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PDGF and its receptors in the developing rodent retina and optic nerve

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SUMMARY

We have used in situ hybridization to visualize cells in the developing rat retina and optic nerve that express mRNAs encoding the A and B chains of platelet-derived growth factor (PDGF-A and PDGF-B), and the alpha and beta subunits of the PDGF receptor (PDGF-αR and PDGF-βR). We have also visualized PDGF-A protein in these tissues by immunohistochemistry. In the retina, PDGF-A mRNA is present in pigment epithelial cells, ganglion neurons and a subset of amacrine neurons. PDGF-A transcripts accumulate in ganglion neurons during target innervation and in amacrine neurons around the time of eye opening, suggesting that PDGF-A expression in these cells may be regulated by target-derived signals or by electrical activity. In the mouse retina, PDGF-A immunoreactivity is present in the cell bodies, dendrites and proximal axons of ganglion neurons, and throughout the inner nuclear layer. PDGF-αR mRNA is expressed in the retina by astrocytes in the optic fibre layer and by a subset of cells in the inner nuclear layer that might be Müller glia or bipolar neurons. Taken together, our data suggest short-range paracrine interactions between PDGF-A and PDGF-αR, the ligand and its receptor being expressed in neighbouring layers of cells in the retina. In the optic nerve, PDGF-A immunoreactivity is present in astrocytes but apparently not in the retinal ganglion cell axons. PDGF-αR^+ cells in the optic nerve first appear near the optic chiasm and subsequently spread to the retinal end of the nerve; these PDGF-αR^+ cells are probably oligodendrocyte precursors (Pringle et al., 1992). RNA transcripts encoding PDGF-B and PDGF-βR are expressed by cells of the hyaloid and mature vascular systems in the eye and optic nerve.

Key words: PDGF, PDGF receptors, retina, optic nerve, neurons, glial cells, blood vessels, in situ hybridization, immunohistochemistry, rat, mouse

INTRODUCTION

Many polypeptide growth factors are expressed in the mammalian CNS during development and in the adult. In order to understand the functions of these factors we need to identify their cellular sources and targets, but this is hampered by the extreme complexity of much of the CNS, and the paucity of markers that can be used to identify neural cells in situ. For these reasons we have concentrated on the peripheral visual system (optic nerve and retina), which is one of the simplest and best-characterized regions of the CNS. The optic nerve carries axons from ganglion neurons in the retina to the brain, and contains glial cells but no neuronal cell bodies. The retina contains several types of neurons and photoreceptors in addition to glial cells. The development, cytoarchitecture and physiology of the retina and optic nerve have been well documented, making these ideal organs for investigating cell-cell interactions.

Platelet-derived growth factor (PDGF) and its receptors are expressed in the developing and mature CNS (Richardson et al., 1988; Sasahara et al., 1991; Yeh et al., 1991; Pringle et al., 1992; Pringle and Richardson, 1993). PDGF is a disulphide-linked dimer of A and B chains, with the structure AA, BB or AB. There are also two types of PDGF receptor subunits with different ligand specificities; the alpha subunit (PDGF-αR) can bind both A and B chains of PDGF while the beta subunit (PDGF-βR) binds only the B chain (reviewed by Heldin and Westermark, 1990). From our studies of gliogenesis in the developing rat optic nerve, a CNS white matter tract, we have proposed specific roles for PDGF in regulating the proliferation and survival of glial cells belonging to the oligodendrocyte lineage (reviewed by Richardson et al., 1990; Barres et al., 1992a). Oligodendrocyte lineage cells express PDGF-αR (Hart et al., 1989; McKinnon et al., 1990) and consequently can respond to all three dimeric isoforms of PDGF (Pringle et al., 1989). We showed previously that cultured cortical astrocytes synthesize and secrete PDGF-A but not PDGF-B, and on this basis suggested that oligodendrocyte precursors in the optic nerve might divide in response to astrocyte-derived PDGF-AA (Pringle et al., 1989). Recently it was found that many CNS neurons express PDGF (Sasa-

hara et al., 1991; Yeh et al., 1991). This raised the possibility that retinal ganglion cells, whose axons project through the optic nerve to the brain, might provide an additional or alternative source of PDGF for glial cells within the nerve. In addition, the fact that some developing and mature CNS neurons also express PDGF receptors (Smits et al., 1991; Hutchins and Jefferson, 1992; Pringle and Richardson, 1993) suggests that PDGF may also play some role in neuronal development, maintenance or function. Mapping the patterns of expression of PDGF and its receptors in the developing CNS will help to identify the sources and targets for PDGF and shed some light on the significance of the cellular interactions mediated by PDGF. In this paper we describe the results of an in situ hybridization survey of the cell types that express PDGF-A, PDGF-B, PDGF-αR and PDGF-βR during embryonic and postnatal development of mouse and rat retinae and optic nerves, and a study of PDGF-A immunoreactivity in these tissues using an antiserum raised against recombinant mouse PDGF-A.

MATERIALS AND METHODS

Preparation of tissue sections

Whole heads of E12 to P5 rats (Sprague-Dawley), whole eyes of P10 to adult rats, and whole heads of P5 agouti mice (H4 strain; MRC Radiobiology unit, Harwell) were used in this study. Four individual rats were processed at each age. Tissues were fixed overnight in cold 4% paraformaldehyde and cryoprotected in 30% (w/v) sucrose in PBS for at least 24 hours. Tissue was frozen in OCT embedding compound (Tissue-Tek; Miles Inc.) and blocks stored at −70°C until required. Cryosections (10 μm) were collected on APES-coated slides (APES is 3-aminopropyltriethoxysilane (Sigma) used at 3% v/v in industrial methylated spirits). The sections were air dried, post-fixed in 4% paraformaldehyde, washed briefly in PBS and then dehydrated through an ascending series of ethanols (30% to absolute) and stored at −70°C.

Preparation of in situ hybridization probes

^{35}S-labelled RNA probes were prepared by in vitro transcription as described previously (Cox et al., 1984). Antisense (i.e. complementary to the mRNA) and sense (homologous to the mRNA) PDGF-αR probes were generated from an approx. 1.5 kb *Sac*I-*Pvu*II fragment coding for most of the extracellular domain of rat PDGF-αR (Lee et al., 1990) cloned into pGEM1 (Promega Biotec); T7 RNA polymerase generated the antisense probe and SP6 polymerase the sense probe. The PDGF-A antisense probe was transcribed with T7 RNA polymerase from an approx. 1.4 kb *Eco*RI fragment encompassing the coding region of a rat PDGF-A cDNA (clone 5.3; Pollock, 1992) in PTZ-18r (Pharmacia); the sense probe was transcribed with T7 polymerase from an approx. 1.0 kb *Eco*RI fragment in the same vector (clone 4.1; Pollock, 1992). (The sequence of rat PDGF-A has been submitted to GenBank under accession number L06894.) The PDGF-B probe was generated from an approx. 740 bp *Hin*dIII-*Eco*RI cDNA fragment spanning the entire coding sequence of rat PDGF-B (H. Mudhar, unpublished data). The PDGF-βR probes were transcribed from an approx. 1.2 kb *Hin*dIII-*Bam*HI cDNA fragment encoding the extracellular and transmembrane domains of rat PDGF-βR (H. Mudhar, unpublished). Both PDGF-B and PDGF-βR fragments were isolated by reverse transcriptase-PCR from RNA of a spontaneously immortalized Schwann cell line (Eccleston et al., 1990) and cloned into Bluescript ks+ (Stratagene). The veracity of these clones was confirmed by sequencing and by northern blot analysis of RNA isolated from E18 rat brain; the PDGF-B chain probe detected a single RNA of 3.5 kb, and the PDGF-βR probe detected a single approx. 6 kb transcript, as expected (Richardson et al., 1988; Matsui et al., 1989). For both PDGF-B and PDGF-βR, transcription with T3 RNA polymerase yielded the antisense probe and T7 polymerase the sense probe.

In situ hybridization

In situ hybridization was performed on two separate occasions, using tissue sections prepared from a total of four animals from each developmental stage. The in situ hybridization protocol was based on that of Lawrence and Singer (1985), with the modifications described by Pringle et al. (1992). Sections were coated with Ilford K5 nuclear emulsion, exposed for 4-10 days in the dark at 4°C and developed in Kodak D-19. Sections were usually lightly counterstained with 5% (w/v) haematoxylin solution (Gills No.3, Sigma). For high magnification microscopy a 20% solution was used.

