Platelet-Derived Growth Factor and its Receptors in Retinal Development

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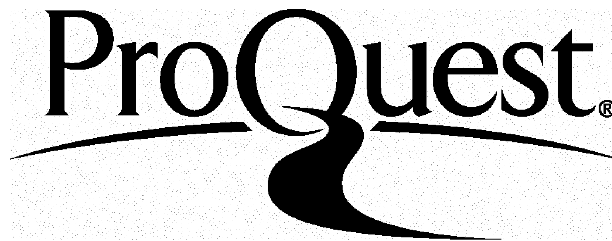

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To my late grandparents

ABSTRACT

Many polypeptide growth factors are expressed in the mammalian central nervous system (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. I have concentrated on the peripheral visual system, the optic nerve and retina, which is one of the simplest and best-characterized regions of the CNS.

Platelet-derived growth factor (PDGF) and its receptors are expressed in the developing and mature CNS. 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 with different ligand specificities. PDGF-A only activates the alpha receptor whereas PDGF-B activates both alpha and beta receptors.

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 PDGF, and the alpha and beta subunits of the receptors. We have also visualized PDGF-A protein in these tissues by immunohistochemistry. In the retina, PDGF-A mRNA is present in retinal ganglion cells (RGCs) and a subset of amacrine neurons. Transcripts accumulate in RGCs 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 electrical activity. In the mouse retina, PDGF-A immunoreactivity is present in the cell bodies, dendrites and proximal axons of RGCs, and throughout the inner nuclear layer (INL). PDGF-alpha receptor mRNA is expressed in the retina by astrocytes in the optic fibre layer and by a subset of cells in the INL. Taken together, our data suggest short-range paracrine interactions between PDGF-A and PDGF-alpha receptor, the ligand and its receptor expressed in neighbouring layers of cells in the retina.

What is the biological significance of these local interactions? There are never any retinal neuroepithelial cell divisions in the INL, so PDGF-A cannot be a mitogen for retinal cells. We tested the possibility that PDGF-A may act as a survival factor, by injecting recombinant PDGF-AA into P-7 rat eyes and counting the pyknotic cell nuclei at P-10, which is the time of maximal naturally-occurring cell death in the INL, during normal development. PDGF-AA reduced the number of pyknotic cell nuclei by approximately 50% as compared to controls. This implies that PDGF-A may be a survival factor in

the retina and furthermore that PDGF-A may be present in limiting amounts in the developing inner nuclear layer (INL). Secondly, what is the role of PDGF in astrocyte development? This question was addressed by neutralizing endogenous retinal PDGF, by using a soluble extracellular portion of the rat PDGF-α receptor. Cos cells transfected with expression vector pRα17, encoding the mutant receptor were injected into the vitreous, behind the lens. As compared to controls, the presence of the soluble extracellular alpha receptor significantly reduced the migration of the astrocytes across the retina and caused their processes to fasiculate.

In the optic nerve, PDGF-A immunoreactivity is present in astrocytes but apparently not in the retinal ganglion cell axons. PDGF-alpha receptor cells in the optic nerve first appear near the optic chiasm and subsequently spread to the retinal end of the nerve: these PDGF-alpha receptor positive cells are probably oligodendrocyte precursors. RNA transcripts encoding the PDGF-B and PDGF-beta receptor are expressed by cells of the hyaloid and mature vascular systems in the eye and optic nerve.

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ABBREVIATIONS

BDNF	Brain derived neurotrophic factor
bp	base pair
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CNTF	Ciliary neurotrophic factor
CNS	Central nervous system
CSF	Colony stimulating factor
dH_2O	deionized water
DNase	Deoxyribonuclease
EDTA	Ethylene Diamine Tertaric Acid
EGF	Epidermal growth factor
FITC	Fluorescein isothiocyanate
GFAP	Glial fibrillary acidic protein
HRP	Horse radish peroxidase
IGF	Insulin-like growth factor
INL	Inner nuclear layer
IPL	Inner plexiform layer
kb	kilobase
M	Molar
mRNA	Messenger RNA
NGF	Nerve growth factor
NGS	Normal goat serum
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PCR	Polymerase chain reaction

PDGF	Platelet-derived growth factor
PI	Propidium iodide
PNS	Peripheral nervous system
RGC	Retinal ganglion cell
RNASe	Ribonuclease
RNASin	Human placental RNASe inhibitor
SH	Src homology
TAE	Tris acetate agarose running buffer
TBS	Tris buffered saline
TGF-α	Transforming growth factor-alpha
TK	Tyrosine kinase
Trk	Tyrosine receptor kinase

CHAPTER ONE

1.1POLYPEPTIDE GROWTH FACTORS

The ability of on organism to grow, survive and replicate is fundamental to its life cycle. In a developing organism, these processes have to be co-ordinated both spatially and temporally; this depends on a balance between positive and negative influences and involves interactions between cell-intrinsic mechanisms and signalling molecules in the extracellular environment. These signalling molecules can either be cell surface or matrix bound, or freely diffusible in the extracellular medium. One major class of signalling molecules are the polypeptide growth factors, which are secreted from cells and subsequently remain soluble or, in certain cases, bind to extracellular components of the extracellular matrix. They interact with cells by binding to specific transmembrane cell-surface receptors, and elicit a wide range of physiological responses. The precise response of a cell is dependent on the biochemical make-up of that cell, and the modulating influences of other signalling molecules in the environment. Although they have been termed growth factors, they can induce many cellular responses apart from mitosis; they can act as differentiation factors, trophic factors and probably fulfill other functions which have yet to be discovered.

1.2 GROWTH FACTOR RECEPTORS

In order to make sense of its immediate environment a cell should posses machinery that can interpret and react appropriately to environmental stimuli. Cells detect signals in the extracellular environment through specific ligand-binding receptor proteins. Broadly speaking, cells possess two types of receptor; intracellular and transmembrane. The best known example of the former are the classic steroid nuclear receptors. Here,

the lipophilic steroid molecule crosses the plasma membrane, binds to its appropriate receptor in the cytoplasm or nucleus of the cell, and modulates transcription by binding directly to sequence elements associated with responsive genes (Yamamoto, 1985). In the case of transmembrane receptors, there are many types, which can be conveniently divided into three broad categories: ligand gated ion channels; G-protein linked receptors and enzyme linked receptors (Kahn, 1976; Snyder, 1985). Growth factor receptors fall into the latter category.

1.3 STRUCTURE OF GROWTH FACTOR RECEPTORS.

Growth factors mediate their many effects by binding to cell surface, membrane-spanning, enzyme-linked receptors, which can be tyrosine, serine or threonine kinases or phosphatases. By far the most well characterised type of receptor is that associated with tyrosine kinase activity. All receptor tyrosine kinases are composed of three major domains; an extracellular, glycosylated portion, a membrane spanning, alpha helical hydrophobic region and a cytoplasmic domain that contains a tyrosine kinase catalytic domain and regulatory autophosphorylation sites. The ligand, by binding to the extracellular domain, activates the cytoplasmic portion which is responsible for transmitting the signals generated to intracellular target proteins that mediate the biological response.

On the basis of sequence similarity and structure, there are at least four subclasses of tyrosine kinase receptors (Fig. 1). Through the availability of cDNA clones for these receptors and subsequent mutational analyses, it has been possible to begin to elucidate their mechanism of action.

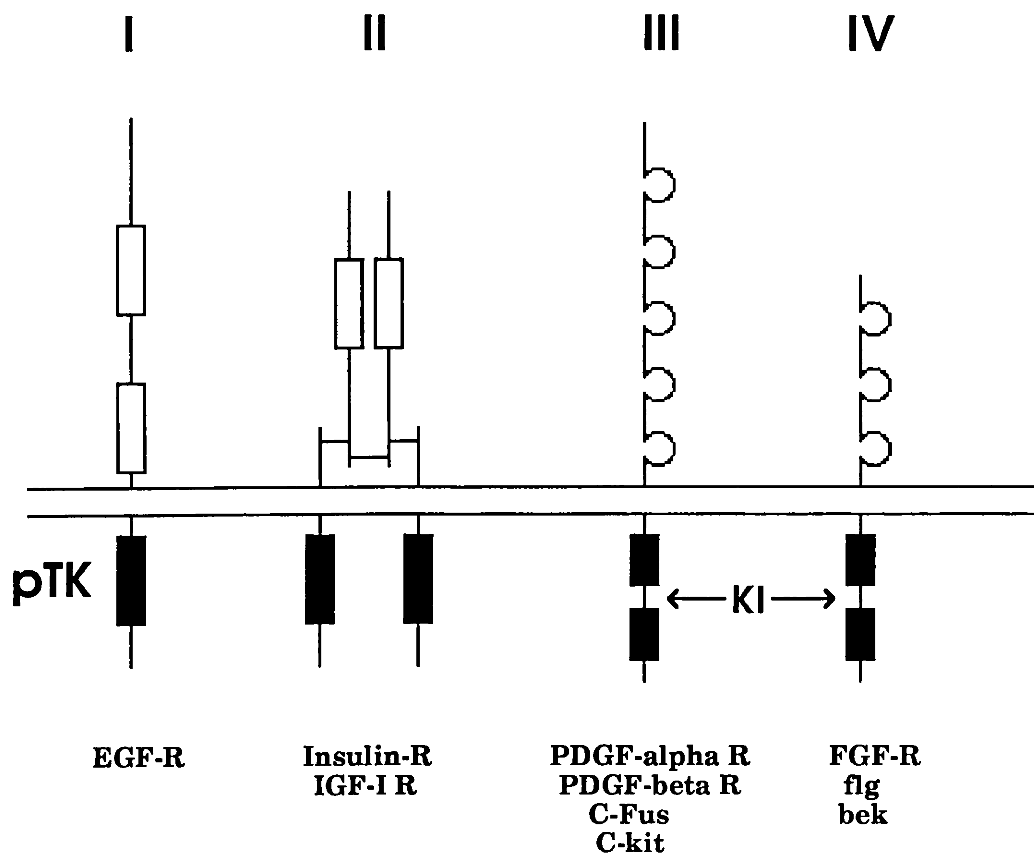


Figure 1. Receptor Tyrosine Kinase Subclasses
(Adapted from Ullrich and Schlessinger, 1988)

pTK=protein tyrosine kinase, KI=kinase insert

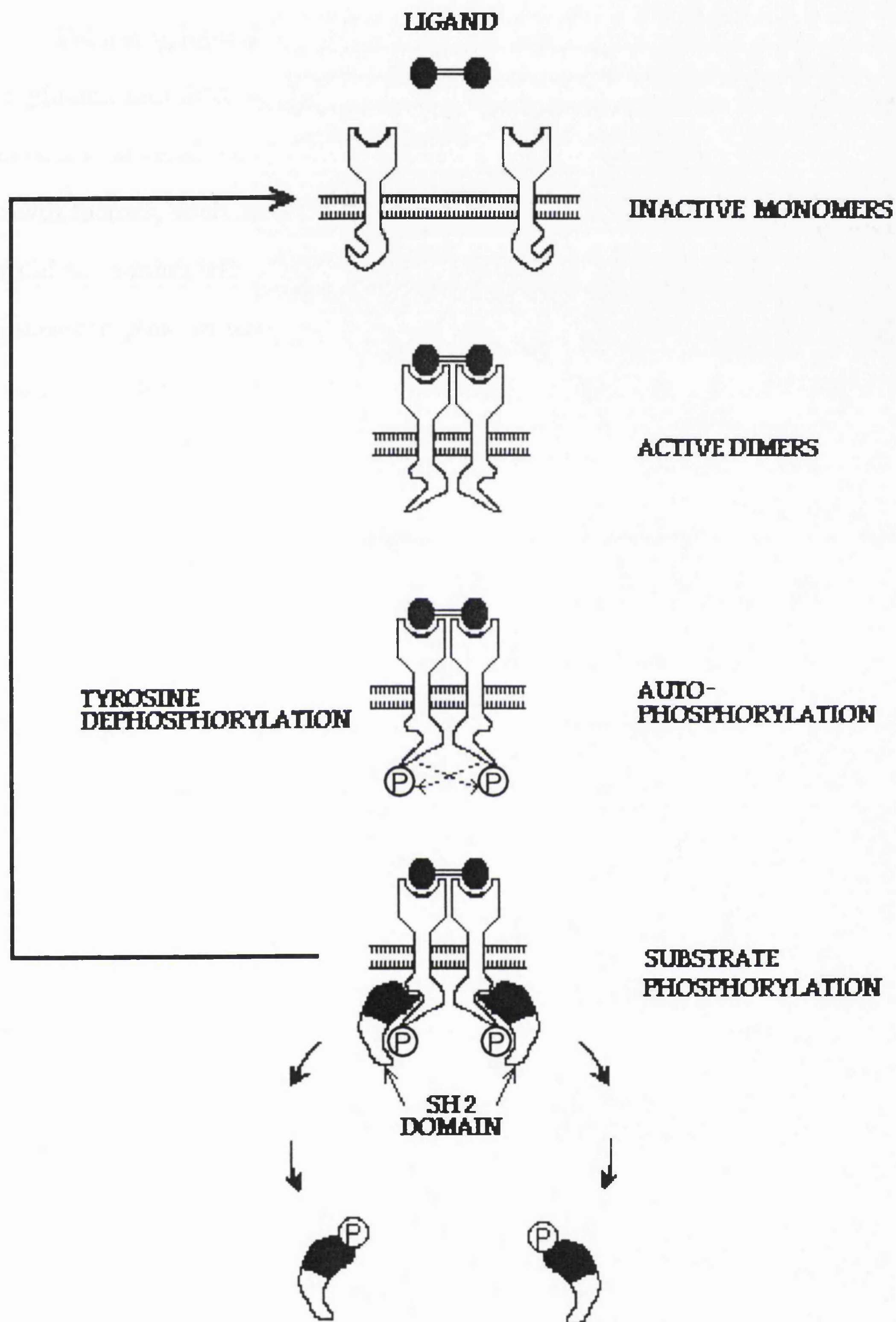


Figure 2. Dimerization Model for Receptor Activation.
(adapted from Ullrich and Schlessinger 1992)

Following ligand binding, receptors undergo dimerization in the plane of the plasma membrane (Schlessinger, 1988; Ullrich and Schlessinger, 1990). There are several ways in which this can be achieved. In the case of dimeric growth factors, such as platelet-derived growth factor (PDGF), binding of the ligand to monomeric, inactive receptors cross links them to form an active dimeric complex. In the case of epidermal growth factor (EGF), dimerization is thought to operate via a conformational change in the extracellular domain that stabilizes the interaction between two occupied receptors (Lax et al., 1991; Hurwitz et al., 1991). The function of dimerization is to activate the intrinsic protein tyrosine kinase activity. On activation, the kinase is responsible for phosphorylating specific tyrosine residues on the cytoplasmic domain of its associated receptor. The phosphorylated residues then act as docking sites for specific cytoplasmic proteins that are themselves phosphorylated and are responsible for transducing the signal (Fig. 2).

A cell that has had its tyrosine kinase receptors activated displays a myriad of cytoplasmic interactions that eventually lead to a physiological response, that is, the transduction of signals from the cell surface to the nucleus. We shall briefly consider some of the proteins involved in this cascade and discuss their properties. The tyrosine-phosphorylated regions in growth factor receptors function as docking sites for cellular proteins such as phospholipase C-gamma (PLC-γ) ,the GTPase-activating protein (GAP) of ras and the 85 kDa subunit (P85) of phospyatidylinositol 3-kinase (PI 3-kinase) and other proteins (Koch et al., 1991). These and other signaling proteins are able to recognize specifically different growth factor receptors, and the association between these proteins is dependent on phosphorylation of the receptor molecules on tyrosine residues (Margolis et al., 1990). This association is mediated by a conserved region of approximately 100 amino

acids, termed src homology 2 domains (SH2). These are recognition motifs for specific tyrosine-phosphorylated peptide sequences. SH2 domains are usually accompanied by another conserved domain of 50 amino acids, termed SH3 domain. The specificity of the interactions between receptors and SH2 domains is determined by the amino acid residues immediately surrounding the phosphorylated tyrosine residues. Proteins containing SH2 and SH3 domains can be divided into two groups. One group contains various catalytic activities within the same polypeptide chain, while the second group of proteins contains only SH2 and SH3 domains. The two types of domain are frequently found within a single protein, although certain proteins contain only SH3 or SH2 domains. SH3 domains have been found in cytoskeletal proteins, such as myosin1B, fodrin and the yeast actin-binding protein ABP-1 (Drubin et al., 1990; Jung et al., 1987). SH3 domains are thought to act as protein binding modules, and that they are involved in linking signals transmitted from the cell surface by protein-tyrosine kinases to 'downstream' effector proteins. It has been shown that deletion of the SH3 domains of the c-Src or c-Abl tyrosine kinases leads to activation of their oncogenic capacity, maybe reflecting a loss of regulation of kinase activity and interaction with proteins (Jackson et al., 1990). SH3 may therefore have regulatory roles, mediating interactions between the kinases and proteins with regulatory functions.

Having described the general properties of SH2 and SH3 domains and alluding to their functions, it is best to illustrate their actions by means of a few examples, as illustrated in the table below.

Molecule	C. Elegans	Species Drosophila	Mammal
Tyrosine kinase receptor	let-23	sevenless	EGF-R
SH3-SH2-SH3 adapter protein	sem-5	Drk	Grb-2
Guanine-nucleotide exchange factor	?	Sos	mSos1&2
Ras GTPase	Ras	Ras	Ras

Table 1. Summary of the known components of the Ras signaling pathway in *C. elegans, Drosophila* and mammals. In all cases, an activated receptor kinase (Let 23 in *C. elegans,* Sevenless in *Drosophila* and EGF receptor in mammals) bind to an adapter protein (Sem5, Drk and Grb 2) that links the nucleotide-releasing factor, Sos to Ras activation. (adapted from Schlessinger, 1993)

The species in which these molecular interactions have been studied will be discussed in future sections, Suffice to say that in each case, an activated receptor kinase is proposed to bind to the SH2 domain of an adapter protein which, in turn, interacts through its SH3 domain with a guanine-nucleotide releasing factor to stimulate the GTPase activity of Ras. It is also crucial to note the conservation of this pathway from invertebrates to mammals. Ras is a crucial mediator of many biological responses stimulated by both receptor

and nonreceptor kinases (Satch et al., 1992).It may be regarded as a crucial molecular switch. It is a small guanine nucleotide-binding protein that is biologically inactive in the GDP bound state, and is activated by exchange of GDP for GTP. Formation of GTP-bound Ras is regulated by guanine nucleotide releasing factors and the GTPase activity of Ras is enhanced by GTPase-activating proteins such as GAP and NF1 (Polakis et al., 1993) . It has been shown that various growth factors stimulate the formation of GTP-Ras thus coupling signals from the cell surface to the downstream kinase cascade (Satch et al., 1992; Polakis et al., 1993). The kinase cascade downstream of Ras includes the serine/threonine kinase Raf, mitogen-activated protein kinase kinase (MAPKK) and mitogen-activated protein kinase (MAPK) (Satch et al., 1992). The activation of this cascade leads ultimately to the phosphorylation of transcription factors.

1.4 IMPORTANCE OF GROWTH FACTORS AND THEIR RECEPTORS IN DEVELOPMENT.

Studies in flies, nematodes and mice have shown that growth factor signalling plays crucial roles during development. Genetic studies in mice have uncovered a whole host of mutations that exhibit pleiotropic developmental defects. For example the White Spotting (W) and Steel (Sl) mouse mutants exhibit anomalies in the melanocytic, haemopoietic and germ cell lineages. It has been shown that there is allelism between the W mutation and *c-kit*, which encodes a transmembrane protein tyrosine kinase, that is similar to the receptors for colony-stimulating factor-1 (CSF-1) and PDGF. The Sl locus has been shown to encode the ligand (kit-ligand or Steel factor) that binds to and activates c-kit (Chabot et al., 1988; Geissler, 1988; Reith et al., 1990). In the Patch mouse mutant which exhibits a deletion of the PDGF-alpha receptor

(and possibly a neighbouring gene(s)), homozygotes for the mutation die before birth after exhibiting certain gross abnormalities, including cleft face, subepidermal blebs, and general oedema (Gruneberg and Truslove, 1960). Heterozygotes exhibit abnormal melanocyte development. In a couple of recent studies null mutations of the transforming growth factor-alpha gene (TGF-α) have been documented. A mouse mutant called Waved-1 (wa-1), which exhibits abnormal skin architecture, wavy hair, and curly whiskers and often develops corneal inflammation is as a result of a mutation of the TGF-alpha gene. Creation of a null mutation by gene targeting in embryonic stem cells (ES) of the TGF-α gene generates a mutant that in the homozygous state, is healthy and fertile but exhibits the same features as the wa-1 mutant (Bruce Mann et al., 1993; Luetteke et al., 1993).

