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# Variable and multiple expression of *Protease Nexin-1* during mouse organogenesis and nervous system development

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## SUMMARY

**Protease Nexin-1 (PN-1) also known as Glia-Derived Nexin (GDN) inhibits the activity of several serine proteases including thrombin, tissue (tPA)- and urokinase (uPA)-type plasminogen activators. These and other serine proteases seem to play roles in development and tissue homeostasis. To gain insight into where and when PN-1 might counteract serine protease activities in vivo, we examined its mRNA and protein expression in the mouse embryo, postnatal developing nervous system and adult tissues. These analyses revealed distinct temporal and spatial *PN-1* expression patterns in developing cartilage, lung, skin, urogenital tract, and central and peripheral nervous system. In the embryonic spinal cord, *PN-1* expression occurs in cells lining the neural canal that are different from the cells previously shown to express *tPA*. In the developing postnatal brain, *PN-1* expression appears transiently in many neuronal cell populations. These findings suggest a role for PN-1 in the maturation of the central nervous system, a phase that is accompanied by the appearance of different forms of PN-1. In adults, few distinct neuronal cell populations like pyramidal cells of the layer V in the neocortex retained detectable levels of *PN-1* expression. Also, mRNA and protein levels did not correspond in adult spleen and muscle tissues. The widespread and complex regulation of *PN-1* expression during embryonic development and, in particular, in the early postnatal nervous system as well as in adult tissues suggests multiple roles for this serine protease inhibitor in organogenesis and tissue homeostasis.**

Key words: protease inhibitor, nervous system, mouse development, *PN-1*

## INTRODUCTION

Serine proteases and their corresponding inhibitors seem particularly well suited for regulating molecular events in developmental processes and/or tissue homeostasis. (i) In *Drosophila*, dorsoventral pattern formation requires *snake* and *easter* whose gene products are homologous to vertebrate serine proteases (DeLotto and Spierer, 1986; Hecht and Anderson, 1992). (ii) Serine proteases are secreted by cells generally as inactive zymogens that require cleavage, often by another serine protease, to yield the active enzyme. (iii) Several serine proteases including thrombin, tissue (tPA)- and urokinase (uPA)-type plasminogen activator have specific receptors on the surface of many cell types (Plow et al., 1986; Nielsen et al., 1988; Verrall and Seeds, 1988; Pittman et al., 1989; Vu et al., 1991). The binding of active proteases or their corresponding zymogens to the matrix or cell surface may provide a reservoir of spatially confined proteolytic activity but may also prevent inhibition of proteases (Hajjar et al., 1987; Knecht, 1988). (iv) Gene expression of both *tPA* and *uPA* occurs during embryonic development (Menoud et al., 1989; Sappino et al., 1989; Sumi et al., 1992). (v) Thrombin provokes potent and diverse effects in many cell types. For some cells, it can serve as a mitogen (Carney and Cunningham, 1978; Shuman, 1986) while, in primary neuronal, astroglial, neuroepithelial and neuroblastoma cells, it can inhibit morphological differentiation (Monard et al., 1983; Hawkins and Seeds, 1986; Gurwitz and Cunningham, 1988; Grand et al., 1989; Nelson and Siman, 1990; Jalink and Moolenaar, 1992; Suidan et al., 1992). Thrombin-mediated inhibition of neurite outgrowth is known to involve cleavage and activation of its seven-transmembrane domain receptor (Jalink and Moolenaar, 1992; Suidan et al., 1992). In the developing nervous system, several serine proteases might cooperate to regulate neurite outgrowth (Monard et al., 1983; Hawkins and Seeds, 1986; Monard, 1988; Pittman et al., 1989), growth of commissural axons (Sumi et al., 1992), synapse elimination (Vrbova and Lowrie, 1988; Hantai et al., 1989) and the formation and maintenance of the neuromuscular junction (Festoff et al., 1991). In the adult, their hydrolytic activity might provoke molecular and cellular changes asso-

ciated with synaptic plasticity and learning (Fazeli et al., 1990; Qian et al., 1993).

To control serine protease activities in such diverse processes, several inhibitors seem to have evolved. One of these is Glia-Derived Nexin (GDN), a $43\times10^3$ $M_r$ protein also known as Protease Nexin-1 (PN-1) (Baker et al., 1980; Guenther et al., 1985; Gloor et al., 1986; McGrogan et al., 1988). GDN/PN-1 can modulate the proteolytic activity of thrombin, tPA, uPA and trypsin (Baker et al., 1980; Guenther et al., 1985; Stone et al., 1987). GDN/PN-1-mediated inhibition of thrombin is specifically enhanced by heparin, mainly produced by mast cells, and by heparan sulphate or chondroitine sulphate (Farell and Cunningham, 1986, 1987; Stone et al., 1987) components of the extracellular matrix (Lindahl and Höök, 1978). Interestingly, heparan sulphate is known to undergo agrin-mediated concentration at synapses including the neuromuscular junction (Nastuk and Fallon, 1993), where GDN/PN-1 has also been shown to be preferentially located (Festoff et al., 1991).

In culture, GDN/PN-1 is secreted by a variety of cell types including fibroblasts, astrocytes, glioma cells (Monard et al., 1973; Baker et al., 1980; Guenther et al., 1985; Rosenblatt et al., 1987) and some neuroblastoma cells (Reinhard et al., unpublished data). Knowledge of the regulation and expression of *GDN/PN-1* in vivo is limited. In rodents, *GDN/PN-1* expression has been shown mainly in parts of the genital tract and in the primary olfactory pathway, tissues rich in serine proteases (Reinhard et al., 1988; Vassalli et al., 1993). Enhanced expression of *GDN/PN-1* mRNA and protein occurs both in the central (CNS) and in the peripheral (PNS) nervous system upon lesion (Meier et al., 1989; Hoffman et al., 1992). Since GDN/PN-1 promotes neurite outgrowth (Monard et al., 1983; Guenther et al., 1985; Gurwitz and Cunningham, 1988) as well as astrocyte stellation (Cavanaugh et al., 1987) by counteracting thrombin, it seems likely that it can fine tune the action of serine proteases directing cytoarchitectural and histoarchitectural plasticity. To gain insight into where and when *GDN/PN-1* might act, in particular in the developing nervous system, we determined its spatial and temporal expression pattern in the mouse. We found the protein expressed in several tissues and cell types in the mouse and several forms of the protein were detected in some organs. For these reasons, we propose to use the more general term of Protease Nexin-1 (PN-1).

## MATERIALS AND METHODS

### Preparation of embryos and brains

Mouse embryos were obtained from matings of superovulated CB6 $F_1$ females with C57BL/6 males. Midday after vaginal plug formation was designated E0.5 and the day of birth was considered P0. For northern and immunoblot analyses, brains and other organs were collected, flash frozen in liquid nitrogen and stored at −70˚C. In situ hybridisation experiments were carried out on mouse embryos collected at developmental stages E7.5 up to E18.5 and on mouse brains collected from birth up to adulthood. For this purpose, embryos were prefixed in utero by perfusion of pregnant females with a cold solution of 4% paraformaldehyde in PBS. Postnatal (<3 weeks of age) and adult mice were also perfused with a 4% paraformaldehyde solution in PBS before dissection of their brain. Embryos and brain tissue were then fixed overnight at 4˚C in a freshly prepared solution of 4% paraformaldehyde in PBS, cryoprotected by an overnight incubation at 4˚C in 30% sucrose in PBS and sectioned.

### Northern blot analysis

Total RNA was extracted from frozen embryos, postnatal and adult brains and adult mouse organs by homogenisation in guanidinium thiocyanate solution using a Polytron as described by Chomczynski and Sacchi, 1987. Samples of 10 μg of total RNA were electrophoresed on 1.2% agarose-formaldehyde gels, and transferred to nylon membrane (Gene Screen, NEN) using 10× SSC. Membranes were hybridised overnight at 45˚C in hybridisation solution (50% formamide, 5× SSC, 5× Denhart's reagent, 10 mM EDTA, 0.1 mg/ml denatured salmon sperm DNA and 1% SDS) containing $10^6$ counts/minutes/mlof a $^{32}$P-labelled rat *PN-1* cDNA probe corresponding to a 1468 bp *Xho*I-*Xba*I DNA fragment (Gloor et al., 1986). Membranes were then washed 3 times for 40 minutes at 45˚C in 1× SSC, 1% SDS and exposed overnight with a Kodak XAR film. Loading and transfer of equal amounts of RNA was confirmed by methylene blue staining of the ribosomal RNA (rRNA) before hybridisation of the membranes.

### SDS gels and immunoblotting

Mouse tissues were homogenised in 10 mM Hepes, containing 0.32 M sucrose, then mixed with 10 mM Tris-HCl pH 6.8, 1% SDS, 4% glycerol and boiled 5 minutes at 95˚C. Protein concentration in the supernatants was determined according to Schaffner and Weissmann (1973), using bovine serum albumin (BSA) as standard. 100 μg of total protein per lane was separated overnight at 4˚C on 10% polyacrylamide/0.3% bisacrylamide gels in 25 mM Tris, 0.2 M glycine, 1% SDS, and transferred onto nitrocellulose membrane (Schleicher and Schuell) at 80 V for 1.2 hours in 25 mM Tris, 0.2 M glycine, 0.1% SDS, and 10% methanol. Membranes were blocked for non-specific antibody binding in 1% BSA in TBS for 3 hours at 37°C, incubated 2 hours at room temperature with a 1:1000 dilution of a mouse anti-rat PN-1 monoclonal antibody (4B3, 26.2 mg/ml) (Meier et al., 1989) in 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.25% BSA, 0.5% Nonidet P40 (TENB-N) containing 5% fetal calf serum and washed with TENB-N. They were then incubated for 1 hour at room temperature with a 1:200 dilution of biotinylated goat anti-mouse IgG (SPA, Milano, Italy), washed and then incubated with a 1:200 dilution of streptavidin-biotinylated alkaline phosphatase complex (Bio Division) in TENB-N containing 5% fetal calf serum. Immunoreactivity was revealed by incubating the membranes in 100 mM Tris-HCl pH 8.8, 100 mM NaCl, 5 mM $MgCl_2$ with 0.3 mg/ml Nitro Blue Tetrazolium (Sigma) and 0.15 mg/ml of 5-bromo-4-chloro-3-indolylphosphate (Boehringer Mannheim).