Immunocytochemistry

Postnatal day 5 (P5) and P15 agouti mice (H4 strain) were killed with CO_2 and promptly perfused through the heart with phosphate-buffered saline (PBS, pH 7.4) followed by 4% (w/v) paraformaldeyde in PBS. The heads were removed and placed in 4% paraformaldeyde for a further 24 hours at 4°C, then the tissue was cryoprotected by immersion for at least 24 hours in a 30% (w/v) solution of sucrose in PBS. The tissue was frozen in OCT embedding compound and the blocks were stored at −70°C until use. Cryostat sections (8 μm nominal thickness) were collected on APES-coated slides, air dried and non-specific immunoglobulin binding sites blocked with a combination of 50% normal goat serum (NGS) in Tris-buffered saline (TBS; 0.15 M NaCl, 50 mM Tris pH 7.4), with 100 mM L-lysine and 2% (w/v) bovine serum albumin (BSA, fraction V; Sigma). The sections were incubated overnight at 4°C with a rabbit antiserum raised against recombinant mouse PDGF-A (Mercola et al., 1990; Wang et al., 1992; 1:150 dilution), followed by biotinylated anti-rabbit IgG (Amersham; 1:100 dilution) for 1 hour at room temperature, followed by fluorescein-conjugated streptavidin (Amersham; 1:100 dilution) for 1 hour at room temperature. As a control, the anti-PDGF antibody was pre-incubated with a 100-fold molar excess of recombinant human PDGF-AA (Peprotech, New York) overnight at 4°C. Antibody-antigen complexes were removed by centrifugation at 400,000 ***g*** for 20 minutes at 4°C in a Beckman TLX ultracentrifuge, and the supernatants used for immunohistochemistry. Parallel pre-incubations were carried out with recombinant acidic or basic FGF, and with recombinant human PDGF-BB (all Peprotech). Pre-incubation with PDGF-AA, but not with the other factors, abolished or caused a marked reduction of the signal. Sections of P5 optic nerve were double-labelled with anti-PDGF-A and monoclonal anti-GFAP (Sigma). Sections were incubated simultaneously with both primary antibodies; the GFAP immunoreactivity was visualized with Texas red-conjugated goat anti-mouse IgG (Cappel) and PDGF-A immunoreactivity visualized as above. Sections of P15 mouse retinae were double-labelled with anti-PDGF-A and monoclonal antibody HPC-1 (Barnstable et al., 1985), a marker for amacrine neurons. Sections were incubated simultaneously in both primary antibodies and visualized as above. All antibodies were diluted in TBS with 100 mM L-lysine, 2% (w/v) BSA and 0.2% (v/v) Triton X-100. Post-incubation washes were with 3 changes of TBS for 10 minutes each. Sections were mounted under glass coverslips in Citifluor anti-fade reagent (City University, London) and viewed in the fluorescence microscope.

Combined in situ hybridization and immunohistochemistry

Sections of rat retinae were incubated with monoclonal anti-GFAP antibody, immunoperoxidase labelled using the Vectastain ABC

kit (Vector Laboratories, Peterborough, UK) and subsequently subjected to in situ hybridization with the PDGF-αR probe. The procedure was essentially as described by Watts and Swanson (1989), except that dithiothreitol was not included in the antibody solutions. The sections were photographed at high magnification under bright-field illumination in two planes of focus optimal for the HRP reaction product and the silver grains. In the latter case, an optical filter was used to obscure the brown HRP reaction product.

Immunoselection of retinal ganglion neurons for in situ hybridization

Retinal ganglion neurons from P0 Sprague-Dawley rats were purified to >99% purity by immunoselection as described by Barres et al. (1988), except that the cells were removed from the final anti-Thy-1 plate by mechanical trituration rather than with trypsin. Cells were counted and plated at 10,000 cells/well in 8-well glass Lab-Tek slides (Nunc-Gibco BRL) and allowed to settle for 20 minutes at room temperature. Cells were fixed in 4% paraformaldeyde in PBS for 15 minutes and processed for in situ hybridization as described above. The cells were hybridized with ^{35}S-RNA probes specific for PDGF-A mRNA and washed according to the protocol of Deneris et al. (1989), modified by the addition of an initial 1 hour wash in 4× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5) at room temperature and a final high-stringency wash in 0.1× SSC at 55°C for 30 minutes.

RESULTS

To map the distributions of RNA transcripts encoding PDGF and its receptors in the developing rodent retina and optic nerve, we performed in situ hybridizations with ^{35}S-labelled 'antisense' probes complementary to the rat PDGF-A, PDGF-B, PDGF-αR and PDGF-βR mRNAs. In parallel, we used 'sense' probes (homologous to the mRNAs) as controls for the specificity of the hybridization reactions; in each case the sense probes gave very little or no detectable in situ hybridization signal over the eye or optic nerve (Fig. 1), whereas the antisense probes gave reproducible signals over cell bodies in these tissues (see below). We also used an antibody against PDGF-A to visualize the distribution of this protein in the retina and optic nerve.

PDGF-A mRNA and immunoreactivity in the retina

Cryosections through the retinae of embryonic and postnatal rats and mice were subjected to in situ hybridization. As reported previously (Orr-Urtreger and Lonai, 1992), many tissues outside of the CNS express PDGF-A mRNA (e.g. skin, muscle, nasal epithelium; see Fig. 2). PDGF-A transcripts first appear in the rat neural retina at E18, in the retinal ganglion cell (RGC) layer (arrowheads in Fig. 2B). No gradient of expression across the retina is apparent in either the mediolateral or dorsoventral directions. By the day of birth (E21/P0), the PDGF-A signal increases appreciably in the RGC layer (Fig. 2C,F). The intensity of this signal is maintained for at least several weeks (Fig. 2D,E), but declines somewhat in the adult (not shown). PDGF-A transcripts can also be detected in the RGC layer of the embryonic and postnatal mouse retina (e.g., Fig. 3A). To confirm that PDGF-A is expressed by retinal ganglion neurons, we purified these cells from newborn rat retinae by immunoselection with a monoclonal anti-Thy-1 (see Materials and methods) and subjected the isolated cells to in situ hybridization. Using the 'antisense' PDGF-A probe, a positive hybridization signal was obtained over 95% of the immunoselected cells (Fig. 2I). The negative control ('sense') PDGF-A probe displayed only background signal (Fig. 2J). To test for the presence of PDGF-A protein, we used a rabbit antiserum raised against recombinant mouse PDGF (Mercola et al., 1990; Wang et al., 1992) to label

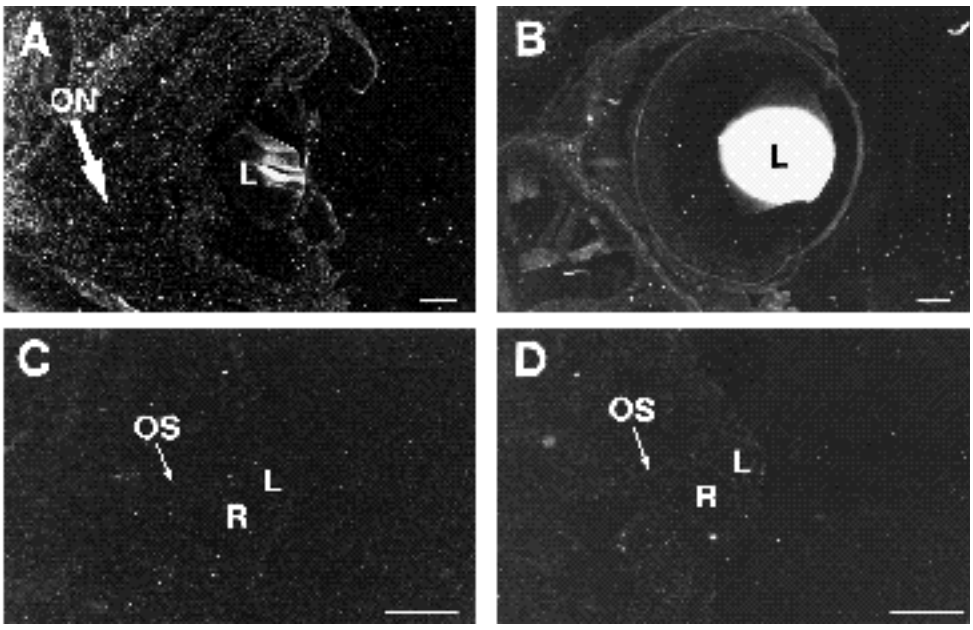

Fig. 1. Negative controls for the specificity of the in situ hybridization probes used in this study. The figure shows dark-field images of in situ autoradiographs generated with 'sense' probes corresponding to PDGF-A (A), PDGF-αR (B), PDGF-B (C) and PDGF-βR (D). A shows an adjacent section to that illustrated in Fig. 2D (PDGF-A antisense probe). B shows an adjacent section to that in Fig. 5F (PDGF-αR antisense probe), only at slightly lower magnification. C shows an adjacent section to the one in Fig. 8B (PDGF-B antisense probe) and D shows an adjacent section to that of Fig. 8A (PDGF-βR antisense probe). In each case, there is no specific hybridization signal over the eye or optic nerve (ON), although there is a low non-specific background over certain extraocular tissues in A. Note that the intense dark-field signal in the eye lens (L) in B is not due to silver grains but to the inherent light-scattering properties of the poorly sectioned lens tissue in this and other figures. OS, optic stalk; R, retina. Scale bars, 500 μm.

sections of adult mouse retinae. PDGF-A immuno-reactivity was detected by immunofluorescence microscopy in the cell bodies of RGCs, and also associated with the dendritic processes and proximal parts of the RGC axons (arrows in Fig. 3B). Several results from control experiments indicate that our immunofluorescence labelling procedure is specific