Many other examples exist of developmental defects being the result of mutations in growth factor signalling pathways.

1.5 DROSOPHILA EYE DEVELOPMENT, CAENORHABDITIS ELEGANS VULVAL INDUCTION-EXAMPLES OF CELL SIGNALLING BY INTEGRAL MEMBRANE PROTEINS.

Two invertebrate developmental systems, the developing *Drosophila* eye and vulval development in *Caenorhabditis elegans,* have been thoroughly studied because of the simplicity of these organisms and the fact that they are accessible to mutational analysis. In both developmental systems, one founder cell instructs the differentiated cell fates of its neighbours by cell-contact mediated signalling events.

During *C. elegans* vulval induction, the anchor cell of the gonad induces three of the six neighbouring, ectodermal precursor cells to generate vulval

tissue (Horvitz and Sternberg, 1991), with the other three going on to form nonspecific epidermis. Several genes are involved in this vulval induction process; *lin-3* encodes an epidermal growth factor (EGF- like) ligand which is expressed on the surface of the anchor cell (Hill and Sternberg, 1992). The *let-23* gene encodes a tyrosine kinase of the EGF receptor subfamily which is the receptor for the *lin-3* ligand (Aroian et al., 1990) and is expressed on the precursor cells. As ligand binds to receptor a series of transduction events ensue, implicating the gene product of *let-60* that encodes a Ras protein, acting downstream of *let-23* (Han and Sternberg, 1990) to control vulval fates and *sem-5* , a gene that encodes a protein that has Src homology domains SH2 and SH3 that is thought to act between *let-23* and *let-60* (Clark et al., 1992). More recently, this cascade of events has been extended to by the identification of *lin-45,* which encodes the cytoplasmic serine-threonine kinase, Raf, and acts downstream of *let-60 ras* .If mutations take place in any of these genes, a “vulva-less” phenotype is observed. Conversely, a lack of activity of negatively acting genes or overactivity of *let-60 ras* leads to a multivulva phenotype (Han et al., 1993).

The adult Drosophila eye is made up of 800 twenty-cell units called ommatidia, with each ommatidium being made up of eight photoreceptor cells, called R1-R8, four lens secreting cells and eight accessory cells. During development the photoreceptors assemble and differentiate in a fixed temporal sequence; R8 is the founder cell in each ommatidium, followed by the pairwise addition of R2/5, R3/4 and R1/6, with R7 appearing last (reviewed in Banerjee and Zipursky, 1990). Cell fate specification of the R7 photoreceptor depends on a signal from R8. This induction is mediated by two cell surface proteins: the ligand, bride of sevenless, Boss (Reinke et al., 1988; Hart et al., 1990), which is the ligand for the product of the *sevenless* (*sev*) (Harris et al.,

1976; Hafen et al., 1987; Bowtell et al., 1988; Basler et al., 1991), a membrane spanning, tyrosine kinase receptor. Boss is expressed only by R8 and its reception requires the function of sevenless in the R7 cell. In mutations affecting *boss* or *sev*, the R7 cell differentiates into a cone cell. Like *C. elegans* vulval induction, the intracellular signal that eventually leads to induction is conveyed via a series of intracellular proteins present in R7. These include products of the genes, from upstream to downstream in the cascade series, *drk*, which is the equivalent of *sem-5* (Olivier et al., 1993) *son-of-sevenless* (Rogge and Karlovitch, 1991) which is believed to be an exchange factor, *ras 1* (Simon et al., 1991) , *gap1* (Gaul et al., 1992) *draf* which encodes a cytoplasmic serine-threonine kinase (Ambrosio et al., 1989) and *seven-in-abstentia* (Carthew and Rubin, 1990) which encodes a nuclear factor that is required in R7.

Common features are therefore emerging from mutational analysis of developmental signalling cascades in worms and flies, and these insights are applicable to cells of higher organisms.

1.6 DEVELOPMENT OF THE VERTEBRATE CENTRAL NERVOUS SYSTEM .

The mature vertebrate central nervous system (CNS) consists of a variety of neurons and non-neuronal cells, principally glial cells. When and how these cells arise is a key question and forms the basis of understanding how regional diversification arises at the anatomical level. The CNS is ectodermal in origin. Neural induction , the process by which the ectoderm becomes induced to become neural tissue, occurs as a consequence of signals emanating from underlying mesodermal tissue (Mangold, 1933). Neural

induction results in the formation of the neural plate, whose lateral borders then roll up, meet and fuse along the midline to form the neural tube. At its earliest stage, the neural tube consists of a central cavity, the forerunner of the central canal and ventricular system of the mature spinal cord and brain; its wall consists of rapidly dividing neuroepithelial precursor cells. Regional diversification occurs, such that in its early stages the rostral part consists of a series of enlargements whose borders demarcate the prosencephalon-forebrain, the mesencephalon-midbrain and the rhombencephalon- hindbrain. At predetermined times during development, the dividing neuroepithelial cells at the ventricular surface give rise to post-mitotic neuronal progenitor cells, which migrate radially into the developing grey matter and differentiate in situ. In this way, the regions undergo further anatomical change to create the mature structures, such as the cerebral cortices, which are derived from the prosencephalon and the cerebellum, which is derived from the rhombencephalon. At a given point in time, the CNS consists of several anatomically distinct regions which are at different stages of maturation.

1.7 THE ROLE OF GROWTH FACTORS IN CNS DEVELOPMENT.

Polypeptide growth factors have now been established as playing key roles in the development of the CNS. Several well-known and characterized growth factors are expressed in the developing CNS; these include IGFs, FGFs, PDGF, EGF and the neurotrophins. The functions of these factors is under investigation and I review what is known about this in the following paragraphs.

A.Insulin-like growth factors

Endocrine hormones like insulin constitute a family of growth regulating factors that includes relaxin and insulin-like growth factors-I and II (IGF-I and II). Insulin is released from the pancreas and affects general metabolism and may also be required for long-term survival ; IGFs are released from the liver to affect the growth and development of an organism. Insulin and IGFs have their own distinct receptors although, high concentrations can cross react with the non-cognate receptor. mRNA and protein for ligand and receptor have been found in the developing and mature nervous system. Insulin and IGFs increase neurite outgrowth of a wide range of isolated central neurons, as well as enhancing their long-term survival in vitro (Aizenman and de Vellis, 1987). Physiological levels of IGF-I and IGF-II induce neurite outgrowth in cultures of motor (Caroni and Grandes, 1990), sensory (Bothwell 1982; Recio-Pinto et al., 1986) and sympathetic (Recio-Pinto et al., 1986) neurons. The survival-enhancing effects of insulin and IGFs are not restricted to neuronal cells, since they can also act as survival factors for oligodendrocyte precursors (O-2A progenitors) and for oligodendrocytes (Barres et al., 1992a; Barres et al., 1993a, 1993b, 1993c). Recent studies have shown, in addition, that IGF-I is a survival factor for axon-deprived oligodendrocytes in vivo (Barres et al., 1993b)

In certain studies insulin and IGFs have been shown to be co-mitogens with other factors for certain cell types although they do not by themselves promote proliferation. For example, neuroepithelial cells survive in vitro in the presence of IGFs alone, but the cells do not proliferate unless a mitogenic growth factor such as bFGF is also present in the growth medium (Drago et al., 1991).

Experiments with transgenic mice have also revealed important

findings regarding in vivo roles for IGF-I. Transgenic mice carrying an extra copy of the IGF gene showed a 55% increase in brain size as compared to controls, owing to an overall increase in cell size and, apparently, cell number (Carson et al., 1993). Further analysis showed that the amount of myelin produced by each oligodendrocyte had increased, although the absolute numbers of oligodendrocytes were not compared between transgenics and controls. In the light of what is known about IGF acting as a survival factor for oligodendrocytes and their precursors (Barres et al., 1992a, 1993a, 1993b, 1993c) it is possible that oligodendrocyte numbers are increased in the transgenics compared to controls.

B. The Neurotrophins.

The neurotrophins now comprise a large family of proteins that play key roles as neuronal survival factors during development. In the course of neural development many more neurons are produced than are actually required and in time those neurons that are not required die. Overall, up to 50% of the cells in certain neuronal populations can die during CNS development. The reason for this mass destruction is to match the number of neurons to the size of the target field and to eliminate inappropriate nerve connections. A neural target is usually innervated by a set number of neurons. The target tissue produces limiting amounts of neurotrophic factor, which the incoming neurons compete for. Those that receive an adequate supply of neurotrophic factor survive and those that do not, die. The first of such factors to be identified was nerve growth factor, NGF (Cohen, 1960), which was isolated and purified from the mouse submandibular gland. At the same time Cohen provided evidence that NGF was required in vivo, since anti-NGF antibodies, injected into newborn rodents, destroyed the peripheral

sympathetic nervous system (Levi-Montalcini and Booker, 1960; Cohen, 1960). It was then confirmed that NGF supported the survival of dissociated sensory and sympathetic neurons in vitro (Levi-Montalcini and Angeletti, 1963).

The neurotrophin family now comprises five related proteins; NGF, brain-derived neurotrophic factor (BDNF) and neurotrophins 3,4 and 5 (NT-3, NT-4 and NT-5). The actions of the neurotrophins are mediated via the *trk* proto-oncogene tyrosine kinase receptors, of which there are 3 known types, TrkA, TrkB and TrkC. NGF mediates its effects via TrkA to which it binds with high affinity, while BDNF acts via TrkB , NT-3 acts via TrkC and the actions of NT-4 and NT-5 are mediated via trkB but also, to a lesser extent through Trk-A. One characteristic feature of the neurotrophins is that they are promiscuous; multiple interactions exist between them and their receptor tyrosine kinases, with principal and weaker cross-reactivities. For example, NT-3 has TrkC as its main receptor but also cross-reacts with Trk-A and TrkB. The same applies to NT-4 and NT-5 as stated above (Review: Glass and Yancopoulos, 1993).

All of the neurotrophins bind to the low affinity NGF receptor, p75, which is a transmembrane glycoprotein, that does not possess a tyrosine kinase domain and is expressed in all NGF-responsive cells. Several roles for this receptor have been proposed, mainly pertaining to the establishment of high affinity binding sites for NGF in association with TrkA. Targeted mutations of p75 leads to deficits in the peripheral sensory nervous system. Mice homozygous for the mutation are viable and fertile and display decreased sensory innervation by calcitonin gene-related peptide and substance P-immunoreactive fibres. The defective innervation is correlated with loss of heat sensitivity and associated with ulceration in distal extremities. Analysis

of sympathetic ganglia or the density of sympathetic innervation of the iris or salivary gland showed no deficits (Lee et al., 1990). The results suggest that the low affinity receptor is implicated in the development and function of sensory neurons.

Recently targeted mutations of the TrkA, TrkB and TrkC genes have displayed some very interesting phenotypes. Mice lacking TrkA have severe sensory and sympathetic neuropathies and most die within one month of birth. They have extensive neuronal cell loss in trigeminal, sympathetic and dorsal root ganglia, as well as a decrease in the cholinergic basal forebrain projections to the hippocampus and cortex. These findings demonstrate that TrkA is the primary mediator of the trophic actions of NGF in vivo and that this signalling pathway plays a crucial role in the development of both the peripheral and the CNS (Smeyne et al., 1994). Homozygous mice defective for TrkC tyrosine protein kinase receptors lack Ia muscle afferent projections to spinal motor neurons and have fewer large myelinated axons in the dorsal root posterior columns of the spinal cord. These mice display abnormal movements and postures, indicating that NT3/TrkC-dependent sensory neurons may play a primary role in proprioception, the sense of position and movement of the limbs (Klein et al.,1994).

All of the neurotrophins support the survival of distinct, but overlapping sets of neurons in vitro (Maisonpierre et al., 1990;Hohn et al., 1990). Most of the neurotrophins and their receptors have been mapped in the CNS, mainly by in situ hybridization with some antibody staining. NGF produced by specific target tissues enhances the survival, differentiation and maintenance of sensory and sympathetic neurons in vitro and in vivo. In the brain, NGF appears to act as a trophic factor in vitro for basal forebrain cholinergic neurons, which project to the hippocampus and neocortex (both

sites of NGF synthesis and release) and striatal (caudatoputamen) neurons (Gnahn et al., 1983;Johnston et al., 1987). Transection of the fimbria-fornix normally leads to degeneration of basal forebrain cholinergic neurons. If NGF is infused into the ventricles after such a lesion, the degeneration is prevented or reversed (Hefti, 1986; Kromer et al., 1987; Hagg et al., 1988; Koliatos et al.,1990; Tuszynski et al., 1990). Regarding cognitive function, if NGF is infused into aged rats, NGF enhances spatial learning and reverses cholinergic neuronal atrophy (Fischer et al., 1987). Although principally a trophic factor, NGF has been found to be a mitogen for certain neural crest derived cells. Adrenal chromaffin cells from postnatal rats proliferate in response to NGF (Lillien and Claude, 1985) and, given further exposure, differentiate into neuronal-like cells. NGF has also been reported to be a mitogen for CNS neuroepithelial stem cells (Catteneo and McKay, 1990). In addition it is a mitogen for NIH 3T3 cells that have TrkA transfected into them.

BDNF has been localized in a variety of species to specific neuronal populations. The highest expression at the mRNA level is seen in the hippocampal formation (Ernfors et al., 1990;Phillips et al., 1990). In other regions studied , BDNF mRNA is also found in pyramidal cells of the cerebral cortex, in many olfactory projection regions and, in the mouse. is expressed at low levels in the hypothalamic mammillary nuclei, the inferior and superior colliculis and cerebellar granule cells (Hofer et al., 1990).

In vitro studies on CNS neurons have demonstrated that BDNF is a neurotrophic factor for embryonic rat retinal ganglion cells (RGCs) (Johnson et al., 1986) but has little effect on postnatal RGCs. In addition to its survival-promoting effect, BDNF also enhances the regeneration of RGC axons in adult rat retinal explants (Thanos et al., 1989), with a small effect on survival in addition. Chick retinal ganglion cells display a distinct window of response to

BDNF; they are most receptive to it during the time when there is greatest competition among the RGCs for a limited target, during the time when the number of axons in the optic nerve is at its peak (Rodriguez-Tébar et al., 1989). Septal cholinergic and dopaminergic neurons of the ventral mesencephalon also display enhanced survival in response to BDNF.

In the peripheral nervous system BDNF enhances the survival and elicits neurite outgrowth of sensory neurons of the dorsal root and nodose ganglia (Lindsay et al., 1985). Aside from the in vitro observations on the effects of BDNF on various neuronal populations, newly established in vivo roles for BDNF have been elucidated. Administration of BDNF protein to the chick embryo rescues developing motorneurons from cell death (Oppenheim et al., 1992). BDNF also rescues spinal motor neurons from axotomy-induced cell death (Yan et al., 1992) and prevents the death of motorneurons in newborn rats after facial nerve section (Sendtner et al., 1992).

Neurotrophin-3 is expressed at a much higher level during development than in the adult (Maisonpierre et al., 1990), suggesting that it too might be a target-derived factor for developing neurons. It is also expressed in regions of the developing CNS where neuronal precursors are undergoing proliferation and terminal differentiation, as in the region of the granule cells in the cerebellum, suggesting that it might be a mitogen or differentiation factor. In vitro NT-3, like BDNF, enhances the survival of dorsal root and nodose ganglia neurons, as well as enhancing the survival and neurite outgrowth of dopaminergic neurons (Gotz et al., 1992). Recently, it has been reported that NT-3 increases the regenerative sprouting of lesioned corticospinal tracts in rats, whereas BDNF has no effect (Schnell et al., 1994). It is also one of a series of survival factors for mature oligodendrocytes in the optic nerve (Barres et al., 1993b) and has recently been shown to be a potent mitogen for

oligodendrocyte precursor cells, in vitro and in vivo (Barres et al., 1993). It is also a mitogen for neural crest cells (Kalcheim et al., 1992).

Some confusion exists as to whether NT-4 and NT-5 are distinct neurotrophic factors in their own right or whether NT-5 is in fact the mammalian counterpart of NT-4 which was originally isolated from Xenopus. Current ideas suggest the latter since mammalian NT-5 is the closest mammalian relative of Xenopus NT-4 and it parallels Xenopus NT-4 in its specific interactions with TrkB but not TrkA or TrkC (reviewed in Glass and Yancopoulos, 1993). Functionally, the effects of NT-4 parallel those of BDNF, in that it exerts activity over dorsal root and nodose ganglia neurons.

C. Ciliary Neurotrophic Factor.

Ciliary neurotrophic factor (CNTF), whose structure bears no resemblance to that of the neurotrophin family, is another factor which plays a role in the development of the CNS. CNTF, leukaemia inhibitory factor (LIF) and interleukin 6 (IL-6) belong to a family of related cytokines (Patterson, 1992) which affect the behaviour of neural and haemopoeitc cells. The CNTF propeptide lacks a signal peptide and is presumably not secreted constitutively, unlike IL-6 and LIF, which do possess secretion signals. CNTF takes its name from the fact that it supports the survival of parasympathetic motor neurons from the chick ciliary ganglion, as well as stimulating their choline acetyltransferase (ChAT) activity (Nishi and Berg, 1979; Unsicker et al., 1987). In addition it supports the survival, in vitro, of sympathetic ganglion neurons (Ernsberger et al., 1989) as well as inducing cholinergic differentiation of mature sympathetic neurons (Saadat et al., 1989; Rao et al., 1992), and enhancing the survival of developing peripheral sensory neurons (Manthorpe et al., 1981) and spinal cord motor neurons (Arakawa et al.,

1990). Isolated neurons from the CNS, such as retinal ganglion cells, are supported by CNTF (Lehwalder et al., 1989) and retinal amacrine cells are induced by CNTF to increase their cholinergic characteristics (Hofmann, 1988). These in vitro observations have been extrapolated to in vivo studies,which have demonstrated CNTF to be a survival factor for lesioned neurons. Infusion of CNTF after a unilateral fimbria-fornix transection, prevents the loss of medial septal cholinergic neurons (Hagg et al., 1992) in the CNS. In the PNS, CNTF, when applied to the distal stump of lesioned rat facial nerve, saves most of the motor neurons that would have otherwise died (Sendtner et al., 1992).

Probably the best characterized role for CNTF is that in triggering the bipotential oligodendrocyte-type2-astrocyte progenitors (O-2As) to differentiate into type-2-astrocytes. In vitro, O-2As differentiate into oligodendrocytes by default, but their conversion to type-2-astrocytes requires the instructive influence of CNTF (Hughes and Raff, 1987). Optic nerve extracts induce O-2A cells to express GFAP, the astrocytic marker, with this inducing activity being greatest from extracts of 3 week old optic nerves (Hughes and Raff, 1987). The GFAP inducing activity and the nerve-derived CNTF activity co-purify and since the effects can be mimicked by purified CNTF , it suggests that CNTF is the molecule that is important in type-2-astrocyte induction (Hughes et al., 1988), with the most likely source being type-1-astrocytes (Lillien et al., 1988). The effect of CNTF is transient; extracellular matrix is required together with CNTF in order to induce stable type-2 astrocyte differentiation (Lillien et al., 1990a, 1990b). Recently it has been shown that CNTF is also a survival factor for oligodendrocytes in vitro (Louis et al., 1993) and in vivo (Barres et al., 1993b, 1993c).

mRNA for CNTF is present in the optic nerve and olfactory bulb, with

lower levels in other regions of the CNS (Stöckli et al., 1991). In the PNS it is found in Schwann cells in the sciatic nerve (Stöckli et al., 1989) . At the CNS sites it is the astrocytes that are found to contain the message and protein and, in vitro, cortical astrocytes produce CNTF-like activity (Rudge at al., 1985). Since CNTF lacks a signal peptide, it is unlikely to be released under normal physiological conditions from astrocytes. Its major role might be in injury so that, if released from ruptured astrocytes (Rudge et al., 1985; Lillien et al., 1988) or Schwann cells, it might be able to promote the survival and enhance neurite outgrowth of neurons and enhance the survival of oligodendrocytes in the CNS. Exogenously introduced CNTF might therefore turn out to have therapeutic value in enhancing the survival of the various CNTF responsive cells, in the event of injury or disease. Indeed, in a mouse model of progressive motor neuronopathy, it has been shown that introducing CNTF enhances the survival of the motor neurones in the spinal cord and considerably prolongs the life of the mutant strain (Sendtner et al., 1992).

