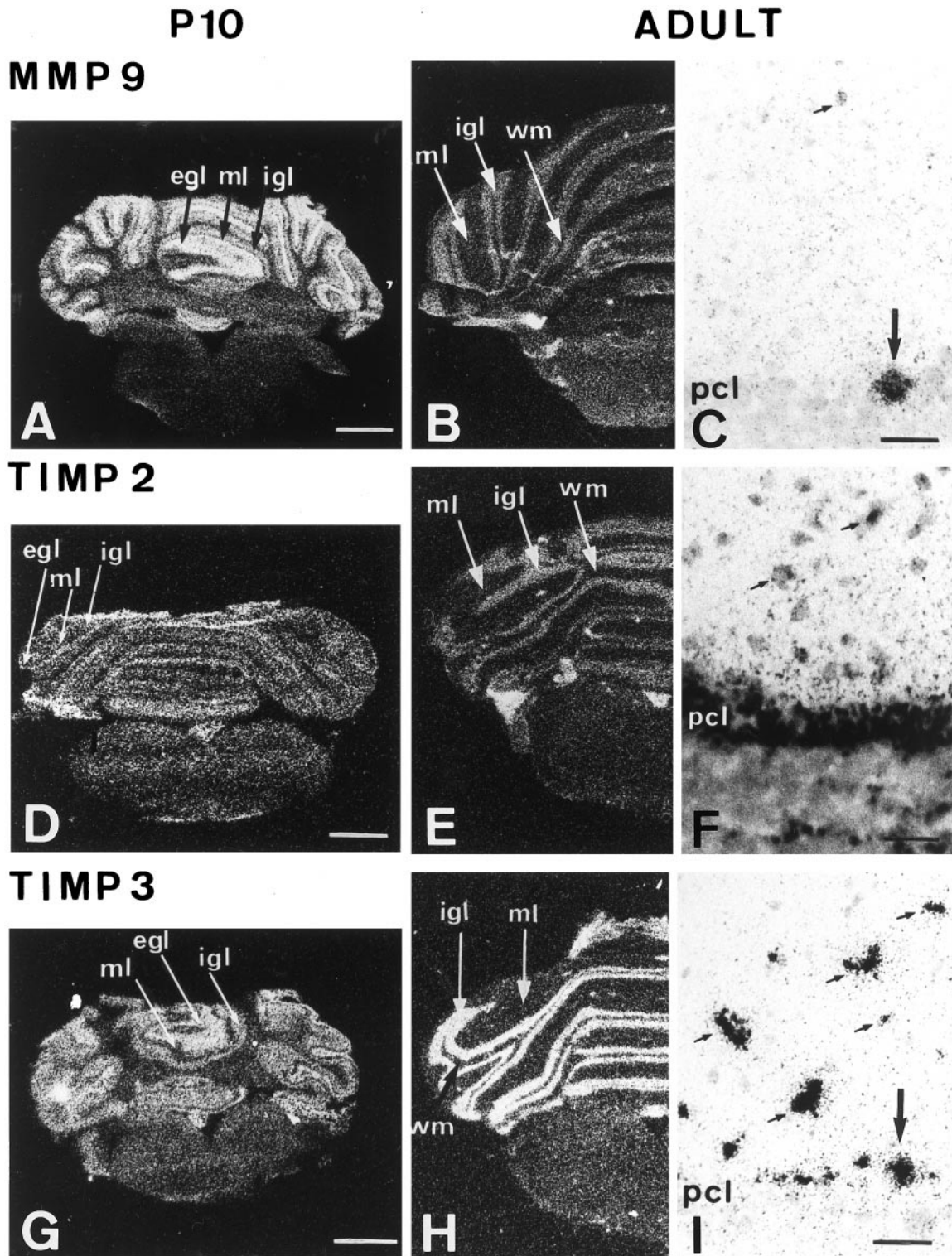
In contrast, despite its high activity revealed by zymography, MMP 2 was confined to PK cell bodies and was undetectable in the EGL and ML at all stages of development. MMP 2 has the unique ability of being activated at the cell surface in response to particular matrix proteins (Deryugina et al., 1997, 1998; Strongin et al., 1995). Its ability to cleave the fibroblast growth factor receptor (FGFR) (Levi et al., 1996) could be of interest in the context of the cerebellum, because FGFR and FGF expression is tightly regulated in the developing postnatal cerebellum in relation to proliferation and migration (Hatten et al., 1988; Tao et al., 1996; Hattori et al., 1997).

The expression of MMPs 3 and 9 in PK cell bodies and their proximal dendrites on P10 and P15 coincides with the arborization of PK cells (confirmed by calbindin-D immunolabeling of the PK dendritic tree; our unpublished observations), when parallel fibers start to form synapses with the outgrowing dendritic spines and the lower stellate cells begin to establish connections (Altman, 1972b). The strong diffuse expression of MMPs 3 and 9 in this area, compared with that in the adult cerebellum, may reflect the release of the enzyme into the pericellular environment of the PK cells. A role for metalloproteinases in axonal growth and pathfinding has recently received support from findings showing that axons stall and fail to extend through the nerve cord in the metalloproteinase gene-defective *Drosophila* mutant, Kuzbanian (Fambrough et al., 1996). In addition, MMP 3 has been shown to mediate PC 12 cell growth cone penetration through basal laminae (Nordstrom et al., 1995), whereas metalloproteinase inhibitors inhibit growth cone retinal activity (Sheffield et al., 1994). Thus, axonal growth might require spatial and temporal regulation of ECM degradation at the cell surface, and the growing dendrites and growth cones may use focalized proteolysis in a similar way to that proposed for other migrating cells (Basbaum and Werb, 1996).