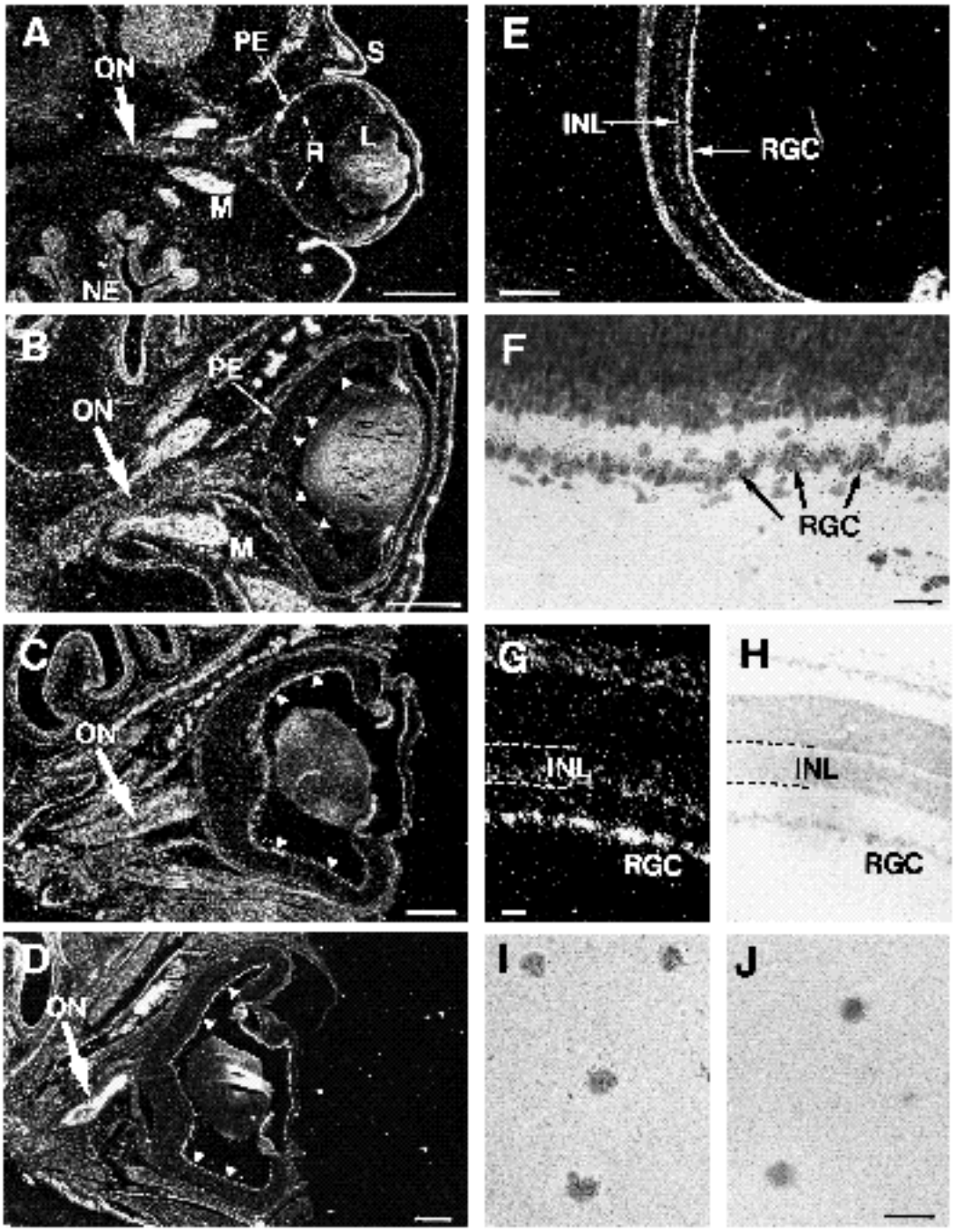

Fig. 2. Distribution of PDGF-A transcripts in the developing rat retina (R) and optic nerve (ON). Horizontal sections were taken through the optic nerves and eyes of developing rats at E16 (A), E18 (B), P0 (C), P5 (D) and P15 (E,G), subjected to the in situ hybridization procedure and photographed in dark-field illumination. In the retina, PDGF-A mRNA is first detectable in the RGC layer at E18 (arrowheads in B), but the signal intensifies markedly by P0 (arrowheads in C). A high magnification view of a P0 retina, photographed in bright-field illumination (F), shows silver grains overlying the RGC cell bodies. In situ hybridization on immunoselected retinal ganglion neurons in vitro (I,J) shows a positive signal over the neuronal cell bodies with the antisense probe (I) but not the sense probe (J). By P15, a second layer of PDGF-A mRNA is present in the inner nuclear layer (INL) of the retina (E). G, H are corresponding dark-field and bright-field images of a P15 retina hybridized in situ with the PDGF-A antisense probe; silver grains are present over retinal ganglion neurons and the innermost third of the INL (the INL is delineated by dotted lines), where amacrine neurons are located. PDGF-A mRNA is present in the optic nerve (ON) at all ages from E14 (A-D); the signal intensity increases between E18 (B) and P0 (C). L, lens; PE, retinal pigment epithelium; S, skin; M, muscle; NE, nasal epithelium; RGC, retinal ganglion cells. Scale bars: A-E, 500 μm; F-J, 20 μm.

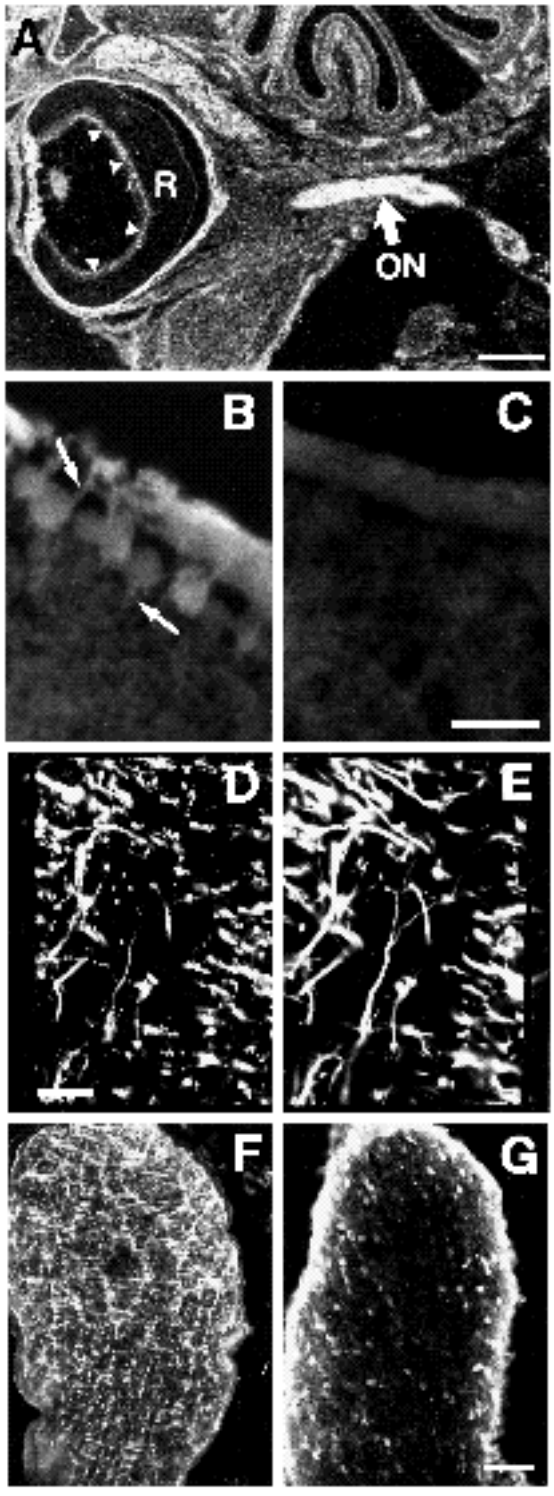

Fig. 3. Distribution of PDGF-A mRNA and protein in the mouse retina (R) and optic nerve (ON). Sections of P5 (A,D-G) or adult (B,C) mice were subjected to in situ hybridization (A) or immunohistochemistry (B-G) with reagents directed against PDGF-A or and/or GFAP. PDGF-A mRNA, visualized by in situ hybridization (A), is expressed in the RGC layer and the optic nerve of the P5 mouse, as in the rat (see Fig. 2D). B shows PDGF-A immunoreactivity in a section of the adult mouse retina, visualized by immunofluorescence microscopy: PDGF-A is associated with the cell bodies, axons (upper arrow) and dendrites (lower arrow) of RGCs (also see Fig. 4A). No signal is obtained in an adjacent section when treatment with the anti-PDGF-A antibody is omitted (C). D and E show PDGF-A immunoreactivity and GFAP immunoreactivity, respectively, in the same section of an interior region of the P5 mouse optic nerve, visualized by immunofluorescence microscopy. PDGF-A is visualized using fluorescein and GFAP with rhodamine. PDGF-A immunoreactivity (D) coincides closely with GFAP immunoreactivity (E). F and G are immunofluorescent micrographs of sections through part of a P5 mouse optic nerve, labelled with anti-PDGF-A antibody either before (F) or after (G) preincubating the antibody with an excess of recombinant PDGF-AA. Labelling of astrocyte processes (F) is largely obliterated by preincubation with recombinant PDGF-AA (G), as is labelling of RGCs (not shown). D and E are confocal micrographs; the others are conventional micrographs. Scale bars: A, 500 μm; B-E, 20 μm; F,G, 100 μm.

for PDGF-A. First, the anti-PDGF-A antibody labels Cos cells that have been transfected with an expression vector encoding mouse or human PDGF-A, but not untransfected Cos cells (Mercola et al., 1990; A. Calver, unpublished data). Second, our secondary antibodies give no fluorescent signal in the absence of the primary anti-PDGF antibody (Fig. 3C). Third, the immunolabelling is abolished or strongly reduced when the anti-PDGF-A antibody is pre-incubated with an excess of recombinant human PDGF-AA (compare Fig. 3F with 3G), but is unaffected by pre-incubating with excess recombinant acidic or basic FGF, or PDGF-BB (not shown).