CNTF mediates its effects via a receptor complex that is thought to consist of three subunits; an alpha chain, that is a membrane bound GPI linked molecule, and two associated subunits called gp130 and LIFRß, which are involved in signal transduction (reviewed by Davis and Yancopoulos, 1993). The receptor is expressed exclusively within the nervous system with one exception being skeletal muscle (Davis et al., 1991).

D. Fibroblast Growth Factors

The fibroblast growth factors constitute a large and growing family of interrelated proteins of which there are currently seven known members. The best-characterized members of the family are acidic FGF (aFGF; Thomas et al., 1984) and basic FGF (bFGF; Lemmon and Bradshaw, 1983; Bohlen et al.,

1984) which have both been studied extensively with regards to CNS distribution and function. The other known family members are INT-2 (Smith et al., 1988), HST/k-FGF (Delli-Bovi et al., 1987; Yoshida et al., 1987), FGF-5 (Zhan et al., 1988), FGF-6 (Marics et al., 1989) and keratinocyte growth factor, KGF (Finch et al., 1989; Rubin et al., 1989). These growth factors are also known as the heparin binding growth factors as they generally have high affinity for heparin and other related molecules, which in turn regulate the activity of the FGFs. One of the salient features of the structures of aFGF and bFGF is that like CNTF, they lack a classic signal sequence for secretion. Thus it is not clear whether aFGF or bFGF are normally secreted from cells and, if so, how. Other members of the FGF family do possess signal sequences, however, and there is evidence for constitutive secretion of these factors.

FGFs exert a wide spectrum of biological actions, stimulating cell proliferation and differentiation, angiogenesis, chemotaxis and repair (reviewed in Burgess and Maciag, 1989) of many cell types. They mediate their effects by binding to distinct, membrane spanning tyrosine kinase receptors (FGFR1-R4) whose cDNA sequences place them in a separate subgroup (Yarden and Ullrich, 1988). The relationships among the different FGFs and the FGFRs is still being worked out but, for example, FGFR1 appears to be the major receptor for aFGF and FGFR2 for bFGF.

In vivo studies using immunocytochemistry and in situ hybridization have shown FGFs (Grothe et al., 1991; Wilcox et al., 1991; Elde et al., 1991; Stock et al., 1992) and their receptors (Wanaka et al., 1991) to be present in the mammalian CNS. Both aFGF and bFGF have been isolated from the embryonic and adult mammalian and chick CNS (Gospodarowicz et al., 1984, 1986; Gimmez-Gallego et al., 1985, 1986), with aFGF being the main form. Both are present in various neuronal subpopulations during development and

in the adult and in some cases immunoreactive protein seems to be associated with extracellular compartments (Fu et al., 1991; Elde et al., 1991). The FGF receptors are expressed early on in development at the ventricular zones but, as differentiation proceeds, expression is restricted to various neurons, such as retinal ganglion cells and hippocampal neurons (Wanaka et al., 1991; Elde et al., 1991). These data suggest that FGFs first play a role in the mitoses of neuroepithelial cells and later in the maintenance of specific neuronal populations.

In vitro studies have shown that FGFs exert effects on a variety of neonatal and postnatal neurons from many brain regions with respect to enhancing their survival, neurotransmitter synthesis and neurite outgrowth (Walicke et al., 1986; Morrison, 1987; Unsicker et al., 1987; Hatten et al., 1988; Ferrari et al., 1989; Grothe et al., 1989; Matsuda et al., 1990). In vivo roles for FGF have not really been established and some of the data is conflicting, especially regarding its role as a putative neurotrophic factor in vertebrates. In vitro studies on glial cells have revealed that FGFs are synthesized by astrocytes (Pettmann et al., 1985) and exert a spectrum of biological actions, including acting as mitogens for astrocytes (Pettmann et al., 1985) and enhancing their expression of glial fibrillary acidic protein (Morrison et al., 1985) and their cell migration (Senior et al., 1986). bFGF, in the presence of insulin, is mitogenic for oligodendrocyte precursors (O-2As) and a combination of bFGF and PDGF exerts a co-operative effect, causing prolonged proliferation of the oligodendrocyte precursors (Bögler et al., 1990) and inhibiting oligodendrocyte differentiation. In addition, bFGF promotes DNA synthesis in oligodendrocytes but, again, only in the presence of IGF-I or a high concentration of insulin (Barres et al., 1992, 1993).

Brain injury caused by direct damage or ischaemia induces increases in

mRNA and protein for bFGF specifically (Nieto-Sampedro et al., 1988: Finkelstein et al., 1988), leading to gliosis and vasoproliferation. Studies of the lesioned nervous system, notably the transected fimbria-fornix and transected adult rat optic nerve, have shown that administration of FGF rescues most cholinergic neurons in the ipsilateral medial septal nucleus (Anderson et al., 1988; Otto et al., 1989) and partially prevents degeneration of ganglion cells in the retina respectively (Sievers et al., 1987). The mechanism of rescue could either be direct or via glial, vascular or inflammatory cells.

E. Platelet-derived Growth Factor

Platelet-derived growth factor is a cationic, glycosylated protein of 30 kD which consists of two polypeptide chains, denoted A and B, connected by disulphide bridges. The two polypeptide chains share ~60% sequence similarity, and can be combined as homo- or heterodimers. Three forms of PDGF therefore exist, PDGF-AA, -AB and -BB (Bowen-Pope et al., 1989; review, Heldin and Westermark, 1989b). PDGF is a mitogen for mesenchymal cells (Ross et al., 1986) but, in addition, stimulates vasoconstriction (Berk et al., 1986) and chemotaxis (Seppa et al., 1982; Grotendorst et al., 1982; Duel et al., 1982). As its name suggests, it is found in high concentrations in blood platelets, which store it in granules and release it at sites of tissue damage where it plays a key physiological role in wound healing, acting as a vasoconstrictor and as a mitogen for vascular and other connective tissue cells. It is also implicated in various pathological processes such as in atherosclerosis, myeloproliferative disease and in neoplasia, where high levels of PDGF are produced by osteosarcomas and gliomas, for example (Nistér et al., 1988).

The effects of PDGF are mediated via membrane spanning, glycosylated, tyrosine kinase receptors of which there are two types, the alpha (PDGF-αR) and beta (PDGF-ßR) subunits. Each of the subunits has different ligand-binding specificities. PDGF-A only activates the alpha receptor whereas PDGF-B activates both alpha and beta receptors (Heldin et al., 1988; Hart et al., 1988). The unoccupied receptors are present in the plasma membrane as inactive monomers but, when a dimeric molecule of PDGF binds, it dimerizes and activates the receptors . The response of a given receptive cell type will therefore depend on the type of receptor subunit(s) that it expresses on its surface and the PDGF isoforms to which that cell is exposed.

F. Roles for PDGF in Development.

1. Embryogenesis.

PDGF and its receptors are expressed in embryos from the earliest stages of development, suggesting that PDGF signalling has an important role in the embryo. PDGF-A and PDGF-αR mRNA are present in Xenopus oocytes and early cleavage stage embryos (Mercola et al., 1988, 1990a, 1990b). Measurements of transcript levels for both chains of PDGF and both receptor subunits have shown that PDGF-A and PDGF-αR are expressed during early postimplantation mouse development, with PDGF-ßR being only detected towards the end of this period and PDGF-B chain expression being absent throughout this period (Mercola et al., 1990). Using the same system, recent evidence suggests that transcripts and protein for both PDGF-A chain and PDGF-αR are coexpressed in all cells of the pre-implantation embryo. Following implantation, ligand and receptor assume a quite different

distribution, in that PDGF-A mRNA is expressed in ectoderm and PDGF-αR mRNA is expressed in juxtaposed mesoderm. Thus, in this system PDGF may elicit an autostimulatory signal in the pre-implantation embryo. Post-implantation, however, a paracrine mechanism is the most likely , with ectoderm affecting aspects of the differentiation and movement of the neighbouring mesoderm (Palmieri et al., 1992).

2. PDGF in Central Nervous System development

PDGF has been established now as playing an important role in the development of the nervous system, in particular in the ontogeny of glial cells in the grey and white matter of the CNS. Since the vertebrate CNS is very complicated with regard to the many different types of neuronal and glial cells, studies have concentrated on a very simple white matter tract in the developing rat CNS, the optic nerve. The optic nerves contain glial cells and other cell types such as endothelial cells and microglial cells, but contain no neuronal cell bodies. They convey the axons of the retinal ganglion cells, whose cell bodies are situated in the retina, to secondary visual centres in the brain. Since the optic nerve does not contain any nerve cell bodies, it makes it ideal for studying glial cell development. It is believed that the optic nerve is a fairly typical CNS white matter tract and developmental mechanisms in the optic nerve are representative of white matter in other regions of the CNS.

In cultures of developing rat optic nerve, three differentiated glial cells can be recognised by morphology and by specific antigenic markers. Two of these cells are astrocytes, called type-1 and type-2. Type-1 astrocytes are flat and cuboidal in shape whereas type 2 astrocytes have a distinct process-bearing morphology. The third type of differentiated cell is the oligodendrocyte, the cell that wraps sheaths of myelin around axons in the CNS. Type-1

astrocytes are derived from a dedicated precursor cell which resides in the neuroepithelium of the developing optic nerve. The type-2 astrocytes and oligodendrocytes are derived from a single precursor cell in vitro, the oligodendrocyte-type-2 astrocyte progenitor, or O-2A progenitor (Raff et al., 1983). Antibody markers can be used to distinguish the various glial cell types in cultures of dissociated rat optic nerves. O-2A progenitors can be recognized by antibody A2B5, which is a cell surface marker (Eisenbarth et al., 1979). Oligodendrocytes label on their surfaces with antibodies to galactocerebroside (GC) (Raff et al., 1978) and both sets of astrocytes label with antibodies to glial fibrillary acidic protein (GFAP) (Bignami et al., 1972; Raff et al., 1983). Type-2 astrocytes but not type-1 astrocytes, also label with A2B5 (Raff et al., 1983).

Studies of dissociated cell suspensions from optic nerves of rats of different developmental ages suggest that type-1 astrocytes first appear at embryonic day 15-16 (E15-16), oligodendrocytes around the day of birth, and type-2 astrocytes, in very small numbers, appear in the second postnatal week (Abney et al., 1981; Miller et al., 1985; Williams et al., 1985). In the intact optic nerve, O-2As proliferate rapidly and, around the day of birth, some of them stop dividing and differentiate into oligodendrocytes while the others continue to proliferate. Proliferation and differentiation continue for several weeks after birth, with oligodendrocyte production being over at around postnatal day 45. When optic nerve cells are cultured in vitro, their behaviour depends on the composition of the culture medium. In defined, low serum medium they do not develop on their normal schedule but differentiate prematurely into oligodendrocytes within 1-2 days.In the presence of 10% fetal calf serum (FCS), they differentiate, within 2-3 days into type-2 astrocytes (Raff et al., 1983, 1984). These observations clearly suggest that

there are signals in the intact optic nerve that keep the O-2As proliferating and prevent premature differentiation, but which are absent in vitro. When O-2As were grown on a monolayer of type-1 astrocytes from rat cortex, or in astrocyte-conditioned medium, they continued to divide and the in vivo timing of appearance of oligodendrocytes was reconstituted, suggesting that type-1 astrocytes are a source of factors that stimulate proliferation and prevent the premature differentiation of O-2As (Raff et al., 1985).

There is strong evidence that PDGF plays an important role in mediating the interaction between type-1 astrocytes and O-2A progenitor cells in vitro: First, recombinant PDGF is a potent mitogen of O-2A progenitor cells in vitro (Noble et al., 1988; Richardson et al., 1988), with PDGF reconstituting the normal development of oligodendrocytes in embryonic rat optic nerve cultures (Raff et al., 1988).Second, cortical type-1 astrocytes contain mRNA for PDGF-A, and the protein can be immunoprecipitated from metabolically labelled cortical astrocyte cultures (Richardson et al., 1988). Since no PDGF-B chain was detected in the astrocyte cultures, the dimeric isoform presented to the O-2As is probably PDGF-AA. Third, the mitogenic activity for the O-2As from type-1 astrocyte conditioned medium cofractionates with PDGF on a size-exclusion column, and this mitogenic activity can be abrogated using anti-PDGF neutralizing antisera (Richardson et al., 1988; Noble et al., 1988). PDGF-A mRNA is present in the developing optic nerve (Pringle et al., 1989; Mudhar et al., 1993) and the protein can be detected by immunohistochemistry in astrocytes in the nerve (Mudhar et al., 1993). Moreover extracts of neonatal optic nerve contain PDGF activity which can be neutralized by anti-PDGF antisera. All these lines of evidence suggest that PDGF-A, presumably incorporated into PDGF-AA homodimers, might be a key player in the development of oligodendrocytes in vivo.

One of the salient features of the O-2A progenitor cells is that no matter how much PDGF is added to them in culture, they do not proliferate indefinitely (Raff et al., 1988). By virtue of an intrinsic clock that counts time or cell divisions, the O-2A progenitor cells differentiate, by default, into oligodendrocytes, after about eight cell divisions (Temple and Raff, 1986). O-2A progenitor cells express PDGF-αR receptors but no PDGF-ßR, as determined by iodinated-PDGF binding to cells in vitro (Hart et al., 1989a). In addition, newly-formed oligodendrocytes also posses functional PDGF-αR, as determined by ^{125}I-PDGF binding and the transient increase in cytosolic calcium and c-Jun and c-Fos expressions in the nucleus following PDGF stimulation. (Hart et al., 1989b, 1992).

1.8 Distribution of PDGF and its receptors in the CNS and its significance.

PDGF and its receptors have been localised to distinct cell types in the developing and mature vertebrate nervous system, by in-situ hybridization and immunohistochemistry. PDGF-A chain mRNA is expressed in astrocytes and, more prominently, by subsets of central and peripheral neurons. This distribution is supported by the presence of immunologically detectable protein (Yeh et al., 1990). PDGF-B chain is expressed by neurons and has been localized by immunohistochemistry to nerve fibre tracts and nerve terminals in primate brains. In transgenic mice carrying a chloramphenicol acetyl transferase (CAT) reporter gene under the transcriptional control of the human PDGF-B gene promoter, CAT activity was detected in regions of the brain that contain neurons, such as the hippocampus, cerebellum and cerebral cortex, but not in the optic nerve which contains glial cells and axons

but no neuronal cell bodies (Sasahara et al., 1990). This supports the view that PDGF-B is expressed by neurons but not glial cells. In contrast, optic nerve astrocytes, are known to express PDGF-A (Mudhar et al., 1993; this thesis).

In situ hybridization with a probe for PDGF-αR mRNA (Lee et al., 1990) revealed PDGF-αR expression in a subset of glial cells during late embryonic and postnatal development and in the adult. The same study provided evidence that PDGF-αR is expressed transiently by some immature neuronal populations but not by mature neurons. This, together with ^{125}I-PDGF binding studies on enriched populations of glial cells in vitro, suggested that, after E 14 in the rat, PDGF-αR might be expressed predominantly or exclusively by oligodendrocyte precursors (Pringle et al., 1992)

Studies on rat spinal cord development further support the idea that PDGF-αR-positive cells may correspond to oligodendrocyte precursors. The changing distribution of the cells that express PDGF-αR between E14 and birth suggests that these cells originate in the ventral half of the cord at E14 and migrate dorsally so as to populate the cross-section of the cord uniformly by E 18 (Pringle and Richardson, 1993). This closely mirrors the manner in which oligodendrocyte precursors are thought to be redistributed in the developing spinal cord between E14 and E18 (Warf et al., 1991). Moreover, it has recently been shown that the early PDGF-αR-positive cells are superimposible on cells that express mRNA encoding 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP), another marker of oligodendrocyte lineage cells (Yu et al., 1994).

PDGF beta-receptors are localised on specific neuronal populations in

the vertebrate CNS (Smits et al., 1991) and certain isolated neurons seem to survive preferentially in the presence of PDGF-BB as compared to control conditions.

These expression data raise the key question of whether neuron-derived PDGF plays a role in the development of the CNS. Since O-2A progenitors proliferate in response to PDGF in vitro, one possibility is that neuron-derived PDGF acts as a mitogen, survival factor or chemoattractant for glial cells. PDGF stimulates O-2A motility in vitro (Noble et al., 1988) and there is evidence that O-2A progenitors may be migratory cells in vivo, moving into developing white matter tracts from germinal zones elsewhere in the CNS (Small et al., 1987; Reynolds and Wilkin, 1988; Pringle et al., 1992; Mudhar et al., 1993). It is possible that PDGF, synthesized in neuronal cell bodies, might be transported into and released from axons. There is evidence that oligodendrocyte development depends on the presence of intact axons; if the optic nerve is cut at birth, the accumulation of oligodendrocytes and their progenitor cells is markedly reduced (David et al., 1984; Barres et al., 1993a, 1993c). This has ben shown to result from decreased long-term survival of oligodendrocyte lineage cells in the transected nerve (Barres et al., 1993a). Moreover, Barres and Raff (1993a) showed that inhibiting electrical transmission in intact optic nerve axons by administering tetrodotoxin (TTX) to retinal ganglion cells in the eye decreased the accumulation of oligodendrocytes in the nerve, but that this effect could be reversed by experimentally delivering PDGF-A to the optic nerve. This raises the possibility that RGCs might be a source of PDGF-A for optic nerve glia, and that neuronal activity is somehow necessary for the delivery of this PDGF. An alternative explanation is that electrical activity is required to induce synthesis or release of PDGF from astrocytes that are resident in the optic

nerve. These are some of the questions that are experimentally addressed in this thesis.

1.9 DEVELOPMENT OF THE VERTEBRATE EYE.

One of the best characterized regions of the CNS is the vertebrate retina, partly because it is one of the most accessible CNS tissues and relatively simple to manipulate experimentally. In its mature form the vertebrate retina is a stratified structure, with each stratum containing a subset of retinal cell types, or a plexus of cellular processes and interconnections (Cajal, 1893; Rodieck, 1973) (Fig. 3). How this neatly layered structure develops is a key question and, because of its accessibility, it is increasingly subject to molecular and cell biological analysis. Below, I review some of the progress that has been made.

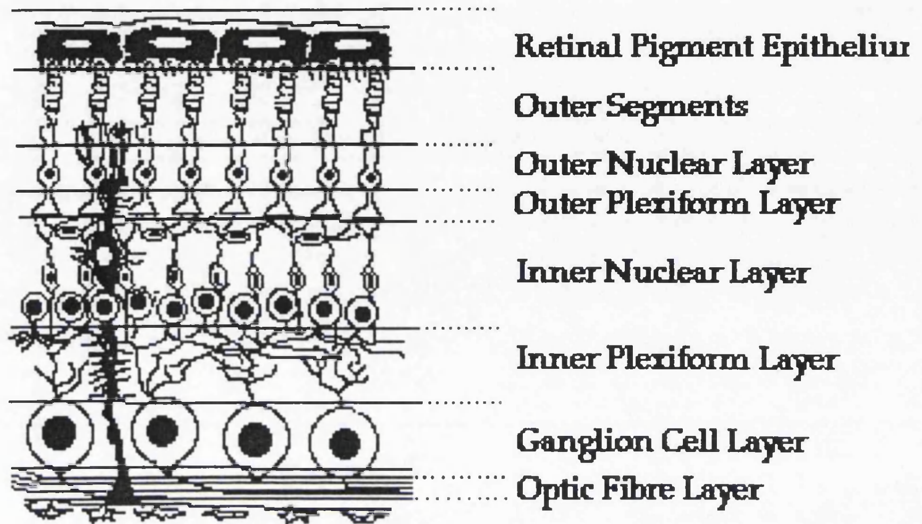
A. Development of the optic cup.

In the early CNS, the optic primordium is recognised as a thickened region of the wall of the developing CNS, which develops into a pair of diverticula, the optic vesicles. These grow out from the ventrolateral walls of the presumptive diencephalic wall, towards the surface ectoderm. The vesicles are at this point connected to the rest of the developing brain by the primordial optic nerve, known as the optic stalk. As the vesicles make contact with the surface ectoderm, two major events take place: the optic vesicle invaginates to create a bi-layered structure called the optic cup and the surface ectoderm thickens to become the lens placode. The bi-layered optic cup becomes the retina, with the outer wall becoming the retinal pigment epithelium (RPE) and the inner part giving rise to the neural retina (Fig 4).

B. Development of the neural retina.

Like all regions of the developing CNS, the very early forerunner of the neural retina consists of a proliferating neuroepithelial sheet, with cells in various phases of the cell cycle and executing interkinetic nuclear migration. The ventricular surface is that closest to the RPE. Initially the RPE and neural retina are separated by the optic ventricle, but this is obliterated as the apposition between the two layers becomes tighter.