### In situ hybridisation

Before sectioning, tissues were embedded in Tissue-Tek OCT compound (Miles). 10 μm cryostat sections were collected on glass slides previously treated with 3-aminopropyltriethoxysilan (Rentropp et al., 1986), and immediately dried for 2 minutes at 50˚C before storage at −70°C in boxes containing silica gel. Before hybridisation, sections were postfixed 10 minutes in 4% paraformaldehyde in PBS, rinsed in PBS, depurinated 20 minutes in 0.2 M HCl at room temperature, treated 30 minutes in 2× SSC at 70˚C and serially dehydrated in ethanol. Sections were then prehybridised for 3 hours at 54˚C in a solution containing 50% formamide, 10% dextran sulfate, 1× salt buffer (0.3 M NaCl, 10 mM Tris-HCl pH 6.8, 10 mM $NaH_2PO_4$, 5 mM EDTA, 0.02% polyvinylpyrrolidon, 0.02% Ficoll 400, 0.02% BSA), 1 mg/ml *E. coli* RNA, 20 mM dithiothreitol (DTT) and 0.4 μM cold αS-UTP. Sense and antisense RNA probes were synthesised from a 1329 bp *Bam*HI-*Xba*I rat *PN-1* cDNA template subcloned into pSPT 18

plasmid using $^{35}$S-UTP (400 Ci/mmol, Amersham) and SP6 or T7 RNA polymerases (Transcription Kit, Boehringer Mannheim). Probes were subsequently hydrolysed for 40 minutes at 60˚C in 80 mM $NaHCO_3$, 120 mM $Na_2CO_3$, 10 mM DTT, pH 10.2. Sections were hybridised in a humidified hermetic chamber overnight at 54˚C in the same solution as that used for prehybridisation, containing $0.5\times10^6$ counts/minutes/ml of [$^{35}$S]UTP-labelled hydrolysed cRNA probe. Slides were then washed 2 times for 1 hour at 54˚C in 50% formamide, 1× salt buffer (see above) and 10 mM DTT and equilibrated 15 minutes in 0.5 M NaCl, 10 mM Tris-HCl pH 7.6, 5 mM EDTA, 10 mM DTT. Sections were subsequently treated for 30 minutes at 37˚C with 50 μg/mlRNase A (Boehringer Mannheim) in equilibration buffer, rinsed 15 minutes in the same buffer and washed while stirring at room temperature for 1 hour in 2× SSC followed by 1 hour in 0.1× SSC. Sections were serially dehydrated in ethanol containing 300 mM ammonium acetate and air dried, dipped in Ilford K5 Photoemulsion diluted 1:1 in water and exposed 1 week at 4˚C. Finally, they were developed for 3 minutes in Kodak D19 developer, fixed for 10 minutes in 30% sodium thiosulfate and counterstained with haematoxylin/eosin (Sigma).

In situ hybridisation experiments using digoxigenin-labeled *PN-1* cRNA probe were performed according to Schaeren-Wiemers and Gerfin-Moser (personal communication). Hybridisation was performed overnight at 68˚C using 0.5 ng of hydrolysed *PN-1* cRNA probe per μl of hybridisation solution and the color reaction was performed overnight in the dark.

### Immunocytochemistry

For immunocytochemical analyses, embryos, postnatal and adult brains were first incubated at room temperature for 1-2 hours in Cryofix solution (Merck) with shaking. The tissues were subsequently fixed for 4 minutes at 55˚C in a microwave oven (H 2500, Biorad). After cryoprotection overnight at 4˚C in 30% sucrose in PBS, tissues were frozen at −70˚C in isopentane either before or after embedding in Tissue Tek OCT compound (Miles). Immunocytochemistry was performed on 10 μm cryostat sections placed on glass slides treated with 3-aminopropyltriethoxysilan (Rentropp et al., 1986). The sections were dried overnight at room temperature then incubated at room temperature for 30 minutes in 15% (v/v) of a saturated (1.2%) picric acid solution in PBS. The picric acid was removed using three washes of 3 minutes in 70% ethanol in PBS and sections were subsequently fully hydrated by 3× 5 minute incubations in PBS. To block non-specific peroxidase activity in tissues, sections were incubated for 30 minutes in 0.3% $H_2O_2$ and 70% methanol in PBS. After 3× 5 minute washes in PBS, 5 minutes in 0.2% Triton X-100 in PBS followed by 3× 5 minutes in PBS containing 0.5% BSA, sections were overlaid overnight at 4˚C with a solution of PBS with 0.5% BSA containing 10-50 μg/ml of the mouse anti-rat PN-1 monoclonal antibody 4B3 or of an isotype matched control antibody (IgG1). Slides were then washed 3× 5 minutes in PBS with 0.5% BSA at 4˚C, before application of the second antibody (biotinylated anti-mouse IgG from sheep, Amersham) at a dilution of 1:200 in PBS with 0.5% BSA. After a 45 minute incubation at room temperature, sections were washed 3× 5 minutes in PBS with 0.5% BSA and incubated with streptavidin-biotinylated horseradish peroxidase complex (Amersham) diluted 1:100 in PBS with 0.5% BSA for 30 minutes at room temperature. After final washes of 2× 5 minutes in PBS and 2× 5 minutes in 1 M $Na_2HPO_4$, 1 M $NaH_2PO_4$, pH 7.2 (PB), the specific binding of the 4B3 antibody was visualised by incubating the slides in the dark for 8 minutes in PB containing 0.5 mg/ml diaminobenzidine and 0.1% $H_2O_2$. The horseradish peroxidase reaction was stopped by rinsing with PB. Sections were counterstained with Nuclear Fast Red and dehydrated using increasing concentrations of ethanol and finally xylol before mounting.

## RESULTS

### *PN-1* expression occurs in many tissues during embryogenesis

The pattern of *PN-1* expression in foetal development was analysed using sagittal and transversal sections of mouse embryos. Embryos were collected from E7.5 onward when *PN-1* mRNA was first detected using northern blot analysis (result not shown). In each in situ hybridisation experiment, the specificity of the signals obtained with the *PN-1* cRNA antisense probe was shown by comparing signals obtained with a *PN-1* cRNA sense probe on adjacent sections. The latter gave consistently negligible non-specific background hybridisation signals (Fig. 1E,F). PN-1 immunoreactivity was detected using the mouse monoclonal antibody 4B3 (isotype IgG1) raised against rat PN-1. The specificity of signals was verified using an isotype-matched anti-SV40 T antigen antibody and, in parallel, by omitting the first antibody.

At E7.5, *PN-1* mRNA was expressed in both embryonic and extraembryonic structures. Extraembryonic tissues that displayed strong hybridisation signals included the ectoplacental cone and the uterine decidua. Weaker hybridisation signals were also detected in the proper embryonic structures (Fig. 1A,B). In E9.5 embryos, *PN-1* mRNA was found in the neuroepithelium of the closing neural tube (Fig. 1C,D). From E10.5 onward, *PN-1* mRNA expression became apparent in many tissues. Initially, these included the cartilage primordia of the snout, ribs and vertebras and a subset of progenitor cells in the floor plate of the mesencephalon and myelencephalon (Fig. 2A-J). At later stages, *PN-1* mRNA was also detected in developing limbs, vertebras, ribs and skull as well as in tissues including lung, epidermis, heart, genital organs, metanephros, tongue and stomach. Expression was also detected in structures arising from the neural crest like dorsal root ganglia and facial connective tissues (Fig. 2E-L). Except for some protein found in rare cells at E14.5, no *PN-1* expression was found in embryonic liver at any developmental stage (Fig. 3A). Immunocytochemical analyses confirmed the expression of PN-1 protein in essentially all tissues expressing *PN-1* mRNA (Fig. 3).

### *PN-1* expression in the embryonic nervous system

#### Early stages

Formation of the neural tube begins at E8 when the two lateral edges of the neural folds come together in the dorsal midline. In the closing neural tube at E9.5, *PN-1* expression was first observed in the neuroepithelial cells (Fig. 1C,D). At E10.5 when the neural tube is completely closed, *PN-1* mRNA expression is restricted to clusters of ventricular progenitor cells in the floor plate of the mesencephalon and myelencephalon as well as the diencephalon (Fig. 2A,B). At later stages, from E13.5 until birth, *PN-1* expression was extended to all progenitor cells in the floor plate of the mesencephalon and myelencephalon (Fig. 2G-J). Interestingly, at E12.5, expression was also found in progenitor cells of the basal and intermediate plate lining the neural canal in the developing spinal cord. In addition, some cells located