Beginning around P10, a subset of cells in the inner nuclear layer (INL) also starts to express PDGF-A mRNA. The signal is highest at the innermost (vitreal) aspect of the INL; at first the signal is relatively weak but by P15 its intensity increases significantly (Fig. 2E,G,H and arrows in Fig. 8A). There is no discernable gradient of expression across the retina. The location of the PDGF-A-expressing cells at the inner aspect of the INL (small arrows in Fig. 8A) suggests that they are amacrine neurons. Note, however, that only a small subset of the cells at the inner surface of the INL expresses detectable PDGF-A mRNA. In an attempt to directly identify the PDGF-A-expressing cells in the INL, we performed double-label immunohistochemistry on sections of P15 mouse retina with anti-PDGF-A antibody and antibody HPC-1, a specific marker of amacrine cells and their processes in the rodent retina (Barnstable et al., 1985; Fig. 4). Strong PDGF-A immunolabelling is present in RGCs and the optic fibre layer (Fig. 4A), as in the adult (compare Fig. 3B). In addition, PDGF-A immunoreactivity is present throughout the INL, apparently at the surfaces of cells or in the extracellular spaces (Fig. 4A). The brightest labelling is most often associated with cells at the inner aspect of the INL; at least some of these brightly labelled cells can be identified as amacrine cells because they also label with monoclonal antibody HPC-1 (Fig. 4B). This pattern of labelling, together with the in situ hybridization data (Fig. 2G,H and Fig. 8A), is consistent with the idea that PDGF-A is synthesized and secreted by a subset of amacrine cells, and subsequently accumulates in the extracellular spaces throughout the depth of the INL.

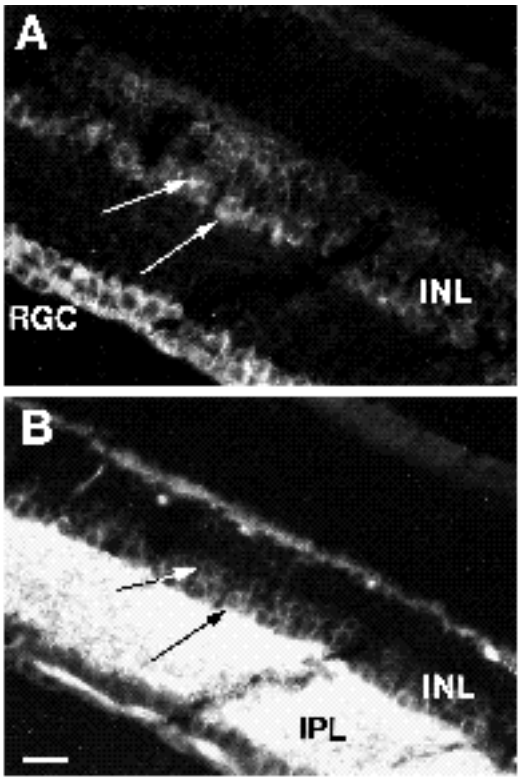

Fig. 4. Double-label immunohistochemistry of P15 mouse retina, with anti-PDGF-A and monoclonal antibody HPC-1, a surface marker of amacrine neurons. Anti-PDGF-A stains the retinal ganglion cell (RGC) layer and the inner nuclear layer (INL; A). Antibody HPC-1 stains the processes of amacrine neurons in the inner plexiform layer (IPL) and their cell bodies in the INL. Arrows indicate HPC-1-positive amacrine neurons that stain intensely for PDGF-A. Scale bar, 20 μm.

In addition to retinal ganglion and amacrine neurons, cells in the retinal pigment epithelium express PDGF-A strongly from E16 onwards (Fig. 2A-D), the level of expression declining gradually after birth.

PDGF-αR expression in the retina

PDGF-αR mRNA is abundant in many mesodermal and neural crest-derived tissues outside of the CNS (Morrison-Graham et al., 1992; Orr-Urtreger et al., 1992; Pringle et al., 1992; Schatteman et al., 1992). PDGF-αR is also expressed strongly in the proliferating anterior lens epithelium at all ages examined (Fig. 5A-H). The intense dark-field signal in the body of the postnatal lens (Fig. 5D-H) is artefactual, being caused by the intrinsic scattering qualities of the sectioned lens tissue. In the retina, cells expressing PDGF-αR first appear at E14 near the exit point of the optic nerve (arrow in Fig. 5A). Between E14 and the day of birth, PDGF-αR expression in this region intensifies and expands to fill the optic nerve head (Fig. 5B-D), then gradually spreads across the inner surface of the retina (Fig. 5D,E). The front of PDGF-αR$^+$ cells extends approximately 0.9 mm from the edge of the optic nerve head at P0, 1.2 mm at P2, 2.1 mm at P5 and reaches the periphery of the retina at around P8 (data not shown). This corresponds closely with the rate of migration of retinal astrocytes from the optic nerve head (Watanabe and Raff, 1988), suggesting strongly that the PDGF-αR$^+$ cells are astrocytes. This was confirmed by combining in situ hybridization with the PDGF-αR probe, and immunohistochemistry with an antibody against glial fibrillary acidic protein (GFAP), on either the same (Fig. 6C,D) or adjacent (Fig. 6A,B) sections. This showed that the PDGF-αR autoradiographic signal is associated with cells that express GFAP, a specific marker of astrocytes in the CNS. PDGF-αR expression in astrocytes in the optic fibre layer and at the optic nerve head persists throughout postnatal life (Fig. 5G,H) into adulthood (not shown). We do not believe that cells in the walls of blood vessels (e.g. endothelial cells) express PDGF-αR. Under high magnification, it is possible to discern structures at the optic nerve head and in the optic fibre layer that resemble cross-sectional and longitudinal views of blood vessels (see Fig. 10): these structures do not label with the PDGF-αR in situ probe. Moreover, we do not see PDGF-αR transcripts in the outer plexiform layer of the retina, where blood vessels are known to be located after the second postnatal week (Engerman and Meyer, 1954), or in any structures resembling blood vessels in the optic nerve or brain (Pringle et al., 1992).

By P2, a second band of PDGF-αR expression emerges in the INL (arrows in Fig. 5E,F). This band of PDGF-αR expression appears to expand outwards from the centre towards the periphery of the retina, reaching the outer edge by about P5. At P5 only a subset of cells in the INL expresses PDGF-αR; these cells are located more often in the central part of the INL than at the edges (Figs 7, 8C). At P15, the PDGF-αR signal is clearly restricted to the outer (choroidal) half of the INL (Fig. 8B).

Astrocytes in the optic nerve express PDGF-A

PDGF-A is synthesized and secreted by cultured rat cortical astrocytes (Richardson et al., 1988). The fact that PDGF-A mRNA (Pringle et al., 1989) and activity (Raff et al., 1988) is also present in the postnatal rat optic nerve led us to suggest that type-1 astrocytes are a major source of PDGF in the nerve. This hypothesis is supported by the results of our present study. At E14, when the optic nerve contains astrocyte precursors but no mature astrocytes, no PDGF-A transcripts are detected by in situ hybridization (data not shown). By E16, when GFAP$^+$ astrocytes are beginning to appear in the nerve (Miller et al., 1985), PDGF-A mRNA is present along the length of the optic nerve except for the developing lamina cribrosa at the extreme retinal end (Fig. 2A). Between E16 and the day of birth (E21/P0) the PDGF-A signal increases (compare Fig. 2B with 2C). PDGF-A expression persists throughout postnatal development (Fig. 2D), but declines somewhat in the adult (not shown). Similar results were obtained in the mouse optic nerve (Fig. 3A, for example). To determine the distribution of PDGF-A protein in the optic nerve we stained sections of P5 mouse optic nerve with our anti-PDGF-A antibody. Intense labelling of many cell processes was observed (Fig. 3F). These processes were generally aligned perpendicular to the long axis of the nerve, suggesting that they might correspond to astrocyte processes rather than RGC axons. To obtain direct evidence for PDGF