Studies of retinae of many species show a concentration of mitotic figures at the ventricular zone, with the mitotic spindles of most of the ventricular zone cells parallel to the ventricular surfaces. The pattern of mitotic divisions that create the cell types of the retina are comparable to those seen at ventricular surfaces in the rest of the neural tube, except that cells born later in their development do not migrate through earlier formed cells as they do, for example, in the developing cerebral cortex (Berry and Rogers, 1965).



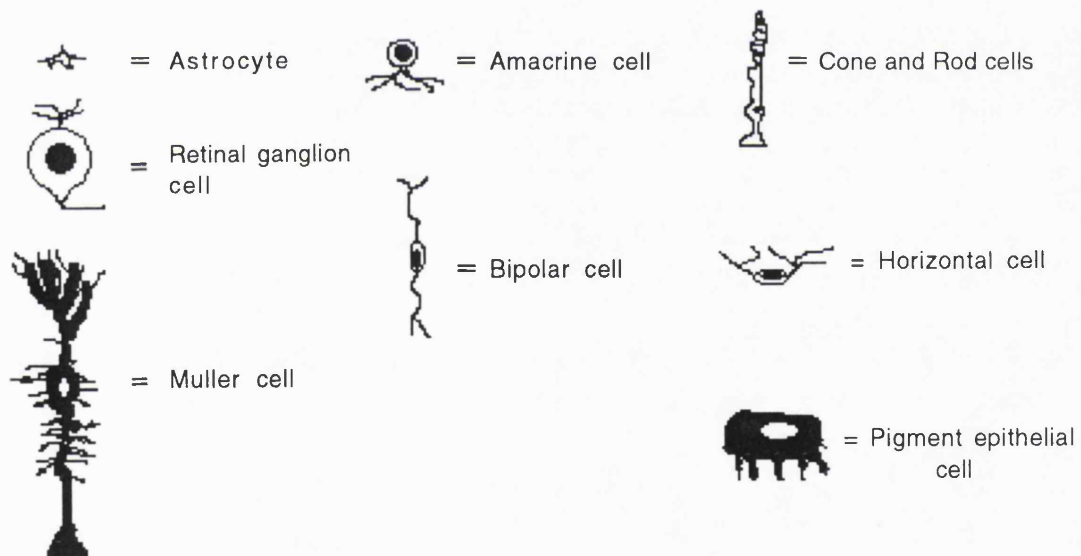


Figure 3. The generalized vertebrate retina

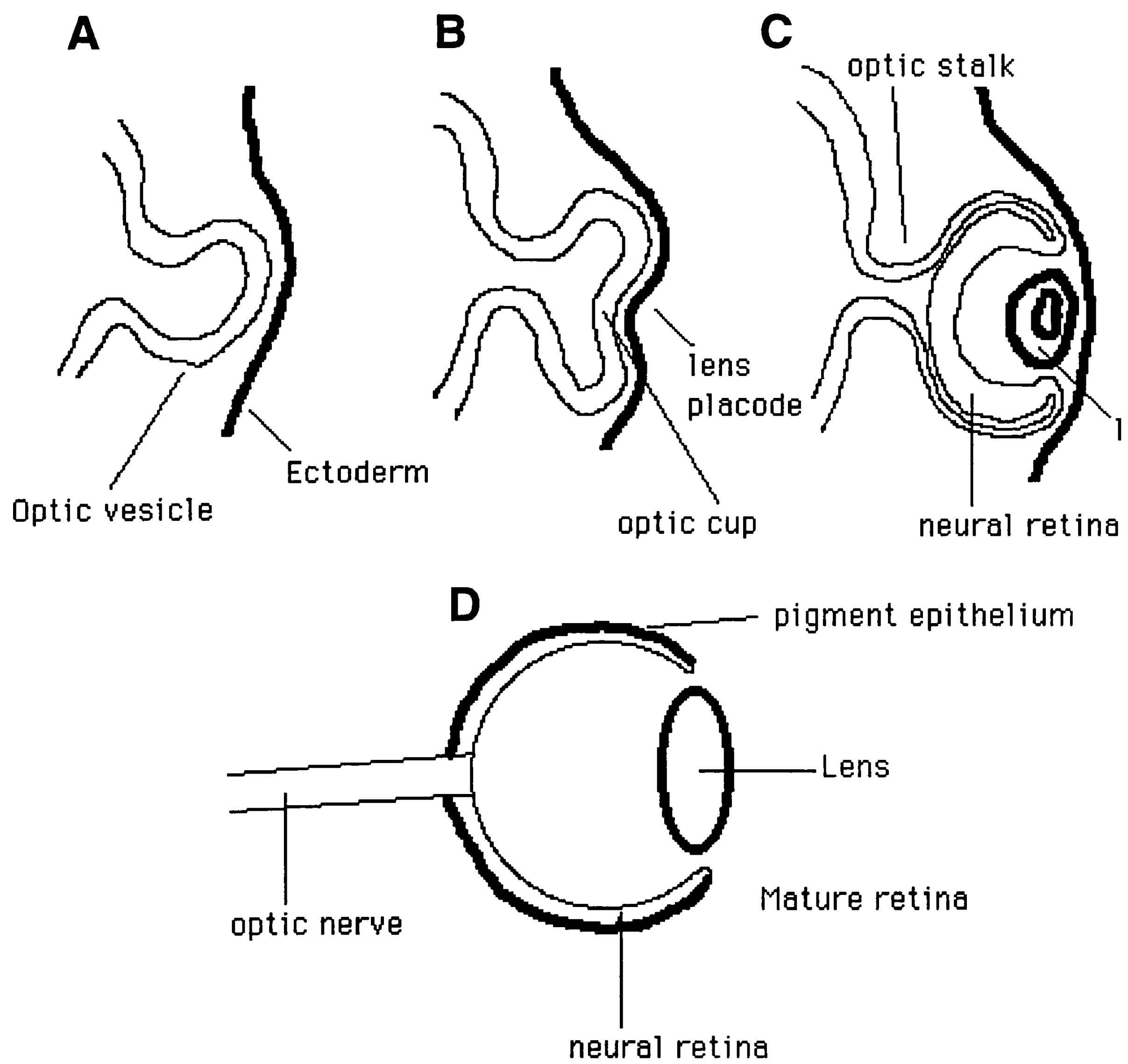


Figure 4. The development of the vertebrate retina.

C. Order of Birth of Retinal Cells.

Across a wide range of animal species, retinal cells are generated in sequence, with the first postmitotic cells observed around the time of optic cup formation. Tritiated thymidine analysis of retinae of many species shows that, generally, there is a strong correlation between the assumption of a particular cell fate and the time at which that cell is born. Invariably, the first cells to be born are the retinal ganglion cells (RGCs) and the last, the bipolar cells (Sidman, 1961; Young, 1985a, 1985b). What determines this order or for what reason RGCs cells should be generated first, is not known.

There are two extreme possibilities: either each differentiated cell type is generated by a distinct subset of precursors, or the precursors are multipotential and capable of generating the complete set of cell types. The available data favours the latter view.

D. Lineage analysis in the retina.

Several studies have been conducted to resolve the lineal relationships among the various retinal cell types. The general strategy has been to label individual retinal precursor cells and then to follow the progeny that are formed as a function of time. One approach has been to inject dye such as HRP (Holt et al., 1988) or fluorescent dextran (Wetts and Fraser, 1988; Wetts et al. 1989) into individual precursors and then to study the progeny that inherit the dye. An inherent fault with this technique is dye dilution with further cell divisions. Another approach has employed replication-defective retroviruses that encode ß-galactosidase or alkaline phosphatase reporter genes. Retrovirus is introduced into the precursors and the progeny identified by histochemically staining for the reporter gene products. Since the viruses are replication defective, there is no lateral spread from the point of infection

and only the daughter cells from a given cell division acquire the virus (Turner and Cepko, 1987; Turner et al., 1990; Fekete et al., 1990, 1991), allowing a clonal analysis to be conducted.

Both techniques have yielded the same results, namely that the retinal precursors are multipotent. Clones consist of all the possible permutations of cell type present in the retina, with variation between clone sizes even in a single injected, or infected retina. In the rat and mouse retinae the majority of the clones consist of rod photoreceptors, which is not surprising since these cells make up 70% of the cells in the rodent retina.

It has been argued that the multipotential nature of the precursors is maintained until the final cell division, since some of the two cell clones that are observed consist of two different cell types for example, a Muller glial cell and a rod photoreceptor cell. What these arguments fail to consider is the contribution of differential cell survival in determining the composition of a clone of cells. Since naturally occurring cell death occurs throughout the various layers of the retina (Young, 1984; Beazely et al., 1987) this is likely to have a profound influence on final size and composition of the clone. The conclusion that the multipotentiality of a precursor is maintained up until its final cell division might, therefore, be misleading. Future studies should hopefully resolve this issue.

E. Determination of cell fate.

A major challenge is to understand how a multipotential retinal precursor decides to give rise to a particular differentiated cell type. Genetic and morphological studies of Drosophila eye development have shown that short range signals from neighbouring cells play a key role in determination of cell fate (Ready et al., 1976; Tomlinson, 1988). It seems not unreasonable

therefore, to consider whether the determination of cell type in vertebrate retinae might not also rely on cell-cell interactions. Evidence to support this idea comes from cell ablation experiments, whereby destruction of a particular group of amacrine cells in the goldfish and frog retinae leads to a compensatory increase in their production from the precursors present at the ciliary margin (Negishi et al., 1982; Reh and Tully, 1987).

Production of the various cell types in the vertebrate retina occurs in a given temporal sequence. Two possibilities exist to explain the rigid timing of cell production. First, environmental cues might change with time, or intrinsic properties of the precursors might change with time. In vitro studies on rod photoreceptor development in the rat retina (Watanabe and Raff, 1990, 1992; Altshuler and Cepko, 1992) have shown that the properties of retinal neuroepithelial cells change as retinal development proceeds. When neonatal cells were mixed with marked embryonic cells, the neonatal cells did not influence the time of appearance of rod cells from the embryonic precursors, but increased considerably the number of rod cells produced from the embryonic cells, when they had matured to a neonatal age in vitro. This suggested that there was a diffusible rod-promoting signal in the neonatal environment. Such a signal could act in several ways; it could stimulate precursor cells to commit to a rod fate; it could promote the differentiation of committed rod precursors or it could enhance the survival of rods or their committed precursors. The most favoured idea is that that diffusible activity influences cell fate determination, as opposed to enhancing survival or terminal differentiation (Altshuler and Cepko, 1992). The rod-promoting molecule has been further characterized and has turned out to be a simple amino acid, taurine (Cepko, unpublished). Such simple signalling molecules might well operate in determining cell fate decisions in the rest of the CNS.

F. Retinal maturation.

The execution of a pattern of connections that is anatomically and physiologically correct is the next step in the development of the retina. As the various cell types are born, they migrate to their appropriate position in the retina and project axons, in the case of RGCs, or local interconnections in the case of the local interneurons. At the gross anatomical level at P5 in the rat retina, the optic fibre layer (OFL), the RGC layer and the inner plexiform layer (IPL) are evident, with a very sparse appearance of the outer plexiform layer (OPL). The inner nuclear layer (INL) and the outer nuclear layer (ONL) have not yet formed. The inner plexiform layer at this stage consists of small diameter profiles separated by space, with no obvious synaptic connections between them. At the laminar level the retina is practically mature by the beginning of the second postnatal week. By this stage, a distinct OPL, INL and ONL have appeared, with the first synapses becoming evident in the OPL and INL. By the end of the second postnatal week, the retina further matures, with many outer segments in the ONL and many ribbon synapses in the OPL. Synaptic ribbons in the IPL give way to the appearance of morphologically varying and mature ribbonless synaptic contacts which are characteristic of bipolar inputs into the RGC dendrites, with associated amacrine cell inputs. The synaptic machinery is therefore complete and primed by the end of the second postnatal week, just before the rats open their eyes (Weidman and Kuwabara, 1968; Horsburgh and Sefton, 1986; Radel et al., 1992).

Retinal ganglion cell axon growth cones follow a stereotyped pathway through the optic nerve head and along the optic nerve to the brain. In the brain of the rat, the major target of RGCs is the contralateral superior colliculus, since over 90% of the 100,000 optic axons terminate there (Linden and Perry, 1983), largely in the superficial grey lamina. Optic axon terminals

are first seen at the contralateral superior colliculus at around E16, with the first synapses appearing at E18 (Lund and Bunt, 1976).

G. Naturally occurring cell death in the developing retina.

In the development of most organs, it is now appreciated that progressive developmental phenomena such as proliferation are balanced by regressive events, such as cell death (Glucksman, 1951). It has long been known that many neurons in the developing vertebrate CNS die soon after they are formed (Hamburger and Levi-Montalcini, 1949; Cowan et al., 1984; Oppenheim, 1991) as a consequence of competing for limiting amounts of survival factors at their targets. For example, in the developing sympathetic nervous system, the neurons compete for limiting amounts of nerve growth factor (NGF) that is released by the target (Levi-Montalcini, 1987; Purves, 1988; Barde, 1989). Those that do not receive sufficient 'neurotrophic' factor die by programmed cell death. The purpose of this mass culling of neurons serves to match the size of the innervating pool of neurons to the target and to eliminate aberrant neuronal connections (Cowan et al., 1984).

The retina is no exception and, during its histogenesis, cell death plays a role in sculpting the final pattern of organisation. Considerable attention has been paid to the death of RGCs in a variety of species. Retinal ganglion cells are produced in excess and during the key period during which the terminals encounter the target, over 50% of them die at least in part due to competition between terminals from both eyes for limited amounts of neurotrophic factor(s). Thus, if one eye is removed at birth, fewer RGCs die in the contralateral retina and, conversely, if the RGCs are deprived of their targets by transecting the optic nerve or removing the central target, increased death is observed. What of the other retinal neurons? The general rule is that those

retinal cells that are generated early undergo their death program early compared to late-born neurons. In the rat naturally-occurring cell death proceeds as a spatial and temporal wave across the retina, from the inner surface to the outer photoreceptors (Beazely et al., 1987). The greatest number of pyknotic nuclei in the RGC layer is found at the day of birth. Most pyknotic cells are seen in the inner and outer halves of the INL at P5-P7 and P10, respectively, and the maximal number of dead cells in the outer nuclear layer (ONL) occurs around P15. The peak numbers of pyknotic cells observed as a proportion of total live cells are: 0.7% in the inner aspect of the INL, 1.2% in the outer aspect of the INL and 0.1% for the ONL (Beazley et al., 1987). By analogy with other neuronal populations, such as RGCs, whose survival is target-dependent, one might reasonably expect that the final number of INL cells (amacrine and bipolar neurons) would depend on the RGCs, since the RGCs represent a major target for these cells. However, it appears that this is not the case, as depleting the entire population of RGCs by transecting the optic nerve at the day of birth has no effect on the final number of cells in the INL. Furthermore, in the rd/rd mutant mouse, where the photoreceptors undergo premature degeneration such that by the twentieth postnatal day degeneration is complete, leading to a total lack of afferent input into the INL (Blanks and Bok, 1977), again there is no effect on the time course of normal cell death amongst the amacrines or bipolars. Since the INL cells make diverse connections among themselves, it is possible that their trophic support comes from within the INL itself.

1.10 Growth Factors in Retinal Development

A. Proliferation

The embryonic rodent retina is in a state of rapid proliferation. The extent of proliferation begins to decline around the day of birth and is over by the end of the first postnatal week (Denham, 1967; Young, 1985). The progenitors at some point make the decision to drop out of division and differentiate into a particular cell type. What determines the proliferative capacity of a retinal neuroepithelial cell? Intrinsic or environmental regulatory mechanisms could operate exclusively or in combination. Embryonic precursors have a much higher proliferative capacity in vitro than postnatal precursors even when embryonic precursors are co-cultured with an excess of postnatal precursor cells, suggesting that the proliferative potential of the precursors changes with development (Watanabe and Raff, 1990). This could be attributable to either an intrinsic change in the precursor, thus altering its response to a given set of external cues, or to changes in environmental mitogens. Before these possibilities can be distinguished, it is necessary to determine what signals influence the proliferation of retinal precursors during development.

B. Roles of FGF, TGF-α and EGF in proliferation.

Early studies showed that retinal extracts were mitogenic and induced morphological changes in cultured bovine lens epithelial cells. This same mitogenic activity was then subsequently found in other ocular tissues and named Eye Derived Growth Factor (EDGF) (Arruti and Courtois, 1978; Barritault et al.,1981). This factor was then shown to be identical to bFGF (Baird et al., 1985). FGF has now been implicated in regulating a wide range of cellular responses in the eye. It can induce differentiation of lens fibre cells in

vitro (Chamberlain and McAvoy, 1989) and is a mitogen for lens and corneal epithelial cells (Gospodarowicz et al., 1977a), corneal endothelial cells (Gospodarowicz et al., 1977b) and retinal capillary cells (Gospodarowicz et al., 1977c). Detailed studies of the rat lens polarity has shown that there are different concentrations of FGF in the ocular media, with high concentrations of FGF being present in the aqueous humour and low concentrations in the vitreous humour. In vitro studies on lens fibre cells has shown that increasing levels of FGF shifts the cellular response from proliferation to migration and then to differentiation (Chamberlain and McAvoy, 1989). In vivo, cells at the proliferative zone of the lens are exposed to low concentrations of FGF and those at the anterior part of the lens, where lens fibres differentiate are exposed to higher concentrations as a consequence of being in contact with the aqueous humour (Schulz et al., 1993), consistent with the in vitro findings. This suggests that FGF is acting rather like a morphogen, providing positional information for the lens cells and establishing and maintaining lens polarity and growth patterns.

Epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α) are mitogens for retinal neuroepithelial cells in monolayer culture (Anchan et al., 1991). aFGF and bFGF, are also mitogens (Lillien and Cepko, 1992). The precursors change in their response to these growth factors with time; embryonic precursors are more responsive to FGFs while older precursors are more responsive to TGF-α. For example, P0 progenitors require 100x more FGF to display a half-maximal response compared to E18 progenitors (Lillien and Cepko, 1992).

C. Cell type determination.

Growth factors have been implicated in influencing cell fate in the retina. Studies of the phenomenon of transdifferentiation, whereby retinal pigment epithelial cells can reconstitute an entire neural retina if the latter is extirpated in vivo, have shown that FGF is important in eliciting this process, certainly in the chick (Park and Hollenberg, 1989). Furthermore, a detailed analysis of this process in vitro has revealed that early chick retinal cells express a marker for ganglion cells during S phase, and that the addition of aFGF increases the proportion of cells expressing the marker, suggesting that aFGF may play a role in the development of retinal ganglion cells from committed precursors (Guillemot and Cepko, 1992).

There is evidence that TGF-α or EGF may influence the differentiation of P0 retinal precursors. Under normal circumstances, P0 precursors give rise to rod photoreceptors in vivo (Young, 1985b) and in vitro (Watanabe and Raff, 1990; Altshuler and Cepko, 1992). If P0, high density cultures are maintained in vitro in the presence of EGF or TGF-α, rod cell differentiation and/or survival is blocked. There is no effect on the generation of amacrine and bipolar cells, indicating that general differentiation is not affected.

D. Cell survival.

Of all the cells in the retina, retinal ganglion cells have received the most attention from the point of cell survival. Many of these studies have been conducted in vitro although, where possible, in vivo observations have confirmed the tissue culture observations. Survival assays should preferably be done on very pure cell populations as the presence of even a small contaminating population can give misleading results (Habu and Raff, 1977). Nevertheless, it has been shown that BDNF (Johnson et al., 1986) and CNTF (Lehwalder et al., 1989) enhance the survival of pure populations of rat RGCs in vitro. The implication is that, during normal development, RGC axon terminals may compete for limiting amounts of BDNF and/or CNTF at their target, the superior colliculus.

The second cell type to which survival studies have been directed are the photoreceptors; elucidation of the factors that can keep these cells alive

might be of therapeutic value as there are several disorders that lead to photoreceptor degeneration in humans. Intraocular injections of basic FGF (Faktorovich et al., 1990), BDNF, CNTF, interleukin 1ß and acidic FGF (LaVail et al., 1992) into the Royal College of Surgeons (RCS) rat, which has inherited retinal dystrophy, reduces considerably the degeneration of photoreceptors that occurs during constant illumination. The mechanism of action of how these growth factors exert their protective effect remains to be elucidated, as is the normal role of these factors during retinal development.