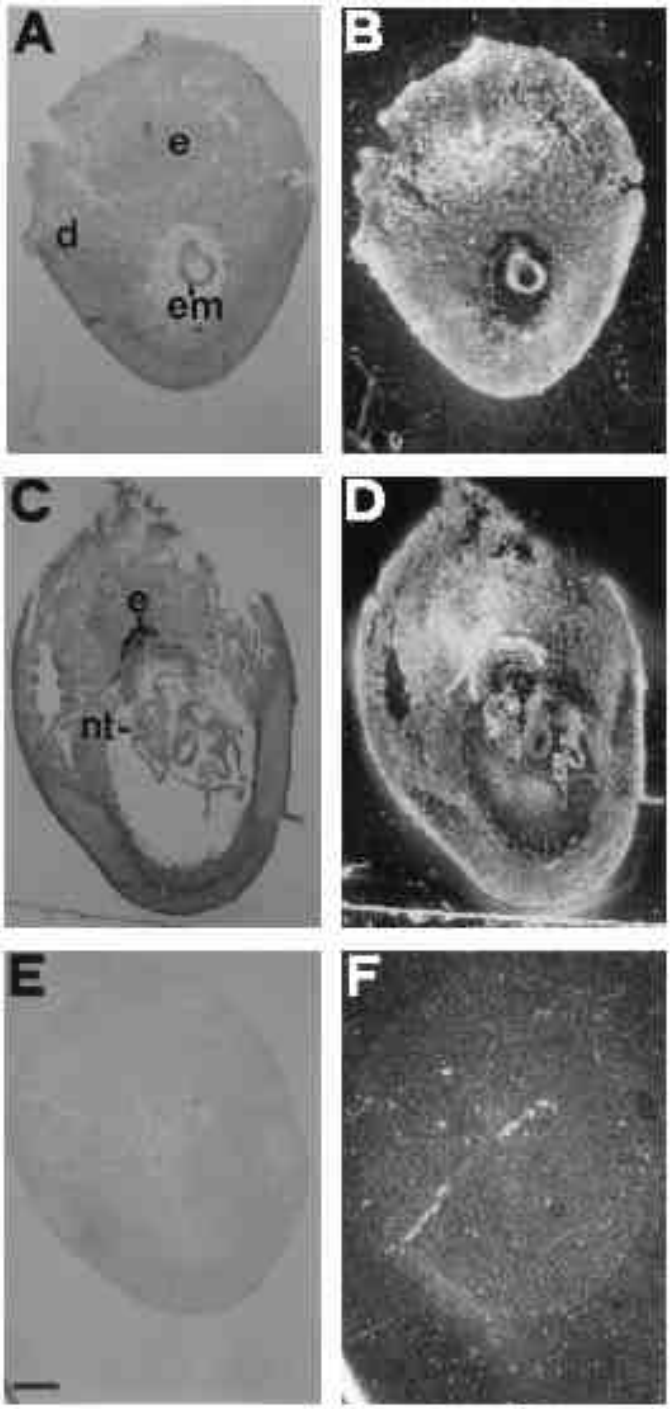


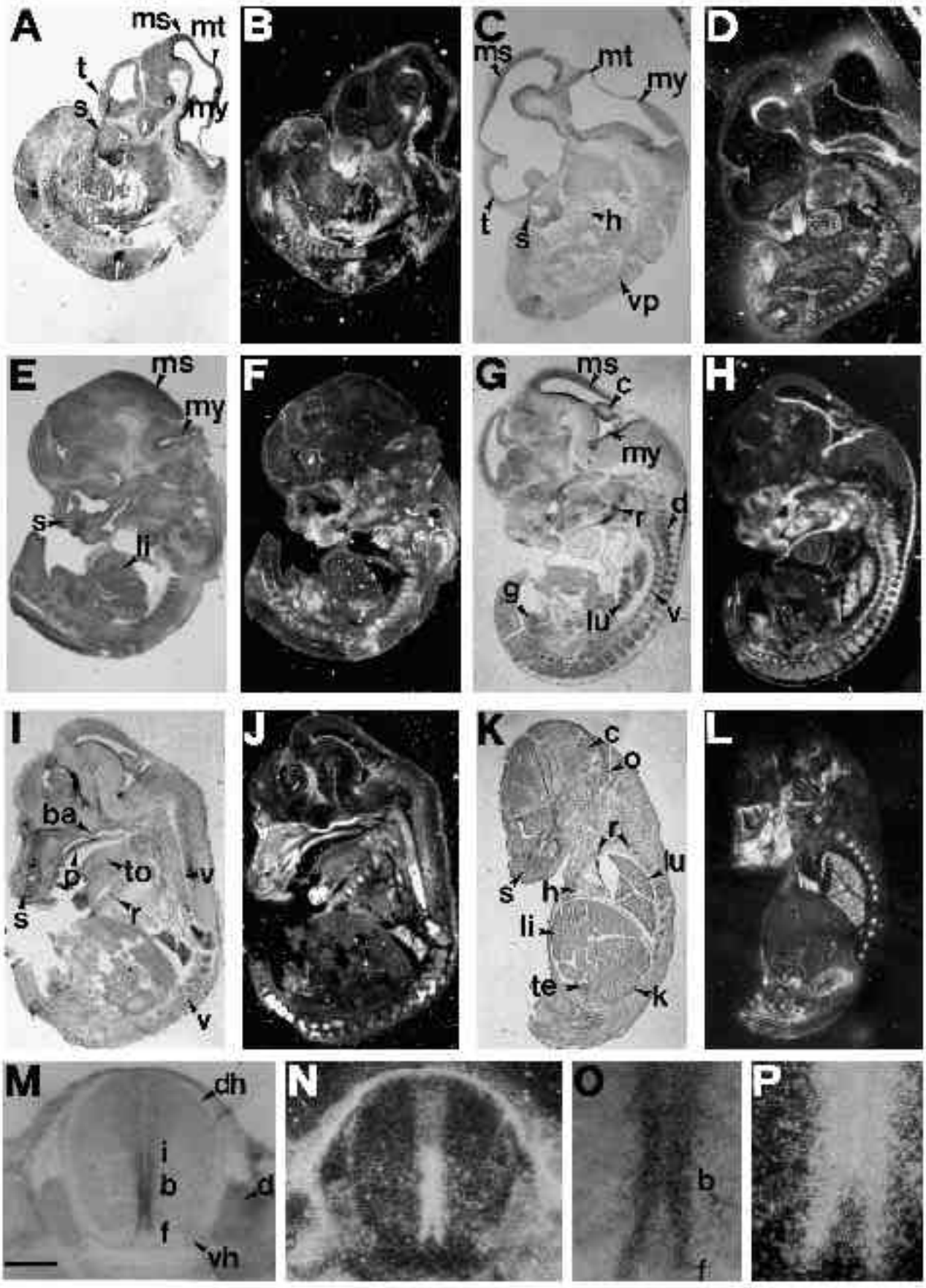

**Fig. 1.** *PN-1* mRNA expression in E7.5 and E9.5 mouse embryos. (A,C) Bright-field and (B,D) the corresponding dark-field images of sections hybridised with the antisense *PN-1* probe. (E,F) Bright and dark-field images of control sections hybridised with the sense *PN-1* probe. (A,B) Transversal section through a E7.5 embryo showing hybridisation signal in the ectoplacental cone and in the embryo. (C,D) Transversal section through a E9.5 embryo. The arrows point to the hybridisation signal in the ectoplacental cone and in the neuroepithelial cells of the closing neural tube. (E,F) Transversal section through a E7.5 embryo. No hybridisation signal was detected using the sense probe. Abbreviations: d, decidua; e, ectoplacental cone; em, embryo; nt, neural tube. Bar, 50 μm.

dorsal to the intermediate plate and virtually all the way up to the roof plate expressed lower but significant levels of *PN-1* mRNA. In contrast, cells in the floor plate completely lacked *PN-1* mRNA (Fig. 2M-P). After E13.5, *PN-1* mRNA could also be detected in structures arising from the rostro-ventral telencephalon that give rise to the olfactory system (Figs 2I-L, 4A-F). No hybridisation signal was detected in the developing neocortex before birth.

### Later stages

During embryonic development, the most interesting *PN-1*-expressing structures to consider are the olfactory bulb and the cerebellum. At E13.5, *PN-1* expression was particularly pronounced in the primitive external plexiform and glomerular zone of the developing olfactory bulb (OB) (Fig. 4A,B). This region is covered two days later by cells derived from the inner ventricular layer that form the primitive layer of olfactory nerve fibers (Hinds, 1968). At E16.5, *PN-1* mRNA was largely restricted to the primitive glomerular layer underlying the newly formed olfactory nerve layer (Fig. 4C,D). This spatially confined distribution of *PN-1* mRNA remained constant during further prenatal and postnatal development of the OB and throughout adult life (Figs 4, 6). In the nasal cavity, neurosensory cells of the olfactory epithelium (OE) sending their axons to the glomerular layer in the OB, also expressed high