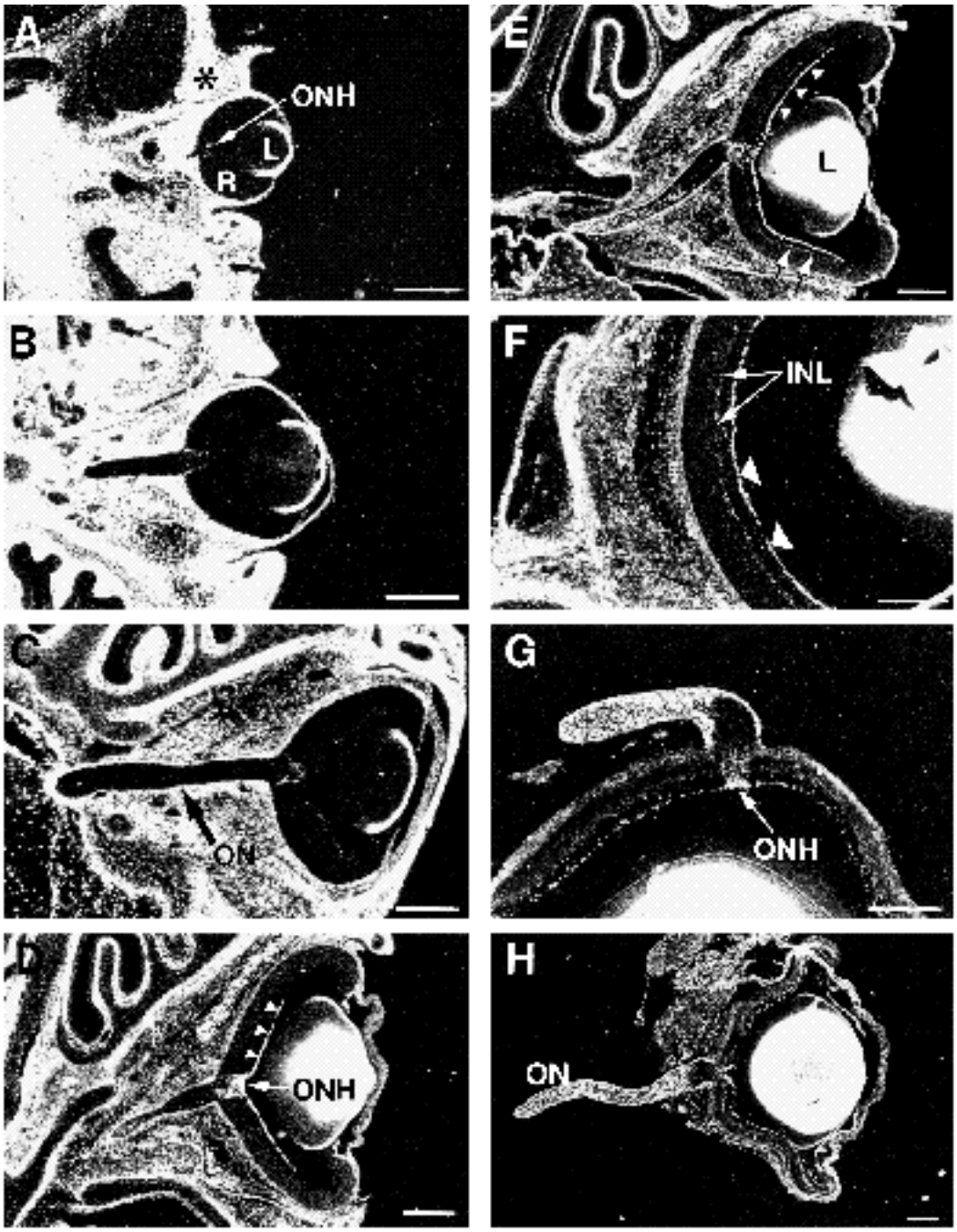

Fig. 5. Distribution of PDGF-αR transcripts in the developing rat retina (R), optic nerve (ON) and lens (L). Horizontal sections were cut through the optic nerves and eyes of rats at E14 (A), E16 (B), E18 (C), P0 (D), P2 (E), P5 (F), P10 (G) and P20 (H); these were subjected to in situ hybridization and photographed under dark-field illumination. PDGF-αR mRNA is observed at the optic nerve head (ONH) from E14 (arrows in A,D and G) and spreads across the inner surface of the retina starting between E18 and P0 (arrowheads in D, E, F). A second layer of PDGF-αR expression develops in the inner nuclear layer (INL) from P2 onwards (arrows in E and F). At all ages examined, PDGF-αR was expressed strongly in surrounding non-CNS tissues (asterisk in A). Scale bars: 500 μm.

in astrocytes, we performed double immunofluorescence labelling experiments on single sections with antibodies against PDGF-A and GFAP. In P5 mouse optic nerve, many cell processes were positive for both PDGF-A (Fig. 3D) and GFAP (Fig. 3E). Since similar patterns of labelling were obtained when either anti-GFAP (not shown) or anti-PDGF-A (Fig. 3F) were used alone, the near coincidence of PDGF-A and GFAP signals was not caused by unwanted cross-reactivities among the antibodies, or to fluorescence breakthrough between channels. No signal was observed when either of the primary antibodies was omitted (not shown). The PDGF-A signal in astrocyte processes was

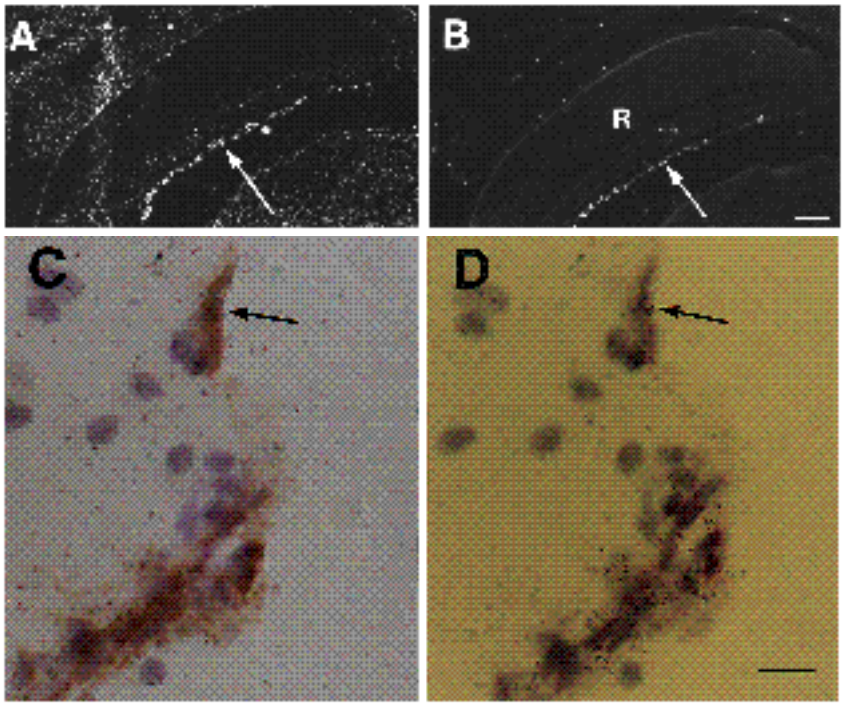

Fig. 6. PDGF-αR and GFAP expression in the rat retina. Consecutive sections through a P9 retina (R) were subjected to in situ hybridization with the PDGF-αR probe (A), and immunohistochemistry with an antibody against GFAP (B). The anti-GFAP antibody was detected with a biotinylated second antibody followed by streptavidin-fluorescein. The PDGF-αR and GFAP signals both lie in the optic fibre layer (arrows in A,B). One section was subjected to combined in situ hybridization to detect PDGF-αR transcripts and immunohistochemistry to detect GFAP (C,D). The GFAP antibody was visualized with a biotinylated second antibody followed by horseradish peroxidase (HRP)-conjugated streptavidin and diaminobenzidine (Vectastain kit). The PDGF-αR autoradiographic signal overlies cells (astrocytes) in the optic fibre layer that stain positive for GFAP. Note that the relatively high level of background in A obscures the PDGF-αR signal in the inner nuclear layer (see Fig. 7). Scale bars: A,B, 100 μm; C,D, 10 μm.

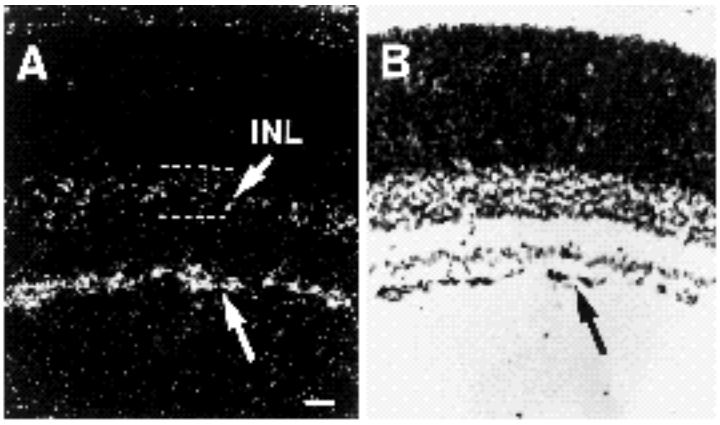

Fig. 7. PDGF-αR transcripts in the P5 rat retina. PDGF-αR transcripts were visualized by in situ hybridization and photographed under dark-field (A) and bright-field (B) illumination. Silver grains are present over cells (astrocytes) in the optic fibre layer (lower arrows) and in the interior of the inner nuclear layer (INL). The dotted lines indicate the limits of the INL. Scale bar, 20 μm.

strongly reduced when the antibody was preincubated with excess recombinant PDGF-AA (Fig. 3G) but was unaffected by preincubating with recombinant PDGF-BB or acidic or basic fibroblast growth factors (not shown). These and other controls for the specificity of the anti-PDGF-A antibody (e.g. see Fig. 3 legend) allow us to conclude confidently that many astrocytes in the optic nerve express PDGF-A. In contrast, the axons of retinal ganglion neurons in the optic nerve did not label with our anti-PDGF-A antibody.

PDGF-αR expression in the optic nerve

The optic nerve is completely devoid of PDGF-αR mRNA before E18, when a very few PDGF-αR$^+$ cells appear at the chiasmal end of the nerve (only one PDGF-αR$^+$ cell is visible in the nerve section illustrated in Fig. 5C). At P0 and P2, there are many more PDGF-αR$^+$ cells in the nerve, and these are distributed in a gradient increasing towards the optic chiasm (Fig. 5D,E). By P5 (not shown), the PDGF-αR$^+$ cells are distributed evenly along the nerve, apart from an exclusion zone close to the eye; this is illustrated for a P15 nerve in Fig. 5H. The exclusion zone includes, but is more extensive than, the lamina cribrosa, which is that part of the nerve that pierces the schlera.

The changing distribution of PDGF-αR$^+$ cells in the developing optic nerve, together with other evidence (for example, the ^{125}I-PDGF binding properties of cultured glial cells; Hart et al., 1989; Pringle et al., 1992) led us to suggest previously that the PDGF-αR$^+$ cells might correspond to oligodendrocyte precursors (Pringle et al., 1992). Oligodendrocyte precursors are thought to migrate into the developing optic nerve from germinal zones in the brain, via the optic chiasm (Small et al., 1987). The results presented here are consistent with that view.