1.11 Aims of this thesis.

We have chosen to study PDGF for following reasons: there are a small number of ligands (three) and receptors (two) and the ligand-receptor interactions have been well worked out. This should greatly facilitate the interpretation of in situ expression studies, for example. Second, PDGF and its receptors have received little if any attention in the developing and adult retina. Therefore, since the retina is accessible and well characterised at the cellular level, we are attempting to understand the functions of PDGF in the retina, in the hope that lessons learned in this system will be more broadly applicable throughout the CNS.

CHAPTER TWO

2.1. IN-SITU HYBRIDIZATION.

A. 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 mice (H4 strain) were used . Animals were perfused with PBS pH 7.4 (PBS contains per litre: 8 g NaCl; 0.2 g KCl; 0.1 g $MgCl_2$; 1.15 g Na_2HPO_4; 0.2 g KH_2PO_4) and then perfused fixed with 4% paraformaldehyde in PBS.Tissues were then further fixed, overnight in cold 4% paraformalehyde and cryoprotected in 30% (w/v) sucrose in PBS for at least 24 hours. Tissue was frozen in OCT embedding compound (Miles-Tissuetek) and blocks placed at -70°C until required. Cryosections of 10μm were collected on baked (slides made RNAse free by baking at 150ºC overnight) APES-coated microscope slides (APES-Sigma is 3-aminopropyltriethoxysilnane, used at 3% v/v in industrial methylated spirits); 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.

B. Preparation of in situ hybridization probes.

^{35}S-labelled probes were prepared by in vitro transcription as described previously (Cox. et. al., 1987). The method used was that according to Promega protocols (see below). Antisense and sense PDGF-alphaR probes were generated from a ~1.5 kb SacI-PvuII fragment coding for most of the extracellular domain of rat PDGF-alphaR (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 a ~1.4 kb EcoRI fragment encompassing the coding region of a rat PDGF-A cDNA (clone 5.3, Pollock 1993) in PTZ-18r (Pharmacia); the sense probe was transcribed with T7 polymerase from a ~1.0 kb EcoR1 fragment in the same vector (clone 4.1) (Pollock , 1992). The PDGF-B probe was generated from a ~740 bp HindIII-EcoRI cDNA fragment spanning the entire coding sequence of rat PDGF-B (c-sis) (H.S. Mudhar, unpublished). The PDGF-ßR probes were transcribed from a ~1.2 kb HindIII-BamHI cDNA fragment encoding the extracellular and transmembrane domains of rat PDGF-ßR (H.S. Mudhar, unpublished). Both PDGF-B and PDGF-ßR 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) vector. The veracity of the 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-ßR probe detected a single ~6kb transcript, as expected (Richardson et al., 1988; Matsui et al., 1989). for both PDGF-B and PDGF-ßR, transcription with T3 RNA polymerase yielded the antisense probe and T7 polymerase the sense probe.

Using bacteriophage T7, T3 and SP6 RNA polymerases (BRL), alpha[^{35}S]-UTP, and linearised DNA templates, ^{35}S-labelled 'sense' and 'antisense' RNA transcripts of high activity (~2 x 10^8 cpm/µg) were generated in vitro. The protocol used was that from 'Promega protocols' and generated approximately 200-300 ng of RNA. The reagents were added in the following order: 4 µl of 5x transcription buffer (200mM Tris-HCl, pH 7.6; 30 mM $MgCl_2$; 10mM spermidine; 50mM NaCl), 2µl of 100mM DTT, 0.5µl (20 units) of RNasin (ribonuclease inhibitor-Promega), 4µl of ATP, GTP, CTP (from a stock solution containing all 3 at 2.5mM-Pharmacia), 2.4 µl of non-radioactive 100µM UTP, 1µl of linearised template DNA at ~ 1µg/µl, 5 µl of alpha-^{35}S-UTP(50µCi at 10 mCi/ml) , 20 units of SP6, T7 or T3 polymerase and dH_20 to bring the final reaction volume to 20µl. The mixture was incubated at 37ºC for 45 minutes and then, a further 20 units of the relevant polymerase added and the tube left for a further 45 minutes. Then, in order to remove the DNA template, 1 unit of RNAse free DNAse (1 unit/µl,Pharmacia) was added , with 20 units of RNasin and 1µl of tRNA, to act as a carrier (tRNA stock at 25mg/ml, Sigma), and incubated at 37ºC for a further 15 minutes. 200 µl of 10mM DTT were added to each sample and two lots of 0.5µl samples removed from each tube; one lot was dissolved in 10 µl of Northern sample buffer, for assessing the full length ofthe transcript, and the other dissolved in 400 µl of 0.5 M NaH_2PO_4 to assess ^{35}S-UTP incorporation. The samples were made 0.3 M with 3M Sodium acetate and the RNA precipitated as described. The pellets were resuspended in 50 µl of 10mM DTT.

To calculate efficiency of incorporation of alpha-[^{35}S]UTP, 50 µl of the probe in the 0.5 M NaH_2PO_4 was applied to 6 GF/C glass microfibre filters (Whatman) per sample. Half of the filters were washed in 500 ml of 0.5M NaH_2PO_4 for a couple of minutes, to remove unincorporated radioactivity. All

filters were dried and placed into scintilliation viles containing scintillation fluid (LKB Ltd.) and counted in a rack ß-counter. Incorporations were usually between 40-80%.

For opimal penetration of tissue sections by the ^{35}S-labelled probes, they required digesting to around 100-150 bp. This was achieved by limited alkaline hydrolysis at 60ºC , by adding to each 50 µl sample, the same volume of 100mM carbonate buffer, pH 10.2 (Na_2CO_3: $NaHCO_3$, in the ratio 6.5:3.5) for **X** mins.

$$\textbf{X}\ \text{minutes} = \frac{Lo - Lf}{KLoLf}$$

where Lo= Original transcript length in kb

Lf= Final transcript length in kb (0.1-0.15)

K= 0.11

After the calculated length of incubation , the reaction was halted by adding 100µl of neutralising buffer (0.2mM sodium acetate, 1% glacial acetic acid, 10mM DTT). At this point, 0.5 µl of the neutralised sample was removed fom each reaction and dissolved in 10 µl of Northern sample buffer, as before. The digested probes were made 0.3M with sodium acetate and precipitated . The probes were dissolved at 1ng/µg/kb in 10 mM DTT and 50% deionised formamide (10x stock) and stored at -20ºC for up to 2 weeks. The sizes of full length and digested probes were estimated by comparison to DNA size markers on formaldehyde agarose gels .

C. In situ hybridization

For tissue sections, the in situ hybridization protocol was based on that of Lawrence and Singer (1985), with the modifications described by Pringle et. al. (1992). Tissue sections were allowed to attain room temperature and then rehydrated through a descending series of alcohols (absolute to 30%) and then washed briefly in PBS. After a 5 minute incubation in proteinase K buffer (50mM Tris-HCl, 5mM EDTA pH 7.5), the sections were exposed to proteinase K (1µg/ml) for 30 mins at 37°C, to deproteinize the sections; enzyme activity was stopped with a 0.2% glycine solution in PBS , the sections briefly washed in PBS and then fixed in 4% paraformaldehyde for 15 minutes. After a 3 minute wash in PBS the sections were acetylated , in order

to reduce the electrostatic non-specific binding of radioactive probes. Sections were preincubated in 0.1 M triethanolamine buffer for 5 minutes and then exposed to to two rounds of 10 minute acetic anhydride incubations (acetic anhydride at 0.4 mls per 200 ml of buffer). After a 3 minute wash in PBS, the sections were dehydrated through an ascending series of alcohols and allowed to dry thoroughly.

D. Hybridization of sections with radioactive probes.

^{35}S-labelled probes were prepared and kept at -20°C as 10x stocks for no longer than 2-3 weeks. If older probes were used, a decrease in signal to noise ratio occured, with a considerable increase and decrease in the background and specific signal respectively. Under all circumstances, therefore, freshly pepared probes were used. Probes were diluted in hybridization buffer and 25µl of diluted probe was placed on each slide. A baked glass coverslip was placed gently on each slide and the probe allowed to disperse . The slides were placed in a box containing blotting paper soaked in wash buffer (0.3M NaCl, 10mM Tris-HCl pH 6.8, 5mM EDTA, 10mM DTT, 50% deionized formamide), the box sealed and the slides hybridized overnight at 55°C.

E. Post hybridisation procedure

After hybridization, slides were incubated in a 4xSSC solution for 15 minutes, in order to loosen the coverslips (20x SSC= 3 M NaCl, 0.3 M sodium citrate, pH 7.5). This was followed by a one hour incubation in 4xSSC + 0.1M DTT, which proved to be an essential step in reducing background signals (Du Pont update on in situ hybridisation; vol 4, No. 5, 1991). Slides were then washed in 'wash buffer' at 65°C for 30 minutes followed by preincubation in RNAse buffer (0.5 M NaCl, 10mM Tris-HCl pH 7.5, 0.1 M EDTA). Unhybridized RNA probe was removed from the tissue sections with a 30 minute incubation in RNAse (20µg/ml) followed by another 30 minute incubation at 65°C in wash buffer and finally, the sections washed in 2x and 0.1x SSC solutions at 45°C for 30 minutes each. Sections were dehydrated through an ascending series of alcohols and air dried.

F. Autoradiography and developing

The slides were coated with Ilford K5 emulsion in a darkroom, the emulsion left to dry for a couple of hours and the slides then placed in a light-tight box containing silica gel dessicant and exposed for 5-15 days at 4ºC. Slides were developed in Kodak D-19 for 2 minutes at 20ºC, development arrested by a 1 minute incubation in a 1% acetic acid solution followed by fixation with 30% w/v sodium thiosulphate. The slides were then washed in distilled water for 30 minutes and counterstained with a 1% haematoxylin solution (Gills No. 3 Sigma; for high power microscopy, a 20% solution was employed) washed again , dehydrated through an ascending series of alcohols (30% to absolute) , cleared in xylene and mounted in XAM (BDH) and the sections examined under bright and dark-field settings using a Leitz microscope.

G. In-Situ Hybridisation on retinal ganglion cells.

Retinal ganglion cells from P-0 Sprague-Dawley rate were purified to greater than 99% purity by immunoselection as described by Barres et al. (1988, see below). Cells were counted and plated at 10 000 cell/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 hybridisation 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). This consisted firstly with the addition of the 1 hour wash in 4x SSC at room temperature, followed by two incubations in RNAse A buffer, the digestion with RNAse A and then a 2x SSC wash at 55ºC for 30 minutes followed by a final high stringency 0.1x SSC wash at 55ºC.

2.2 COMBINED IN SITU HYBRIDIZATION AND IMMUNOCYTOCHEMISTRY.

Sections of rat retina were cut in the normal manner and collected on baked APES-coated slides. Immunocytochemistry was first performed on the sections of interest , followed by in situ hybridization. The procedure used was essentially as described by Watts and Swanson (1989). The most important feature of this protocol was the preservation of the tissue mRNA during the

immunocytochemical steps; appropriate measures were therefore taken to minimise degradation of the target mRNA. Sections of P-8 rat retinae were stained with monoclonal anti-GFAP antibody (Sigma); sections were pre-blocked for 1 hour at 4ºC, with TBS (Tris-buffered saline, 50mM Tris-HCl pH 7.4, 0.9% NaCl, DEPC treated) containing 5mg/ml heparin, to act as an RNAse inhibitor (from Bovine Intestinal Mucosa, Sigma), 50 units/ml RNAsin (Promega) and 2% BSA (Fraction V crystalline, Sigma). Sections were then washed with several changes of TBS and incubated in the primary antibody. Anti-GFAP monoclonal antibody was used at 1:400 and diluted in TBS, containing 5mg/ml heparin and the sections incubated overnight at 4ºC in a humidified chamber. Sections were then incubated in the secondary antibody , biotinylated anti-mouse IgG, used at 1:100 (Amersham), diluted in the same buffer as before, for 1 hour at room temperature. The immunoperoxidase method was used to visualise the GFAP , using the Vectastain AB kit (Vector Laboratories, Peterborough, UK). After the second antibody, the sections were washed with TBS and incubated for 1 hour at room temperature with AB (Avidin-Biotin) reagent, made up in 10 ml of TBS with no heparin and allowed to stand for at least 30 minutes before being used. Sections were washed again and the immunoreactivity developed using the DAB substrate kit for horseradish peroxidase (Vector Laboratories). This reagent was made up by adding 2 drops of buffer stock solution, 4 drops of DAB stock solution and 2 drops of hydrogen peroxide to 5 mls of DEPC treated dH_20 . The tissue sections were incubted with this substrate at room temperature until a suitable dark brown reaction product developed. The reaction was then halted using several washes of DEPC treated water. Sections were then dehydrated through an ascending series of alcohols, air dried and hybridized with the PDGF-alpha receptor probe as above. Post-hybridization washes were exactly as described for in situ hybridization on retinal ganglion cells (see above). The slides were dipped in emulsion and developed as previously described.

2.3 IMMUNOCYTOCHEMISTRY

Postnatal day 5 (P5) and P15 agouti mice (H4 strain; MRC Radiobiology Unit Harwell) were killed with CO_2 and promptly perfused through the heart with phosphate-buffered saline (PBS, pH 7.4) followed by 4% (w/v) paraformaldehyde in PBS. The heads were removed and placed in 4% paraformaldehyde 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 tisue was frozen in OCT embedding compound (Miles, Tissue-Tek) and the blocks were stored at -70°C until use. Cryostat sections (8μm nominal thickness) were collected on APES (Aminopropyltriethoxysilane; Sigma)-coated slides , air dried and non-specific immunoglobulin sites blocked with a combination of 50% normal goat serum (NGS) in 50mM Tris-buffered saline (TBS, pH 7.4), with 100mM L-lysine and 1% (w/v) bovine serum albumin (BSA), for 30 minutes at room temperature. The sections were washed 3 times with PBS and then incubated in TBS containing 0.4% Triton X-100, for 15 minutes. After a further wash in PBS, The sections were incubated overnight at 4°C in a humidified chamber, with a rabbit antiserum raised against recombinant mouse PDGF-A (Palmieri et al., 1992) at 1:150, followed by biotinylated anti-rabbit IgG (Amersham; 1:100 dilution) for 1 hour at room temperature, followed by fluorescein-conjugated stretavidin (Amersham; 1:100 dilution) for 1 hour at room temperature.

A. Specificity of antibody

As a control, the anti-PDGF-A antibody was preincubated with a 100-fold molar excess of recombinant human PDGF-AA (Peprotech, New Jersey) overnight at 4°C on a rotating wheel. Antibody-antigen complexes were removed by centrifugation at 400 000xg for 20 minutes at 4°C in a Beckman TLX ultracentrifuge, and the supernetents used for immunocytochemistry. 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 other factors, abolished or caused a marked reduction of the specific labelling.

B. Double-labelling of sections

Sections of P-5 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 Rhodamine-conjugated goat anti-mouse IgG (Cappel) and PDGF-A immunoreactivity visualized as above. Sections of P-15 mouse retinae were double-labelled with anti PDGF-A and monoclonal antibody HPC-1 (Barnstable et al., 1985), a marker for amacrine neurons in the rat retina. The labelling in the mouse retina resembled exactly previous reports of the

distribution of staining in the rat retina (Barnstable et al., 1985). sections were incubated simultaneously in both primary antibodies and visualized as above. All antibodies were diluted in TBS with 100mM L-lysine, 1% (w/v) BSA and 0.2% Triton X-100. Post-incubation washes were with 3 changes of PBS for 10 minutes each. Sections were mounted under glass coverslips in Citifluor anti-fade reagent (City University, London) and viewed in the fluorescence microscope.

C. Propidium iodide labelling of tissue sections.

Cryosections were permeabilised with 70% ethanol at -20°C for 20 minutes, after which they were rinsed with PBS. In order to stain cell nuclei and identify apoptotic cells, sections were incubated with 5μg/ml propidium iodide (PI-Sigma) and 0.1 mg/ml DNAse free RNAse (Sigma), in PBS for 20 minutes at 37°C (Barres et. al., 1992a). The slides were then rinsed in PBS and the sections mounted under citifluor anti fade reagent (City University, London), and viewed using a Zeiss Universal fluorescence microscope.

D. BrDU incorporation

Rat pups that had received intravitreal injections of growth factor were given a single intraperitoneal injection of BrDU (0.1 mg/ml body weight; Boehringer Manheim) 2 hours before they were sacrificed. BrDU powder was dissolved in MEM-H to the appropriate concentration. After fixation, cryoprotection and sectioning , sections were rehydrated with PBS, followed by post-fixation with 70% ethanol for 10 minutes at -20°C. This was followed by DNA denaturation by incubation in 2M HCl. for 10 minutes followed by 0.1 M sodium borate buffer, pH 8.5, for 10 minutes. Non-specific immunoglobulin sites were blocked on tissue sections with 50% normal goat serum, in TBS, containing 1% BSA and 100mM l-lysine, for 30 minutes at room temperature. after a brief rinse in PBS, the sections were incubated in BU-1, a monoclonal anti-BrDU antibody (kind gift of Dr. J. A. Katzman; Gonchoroff et al., 1985; Griepp et al., 1985). The concentrated supernatent was diluted 1:10 and sections incubated overnight at 4°C in a humidified chamber. Staining was visualized by using biotinylated anti-mouse IgG (Amersham, 1:100) followed by Streptavidin fluorescein (Amersham, 1:100). Washes between incubations were with 3 changes of PBS and all antibodies diluted in TBS. Sections were then processed for propidium iodide staining and mounted.

2.4 WHOLEMOUNT IMMUNOHISTOCHEMISTRY

Retinal wholemount staining was carried out as follows, the protocol being that essentially described in Dreher and Stone, 1987: Rats were perfused with PBS, pH 7.4 and then with 4% paraformaldehyde in PBS. The eyes were enucleated and placed in 4% paraformaldehyde for 1 hour at 4ºC, after which the retinae were carefully removed, as a complete cup and fixed for a further 1 hour at 4ºC. The retinae were then washed, twice, for 10 minutes each with 1% Triton-X-100 in PBS; this step and all subsequent washing steps were carried out on a gently rotating horizontal shaker. After a final rinse in PBS, they were incubated in monoclonal GFAP antibody (Sigma) diluted at 1:400, overnight at 4ºC. After 24 hours, the retinae were washed twice for 10 minutes each in PBS and then incubated with Biotinylated anti-mouse Ig secondary antibody, at 1:100 (Amersham). The GFAP staining was visualised using Streptavidin Fluorescein antibody at 1:100 (Amersham); all dilutions of antibody were in TBS containing 100mM L-lysine. After final washes, each retinal cup was taken and carefully positioned on a glass slide with Teflon multiwells. Six radial incisions were made, with a pair of microscissors, to flatten out the retina and then, they mounted in Citifluor anti-fade reagent and the wholemounts observed using a Leitz Fluorescence microscope.

2.5 INTRAVITREAL INJECTIONS OF GROWTH FACTORS AND COS-CELLS.

Newborn and postnatal day 7 rat pups were administered with a single injection of factor or cells into the eye. Pups were anaesthetized with ether, if P7 or cooled at 4ºC, if newborns, and the eyelids gently eased apart along the natural crease of skin overlying the eyeball using a pair of microscissors, under a dissecting microscope. Using a Hamilton syringe fitted with a 34 guage needle, 0.5µl of the factor or using a 32 guage needle 0.5µl of cells concerned were injected over 2 minutes just posterior to the corneo-scleral junction, behind the vascular network that surrounds the lens, into one of the eyes. The needle was inserted to a 2mm depth into the centre of the vitreous cavity of the eye, behind the lens. After the completion of the injection, the needle was left in position for 30 seconds, with a concomittant reduction in the

pressure that was used to stabilize the eyelids in the open position; this was important in reducing reflux of the factor or cells along the needle tract. The needle was then retracted gently and the eyelids closed with a suture. Controls were always conducted in parallel on the same litter, consisting of PBS, or mock transfected cells and uninjected animals.

2.6 Preparation of Coverslips and plastic wells with various substrata

Plastic wells or glass coverslips (that had been sterilized by baking at 150ºC) were coated with Poly-L-lysine (PLL-Sigma). The PLL was made up as a 100x stock and used at a final concentration of 50µg/ml. The solution was left on the surface of the plastic well or glass coverslips for 1 hour, after which it was aspirated off and the surface allowed to dry thoroughly in a sterile hood. To prepare merosin and laminin coated coverslips, 20µl of stock laminin (Sigma) or stock merosin (Gibco-BRL) were diluted up to a maximum volume of 2 ml in serum free Ham's F-12 medium (Gibco) and PLL coated surfaces coated with O.5 ml of the solution. Coated surfaces were left to incubate overnight at 37ºC/5% CO_2.