**Fig. 2.** *PN-1* mRNA expression during mouse embryonic development. (A,C,E,G,I,K,M,O) Bright-field and (B,D,F,H,J,L,N,P) the corresponding dark-field images. (A,B) Parasagittal section through a E10.5 embryo. (C,D) Sagittal section through a E11.5 embryo. The hybridisation signal was detected in subsets of neuroepithelial cells in the floor plate of the mesencephalon and myelencephalon, in the snout, in the heart and in the vertebra primordium. (E,F) Sagittal section through a E12.5 embryo. *PN-1* mRNA was detected in the snout and in the vertebra primordium. No signal was seen in the liver at any embryonic stage. (G,H) Parasagittal section through a E13.5 embryo where all the neuroepithelial cells in the floor plate of the mesencephalon and myelencephalon expressed *PN-1* mRNA. *PN-1* expression was also seen in the cerebellar plate, lung, dorsal root ganglia, genital tubercle, cartilaginous ribs and vertebras, and in some areas of the skin. (I,J) Parasagittal section through a E14.5 embryo showing a signal also in the cartilage primordium of the snout, the basisphenoid bone and the palate. (K,L) Sagittal section through a E16.5 embryo. *PN-1* mRNA was found in the cerebellum, the lung, the heart, the kidney, the testis and the cartilage of the occipital bone, snout and dorsal part of the ribs. No hybridisation signal was seen in the ossified ventral part of the ribs. (M,N) Transversal section through a E12.5 embryo at the level of the $8^{th}$ and $10^{th}$ thoracic prevertebras showing the developing spinal cord and dorsal root ganglia. A hybridisation signal was detected in neuroepithelial cells of the basal and intermediate plate lining the neural canal. No signal was seen in the floor plate. (O,P) Detail of pictures M and N showing expression in neuroepithelial cells of the basal plate in the spinal cord. Abbreviations: b, basal plate; ba, basisphenoid bone; c, cerebellar primordium; d, dorsal root ganglion; dh, dorsal horn; f, floor plate; g, genital tubercle; h, heart; i, intermediate plate; k, kidney; li, liver; lu, lung; ms, mesencephalon; mt, metencephalon; my, myelencephalon; o, occipital bone; p, palate; r, rib; s, snout; t, telencephalon; te, testis; v, vertebra; vh, ventral horn; vp, vertebra primordium. Bar. (A-H) 1.5 mm; (I,J) 2 mm; (K,L) 2.4 mm; (M,N) 0.3 mm; (O,P) 0.07 mm.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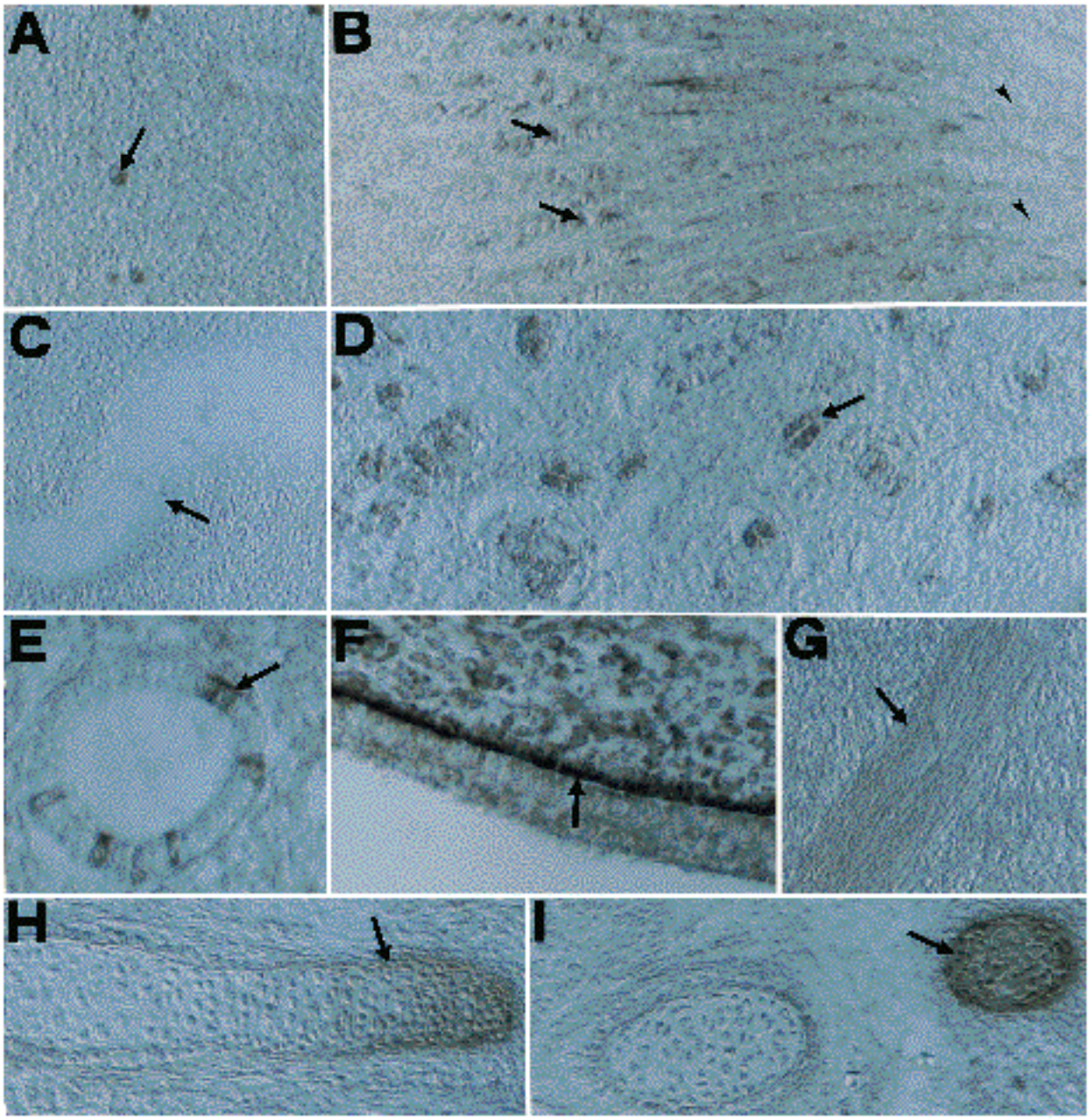


**Fig. 3.** Immunocytochemical analysis of PN-1 expression in E14.5 embryos. Immunocytochemistry was performed as described in Materials and Methods. Tissues shown include (A) foetal liver where only rare cells showed PN-1 immunoreactivity, magnification ×200; (B) developing striated intrinsic muscle in tongue with proliferating myoblasts showing expression (arrow) but myotubes mostly lacking PN-1 immunoreactivity (arrow tip), ×200; (C) olfactory epithelium, which at this stage lacked PN-1 protein (arrow) and mRNA (see Fig. 4A,B), ×200; (D) kidney with PN-1-positive cells confined mostly to primitive glomeruli (arrow) dispersed throughout much of the kidney at this stage, ×200; (E) lung showing a typical bronchiolar structure in which a few discrete cells expressed PN-1 (arrow), ×400; (F) skin with strong PN-1 immunoreactivity predominantly in the basal lamina (arrow), ×400; (G) axon bundles positive for PN-1 in the developing midbrain region probably also including axons (arrow) from layer V cortical neurons projecting subcortically, ×200; (H) cartilage strongly positive for PN-1 (arrow), ×200; whereas (left side of I) the ossified embryonal bone totally lacked PN-1 immunoreactivity, ×200.

levels of *PN-1* mRNA starting around E16.5 (Fig. 4C,D). Earlier in development, the OE did not express detectable *PN-1* mRNA or protein (Figs 3C, 4A,B). Therefore, in the mouse olfactory system, distinct cell populations expressed *PN-1* during development, in particular presynaptic neurosensory cells and cells in the glomerular layer. Mitral cells did not express detectable levels of either *PN-1* mRNA or protein.

In the developing cerebellum at E13.5, cells in the cerebellar anlage expressed *PN-1* mRNA. At E17, when the cerebellar plate is composed of a ventricular germinal layer, a middle mantle and the external granular layer (EGL), cells

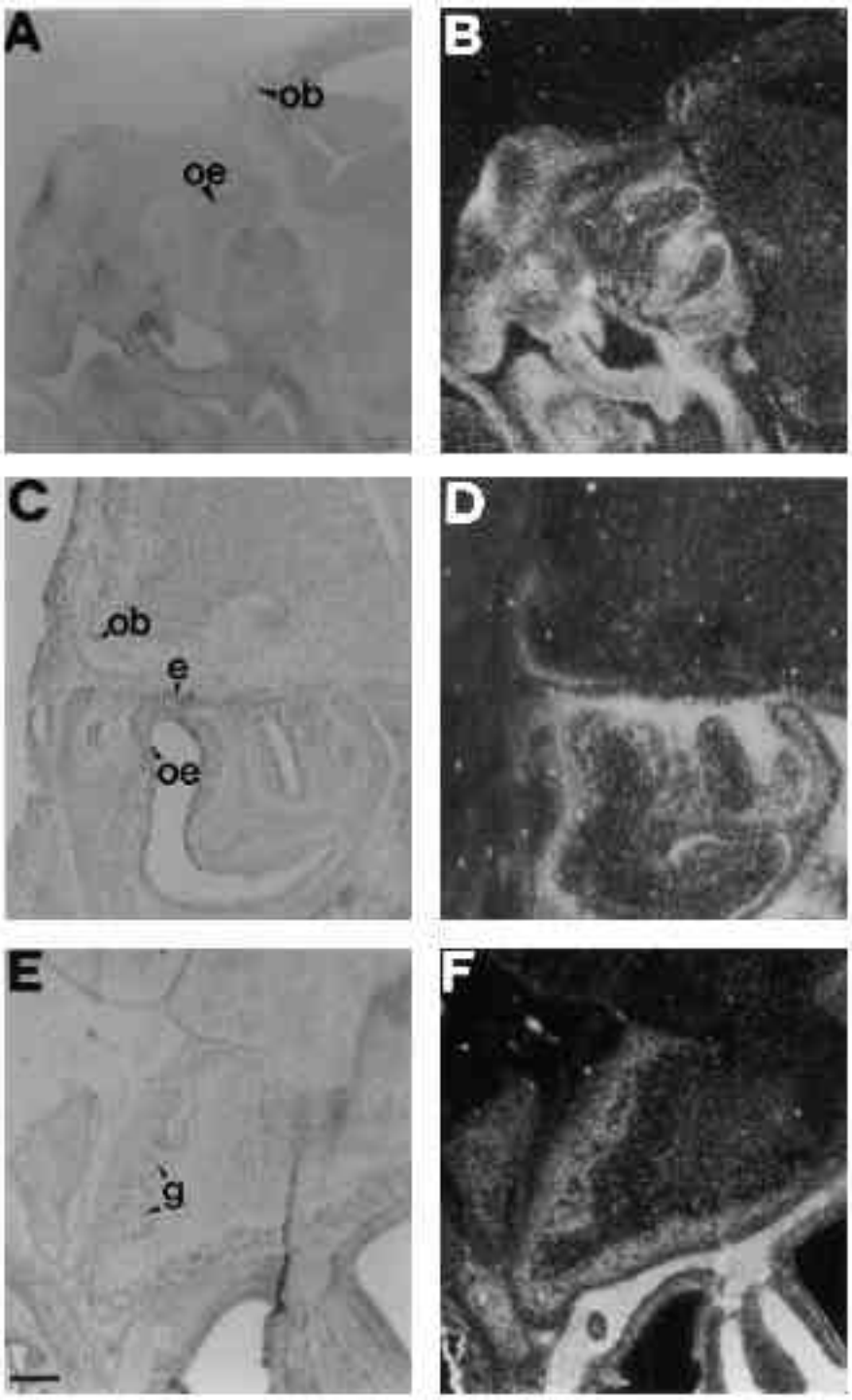


**Fig. 4.** *PN-1* mRNA expression in the embryonic olfactory bulb. (A,C,E) Bright-field and (B,D,F) the corresponding dark-field images. (A,B) Parasagittal section through a E13.5 embryo head showing *PN-1* mRNA expression in the developing olfactory bulb (OB). No hybridisation signal was detected in the olfactory epithelium at this stage. (C,D) Parasagittal section through the telencephalon and nasal cavity of a E16.5 embryo. A strong hybridisation signal was seen in the OB as well as in the olfactory epithelium lining the nasal cavity and in the ethmoid bone. (E,F) Parasagittal section through the OB of a mouse head at birth. *PN-1* mRNA is mainly expressed in the glomerular layer. Abbreviations: e, ethmoid bone; oe, olfactory epithelium; ob, olfactory bulb; g, glomeruli. Bar, (A,B,E,F) 0.1 mm; (C,D) 0.4 mm.

in the plate still showed significant levels of expression. Later on, however, during the major wave of granule cell migration (P3 to P20), when also the differentiation of Purkinje cells is proceeding rapidly, *PN-1* expression was no longer prominent in any layer except for a transient expression in Purkinje cells around P11 (see below and Fig. 8F).