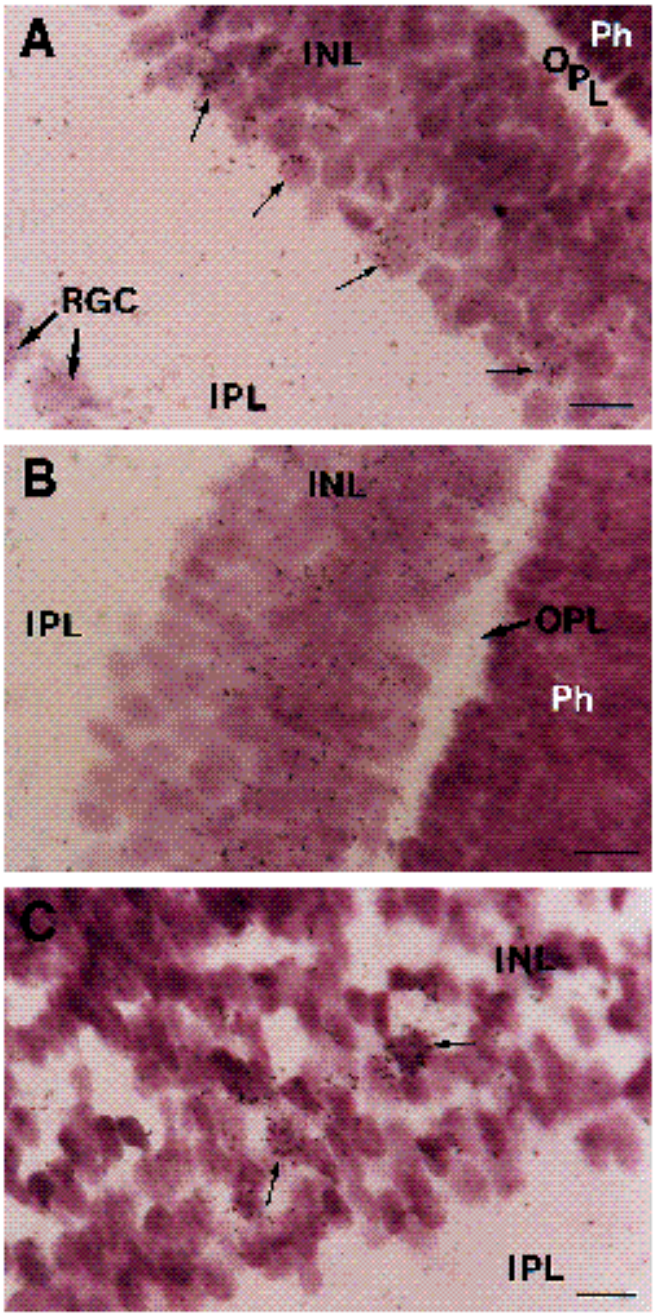

Fig. 8. High magnification bright-field images of in situ autoradiographs with the PDGF-A and PDGF-αR antisense probes, showing the distributions of silver grains over cells in the inner nuclear layer (INL) of the rat retina. The sections have been counterstained with haematoxylin, which stains the cell nuclei. A shows a section of a P15 rat retina (the same one illustrated in Fig. 2E), hybridized with the PDGF-A antisense probe. Silver grains lie over a subset of cells at the inner (vitreal) surface of the INL (small arrows) and over retinal ganglion cells (RGC; large arrows). B shows a section of a P15 rat retina (the same section as in Fig. 5H) hybridized with the PDGF-αR antisense probe. Silver grains overly cells in the outer (choroidal) half of the INL. C shows a section through a P5 rat retina (the same section as in Fig. 7) hybridized with the PDGF-αR antisense probe. At this age the developing INL is not so well delineated as at P15. Silver grains are associated with cells in the interior of of the INL. IPL, inner plexiform layer; INL inner nuclear layer; OPL, outer plexiform layer; Ph, photoreceptors. Scale bars, 20 μm.

PDGF-B and PDGF-βR expression in the developing microvasculature of the retina and optic nerve

The patterns of PDGF-B and PDGF-βR expression in the developing retina and optic nerve are very similar. This suggests that ligand and receptor are expressed by the same, or closely associated cells. In the E14 retina, PDGF-βR and PDGF-B are expressed in small islands of cells in contact with the back of the lens and at the vitreal surface of the retina (Fig. 9A,B). Cells in these locations continue to express PDGF-βR and PDGF-B during postnatal development (Fig. 9C,D) and into adulthood (not shown). At P15 another zone of cells that express PDGF-βR and PDGF-B is apparent in the retina, in the outer plexiform layer (OPL; small arrows in Fig. 9E-H). At high magnification (Fig. 10), it is evident that the PDGF-βR (Fig. 10A) and PDGF-B (Fig. 10B) signals are frequently associated with rows of cells that possess elongated nuclei. We presume that these rows of cells correspond to blood vessels, which are known to be present in the retina both in the optic fibre layer and the outer plexiform layer (Engerman and Meyer, 1954). It is worth noting that not all cells in these presumptive blood vessels express PDGF-B, and not all cells express PDGF-βR, suggesting that PDGF-B and PDGF-βR may be expressed by different subsets of endothelial cells and/or other cells in the walls of blood vessels.

PDGF-B and PDGF-βR transcripts are also present in groups of cells in the optic nerve. The distributions of cells expressing PDGF-B and PDGF-βR are similar and appear about the same time (around birth). As in the retina, the positive cells often have elongated nuclei and sometimes are arranged in rows, strongly suggestive of blood vessels (Fig. 10C). We think it likely that PDGF-βR is expressed by capillary endothelial cells in the optic nerve and retina, since purified endothelial cells from brain have been shown to express PDGF-βR in vitro (Smits et al., 1989). PDGF-B might also be expressed in endothelial cells or in nearby cells such as pericytes.

DISCUSSION

We have presented the results of an in situ hybridization study of PDGF and its receptors during development of the rodent retina and optic nerve. The reason for undertaking this study was the hope that, in a relatively simple, well-characterized and structurally ordered part of the CNS such as the retina, we might be able to identify the specific types of neurons or glial cells that express PDGF and/or PDGF receptors and infer something about their biological functions in the CNS.

PDGF-A and PDGF-B mRNAs are present at distinct sites in the retina, so it seems likely that most PDGF in the