2.7 Culturing and Harvesting Cos cells

Vials of Cos cells were rapidly thawed at 37ºC and the cells added to 10 ml of DMEM-10% FCS. The cells were spun down for 5min at 800g and resuspended in 10 ml of DMEM-10% FCS. One vial of cells was plated per 10cm tissue culture dish and the cells grown at 37ºC/5% CO_2 until confluent. The cells were then split 1:8 until they reached 70% confluency, the stage at which they were ready for transfecting with DNA.

2.8 Transfection of Cos cells by Electroporation

Cos cells were washed, once with calcium-magnesium free DMEM (CMF-DMEM) and then trypsinized off the plates, by adding 1 ml of trypsin to 3.5 ml of CMF-DMEM with 0.5 ml of EDTA pH 7.5, to the cells and incubated for 5 minutes at 37ºC/CO_2. The cells were gently taken off the surface of the plates by gentle pipetting and then transfered to 1ml of FCS. Cells from each plate were spun down at 800g for 5 minutes and the resulting pellet resuspended in 10ml Hepes buffered saline, pH 7.05 (HBS-20mM Hepes,

137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4 and 6mM, D-glucose). The cells were spun down again and resuspended in fresh HBS such that the cells were at a concentration of $8x10^6$/ml. Bio-Rad 0.5 ml sterilized cuvettes were filled with 250μl of HBS to which was added 20μg of DNA followed by agitation. 250μl of the Cos cell suspension was added and the cuvettes placed on ice for a few minutes. The cells were transfected , using a Bio-Rad electroporator at 300volts, at a capacitance setting of 125μFa and a time constant of 4.9 μsec. After transfection the cells were placed on ice momentarily and plated at $2x10^6$/dish in 10 ml of DMEM-10% ; the dish contained 3-4 glass coverslips which were subsequently stained to assess the efficiency of transfection. The cells were grown overnight at 37ºC/5% CO_2 and then harvested as above for injection purposes. The cells were resuspended in MEM-H, spun down, and then resuspended in a fresh lot of MEM-H at $2x10^6$/ml ready for injection.

2.9 Immunohistochemistry on transfected Cos cells.

Cos cells were transfected with pRα17 (Krueger et al., 1994 submitted). In order to assess the transfection efficiency, Cos cells were stained with monoclonal 9E10 (anti-c-myc) (Evan et al., 1985) antibody and cells in representative fields counted. The staining was carried out as follows: Cells were fixed with fresh 4% paraformaldeyde for 5 minutes, washed 3 times in MEM-H and non-specific antibody sites blocked with a 1:1 mix of normal sheep serum (Gibco) and TBLS containing 0.2% Triton X-100, for 30 minutes. After washing, the cells were incubated with monoclonal 9E10 supernatent for 30 minutes , washed 3 times again and then then incubated with goat anti-mouse fluorescein (Capell), for the same length of time. Coverslips were given a final wash and then mounted onto glass slides with Citifluor, and cells visualised with a Zeiss fluoresence microscope. To assess the efficiency of transfection, three representative fields were selected, per coverslip and all the cells counted, under phase optics. Then, under the blue filter, the number of fluorescing cells were counted and expressed as a percentage of the total number. In general, with constant conditions, the usual transfection efficiency was between 20-40% for electroporation. For further details for sections 2.7, 2.8 and 2.9, see Krueger et al., 1994.

2.10 Immunopanning of retinal cell types.

Retinal ganglion cells were purified from the rat retina using the simple technique of immunopanning (Mage et al., 1977; Wysocki and Sato, 1978; Barres et. al., 1988).

A. Purification of retinal ganglion cells.

The protocol used to purify this cell type was based on a modification of the procedure devised by Barres et. al., (1988). The secondary antibody was affinity-purified goat anti-mouse IgG (H+L chain-specific, Sigma) and the primary was MRC OX-7, anti Thy-1 supernatent (Lake et al., 1979). Petri dishes (10 cm; Falcon) were incubated with 5 ml of 50mM Tris pH 9.5 buffer with at least 50µg of the secondary antibody, for 12 hours at 4°C. The dishes were then washed three times with PBS and then incubated with 5 ml of MRC OX-7 supernatent diluted 1:4 with HEPES-buffered minimal Eagle's medium (MEM-HEPES, Flow-Labs) containing BSA (Sigma A4161) at a final concentration of 0.2%, for at least 1 hour at room temperature. The inclusion of BSA blocked non-specific adherence of cells to the panning plate. Just prior to putting on the cell suspension , the plates were rinsed with MEM-HEPES and this medium left on until the plates were used.

B. Preparation of the retinal cell suspension.

Newborn rats were decapitated and the eyes quickly enucleated and then placed in EBSS (Earle's balanced salt solution -Gibco). Neural retinae were carefully dissected free from all other tissues,under a dissecting microscope, and pooled in a bijous containing a small volume of calcium/magnesium free EBSS. Towards the end of the dissection, 2ml of retinal buffer (RB) , containing 1.5mg/ml L-lysine were placed in a 37°C water bath to equilibrate. After 5 minutes a papain suspension (papain - Worthington) was added to this buffer at a final concentration of 10µl/ml of buffer. A few drops of sodium hydroxide were added to adjust the pH and the papain then allowed to dissolve in the buffer for 10 minutes at 37°C. After 10 minutes , the excess EBSS was gently aspirated off the pooled retinae and the papain mix added by filtering it through a 22µm acrodisc. The retinae were incubated for 20 minutes in the papain mix at 37°C , with gentle agitation after 10 minutes . Then, the retinae were carefully removed from the enzyme mix and pipetted into 5ml EBSS solution containing 15mg soya bean trypsin

inhibitor (Boerhinger-Manheim) and 5 mg of BSA (Sigma A4161). The retinae were then gently triturated , using a 1ml Gilson pipette and a blue tip, until a uniform suspension was achieved. The cells were gently spun down for 5 minutes and resuspended in 5 ml of Bottenstein and Sato's defined medium in Ham's F-12 containing which had been equilibrated at 37ºC . The cells were filtered through a 20μ sterile mesh and then placed on the panning plate.

C. Removing adherent cells from the panning plate

For all of the experiments conducted, at least 50 retinae were always used to yield a high number of retinal ganglion cells (maximum of 1 x 10^6 from 50). To purify such a high number of cells, two panning plates were employed in parallel. After the addition of the cell suspension to the plates, the progress of the cells was monitered halfway through, by inspection under a microscope. After inspection, the suspension was swirled a few times in order that all cells could gain access to the surface of the plate. After 20 minutes, the non-adherent cells were removed from the plates with moderate agitation. This was achieved by washing the cells with warm, equilibrated Ham's F-12 several times, with inspection under the microscope to follow the progress of the non-adherent cells. When the plates were free of all floating cells, the attached RGCs were removed by gently and thoroughly pipetting warm F-12 onto them several times, until all of the cells had come off the surface of the plate. The cells were then transfered to a 15 ml Falcon tube , spun for 10 minutes and resuspended in an appropriate amount of growth medium for plating .

2.11 Culturing a Schwann Cell line.

A spontaneously immortalized Schwann cell line (Eccleston et al., 1990) was grown, for subsequent RNA extraction for use in cDNA sysnthesis. A vile of frozen cells was taken and thawed rapidly at 37ºC in a water bath. The cells were added to a 15 ml Falcon tube containing 10 ml of DMEM with 10% heat inactivated foetal calf serum (Dulbecco's Modified Eagle's Medium, Flow Laboratories), the tube inverted and the cells spun down at 1500g for 5 minutes. The medium was aspirated off and the pellet resuspended in 1 ml of medium. To this was then added 4ml of medium and the cells initially grown in a 50 ml Falcon flask, at 37ºC/5% CO_2. When the cells became confluent they were trypsinized off the surface of the flask with 0.1 % trypsin in DMEM

containing Versene . The old medium was sucked off and the cells given a brief rinse in Versene to remove any serum. 2 ml of Versene containing 0.5 ml of trypsin were then added to the cells and the flask incubated at 37ºC/5% CO_2 for 5 minutes. The trypsin was gently aspirated off , the flask agitated several times, by repeated patting with the hand and the cells removed by repeated pipetting with 2 ml of DMEM/F10. The cell suspension was then squirted into a larger flask .

2.12 BACTERIOLOGY

Methods in this section were based essentially on detailed protocols in Sambrook et al. (1989), unless otherwise stated.

A. Bacterial strains

For general cloning and sub-cloning of recombinant plasmids *Escherichia coli (E.coli)* strains DH5 [F^-, *rec* A1, *end* A1, gyrA96, *thi*-1, *hsd*R17, (r_{k^-} , m_{k^-}), *sup*E44, *rel*A1 λ^-] (Hanahan, 1985) and XL1-Blue [*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, lac,[F ' *pro*AB, *lac*I^q ZΔM15, Tn 10 (tet^r) (Bullock et al., 1987) were used .

B. Growth media and agar plate

Bacteria were grown in Luria Broth (LB) containing 10g bacto-tryptone, 5g yeast extract and 10g NaCl per litre, or on LB-agar plates (LB + 15g bacto-agar/litre). In all cases Ampicillin (Amp) at a final concentration of 100µg/ml was added after LB had cooled to below 50ºC. Amp was prepared as a 1000x stock (100mg/ml) by dissolving the sodium salt in deionised water (dH_2O), filter sterilised through a 0.22µm filter (Millipore Corp.) , aliquoted and stored at -20ºC. All strains of *E. coli* were grown at 37ºC and all liquid cultures were continuously agitated in a rotating shaker.

C. Preparation of Competant Bacteria

A modification of Hanahan's (1985) protocol was employed to prepare bacteria that could then be subsequently stored at -70ºC. From either a 100µl glycerol bacterial stock or from an individual colony on a LB-agar plate, DH5

or XL1-Blue *E. Coli* strains were grown in 100 ml of LB, with constant agitation, until an O.D. reading of 0.4-0.6 was attained. The bacteria were then spun at 3000 rpm, for 15 minutes at 4ºC. The supernatent was completely discarded and all subsequent steps carried out on ice. The pellet was resuspended in 50ml (half original volume) of TFB 1 (Transformation Buffer 1: 15% glycerol, 100mM $CaCl_2$, 50 mM $MnCl_2$, 30mM K Acetate, pH adjust to 5.8 with 0.2 M acetic acid) and allowed to incubate on ice for 20 minutes. The bacteria were then re-pelleted at the same speed as above , the supernatent completely discarded and the bacterial pellet resuspended in 4 ml (one twentififth of the original volume) TFB-2 (Transformation Buffer 2: 10mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, pH adjust to 6.5 with 1M KOH) and left for a further 20 minutes on ice. This suspension was aliquoted into eppendorf tubes and snap frozen on a slurry of ethanol and dry-ice, and the aliquots stored at -70ºC. The efficiency of these bacteria, as determined by transforming with either Bluescript or pUC-8 plasmids (50 pg-1 ng) was determined to be between 10^7-10^8 colonies/µg supercoiled plasmid DNA. For experimental purposes, aliquots were allowed to thaw gently on ice.

D. Transformation of bacteria with DNA.

50-100 ng of plasmid DNA were added to 100µl of competant bacteria, briefly mixed and incubated on ice for 30 minutes. This was followed by a brief heat shock at 37ºC for 5 minutes, followed by incubation in 1 ml of LB at 37ºC for 45 minutes , with constant agitation. The cells were then pelleted at 3000g for 5 mintes, resuspended in 100µl of LB and the bacteria spread onto LB-agar plates containing AMP. and incubated overnight at 37ºC. In parallel 10 ng of pUC-9 plasmid was used to check the transformation efficiency of the bacteria . After overnight incubation, individual colonies were selected, picked with sterile yellow Gilson tips and placed into 10ml of LB containing AMP.. These colonies were grown up overnight at 37ºC on a rotational shaker and processed for DNA mini- preps.

Wherever the cloning site of a plasmid formed part of the ß-Galactosidase gene it was possible to screen for inserts by assesing loss of ß-Galactosidase activity. Plasmids with inserts gave white plaques, whereas native plasmid gave blue plaques, when grown on LB-agar plates spread with 40µl of a 2.5% solution of X-gal (5-bromo-4-chloro-3-indolyl-ß-D-

galactopyranoside) in dimethylformamide and 10μl of a 0.1M solution of IPTG (Isopropyl ß-D-thiogalactopyranoside), all in a total volume of 80μl, made up with dH_2O , per plate.

2.13 MOLECULAR BIOLOGY

A. Phenol/Chloroform extractions

Samples to be extracted were made 0.5M with NaCl and equal volumes of UNC-phenol [UNC-phenol made up by dissolving 500g of phenol in 250ml 1M Tris-HCL pH 7.5, 28ml m-cresol, 1.1ml 2-ß-mercaptoethanol (2-ME), 555mg ∂-hydroxyquinoline] and chloroform containing 4% v/v iso-amyl alcohol were added. The combined samples were then vigorously vortexed and spun at 10 000g for 15 minutes. The resultant upper aqueous phase was carefully removed and transferred to a separate tube. This phase was then re-extracted with an equal volume of chloroform, containing the same level of iso-amyl alcohol as before, centrifuged and the upper aqueous phase collected.

B. Ethanol Precipitation of nucleic acids

Samples were made 0.5M with NaCl and twice the volume of cold, 95% ethanol added (-20ºC). The solution was mixed and incubated on dry-ice for 30 minutes. DNA/RNA was pelleted by spinning at 10 000g for 15 minutes at 4ºC. The resultant pellet was washed with 70% ethanol and then air dried before being dissolved in a suitable buffer. With samples containing 5-10μg of DNA/RNA, 25μg of Baker's yeast transfer-RNA (tRNA) was added to act as a carrier.

C. Restriction digest of DNA

In general, 2μg of DNA was digested in a final reaction volume of 20μl, for a minimum of 1 hour at 37ºC. Enzymes were used in buffers containing 10mM Tris-HCl pH 7.5, 10mM $MgCl_2$ and 1mM Dithiothreitol (DTT), and according to the enzymes of choice, four different NaCl concentrations were employed; 0, 50, 100, 150 mM. In general NEB enzymes were used with the appropriate NEB buffer, labelled 1 to 4 and if necessary, acetylated BSA (Bovine serum albumin , fractionV, 10x stock, NEB) was also included in the

reaction.

D. Ligation of DNA

Ligations were carried out using 50ng of vector and a 4-fold molar excess of DNA insert. Ligations were carried out at 14ºC overnight with 1 unit of T4 ligase per reacton in ligase buffer (10x buffer containing 500mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 100mM DTT, 10mM Spermidine, 10mM Adenosine Tri-Phosphate (ATP) and 1mg/ml BSA) in a total volume of 20μl. In parallel several control ligations , consisting of no vector, uncut vector alone, no enzyme and cut vector in the presence of the enzyme were carried out in four separate tubes and also incubated overnight at the same temperature. Competant bacteria (XL1-Blue *E. Coli*) were then transformed with DNA and then spread on LB-agar plates containing AMP. In all cases, blue/white colony selection methods were employed, as noted above. Individual white plaques were picked with a yellow Gilson tip, grown in 10 ml of LB/AMP overnight at 37ºC in a rotational shaker and processed for DNA mini preps.

E. Screening of Bacterial Colonies

F. DNA Mini preparation

Individual white colonies from transformed bacteria were selected from amongst the blue colonies on and LB-Agar plate and grown up overnight at 37ºC as described. 1.5 ml of the overnight cultures were taken and the bacteria pelleted at 10 000g for 30 seconds at 4ºC in eppendorf tubes. The pellets were washed, by resuspension in 1ml of STE (0.1 M NaCl, 10mM Tris-HCl pH 8, 1mM EDTA pH 8) and the bacteria then pelleted as before. Supernatents were discarded and 100μl of solution 1 was added (solution 1= 25mM Tris pH 8, 10mM EDTA, 50mM glucose), tubes then vortexed briefly and left to stand at room temperature for 5 minutes. 200μl of solution 2 were added (solution 2= 0.2M NaOH, 1% (w/v) SDS) with thorough mixing by inversion and the tubes incubated on ice for a further 5 minutes. This was followed by the addition of 150μl of solution 3 [solution 3= 3M potasium acetate, 2M acetic acid pH 4.5 (4ºC)], the resulting solution , inverte several times and left for a further 5 minutes on ice. Eppendorfs were then spun at 10 000G for 10 minutes at 4ºC and the supernatents (approx. 440μl per tube)

extracted twice with phenol/chloroform and then once with chloroform as described. The DNA was then ethanol precipitated as described , washed in 70% ethanol and dissolved in 20 of TE (10mM Tris pH 7.5, 1mM EDTA). 5μl of DNA was removed per sample and cut with the appropriate restriction enzymes and the products then analysed on an agarose gel.

G. 'Maxi' Plasmid preparations

Plasmid DNA was prepared using a scaled up version of the alkaline lysis method described in the Promega Biological Research Products catalogue (1992). 200 ml of LB containing AMP. was inoculated with 0.5 ml of an overnight bacterial culture and grown overnight at 37ºC, in a rotational shaker. Bacteria were pelleted at 5000G for 15 minutes at 4ºC, the supernatents discarded and the pellet resuspended thoroughly, in 6 ml of freshly prepared ice-cold lysis buffer (25mM Tris-HCl pH 7.5, 10mM EDTA, 15% (w/v) sucrose) and left on ice for 20 minutes. Bacteria were then lysed by the addition of 12 ml of 0.2 M NaOH and 1% (w/v) SDS, with careful and thorough mixing and left on ice for 10 minutes. This was followed by the addition of 7.5 ml of 3M potassium acetate, pH 4.6, with thorough mixing ad incubation on ice for a further 10 minutes. The contents were centrifuged at 10 000G for 15 minutes, the supernatents retained, taking care to avoid the white precipitate, and 5μl of RNase A (10mg/ml) added and the supernatent incubated at 37ºC for 60 minutes. The supernatent was then extracted twice with phenol/chloroform and then once with chloroform and the DNA precipitated as described. The DNA pellet was then dissolved in 1.6 ml of H_20 and 0.4 ml of 4M NaCl , followed by the addition of 2 ml of a 13% (w/v) Polyethylene Glycol 6000 (PEG) solution. The mixture was mixed and then incubated on ice for 60 minutes, then centrifuged at 10 000G for 20 minutes, in several eppendorf tubes, the pellets washed with 70% ethanol, air-dried and dissolved in an appropriate volume of TE. The DNA and protein contents were measured on a spectrophotometer at 260 and 280 nm respectively (A_{260}, A_{280}).

$$\text{DNA concentration (mg/ml)} = \frac{A_{260} \text{x Dilution factor x Epsilon}}{1000}$$

Epsilon is the extinction co-efficient for the aborbance of a nucleic acid at 260 nm as determined at 20ºC. For double stranded DNA it has a value of 50/μgcm, for single stranded DNA and RNA, 40/μgcm and for oligonucleotides, 25/μg cm. .

H. Purification of DNA fragments

In all cases the GeneClean II (Bio 101 Inc.) kit was employed to purify DNA fragments. Fragments of interest were run on an agarose gel, excised carefully and weighed. For every 0.1g of gel, 0.3 ml (3 volumes per mass) of NaI solution was added followed by 5 μl of glass milk. In general, if 5μg of DNA or less were to be purified, 5μl was sufficient; if more than 5μg was to be purified, more was usually added. The mixture was shaken well and left for 10 minutes on ice, with intermittant shaking. The glass milk, to which the DNA adhered was pelleted by spinning for 5 seconds at 10 000G and then washed 3 times with 0.5 ml of New wash buffer (concentrate of Tris, NaCl and EDTA made up in absolute ethanol, Bio101 Inc.) by resuspending and spinning. After the final wash, the wash buffer was thoroughly aspirated off and the glass pellet resuspended in 10μl of water, vortexed briefly and the DNA recovered by incubating at 55ºC for 3 minutes. The glass milk was separated from the DNA by spinning for 1 minute at 10 000G and the DNA taken off and stored.

I. NaOH blotting procedure

DNA was vacuum blotted onto Zeta Probe membrane (BioRad). DNA was denatured and blotted using 0.5 M NaOH , 1.5 M NaCl as solvent, followed by neutralization with 0.5 M Tris-HCl, 1.5 M NaCl. The blot was air dried and baked at 80ºC for 1 hour prior to pre-hybridization.