### *PN-1* expression during early postnatal development and in the adult CNS

In the early postnatal brain, *PN-1* mRNA expression increased from P0 to P11, reached maximal levels around P11 and only slightly declined thereafter (Fig. 5A). Interestingly, it was already observed in the rat system that *PN-1* mRNA reached a peak at P12 during postnatal brain development (Gloor et al., 1986). Immunoblot analyses also revealed quantitative changes in PN-1 protein synthesis (Fig. 5B). Like the mRNA, PN-1 protein levels increased in early (P0-P11) postnatal brain tissue, reaching maximal levels at P14. From P14 onward, PN-1 protein levels sharply declined and, in adult brain tissue, little PN-1 protein was

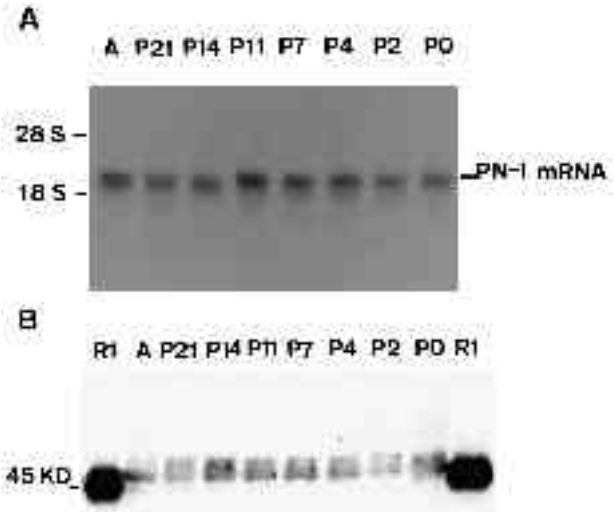


**Fig. 5.** Analyses of *PN-1* expression during postnatal brain development. (A) Northern blot analysis: each lane represents 10 μg of total RNA of postnatal (P) or adult (A) mouse brains. *PN-1* mRNA expression was monitored using a $^{32}$P-labelled rat *PN-1* cDNA probe (see Materials and Methods). Positions of 28 S and 18 S rRNAs are indicated on the left. (B) Immunoblotting: 100 μg of postnatal or adult mouse brain homogenates were loaded in each lane and PN-1 immunoreactivity was revealed using the 4B3 monoclonal antibody raised against rat PN-1. Location of the $45\times10^3$ $M_r$ protein marker is indicated on the left. Abbreviations: A, adult mouse brain; P0-P21, postnatal mouse brains from birth to 21 days after birth. R1 represents 40 ng of purified PN-1 from rat C6 glioma cell conditioned medium (Guenther et al., 1985).

detected. Two major bands were seen in brain tissue (relative molecular mass ($M_r$) : 48 and $46\times10^3$). These different molecular forms of PN-1 include, most likely, molecules bearing post-transcriptional modifications and possibly proteolytic cleavage products (Nick et al., 1990). The determination of the nature and possible functions of these different $M_r$ forms of PN-1 will require further experiments.

The analysis of *PN-1* expression pattern in the postnatal mouse revealed several peculiar features. At P0, *PN-1* mRNA and protein were detected throughout the brain at low (neocortex, cerebellum) or medium-to-high levels (forebrain, midbrain and hindbrain). Within each of these regions, no distinct cell layers appeared to express *PN-1* except the primitive glomerular structures in the OB (Fig. 4E,F) which, at birth, are not yet fully differentiated from the outer plexiform layer. From P2 onward, glomerular structures expressing high levels of *PN-1* mRNA were visible lying superficially to the outer plexiform layer (Fig. 6A-D). In the adult brain, *PN-1* mRNA and protein were expressed mainly in the glomerular region of both the main olfactory bulb (MOB) and the accessory olfactory formation (AOF) (Fig. 6E-H). The AOF is located dorsal and slightly medial, between the MOB and the anterior olfactory nucleus. The sensory epithelium of the AOF is contained in the vomeronasal organ, which is located in the rostral floor of the nasal cavity. The glomeruli of the AOF are smaller and jumbled in contrast to the almost single row arrangement seen in the MOB. In both the main olfactory mucosa and the vomeronasal organ, there is a high turnover of neurosensory cells. Their axons constitute the olfactory and vomeronasal nerves, which synapse upon specialised dendritic arborisations arising from mitral, tufted and periglomerular cells in the MOB and the AOF. Immunoreactivity for PN-1 in these regions is abundant. However, more refined analyses will be necessary to determine the exact nature of all PN-1-positive cell types.

In the P2 brain, *PN-1* mRNA began to appear in some distinct neuronal cell populations in the neocortex, in particular in pyramidal neurons of the layer V. *PN-1* mRNA and protein expression was found as well in subsets of cells in striatum, substantia nigra, thalamus, diagonal band of Broca, cerebellum and cerebellar nuclei, superior and inferior colliculus and brain stem (Fig. 7A; result not shown for the protein). From P4 to P7, *PN-1* expression was even more widespread, in particular in neurons juxtaposing the corpus callosum and in several other neocortical layers (Figs 7B, 8A,B). In the striatum and globus pallidus, expression was quite prominent from P2 to P7 (Figs 7A,B, 8C). In P21 striatum, *PN-1* expression was most important in the matrix (almost no expression was found in the patch compartment) and the homogeneous distribution of the protein throughout this compartment suggests that it might be expressed mainly by glial cells. In the adult striatum, a low number of single cells throughout the matrix compartment still expressed high levels of *PN-1* (Figs 7D, 8I). In the P7 brain, PN-1 protein was also detected in hippocampal neurons but disappeared by P11 (Fig. 8D,E). Around P11, some protein was found in cerebellar Purkinje neurons as well (Fig. 8F), but in adults, only an occasional Purkinje cell showed PN-1 immunoreactivity (result not shown).

In the adult brain, *PN-1* mRNA is distributed mainly in a diffuse manner throughout the tissue (Fig. 7D), a pattern consistent with expression primarily by glial cells. In addition, high levels of mRNA were detected in a few subsets of cells including pyramidal neurons of layer V in the neocortex, cells in the glomerular layer of the OB, in the cerebellar nuclei, in the striatum and in the diagonal band of Broca (Fig. 7D,E). Although levels of *PN-1* mRNA expressed in the adult brain remained high, immunocytochemical and immunoblot analyses showed that PN-1 protein levels were low compared to levels detected in the early postnatal brain. Only cells in the glomerular layer of the MOB and AOF, pyramidal neurons of the layer V in the neocortex and a few cells in the striatum contained detectable PN-1 protein (Figs 6G,H, 8I and results not shown). Therefore, postnatal brain development is accompanied by significant quantitative changes in *PN-1* expression.

### *PN-1* expression in other adult mouse tissues

Northern blot analysis of total RNA extracted from adult mouse tissues revealed the presence of *PN-1* mRNA of uniform size in many tissues (Fig. 9A). In agreement with observations made by Vassalli et al. (1993), the seminal vesicle is a very rich source of *PN-1* mRNA. There, high levels of *PN-1* mRNA were detected in cells of the epithelium, while PN-1 protein was predominantly found at the apical side of these cells : most of it is secreted in the seminal fluid (Fig. 9Ca,b,c,d). In the reproductive mouse system,

*PN-1* mRNA and protein were also found in testis, mainly in interstitial cells between the seminiferous tubules (results not shown for the mRNA and Fig. 9A,B,Ce). Other tissues, including brain (predominantly in the OB), heart, kidney, lung, testis and thymus, also expressed significant levels of *PN-1* mRNA and protein. In contrast, barely detectable levels of mRNA and protein were found in the large intestine, skin, spleen, stomach and tongue. Finally, no mRNA or protein were detected in liver and small intestine (Fig. 9A,B). Interestingly, in some tissues, *PN-1* mRNA levels did not correspond to the amount of protein detected by immunoblot analyses. In adult spleen, for example, significant levels of PN-1 protein were found by immunoblotting, but very low levels of *PN-1* mRNA were detected by northern blot analysis (Fig. 9A,B). The opposite situation was observed in adult skeletal muscle where some *PN-1* mRNA was detected by northern blot analysis whereas no protein was found by immunoblotting. In muscle tissue, nevertheless, PN-1 protein is known to be present at the neuromuscular junction and to colocalise with acetylcholine receptors (Festoff et al., 1991). Therefore, the absence of detected PN-1 immunoreactivity is probably explained by the low amounts of protein present in this organ; these amounts were insufficient to be detected by the procedure used here.

As already noted for the postnatal brain (Fig. 5B), there are variant species of PN-1 protein in several tissues (Fig. 9B). Two distinct PN-1 protein bands (48 and $43\times10^3$ $M_r$) were reproducibly detected in lung and spleen. A third form at $44\times10^3$ $M_r$ was detected in the OB. Immunoblot analyses using isotype-matched antibodies or omitting the first antibody did not reveal any signal around $40\text{-}48\times10^3$ $M_r$, indicating that the above signal represents different molecular forms of PN-1 (result not shown). The immunoblot also seems to indicate that the PN-1 levels could be higher in adult rat brain than in adult mouse brain. This difference could however be due to the fact that the monoclonal antibody 4B3 used was raised against purified rat PN-1. Finally, note that rat and mouse brain PN-1 display slighly different electrophoretic mobilities by immunoblotting. This might reflect differences in post-translational modifications or in primary amino acid sequence since the complete mouse cDNA is not known. Irrespective, these observations suggest that the different molecular forms of PN-1 might play relevant roles in the homeostasis of several tissues.