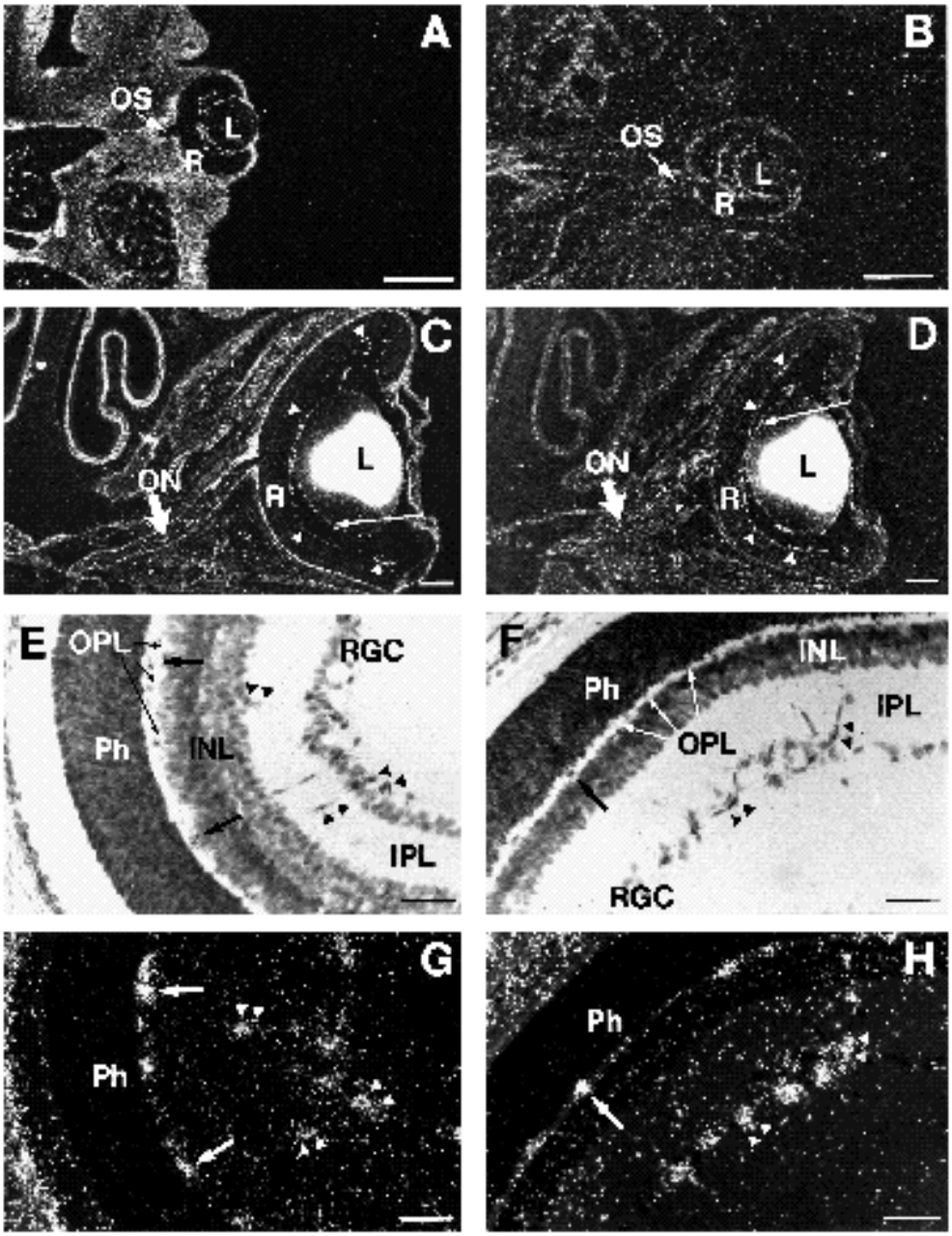

Fig. 9. Distribution of PDGF-βR and PDGF-B transcripts in the developing rat retina. Horizontal sections through rats at E14 (A,B), P2 (C,D) and P15 (E-H) were subjected to in situ hybridization with a probe against rat PDGF-βR or PDGF-B and photographed under dark-field or bright-field illumination. A and B depict nearby (not adjacent) sections of the same animal, while C and D depict adjacent sections of the same animal. E and G are corresponding bright-field and dark-field images of the same section through a P15 rat retina, hybridized with the PDGF-βR antisense probe. F and H are corresponding bright-field and dark-field images of the same section through a P15 rat retina hybridized with the PDGF-B antisense probe. In the eye, both PDGF-βR (A,C,E,G) and PDGF-B (B,D,F,H) are expressed in cells at the posterior surface of the lens (L; thin arrows in C,D), towards the inner surface of the retina (R; arrowheads in C,D,E-H) and, from about P15, in the outer plexiform layer (OPL; small arrows in E,G and F,H). PDGF-βR and PDGF-B are also expressed in cells in the optic nerve (ON; C,D); these cells sometimes appear to be arranged in parallel rows resembling blood vessels (see Fig. 10). OS, optic stalk; RGC, retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer, Ph, photoreceptors. Scale bars: A-D, 500 μm; E-H, 50 μm.

retina is assembled as PDGF-AA or PDGF-BB homodimers. Likewise, cells in the retina appear to express either PDGF-αR or PDGF-βR, but not both. We cannot exclude the possibility that some cells in the optic nerve might synthesize both A and B chains of PDGF, or both receptor subunits. It seems likely from in vitro studies, however, that oligodendrocyte lineage cells in the nerve express PDGF-αR but not PDGF-βR (Hart et al., 1989; McKinnon et al., 1990; for a review of PDGF and its receptors, see Heldin and Westermark, 1990).

Neurons in the retina express PDGF-A

PDGF-A is expressed by retinal ganglion neurons and a subset of amacrine neurons. This is consistent with a previous report that many neurons in both the CNS and the PNS express PDGF-A (Yeh et al., 1991). Expression of PDGF-A is not a general property of neurons, however, for the other major classes of neurons in the retina (bipolar neurons and photoreceptors) do not express PDGF-A. PDGF-B is not expressed by any neurons in the retina, although it is expressed by some neurons and neuronal precursors elsewhere in the CNS (Sasahara et al., 1991; H. Mudhar and W. Richardson, unpublished data).

Retinal astrocytes and cells in the INL express PDGF-αR

The PDGF-αR$^+$ cells at the optic nerve head and at the vitreal surface of the retina are astrocytes. Retinal astrocytes migrate into the retina from the optic disc (Ling and Stone, 1988; Watanabe and Raff, 1988), starting just before birth in the rat (Watanabe and Raff, 1988). This migration coincides closely with the spread of PDGF-αR$^+$ cells across the retina from the optic nerve head. The retinal vasculature develops radially from the optic nerve head on a very similar time scale to astrocyte migration (Ling and Stone, 1988); however, the PDGF-αR in situ hybridization signal coincides with GFAP$^+$ astrocytes and is absent from other cells in the optic fibre layer, including cells in the walls of blood vessels. It is noteworthy that retinal astrocytes appear to differ significantly from optic nerve astrocytes; retinal astrocytes express PDGF-αR but do not express detectable PDGF-A, whereas their optic nerve counterparts express PDGF-A but not PDGF-αR (see below).

PDGF-αR$^+$ cells in the interior of the INL are most likely either bipolar neurons or Müller glia. We favour the latter possibility, because the PDGF-αR$^+$ cells in the INL appear to be a minority of the cells in that region, as are the Müller glia. Also, there is some evidence that cultured Müller glia respond to PDGF by proliferation and chemotaxis (Uchihori and Puro, 1991), suggesting that they possess PDGF receptors, at least in vitro.

Evidence for short-range interactions between PDGF-A and PDGF-αR

We found that PDGF-A and its receptor PDGF-αR are expressed by separate but adjoining layers of cells in the retina (see Fig. 11). This arrangement strongly suggests that PDGF-A and PDGF-αR mediate local, paracrine interactions between cell bodies in the retina. Juxtaposed layers of cells that express PDGF-A or PDGF-αR also occur frequently in the embryo outside of the CNS (Orr-Urtreger and Lonai, 1992).

What is the biological significance of these local interactions? One possibility is that RGC-derived PDGF-A might stimulate proliferation and/or migration of retinal astrocytes from the optic nerve head across the surface of the retina, in contact with the optic fibre layer (RGC axons). Retinal astrocytes or their precursors start to express PDGF-αR at the optic nerve head at E14, before PDGF-A mRNA can be detected anywhere in the retina, but they do not start to migrate towards the periphery of the retina until around E18, coinciding with the appearance of PDGF-A mRNA in the cell bodies of RGCs (see Fig. 12). PDGF-A and PDGF-αR continue to be expressed by RGCs and astrocytes throughout life, so perhaps RGC-derived PDGF is required for long-term survival of astrocytes and/or to maintain appropriate matching of astrocyte numbers to the RGC population.

PDGF-αR is also expressed by cells in the interior of the INL, possibly Müller glia, starting around P2. Amacrine neurons in the inner aspect of the INL subsequently start to express PDGF-A around P10. There is never any cell division in the INL, and even retinal stem cell divisions at the outer surface of the neural retina are over by P7 (Sidman, 1961), so amacrine cell-derived PDGF-A cannot be a mitogen for retinal cells. It is possible that PDGF might act as a survival factor for PDGF-αR$^+$ cells in the INL, because naturally occurring cell death in the choroidal half of the INL peaks just before the onset of PDGF-A expression in amacrine cells, and declines thereafter (Beazley et al., 1987; see Fig. 12).

Electrical activity or target interactions may regulate PDGF-A expression in retinal neurons

Ganglion neurons and amacrine neurons are generated early in retinal development; in the mouse, production of RGCs is essentially over by E16, and amacrine neurons by P0 (Sidman, 1961; Young, 1985). Therefore, RGCs have been in existence for several days, and amacrine neurons for over a week, before they begin to express detectable levels of PDGF-A mRNA. Both classes of neurons begin by expressing low levels of PDGF-A mRNA for a few days, then noticeably up-regulate its expression (see Fig. 12). This could result from either transcriptional or post-transcriptional controls on PDGF-A gene expression.

What controls the onset of PDGF-A expression in these neurons? One possibility is that PDGF-A expression is a direct or indirect consequence of electrical activity in the neurons. Up-regulation of PDGF-A expression in RGCs occurs during the period, around birth, when the RGC axons encounter their target fields in the superior colliculi (Lund and Bund, 1976). Interactions with the target field are known to influence the neurotransmitter phenotype of sympathetic neurons (reviewed by Landis, 1990), and there is circumstantial evidence that target-derived NGF can regulate expression of neuropeptides in sensory neurons (Lindsay and Harmar, 1989). Up-regulation of PDGF-A expression in amacrine neurons between P10 and P15 may be related to the anatomical and synaptic maturation of the retina that occurs during the second postnatal week (Weidman and Kuwabara, 1968) and/or the development of the light-induced response, which peaks around the time of eye opening at the end of the second postnatal week (Ratto et al., 1991; Radel et al., 1992).

PDGF-αR marks oligodendrocyte progenitors in the optic nerve

PDGF-αR is expressed in the optic nerve and elsewhere by oligodendrocyte precursors and newly formed oligodendrocytes (Hart et al., 1989; McKinnon et al., 1990; Pringle et al., 1992). Oligodendrocyte lineage cells respond to PDGF by proliferation (Richardson et al., 1988; Raff et al.,