J. Filter hybridization

DNA that had been blotted onto Zeta Probe (BioRad) was pre-hybridized with 50% deionized formamide, 5x Denhardt's, 6x SSC, 1mM EDTA, 1% SDS (w/v) and 100μg/ml of freshly boiled Salmon sperm DNA., for 2-4 hours. 32P labelled DNA probe that had been boiled for 5 minutes was then added to the membrane and left to hybridize overnight at 42ºC in a Hybaid™ hybridization oven. Post-hybridization washes consisted of an initial 5 minute wash in 2x SSC, 0.5% SDS, followed by another 2x SSC, 0.1% SDS

wash , both at room temperature, followed by a 37ºC wash for 1 hour in 0.1x SSC, 0.5% SDS and a final high stringency wash at 68º with 0.1x SSC, 0.5% SDS. The blot was wrapped in Saranwrap and exposed to pre-sensitised Ilford hyperfilm.

K. Preparation of DNA probes

cDNA was digested with the appropriate restriction enzymes and purified as above. DNA fragments were then labelled with ^{32}P to a specific activity of approximately 2 x 10^8 cpm/μg by random priming (Feinberg and Vogelstein, 1984).. The reagents were added in the following order: 10 μl Oligo labelling buffer (OLB), 2μl BSA (10mg/ml stock, DNase free), 10/50 ng of cDNA which had been boiled for 3 minutes and chilled briefly on ice, 50 μCuries ^{32}P dCTP, 2 units (0.5μl) of *E. Coli* DNA polymerase I (large fragment, Klenow) and x ml of H_20 to bring the final voulme to 50μl .

Oligo labelling buffer was prepared by mixing solutions A:B:C in the ratio 10:25:15.

Solution A: 1ml of 1.25 M Tris-HCl pH 7.5; 0.125 M MgCl2 containing 18μl 2-Mercaptoethanol (2-ME), 500μM dATP, 500μM dTTP and 500μM dGTP.

Solution B: 2M Hepes pH 6.6

Solution C: Pd(N)6 dissolved at 90 OD units per ml. This is the primer for the DNA polymerase and consists of 6 randomly coupled oligonuclotides.

The labelling reaction was carried out at room temperature for a minimum of 4 hours . Unincorporated nucleotides were separated from ^{32}P labelled DNA by spinning the DNA through a G-50 Sepahdex column in TE, made in a 1 ml syringe barrel. Unincorporated nucleotides were retained by the G-50 whereas labelled DNA passed through the column. Just prior to hybridizing , the DNA was denatured into single strands by boiling it for 5 minutes.

L. Electrophoresis of Nucleic Acids

Nucleic acids to be analysed had 1 x loading buffer added to them (0.025% w/v bromophenol blue, 0.025% w/v xylene cyanol, 2.5% w/v ficoll-400) and were electrophoresed through 1% agarose gels, in horizontal submarine set up (Pharmacia). The agarose (Sigma) was melted in 1 x TAE buffer (50x : 242g Tris-base, 57 ml glacial acetic acid, 100ml of 0.5 M EDTA disodium salt, pH 8 , per litre of stock), allowed to cool below 40ºC and poured into a plastic gel tank, fitted with well combs. Gels were stained with a 5μg/ml Ethidium Bromide solution (EtBr.) made up in water, for 5 minutes and nucleic acids visualised using a UV transilluminator. Photographs were taken using Polaroid 665 or 667 type film.

M. DNA Sequencing (Double stranded method)

N. Sequencing Gels

The components required to make up a sequencing gel were purchased from Flowgen, consisting of Sequencing dilution solution, sequence concentrate and sequencing buffer mixed in the proportions 52.5 ml: 15 ml: 7.5 ml respectively. To this was added 0.6 ml of 10% (w/v) ammonium persulphate and 30μl of TEMED (Sigma). The gel mixture was carefully poured between siliconized glass sequencing plates with a large 50 ml syringe and left to solidify. All sequencing reactions were performed using the reagents and protocols from the T7 Sequencing™and the Deaza G/A T7 Sequencing™Mixes, kits from Pharmacia P-L Biochemicals. For the annealing step, 1.5-2.0μg of DNA from a maxi-prep were taken and made up to 8μl with dH20. DNA was denatured with 2μl of 2M NaOH and left for 10 minutes at room temperature. 3μl of 3M NaAcetate (pH 4.5), 7μl of dH20 and 60 μl of 100 % ethanol were added and the DNA precipitated for 15 minutes on dry ice. The DNA was pelleted , washed, air dried and dissolved in 10μl of dH_20. To this 10μl of template DNA , 2μl of annealing buffer and 2μl of primer solution were added and the contents incubated at 37ºC for 20 minutes and then at room temperature for a further 10 minutes. Four 0.5 ml eppendorf tubes were labelled , A, C, G, and T and 2.5μl of each of the 'N' mixes placed in the appropriate tubes and prewarmed to 37ºC . Using cold enzyme dilution buffer (20 mM Tris-HCl, pH 7.5, 5% (w/v) glycerol, 0.1 mg/ml BSA and 5mM DTT) sufficient T7 DNA polymerase was diluted for the sequencing reaction, made to a concentration of 1.5 units/μl. To the annealed samples were added 3μl of

Deaza dATP labelling mix, 1μl (10μCi) of α -35S-dATP and 2μl of the diluted enzyme and the samples left at room temperature for 5-7 minutes. 4.5 μl of this reaction was then transfered into each of the four pre-warmed sequencing mixes and incubated at 37ºC for 5 minutes. 5μl of Stop Solution was added to each tube, the contents mixed and 3μl removed from each tube and transfered to a fresh tube and each 3μl aliqout heated to 78ºC for 2 minutes. 2 μl were then loaded into the sequencing gel, The sequencing gel was pre-warmed for at least 30 minutes and run for 2-4 hours at 1300 V,with 1xTBE as running buffer (10xTBE: 890mM Tris-base, 890mM boric acid, 250mM EDTA, pH adjusted to 8.3 with acetic acid), in a BRL sequencing gel electrophoresis tank, with 0.4 mm spacer combs.

O. Removal and de-blocking of oligos. from the column

Two 5 ml syringes were taken and 2 ml of fresh 30% (w/v) ammonium hydroxide were taken up into one . The empty syringe was attached to the one end of the column. The other syringe was attached to the other end and the column gently flushed several times with the ammonium hydroxide. 1 ml of the solution was left in the column for 30 minutes at room temperature and the whole process of flushing and incubating repeated 3 times. The oligos were de-blocked at 55ºC overnight, and allowed to cool to 4ºC the next day. The ammonium hydroxide was lyophilised on a speedivac and the oligos dissolved in 200 μl of H20 .They were made 2.5 M with ammonium acetate and precipitated with ethanol by adding 2.5x the original sample volume. The pellets were spun out at 10 000G for 20 minutes, washed with 70% ethanol, air dried and dissolved in a suitable volume of H_20. The absorbance of the oligos was measured at 260 nm as noted previously.

P. RNA Extraction

RNA was extracted from an immortalized Schwann cell line (Eccleston et al., 1990) acccording to the technique of Chomczynski and Sacchi, (1987). Cells were disolved in solution D (Stock solution: 25g of guanidinium isothiocyanate, 29.3 ml of water, 1.76 ml 0.75 M sodium citrate, pH 7, 2.64 ml 10% sarkosyl. Solution D is made up by adding 360 μl of 2-mercaptoethanol to 50 ml of this stock solution). To the resulting mixture, was added 0.1 volumes of 2M sodium acetate pH 4, one volume of phenol amd 0.2 volumes of

chloroform/isoamyl alcohol. The mixture was mixed in a tube after the addition of each solution and then left on ice for 15 minutes. The contents were then spun for 20 minutes at 10 000 revs/min, the resulting aqueous phase removed and an equal volume of isopropanol added. The solution was left on dry ice for 1 hour and then spun for 20 minutes at 10 000 revs/min. The pellet was dissolved in a small volume of solution D, an equal volume of isopropanol added again to precipitate the RNA and finally spun at the same speed. The pellet was washed in 70% ethanol, dried and resuspended in DEPC treated water and the RNA stored at -70ºC.

Q. cDNA synthesis

Cold and radioactive first strand cDNA synthesis was carried out on no more than 1µg of total RNA, using the GibcoBRL, cDNA synthesis protocol. To 1µl of Oligo $(dT)_{12\text{-}18}$ (500µg/ml) or 0.5µl of random primer of the same concentration, was added 1µg of total RNA. The volume was brought up to 11µl, with sterile, distilled water and the mixture heated to 70ºC for 10 minutes and then chilled on ice. To this mixture was added 4µl of 5x reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2µl of 0.1 M Dithiothreitol, 1µl of mixed dNTP stock (10mM each of dATP, dGTP, dCTP and dTTP at pH 7) and if a radioactive cDNA sample was to be prepared, 1µl 0f [α-^{32}P]dCTP. The contents of the tube were mixed by gentle vortexing, the tube briefly spun to collect the reaction and then the mixture incubated at 37ºC for 2 minutes. 1µl of Moloney Murine Leukemia Virus RNase H- reverse transcriptase (M-MLV H-RT) was then added to the tube, with mixing, followed by a 1 hour incubation at 37ºC. The reaction was terminated by heating the mixture to 70ºC for 5 minutes. The cDNA was then aliquoted and stored at -20ºC.

R. Polymerase Chain Reaction

Four separate cDNAs were amplified from two separate RNA samples. Firstly, rat PDGF-B chain and rat PDGF-ß receptor cDNAs were cloned from cDNA synthesized from total RNA, isolated from an immortalized Schwann cell line (Eccleston et al., 1990). The primers used to amplify these species were as follows:

1.<u>For rat PDGF-B chain.</u>

Flanking primers:

B1, upstream primer : 5' (GAAGGNGATCNATTCCNGAAG) 3'
G C C G
A

B2, downstream primer: 5' (GGGAAGCACCATTGGCCGTCC) 3'

Nested primers :

Sal I **Hind III**

B3, upstream primer: 5' (CGGˇTCGACAˇAGCTTCCNGAAGAACTNTATA
G G CG
AAATG) 3'
G

Sal I **Eco RI**

B4, downstream primer: 5' (GCGˇTCGACGˇAATTCAATCAGGCAT

CGAGACAGACG) 3'

<u>2. For rat PDGF-ß receptor</u>

Flanking primers:

P1, upstream primer: 5' (GATACNGGNGAATATTTTTG) 3'
C G C C

P2, downstream primer: 5' (TCNCCATANCGACAATATTC) 3'
G G G C

Nested primers:

Sal I **Hind III**

P3, upstream primer: 5' (CGGˇTCGACAˇAGCTTCCNTATGATCATCAAC
C C C G

GNGG) 3'

Sal I **Bam HI**

P4, downstream primer: 5' (GCG'TCGACG'GATCCTAAATATATTCAT
G G C G
T

GNCCATC) 3'
G

S. Amplification of PDGF-B chain and PDGF-ßR cDNAs.

The buffer used was a 5x stock of that described in Jeffrey's et al., 1988, with final concentrations being, 45mM Tris-HCl, pH 8.8, 11mM ammonium sulphate, 4.5mM magnesium chloride, 6.7mM 2-mercaptoethanol, 4.5μm EDTA amd 100μg/ml BSA (DNAse free). To 1μl of cDNA was added 2 μl of each flanking primer, final concentration of 50 pM, 4μl of dNTPs, 10 μl of 5x reaction buffer and 31μl of water, to bring the final reaction volume to 50μl. The reaction was overlayed with 50μl of paraffin oil, heated for 5 minutes at 95ºC, in a Bio-Rad, Hybaid PCR machine, and 0.2μl of DNA Taq-polymerase (Amplitaq-Cetus Inc.) quickly added to each reaction. The tubes were carefully agitated, heated again to 95ºC for 2 minutes and then followed by the following cycles: melting temperature, 95ºC for 30 seconds, annealing temperature of 55ºC for 2 minutes and an elongation temperature of 72ºC for 2 minutes, for 30 cycles. 5μl of the the product of the first 30 reactions was then exposed to a further 30 cycles, using the nested primers, in the same reaction volume.

T. Northern blot Analysis

Total RNA was prepared by homogenising embryonic brain tissue in guanidinium isothiocynate and pelleted through caesium chloride (Chomczynski and Satchi, 1987). Equal quantities of RNA were analysed in 1% agarose gels containing 6% v/v formaldehyde, with 1x MOPS as running buffer, then transferrd to Zeta Probe membranes (BioRad) by vaccuum blotting and the integrity of the RNA confirmed by staining with methylene blue. After subsequent destaining the membranes were hybridized at 42ºC in 5x SSC and washed at a final stringency of 0.1x SSC at 65ºC.

Filters were probed with fragments of recombinant DNA which had been purified by electrophoresis through agarose gels and were labelled by the random primer extension method, as previously described. The used fragments were the same as for the in situs, namely, the 1.2 kb fragment for the PDGF ß-receptor and the 0. 74 kb fragment for the PDGF-B chain.

CHAPTER THREE

PLATELET-DERIVED GROWTH FACTOR AND ITS RECEPTORS IN THE RODENT RETINA.

3.1 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 I 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. A number of well-characterized growth factors are expressed in the retina, including several members of the fibroblast growth factor (FGF) family (Caruelle et al., 1989; Noji et al., 1990; Fu et al., 1991; Fayein et al., 1990; Wilkinson et al., 1989), transforming growth factor alpha (TGF-alpha) (Anchan et al., 1991), TGF-ß2 (Millan et al., 1991), epidermal growth factor (EGF) (Anchan et al., 1991), insulin and the insulin-like growth factors (IGF-I and IGF-II) (Hansson et al., 1989; Meimaridis et al., 1990; Ocrant et al., 1991) and nerve growth factor (NGF) (Chakrabarti et al., 1990). In addition, receptors for FGF (Wanaka et al., 1991), IGF-I (Ocrant et al., 1989; Bassnett and Beebe, 1990), EGF (Anchan et al., 1991) and NGF (Carmignoto et al., 1991) are expressed in the retina.

Platelet-derived growth factor (PDGF) and its receptors are also 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-ßR) 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). It has been previously shown that cultured cortical astrocytes synthesize and secrete PDGF-A but not PDGF-B, and on this basis wec 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 (Sasahara 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. Here I describe the results of an in situ hybridization survey of the cell types that express PDGF-A, PDGF-B, PDGF-αR and PDGF-ßR 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.

3.2 Results

To map the distributions of RNA transcripts encoding PDGF and its receptors in the developing rodent retina and optic nerve, I performed in situ hybridizations with ^{35}S-labelled "antisense" probes complementary to the rat PDGF-A, PDGF-B, PDGF-αR and PDGF-ßR mRNAs. In parallel, I 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

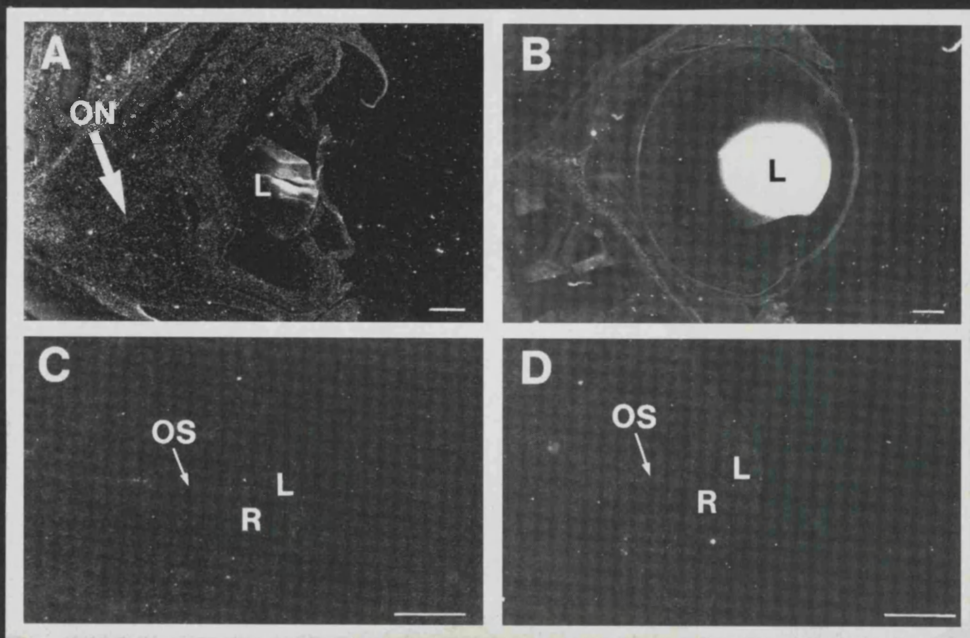


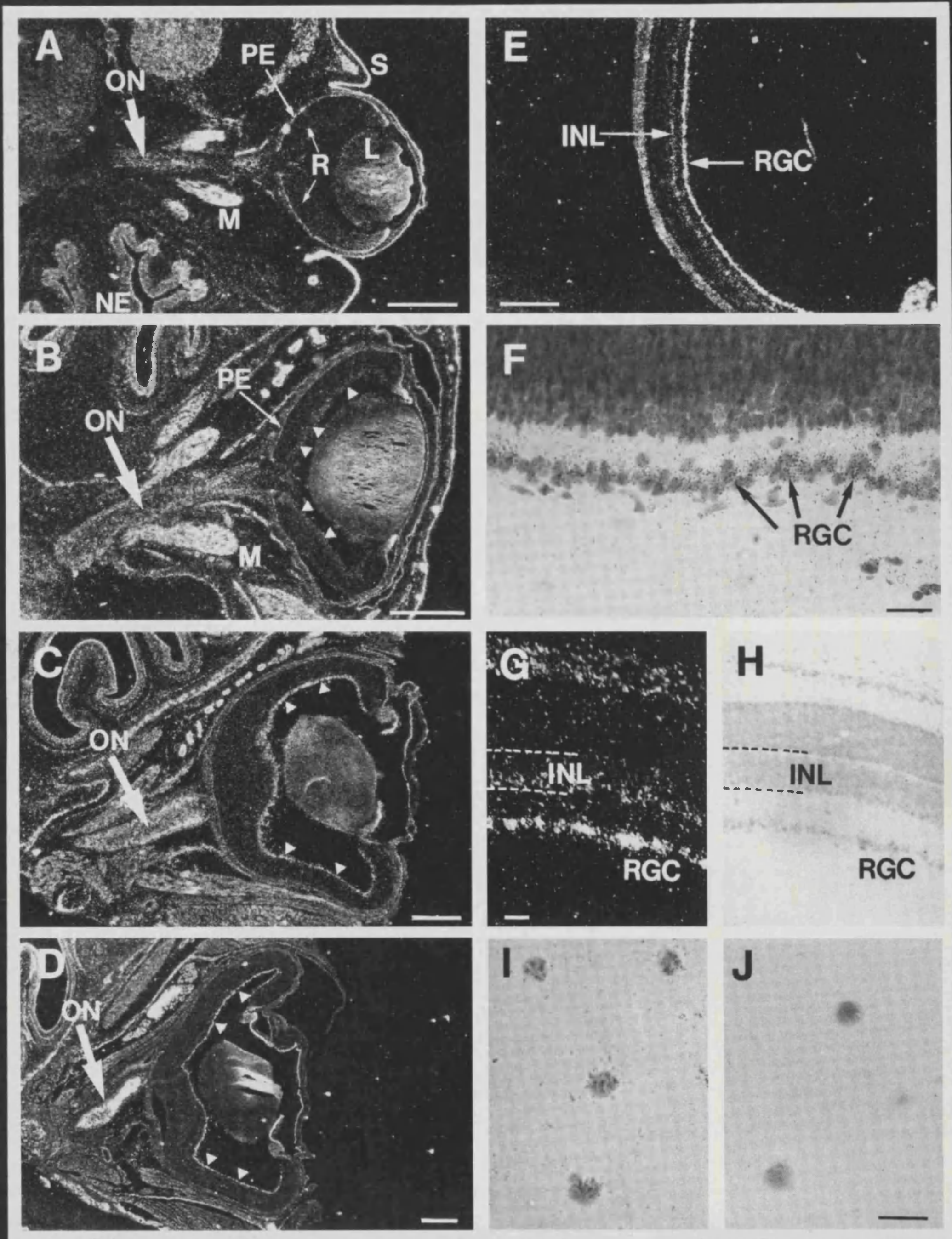
Figure 5. Negative controls for the specificity of the in situ hybridization probes used in this study. The figure shows dark-field images of in situ autoradiographs generated with "sense" probes corresponding to PDGF-A (panel A), PDGF-αR (panel B), PDGF-B (panel C) and PDGF-ßR (panel D). Panel A shows an adjacent section to that illustrated in Fig. 6D (PDGF-A antisense probe). Panel B shows an adjacent section to that in Fig. 9F (PDGF-αR antisense probe), only at slightly lower final magnification. Panel C shows an adjacent section to the one in Fig. 12B (PDGF-B antisense probe) and panel D shows an adjacent section to that of Fig. 12A (PDGF-ßR 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 panel A. Note that the intense dark-field signal in the eye lens (L) in panel 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; ON, optic nerve; L, lens; R, retina. Scale bars: 500 μm.

no detectable in situ hybridization signal over the eye or optic nerve (Fig. 5), 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.