## DISCUSSION

The present study has revealed an unexpected complexity of *PN-1* gene regulation during embryonic development as well as in early postnatal and adult life. *PN-1* mRNA and protein expression levels did not correspond in some tissues (see e.g. spleen and skeletal muscle; Fig. 9). Furthermore, different molecular weight forms of PN-1 protein were reproducibly detected in several mouse tissues by immunoblotting. So far, only a single *PN-1* mRNA species of uniform size has been detected throughout all tissues. Therefore, the different $M_r$ forms of PN-1 probably represent heterogeneities in post-translational modifications, for example, glycosylation: rat PN-1 contains two putative N-linked glycosylation sites (Sommer et al., 1987). Additional heterogeneity in molecular forms might also occur by limited proteolytic cleavage. Elastase and thrombin can generate clipped forms of rat PN-1 in vitro (Nick et al., 1990). Finally, the existence of mRNA splice variants, not resolved by northern blot analysis, cannot be ruled out. Human PN-1, for example, exists in two forms distinct by one amino acid generated from mRNA splice variants (McGrogan et al., 1988).

### *PN-1* expression in neuronal cells

We have observed that *PN-1* is expressed, albeit mostly in a transient fashion, by a variety of neuronal cell populations during the first few weeks of postnatal brain development. The finding that, in addition to glial cells, neurons can express *PN-1* is in agreement with and extends similar observations made in the rat brain; it is also supported by results showing the constitutive synthesis of PN-1 by rat B104 neuroblastoma cells (Reinhard et al., unpublished data). The widespread neuronal expression during the first few postnatal weeks contrasts sharply with the situation in the adult brain where high levels of *PN-1* mRNA and protein were found only in very specific structures. For example, in postnatal and adult brain, *PN-1* expression was found in pyramidal cells of layer V in the neocortex. Expression in these neurons was prominent first around P2, a time when corticospinal axons undergo interstitial branching to form their corticopontine connections (O'Leary and Stanfield, 1985; O'Leary and Terashima, 1988). High levels of *PN-1* expression during this period would suggest a role in the maturation of the connections. Furthermore, *PN-1* mRNA and protein were also found at high levels in the glomerular structures of both the MOB and the AOF where axons of olfactory receptor neurons synapse with mitral, tufted and periglomerular cells. In the glomeruli, ingrowing axons of olfactory neurons are continuously renewed throughout life and have to establish novel synaptic connections. The postsynaptic structures of the mitral, tufted and periglomerular cells must also undergo remodelling but their dendritic structures within the glomeruli are not lost. Although speculative, high levels of PN-1 within the glomerular structures might serve to create a favorable milieu allowing maintenance of the specialised dendritic structures. The persistence of *PN-1* expression in neurons like pyramidal cells of neocortical layer V in the adult brain would also indicate that *PN-1* might be important for the maintenance of the connections.

In contrast to a limited neuronal expression in the adult brain, expression of *PN-1* was found in various neurons within the first three postnatal weeks. For example from P4 to P7, expression of *PN-1* was detected throughout several neocortical layers. In rodents, all neocortical neurons appear to be generated by birth but migration of cortical cells is not complete until the end of the first postnatal week (Altman and Bayer, 1991). Also, already before or around birth, thalamic axons reach their cortical target zones (Lund and Mustari, 1977). These axons accumulate in the subplate beneath the appropriate region of the cortex and do not penetrate the cortical plate until some time later (Shatz et al., 1988). Once appropriate contacts are established, thala-

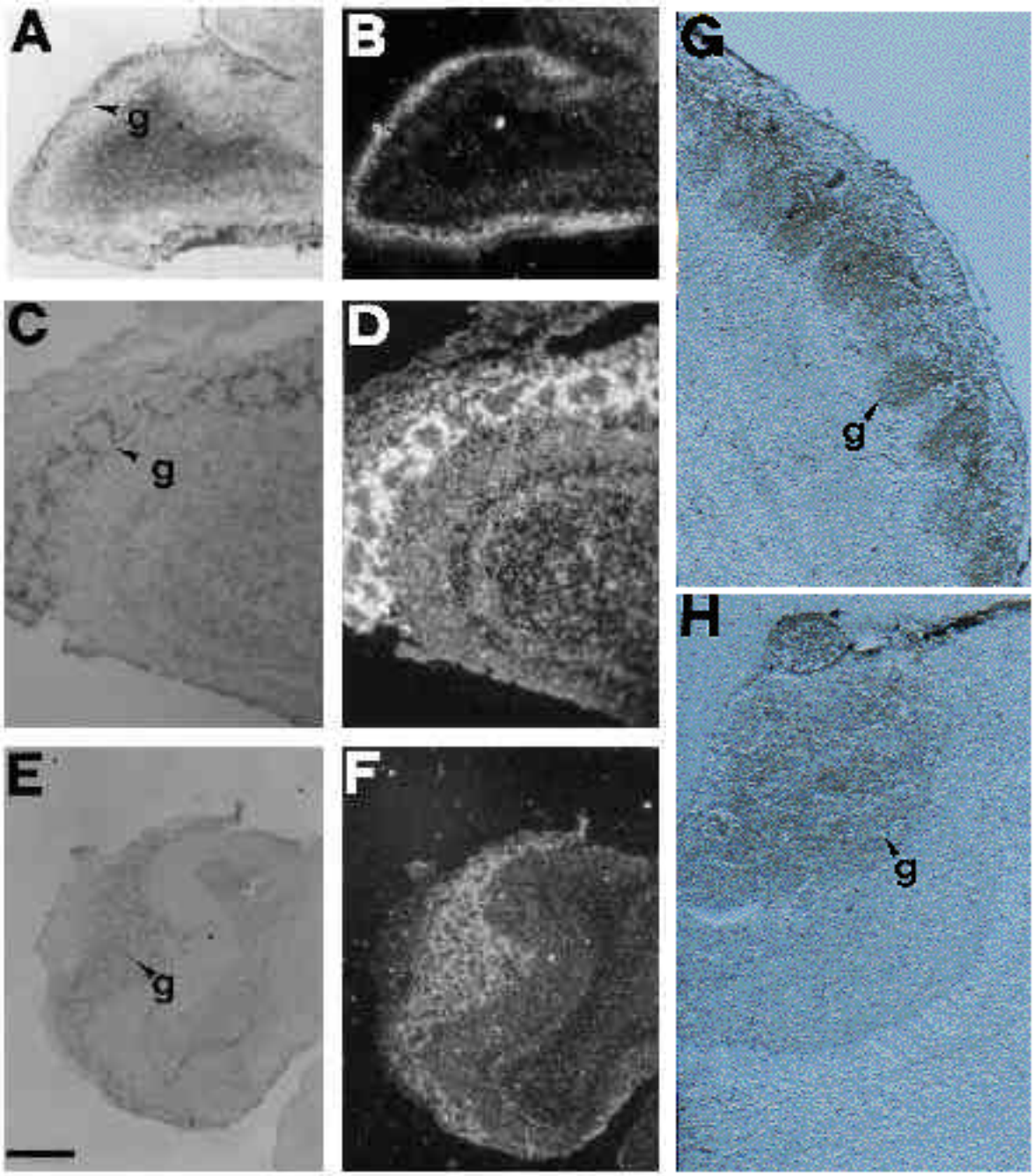


**Fig. 6.** Postnatal expression of *PN-1* mRNA and protein in the main and accessory olfactory bulb. (A,C,E) Bright-field and (B,D,F) the corresponding dark-field images. (A,B) Parasagittal section through the OB of a P2 brain. (C,D) Parasagittal section through the OB of a P14 brain. (E,F) Parasagittal section through the OB of an adult brain. *PN-1* mRNA is mainly expressed in the glomerular layer of the bulb. (G,H) Immunocytochemical localisation of PN-1 in the glomeruli of the main bulb (G) and the accessory bulb (H) of an adult mouse brain, ×150. Abbreviations : g, glomeruli. Bar: (A,B) 0.3 mm; (C,D) 0.2 mm; (E,F) 0.45 mm.

mocortical inputs terminate mainly in layer IV cells of the cortex and layer VI cells send corticofugal projections back to the same thalamic nucleus (for review see Blackmore and Mólnár, 1990). Since the rather immature thalamocortical innervation in rodents at birth (Lund and Mustari, 1977) is followed by an early postnatal maturation of these pathways, a prominent expression of *PN-1* during this period could suggest a role in either the maturation of neurons or in the stabilisation of proper connections. In this respect, it is also interesting to note that *PN-1* expression was quite prominent in Purkinje cells around P11 but not later on. Around P3, the somatic spines of Purkinje cells receive input from climbing fibers and, beginning around P10, these are replaced by inhibitory basket-cell synapses while the climbing fibers shift their excitatory synapses to the Purkinje cell dendrites. *PN-1* expression in hippocampal neurons around P7 is also suggestive of a stabilising role since mossy fibers supplied by dentate granule cells terminate on dendrites close to the