### Expression of TIMPs 1, 2, and 3 in relation to MMPs in the rat developing cerebellum

TIMPs 1, 2, and 3 are equally potent against all active forms of these enzymes (Goldberg et al., 1989; Kleiner and Stetler-Stevenson, 1993). Nevertheless, TIMP 1 or TIMP 2 preferentially complex with the proenzyme form of MMP 9 or MMP 2, respectively (Goldberg et al., 1992; Strongin et al., 1993). The colocalization of MMPs 3 and 9 with TIMPs 1, 2, and 3 in PK cell





**Figure 5.** Differential distribution of MMP 9 (*A–C*), TIMP 2 (*D–F*), and TIMP 3 (*G–I*) transcripts in the developing and adult rat cerebellum. *In situ* hybridization was performed on frontal rat cerebellum sections (12  $\mu$ m) using antisense MMP 9, TIMP 2, and TIMP 3 mRNA oligoprobes provided by Biognostik. Autoradiographic film images (*A, B, D, E, G, H*) were obtained by exposure for 5 d on Hyperfilm- $\beta$ max film (Amersham). The exposure time for the emulsion-coated sections (*C, F, I*) was 6 weeks. On P10, MMP 9 mRNA was intensely expressed in the whole EGL and weakly expressed in the ML, PCL, and IGL (*A*). In the adult, the global labeling was restricted to the PCL and IGL (*B*). MMP 9 mRNA subcellular expression was detected in ML interneurons (*small arrows*) and PK cell somata (*large arrows*) (*C*). TIMP 2 mRNA expression was mainly observed in the EGL on P10 (*D*) and in the PCL and IGL in the adult (*E*). On emulsion-coated sections, TIMP 2 was visible in the ML interneurons (*small arrows*), in the border of PK cell somata, and in the IGL (*F*). On P10, TIMP3 transcript expression was high in the EGL (*G*). No expression of TIMP 3 was seen in the ML on autoradiographic films, but labeling for TIMP 3 was seen in the PCL and IGL of the 10 d cerebellum (*G*). In the adult, labeling was faint in the ML and high in the PCL and IGL (*H*). TIMP 3 mRNA was seen in the ML interneurons (*small arrows*) and in some PK cell somata (*large arrow*) (*I*). WM, White matter. Scale bars: *A, D, G*, 1 and 3 mm; *C, F, I*, 40  $\mu$ m.



bodies at each stage of cerebellar development and in the EGL on P10 suggests an exquisite balance between the enzymes and their inhibitors, which controls the activation and stability of gelatinases, allowing controlled focalized proteolysis, crucial for granular migration and synaptogenesis.

Interestingly, TIMP 1 (and only TIMP 1) is expressed on P15 in clusters of PK cell bodies, distributed in alternating longitudinal stripes. A similar heterogeneity has been seen during postnatal development in the expression of certain proteins, such as homologs of segment-polarity proteins (Oberdick et al., 1993), and adhesion–guidance molecules (Chedotal et al., 1996; Arndt and Redies, 1998). The latter are considered to be recognition signals involved in the formation of the climbing fiber projections emerging from the inferior olive and pointing to the PK cells (Wassef et al., 1992). As TIMP 1 expression coincides with maturation of the climbing fiber afferent system (Mason and Gregory, 1984; Sotelo et al., 1984), this molecule could be involved in olivocerebellar connections.

Independent of its inhibitory effect on MMPs, there is evidence that TIMP 2 may act as a cytokine and stimulate cell proliferation (Hayakawa et al., 1994; Corcoran et al., 1995). This could be relevant to the TIMP 2 expression seen in the EGL (an idea further supported by the absence of MMP 2) at a time when the proliferation and migration of granular cells peak (Rex et al., 1998). In addition, TIMP 3 expression presents a unique pattern localized along the dendrites of PK cells. Unlike TIMPs 1 and 2, which are secreted in a soluble form (Polette et al., 1994), TIMP 3 is associated with the ECM (Leco et al., 1994) and inhibits MMPs 1, 2, 3, and 9 (Apte et al., 1995; Will et al., 1996). In addition, TIMP 3 inhibits the adhesion of melanoma cells to collagens and fibronectin (Ahonen et al., 1998). Thus, in addition to balancing MMP activity during PK cell maturation, TIMP 3 could reduce the attachment of PK cell dendrites to the ECM, facilitating arborization of PK cells and synaptogenesis. As TIMP 3 is restricted to a few tissues that exhibit high metabolic activity or rapid turnover (Zeng et al., 1998), its specific distribution in PK dendrites in the adult cerebellum suggests that TIMP 3 expression could facilitate cerebellar plasticity.

### Expression of MMPs 2, 3, and 9 and TIMPs 1, 2, and 3 in the adult cerebellum

The adult cerebellum is an area of plasticity that supports the learning of specific motor tasks (Gilbert, 1974). This plasticity is associated with an increase in the number of synapses per PK cell (Black et al., 1990). Several proteases, such as calpain and plasminogen activator, which are implicated in synaptic plasticity (Denny et al., 1990; Huang et al., 1996) and induced in PK cells (Seeds et al., 1995, 1996), act by degrading cell surface receptors and cell adhesion molecules to eliminate synapses or ECM molecules, thus exposing cryptic adhesion sites for new synapse formation. Thus, MMPs 3 and 9, which are activated by the plasminogen–plasmin system, and TIMPs 2 and 3, present in the adult cerebellum in which extracellular matrix remodeling is still important, may play a role in synaptic integrity and plasticity.

In conclusion, the differential expression patterns of the various MMPs and TIMPs provides a new insight into the focalized and controlled proteolysis occurring during cerebellar development. The spatiotemporal regulation of MMPs–TIMPs may be crucial in granular cell migration and the maturation of PK cells and, more generally, in brain maturation and plasticity.

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