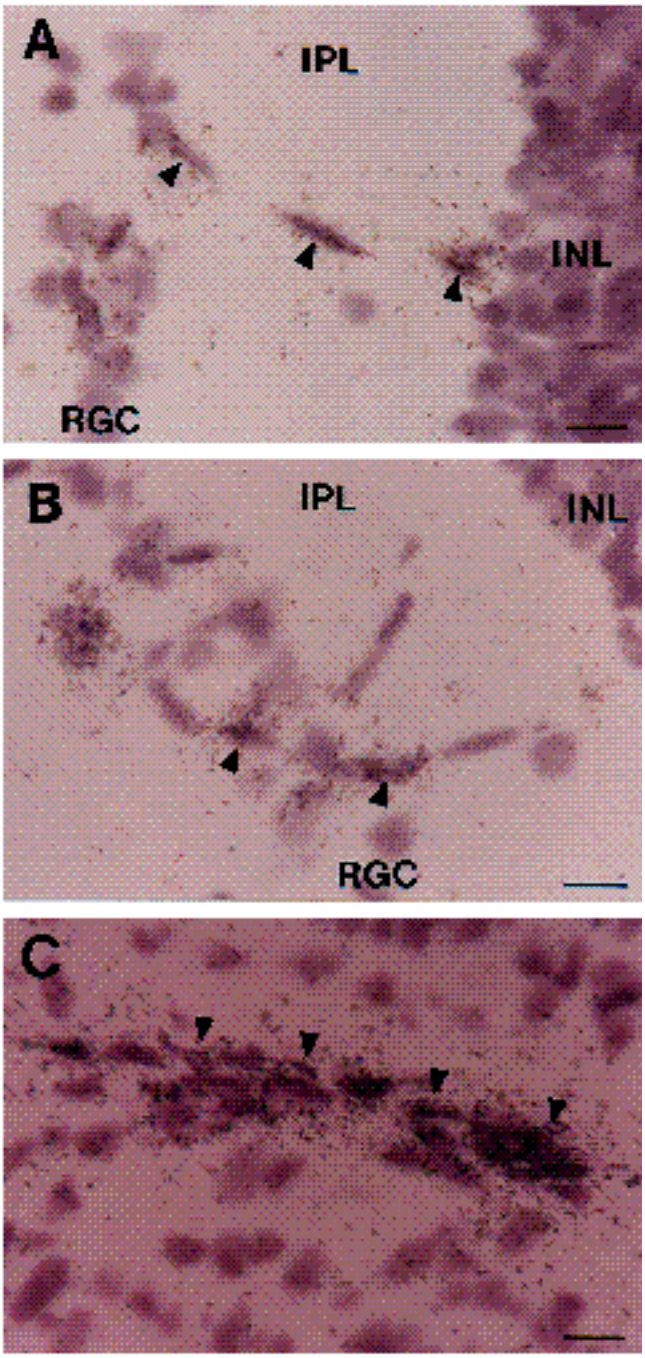

Fig. 10. High magnification bright-field images of in situ autoradiographs with the PDGF-B and PDGF-βR antisense probes on sections of P15 rat retina and optic nerve. The sections have been counterstained with haematoxylin, which visualizes the cell nuclei. A shows a section through a P15 rat retina hybridized with the PDGF-βR antisense probe. Only the inner part of the retina, encompassing the retinal ganglion cell (RGC) layer, the inner plexiform layer (IPL) and the inner (vitreal) aspect of the inner nuclear layer (INL) is shown. Silver grains lie over elongated nuclei in the RGC layer, the IPL and at the border of the INL (arrowheads); these cells often appear in rows and are probably associated with blood vessels (see text). Not all elongated nuclei in presumptive blood vessels express PDGF-βR, however. This section is a higher magnification view of the one in Fig. 9F and H, which shows that cells expressing PDGF-βR are also present in the outer plexiform layer. B shows a section through a P15 rat retina hybridized with the PDGF-B antisense probe. As in A, only the inner part of the retina (RGC layer, IPL and part of the INL) is illustrated. Silver grains lie over cells that sometimes have elongated nuclei (arrowhead), and are often close to other cells with elongated nuclei that are unlabelled. These PDGF-B-expressing cells, like the PDGF-βR-expressing cells, are probably associated with blood vessels. C shows a section through a P2 rat optic nerve, hybridized with the PDGF-βR antisense probe. As in the retina, silver grains often overly rows of cells with elongated nuclei that we interpret as blood vessels. Scale bar, 10 μm.

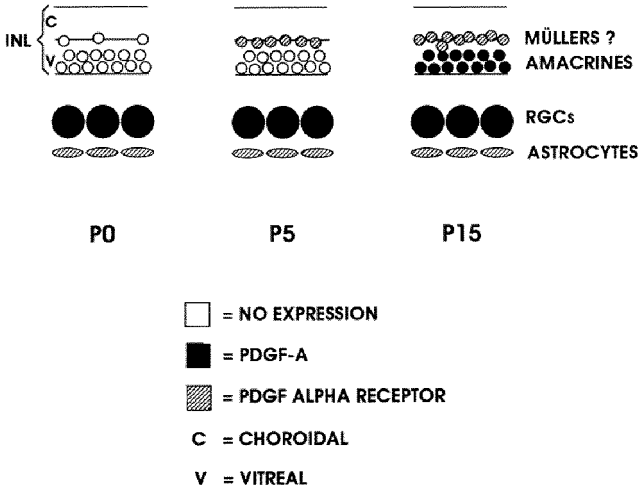

Fig. 11. PDGF-αR and PDGF-A transcripts in the neural retina during development. INL, inner nuclear layer; RGCs, retinal ganglion cells. PDGF-αR and PDGF-A are expressed in distinct but neighbouring populations of cells, suggesting local paracrine interactions between neurons (RGCs, amacrines) and glial cells (astrocytes, putative Müller glia) in the retina.

1988; Noble et al., 1988; Pringle et al., 1989) and survival (Barres et al., 1992b). Based on the temporal and spatial distributions of PDGF-αR$^+$ cells in the developing rat CNS, we previously suggested that expression of PDGF-αR may be restricted to cells of the oligodendrocyte lineage, at least during the later stages of neurogenesis (Pringle et al., 1992). Oligodendrocyte precursors are thought to migrate into the developing optic nerve and other white matter tracts from germinal zones in the brain (Small et al., 1987; Reynolds and Wilkin, 1988; LeVine and Goldman, 1988). The way that PDGF-αR$^+$ cells accumulate in the nerve, starting at the optic chiasm and progressing towards the eye, supports this idea (Pringle et al., 1992 and this paper, Fig. 5C,E,H).

The source of PDGF-A in the optic nerve: astrocytes or axons?

We previously presented circumstantial evidence that type-1 astrocytes in the rat optic nerve express PDGF-A (Richardson et al., 1988; Pringle et al., 1989). The results of double-labelling with antibodies against PDGF-A and GFAP now confirm that view (Fig. 3D,E). We might have expected to detect PDGF-A in the optic nerve axons, since

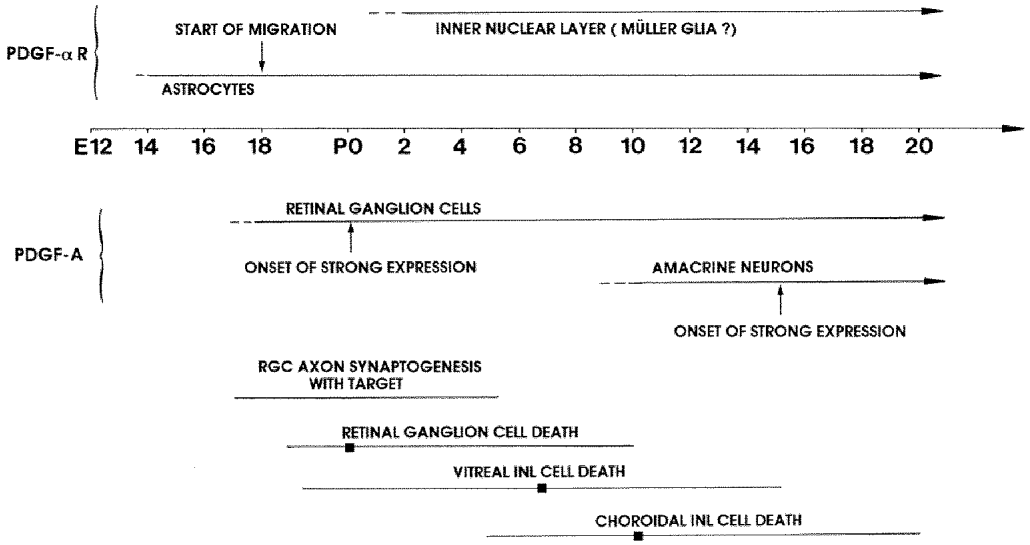

Fig. 12. The dynamics of PDGF-αR and PDGF-A expression in the developing retina compared to some other key developmental events. Dotted lines represent uncertainty about the precise time of onset of gene expression. Black boxes on lines indicate the peaks of cell death in the corresponding cell layers. The lower part of the Figure is adapted from Beazley et al. (1987).

PDGF-A immunoreactivity is associated with RGC axons in the optic fibre layer of the retina (Figs 3B, 4A). It would make sense if RGC axons were a source of PDGF for the oligodendrocyte precursors with which they interact, but our failure to detect PDGF-A immunoreactivity in optic nerve axons argues against this idea. It is possible that RGC-derived PDGF is only transported a short distance into the axons and does not reach the optic nerve, or its concentration in optic nerve axons may be below the limit of detection with our antibody. Alternatively, the PDGF-A that is associated with RGC axons in the retina may be surface-bound, not intracellular.

PDGF-B and PDGF-βR in developing and mature blood vessels

In the eye and optic nerve, the distribution pattern of PDGF-B mRNA is very similar to that of PDGF-βR mRNA. Blood vessels are known to be present at the sites of PDGF-B and PDGF-βR expression in the retina (Engerman and Meyer, 1954). Capillary endothelial cells from brain have been shown to express PDGF-βR in culture (Smits et al., 1989), and our in situ hybridization studies of the rat brain and spinal cord (H. Mudhar and W. Richardson, in preparation) strongly suggest that the same is true in vivo, so it seems reasonable to suppose that the PDGF-βR^+ cells that we see in the retina and optic nerve are endothelial cells. The co-localizing PDGF-B transcripts could be co-expressed in endothelial cells, or in another cell type associated with blood vessels, such as pericytes. There is evidence that capillary endothelial cells in the human placenta co-express PDGF-B and PDGF-βR (Holmgren et al., 1991), suggesting that these cells might undergo autocrine growth stimulation during development.

Blood vessels are closely associated with astrocytes at the inner surface of the rat retina; since retinal astrocytes express PDGF-αR they could conceivably respond to PDGF-BB from vascular cells as well as PDGF-AA from RGCs. Networks of cell-cell interactions such as this could be important for ensuring proportional growth and survival of interdependent tissue elements.

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