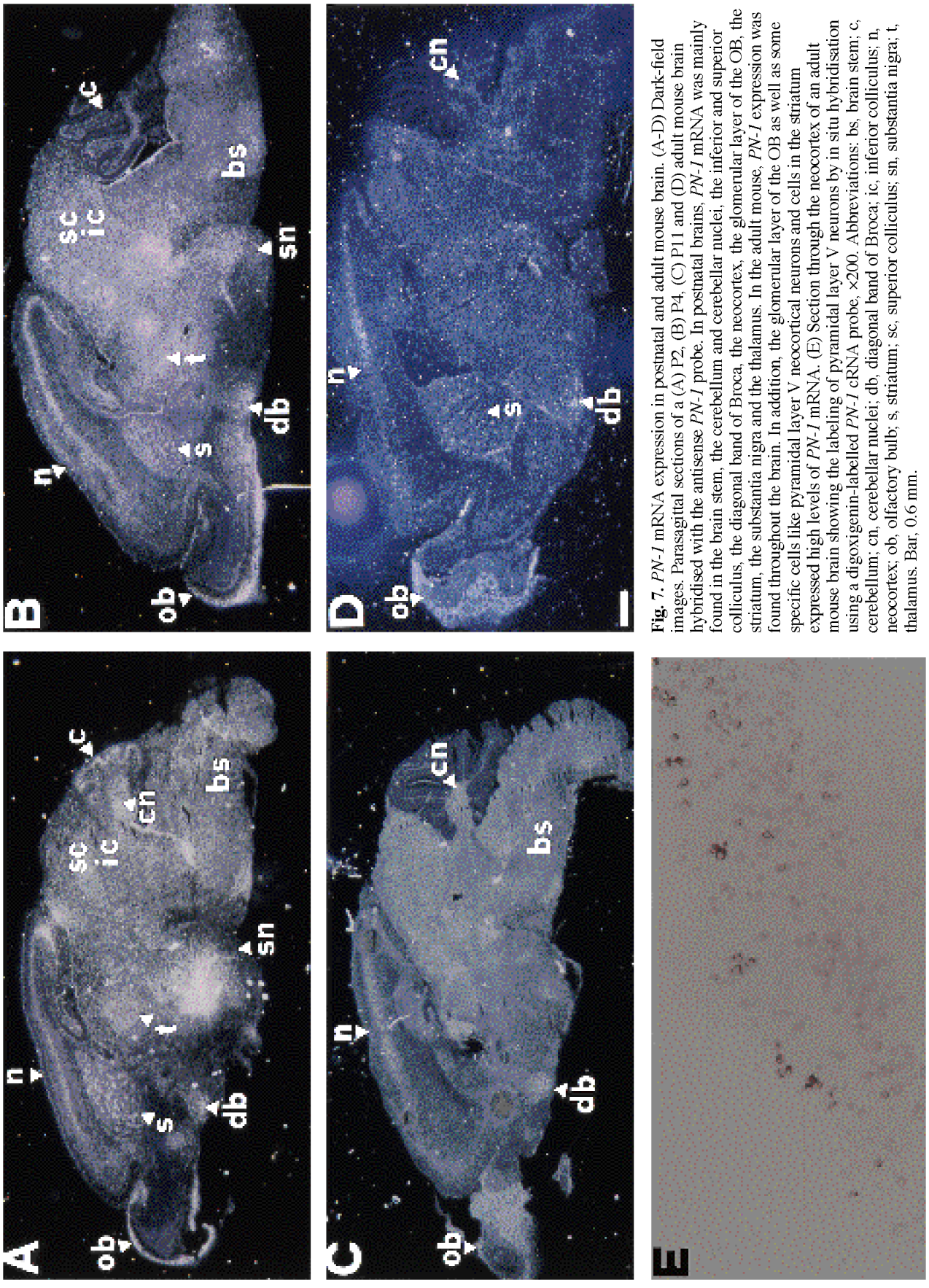


**Fig. 7.** *PN-1* mRNA expression in postnatal and adult mouse brain. (A-D) Dark-field images. Parasagittal sections of a (A) P2, (B) P4, (C) P11 and (D) adult mouse brain hybridised with the antisense *PN-1* probe. In postnatal brains, *PN-1* mRNA was mainly found in the brain stem, the cerebellum and cerebellar nuclei, the inferior and superior colliculus, the diagonal band of Broca, the neocortex, the glomerular layer of the OB, the striatum, the substantia nigra and the thalamus. In the adult mouse, *PN-1* expression was found throughout the brain. In addition, the glomerular layer of the OB as well as some specific cells like pyramidal layer V neocortical neurons and cells in the striatum expressed high levels of *PN-1* mRNA. (E) Section through the neocortex of an adult mouse brain showing the labeling of pyramidal layer V neurons by in situ hybridisation using a digoxigenin-labelled *PN-1* cRNA probe, ×200. Abbreviations: bs, brain stem; c, cerebellum; cn, cerebellar nuclei; db, diagonal band of Broca; ic, inferior colliculus; n, neocortex; ob, olfactory bulb; s, striatum; sc, superior colliculus; sn, substantia nigra; t, thalamus. Bar, 0.6 mm.

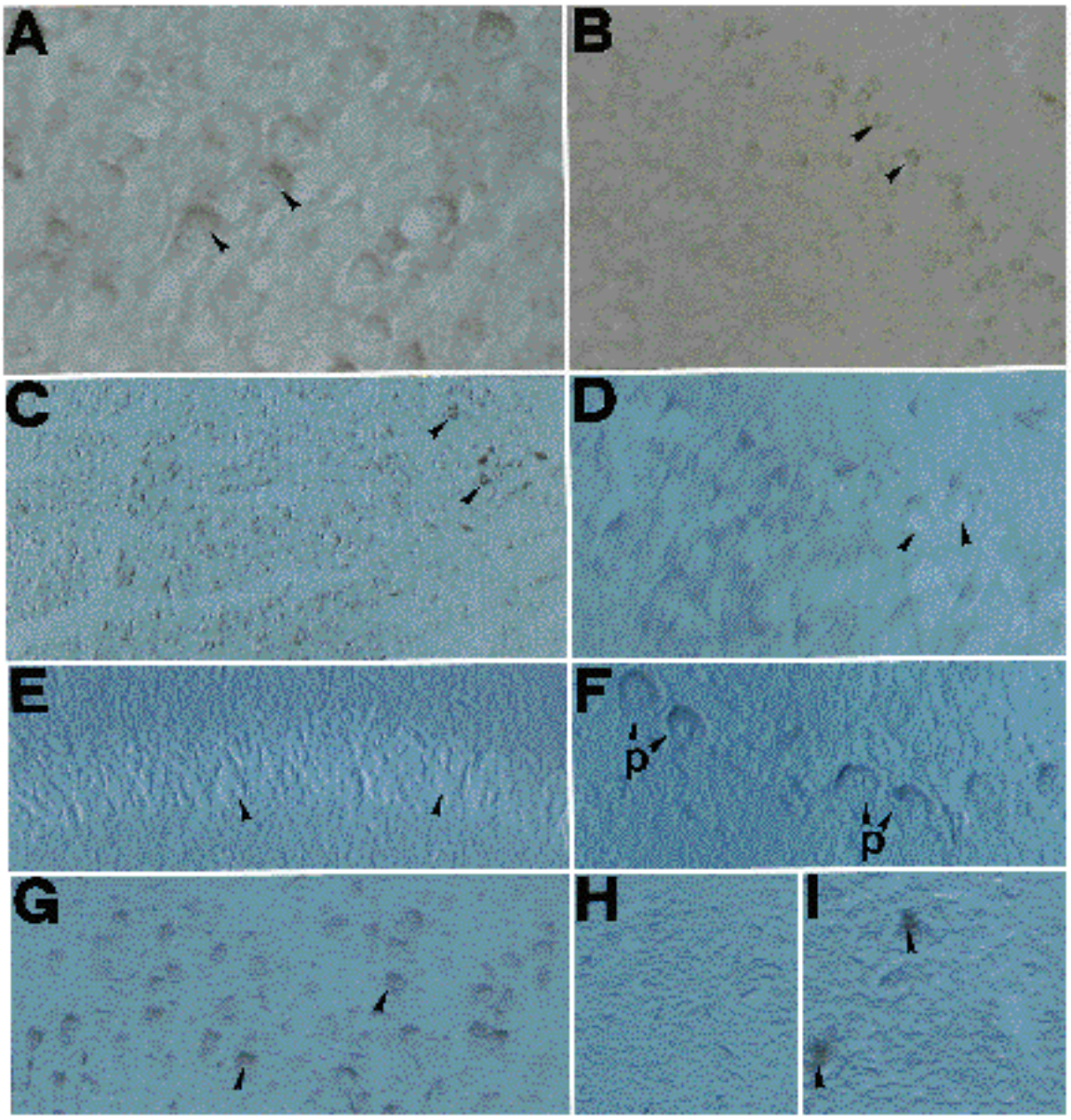


**Fig. 8.** Immunocytochemical localisation of PN-1 in the postnatal mouse brain. Cells containing PN-1 are shown (A) in layer V of the neocortex at P7, ×400; (B) in layers next to the corpus callosum at P7, ×200; (C) in the globus pallidus at P7, ×100; (D) in the hippocampus at P7, ×400; whereas (E) no PN-1 was found in this region at P21, ×200; (F) in Purkinje cells at P11, ×400; (G) in pyramidal layer V neurons of the adult neocortex, ×200; and (I) in adult striatum with a few highly positive cells in the matrix compartment, ×400. (H) Section through the neocortex incubated with an isotype-matched control antibody, ×200. Abbreviations: p, Purkinje neurons.

CA3 cell soma around P9-10 in the rat (Singh, 1977). Also the perforant path fibers from the entorhinal cortex to the hippocampus mature around this time. When correcting for the two-day shorter gestation period in mice, the timing of *PN-1* expression seems again to coincide predominantly with a phase when maturation of connections is finishing.

### Regulated *PN-1* expression in the developing nervous system

A temporally and spatially regulated expression of *PN-1* was noted in a number of quite distinct embryonic nervous system structures. In the developing cerebellum at E13.5, cells in the rostral and caudal region of the rhombic lip, a part of the metencephalon that develops into the cerebellar primordium, expressed *PN-1* mRNA (Fig. 2G,H). Cells migrating from the ventricular germinal zone of the rhombic lip give rise first to Purkinje cells (E10-E13) and, later, to the cerebellar neurons (granule, stellate and basket cells and Golgi type II neurons) and to glial cells. During the period E13-E15, the EGL, a second germinal zone, is formed from cells migrating over the surface of the cerebellar plate (Miale and Sidman, 1961). Furthermore, cells migrating from the caudal part of the rhombic lip form the inferior olivary nuclei, cochlear nuclei and pontine nuclei (Jacobson, 1991). At E14.5, *PN-1* mRNA levels in the rostral and caudal regions of the rhombic lip diminished (Fig. 2I,J) and,

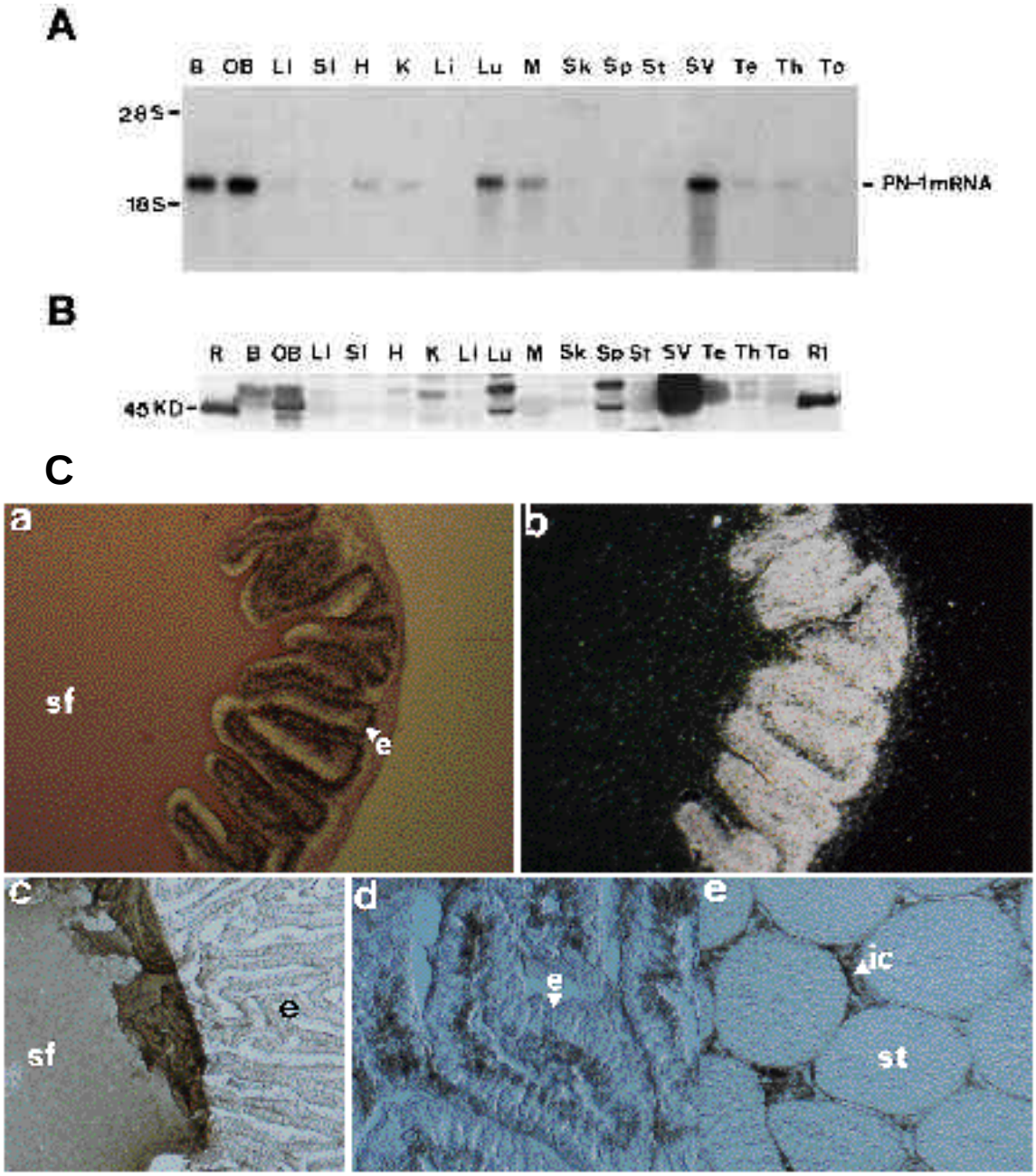


**Fig. 9.** A comparative analysis of *PN-1* expression in adult mouse brain and other tissues. (A) Northern blot analysis: each lane represents 10 μg of total RNA of adult mouse tissues. *PN-1* mRNA expression was monitored using a $^{32}$P-labelled rat *PN-1* cDNA probe (see Materials and Methods). Positions of 28 S and 18 S rRNAs are indicated on the left. (B) Immunoblotting: 100 μg of adult mouse tissue or rat brain (R) homogenates were loaded in each lane and PN-1 immunoreactivity was revealed using the 4B3 monoclonal antibody raised against rat PN-1. R1 represents 40 ng of purified PN-1 from rat C6 glioma cell conditioned medium (Guenther et al., 1985). Location of the $45\times10^3$ $M_r$ protein marker is indicated on the left. Abbreviations: B, brain; LI, large intestine, SI, small intestine; H, heart; K, kidney; Li, liver; Lu, lung; M, skeletal muscle; OB, olfactory bulb; R, adult rat brain; Sk, skin; Sp, spleen; St, stomach; SV, seminal vesicle; Te, testis; Th, thymus; To, tongue. (C) *PN-1* mRNA and protein in the reproductive system: (a) Bright-field image and (b) the corresponding dark-field. (a,b) Adult mouse seminal vesicle showing *PN-1* mRNA expression in the epithelium. (c-e) PN-1 immunoreactivity in (c) seminal vesicle fluid, ×100, (d) seminal vesicle epithelium at the apical surface of the epithelial cells, ×400 and (e) testis, ×100, only in interstitial cells between the seminiferous tubules. Abbreviations: e, epithelium; ic, interstitial cell; sf, seminal fluid; st, seminiferous tubule.

by E17, cells in the cerebellar plate showed significant levels of *PN-1* mRNA (result not shown). At this stage (E17), the plate is composed of an inner ventricular germinal layer, a middle mantle and the EGL. Cells in the deeper stratum of the mantle layer differentiate to form the large neurons of the roof nuclei and cells of the more superficial stratum develop into Purkinje neurons. The latter differentiate rapidly after the granule cells migrate from the EGL to the

internal granular layer (IGL). This occurs from P3 to P20 (Miale and Sidman, 1961) when *PN-1* expression in the cerebellum was no longer prominent in any of the cellular layers except for a transient expression in Purkinje cells around P11.

A second example of *PN-1* regulated expression occurs in the embryonic mesencephalon and myelencephalon. On E10.5, clusters of neuroepithelial cells in the floor plate showed high levels of *PN-1* mRNA expression. From E13.5 until birth, expression was more widespread and found in most of these neuroepithelial cells. Again, between E11.5 and E15.5, we found no clear expression in postmitotic cells that migrated out of the neuroepithelial zone. Migrating cells also did not express *PN-1* in the developing spinal cord. Whether the initially restricted expression in clusters of floor plate cells indicates a distinct developmental potential remains to be seen (Temple, 1990; Jacobson, 1991).

### Differential expression of *PN-1* and serine proteases in the developing mouse

Our results showed that *PN-1* mRNA and protein are expressed in a variety of embryonic and adult mouse tissues. Because PN-1 is a powerful serine protease inhibitor, it was of interest to compare its expression pattern with those of some characterised serine proteases. In the male genital tract, *PN-1* and *uPA* expression correspond remarkably well. We showed that *PN-1* is mostly expressed in the seminal vesicle with the protein found at the apical surface of the secreting epithelial cells and in the seminal fluid. A recent search for uPA inhibitors in the seminal vesicle revealed that PN-1 acts as a ligand for uPA in this tissue (Vassalli et al., 1993). Therefore, it was suggested that the interaction of PN-1 with uPA participates in regulating proteolysis in the lumen of the seminal vesicle.

In the developing spinal cord, *PN-1* expression revealed a pattern strikingly complementary to the pattern reported for the serine protease *tPA* (Sumi et al., 1992). Our results showed that *PN-1* mRNA was prominent in neuroepithelial cells of the basal and, to a lesser extent, the intermediate plates but absent in the floor plate. *tPA* expression, on the other hand, is known to occur specifically and exclusively in the floor plate cells. *tPA* mRNA is first detectable at E10.5 and reaches high levels by E13.5 up to E17 (Sumi et al., 1992). Cells in the floor plate of the developing spinal cord act as guide posts for growing commissural fibers from medially located cell bodies (Tessier-Lavigne et al., 1988; Yaginuma and Oppenheim, 1991). Sumi et al. (1992) suggested that the pronounced expression of *tPA* in floor plate cells might serve to activate a latent or remove a redundant set of receptors or molecules on commissural axons. In line with this hypothesis, it seems likely that the expression of *PN-1* in lateral plate neuroepithelium dorsal of the floor plate prevents the action of *tPA* from acting on molecules on pre- and post-floor plate axonal surfaces. The PN-1 produced by lateral plate cells and the tPA produced by floor plate cells might create two mutually exclusive molecular gradients thereby defining target fields. This finding provides one possible molecular mechanism to restrict the action of *tPA* in spinal cord development.

In addition, prothrombin mRNA is expressed in rat embryos and in the developing brain (Dihanich et al., 1991). The active serine protease thrombin might be generated from its zymogen in the brain and PN-1 might block its activity. It is also interesting to note that *PN-1* is expressed by ventricular germinal cells in the floor plate of the mesencephalon and myelencephalon. These cells occupy the most internal layer which is in close contact with the cerebrospinal fluid that contains many proteins including a variety of proteases (Saunders and Møllgård, 1981). PN-1, here, may serve to inhibit such proteases and may also play a role in the preservation of the location of ventricular cells before migration and differentiation. In addition, *PN-1* expression occurred in several other embryonic and adult mouse tissues many of which, like the brain, express one or more specific serine proteases (unpublished results, Larsson et al., 1984; Rickles and Strickland, 1988; Sappino et al., 1989; Kristensen et al., 1991; Vassalli et al., 1993). In particular, during embryogenesis, *PN-1* mRNA and protein were found in high amounts in the cartilage forming the limbs, vertebras, ribs and skull.

The complex and regulated appearance of *PN-1* mRNA and protein in different organs and in different structures of the brain at distinct stages of development suggest that it probably inhibits several serine proteases depending on cell type and tissue. The present results describing the temporal and spatial expression of *PN-1* lay the groundwork for further study of the biological relevance of the balance between proteases and their inhibitors in specific and well-defined developmental events.

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