A. PDGF-A mRNA and immunoreactivity in the retina

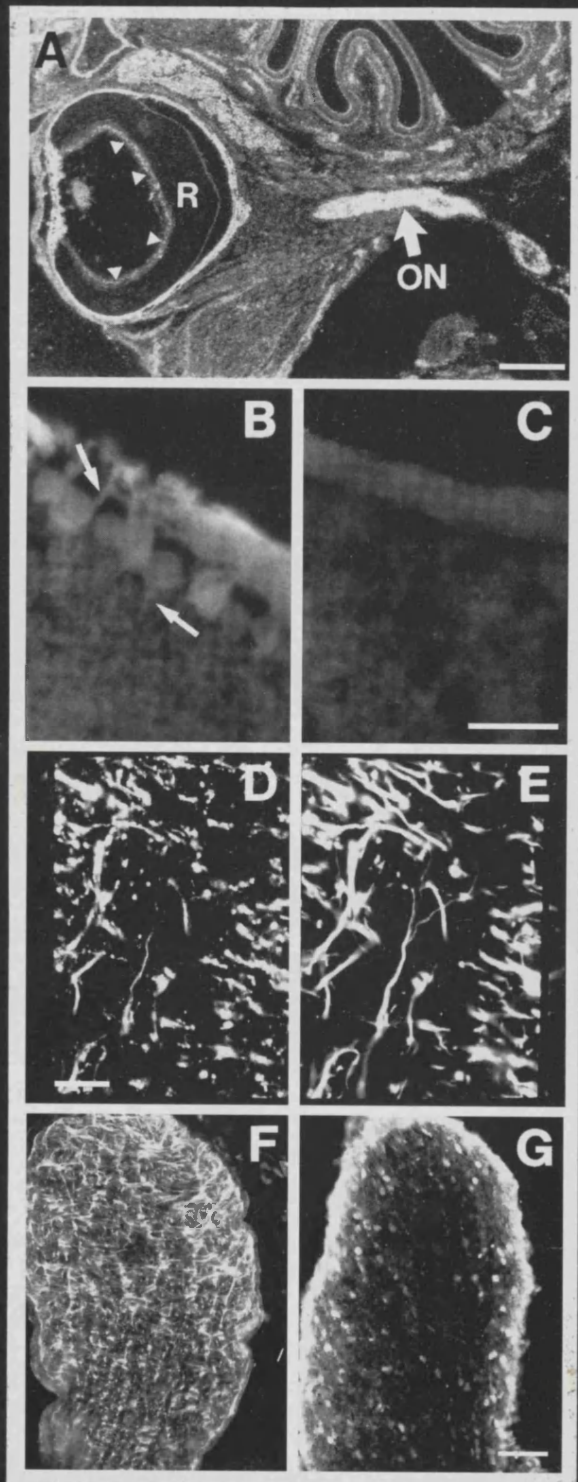
I cut cryosections through the retinae of embryonic and postnatal rats and mice, and subjected them to in situ hybridization as described in Materials and methods. As reported previously (Orr-Urtreger and Lonai, 1992), many tissues outside of the CNS express PDGF-A mRNA; of particular note in the sections displayed in Fig. 6 are the developing extraocular muscles flanking the optic nerve, the nasal epithelium and the skin of the embryo. PDGF-A transcripts first appear in the rat neural retina at E18, in the retinal ganglion cell (RGC) layer (arrowheads in Fig. 6B). 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. 6C, F). The intensity of this signal is maintained for at least several weeks (Figs 6D, 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. 7A). To confirm that PDGF-A is expressed by retinal ganglion neurons, I 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. 6I). The negative control ("sense") PDGF-A probe displayed only bacground signal (Fig. 6J). To test for the presence of PDGF-A protein, I used a rabbit antiserum raised against recombinant mouse PDGF (Mercola et al., 1990; Wang et al., 1992) to label 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. 7B). Several control experiments indicate that our immunofluorescence labelling procedure is specific for PDGF-A. First, the anti-PDGF-A antibody labels Cos cells that have been transfected with an

Figure 6. 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 and G), subjected to the in situ hybridization procedure and photographed in dark field illumination. PDGF-A mRNA is present in many extraocular tissues including developing muscle (M), nasal epithelium (NE) and skin (S). 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 (panel 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 (panel E). Panels 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 (panel B) and P0 (panel C). ON, optic nerve; R, retina; L, lens; PE, retinal pigment epithelium; S, skin; M, muscle; NE, nasal epithelium; RGC, retinal ganglion cells; INL, inner nuclear layer. Scale bars: panels A-E, 500 μm; panels F-J, 20 μm.



A
ON
PE
S
R
L
M
NE
E
INL
RGC
B
PE
ON
M
F
RGC
C
ON
G
INL
RGC
H
INL
RGC
D
ON
I
J

Figure 7. 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 (panel A), is expressed in the RGC layer and the optic nerve of the P5 mouse, as in the rat (see Fig. 6D). Panel 7B 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. 7A). No signal is obtained in an adjacent section when treatment with the anti-PDGF-A antibody is omitted (Fig. 7C). Panels 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 and confocal microscopy. PDGF-A is visualized using fluorescein and GFAP with rhodamine (see Materials and methods). PDGF-A immunoreactivity (D) coincides closely with GFAP immunoreactivity (E). Panels F and G are immunofluorescent micrographs of sections through part of a P5 mouse optic nerve, labelled with anti-PDGF-A antibody either before (panel F) or after (panel G) preincubating the antibody with an excess of recombinant PDGF-AA (see Materials and methods). Labelling of astrocyte processes (panel F) is largely obliterated by preincubation with recombinant PDGF-AA (panel G), as was labelling of RGCs (not shown). Panels D and E are confocal micrographs; the others are conventional micrographs. Scale bars: panel A, 500 μm; panels B-E, 20 μm; panels F,G, 100 μm.


A
R
ON
B
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expression vector encoding mouse or human PDGF-A, but not untransfected Cos cells (Mercola et al., 1990; A. Calver, unpublished). Second, our secondary antibodies give no fluorescent signal in the absence of the primary anti-PDGF antibody (Fig. 7C). 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. 7F with 7G), 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. 6E, G, H and arrows in Fig. 12A). 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. 12A) 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, double-label immunohisto-chemistry was performed 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. 8). Strong PDGF-A immunolabelling is present in RGCs and the optic fibre layer (Fig. 8A), as in the adult (compare Fig. 7B). In addition, PDGF-A immunoreactivity is present throughout the INL, apparently at the surfaces of cells or in the extracellular spaces (Fig. 8A). 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. 8B). This pattern of labelling, together with the in situ hybridization data (Fig. 6G, H and Fig. 12A), 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.

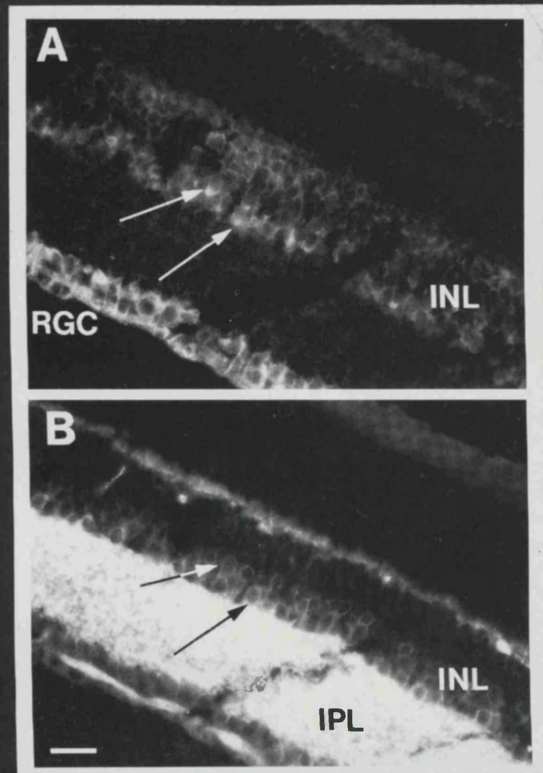


Figure 8. Double-label immunohistochemistry of P15 mouse retina, with anti-PDGF-A and monoclonal antibody HPC-1, a surface marker of amacrine neurons. Anti-PDGF-A stains the retinal ganglion cell (RGC) layer and the inner nuclear layer (INL) (panel A). Antibody HPC-1 stains the processes of amacrine 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. 6A-D), the level of expression declining gradually after birth.

B. 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. 9A-H). The intense dark-field signal in the body of the postnatal lens (Fig. 9D-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. 9A). Between E14 and the day of birth PDGF-αR expression in this region intensifies and expands to fill the optic nerve head (Fig. 9B-D), then gradually spreads across the inner surface of the retina (Fig. 9D, 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. 10C, D) or adjacent (Fig. 10A, 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. 9G, H) into adulthood (not shown). I 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

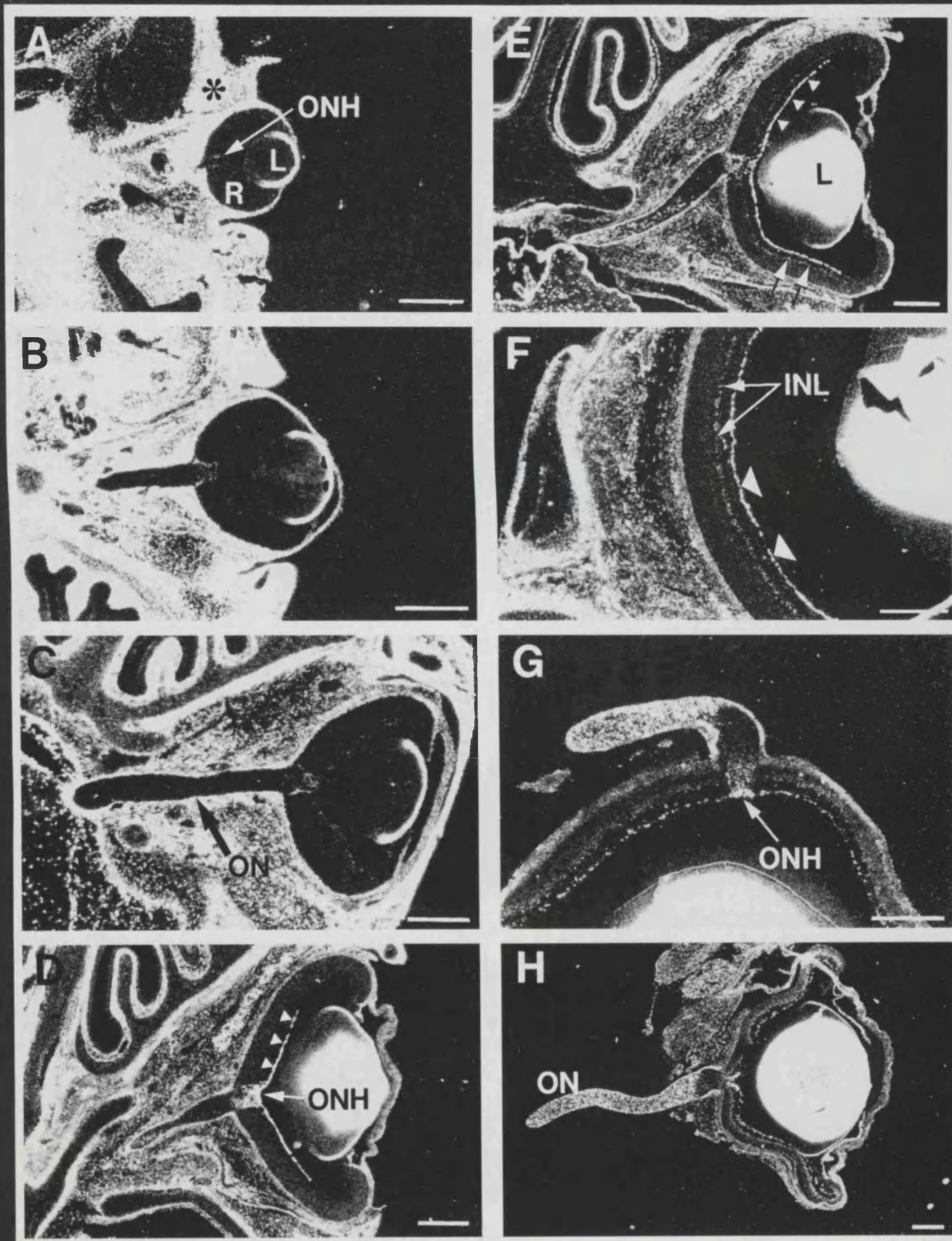


Figure 9. Distribution of PDGF-αR transcripts in the developing rat retina (R), optic nerve (ON) and lens (L). Horizontal sections were cut through the optic nerves and eyes of rats at E14 (A), E16 (B), E18 (C), P0 (D), P2 (E), P5 (F), P10 (G) and P20 (H); these were subjected to in situ hybridization 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.

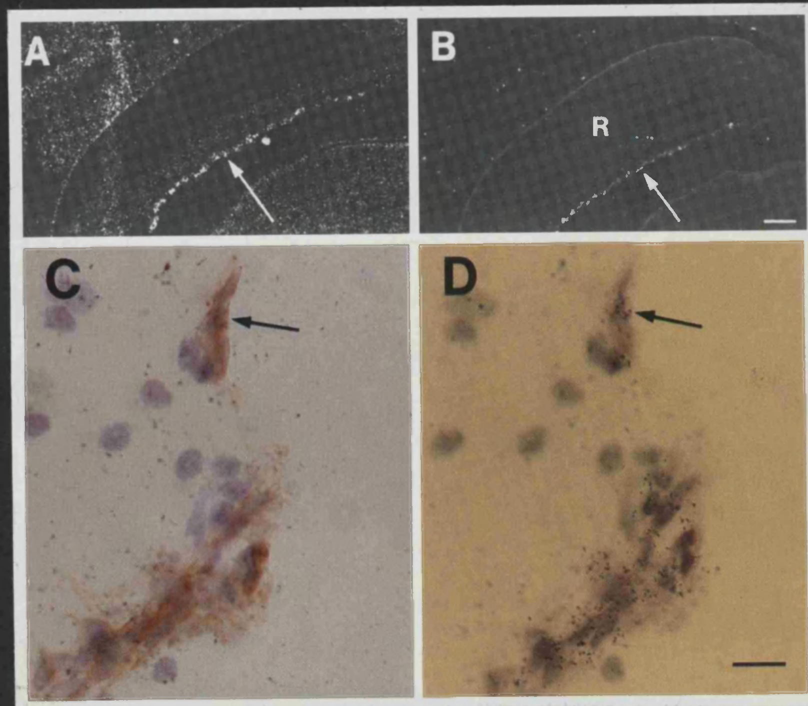


Figure 10. PDGF-αR and GFAP expression in the rat retina. Consecutive sections through a P9 retina (R) were subjected to in situ hybridization with the PDGF-αR probe (panel A), and immunohistochemistry with an antibody against GFAP (panel B). The anti-GFAP antibody was detected with a biotinylated second 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 (panels C, D). The GFAP antibody was visualized with a biotinylated second antibody followed by horseradish peroxidase (HRP)-conjugated streptavidin and diaminobenzidine (Vectastain kit). The sections were photographed at high magnification under bright field illumination, in two planes of focus (C, D) to optimize detection of the HRP reaction product and the silver grains, respectively. In addition, the micrograph in panel D was taken through an optical filter to obscure the brown HRP product and accentuate the silver grains. 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 panel A obscures the PDGF-αR signal in the inner nuclear layer (see Fig. 6). Scale bars: panels A, B, 100 μm; panels C, D, 10 μm.

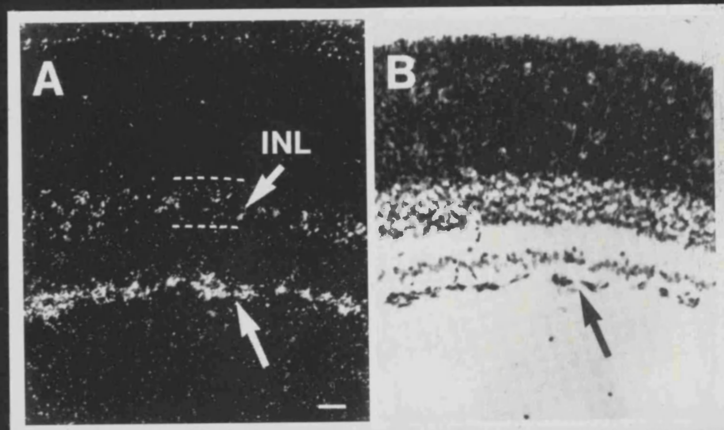
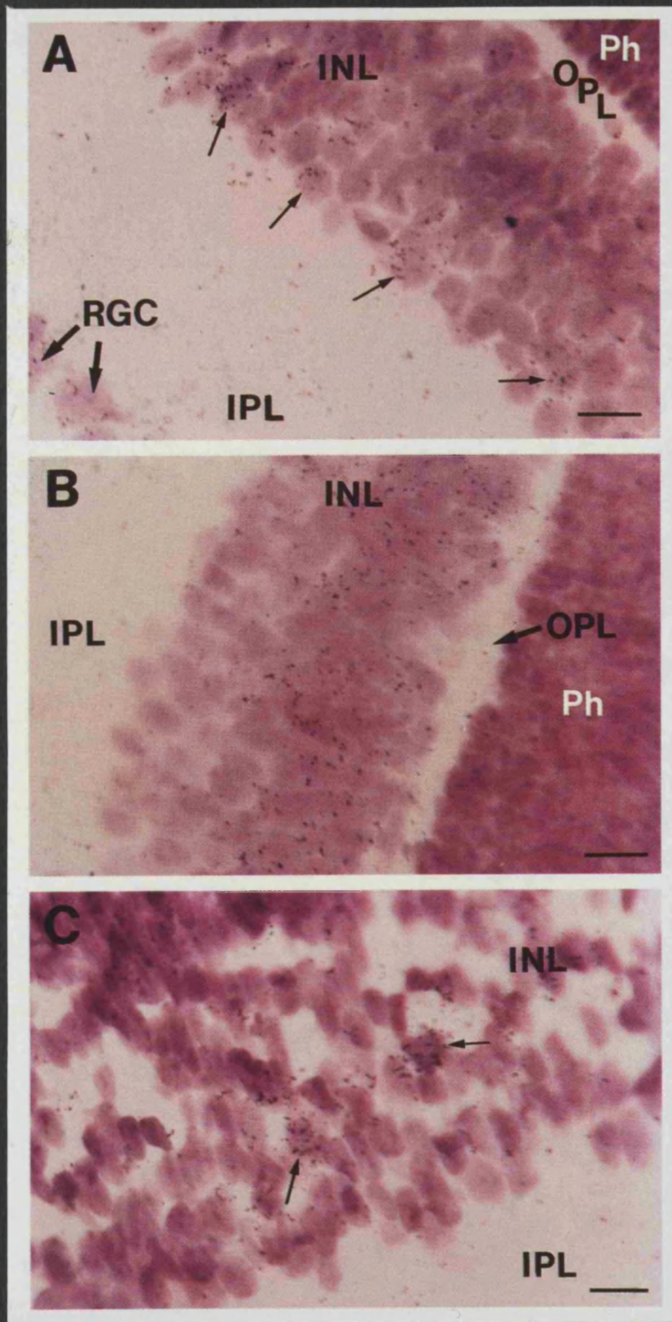


Figure 11. PDGF-αR transcripts in the P5 rat retina. PDGF-αR transcripts were visualized by in situ hybridization and photographed under dark field (A) and bright field (B) illumination. Silver grains are present over cells (astrocytes) in the optic fibre layer (lower arrows) and in the interior of the inner nuclear layer (INL). The dotted lines indicate the limits of the INL. Scale bar, 20 μm.

Figure 12. High magnification bright-field images of in situ autoradiographs with the PDGF-A and PDGF-aR 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. Panel A shows a section of a P15 rat retina (the same one illustrated in Fig. 6E), 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). Panel B shows a section of a P15 rat retina (the same section as in Fig. 9H) hybridized with the PDGF-αR antisense probe. Silver grains overly cells in the outer (choroidal) half of the INL. Panel C shows a section through a P5 rat retina (the same section as in Fig.11) 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. RGC, retinal ganglion cell layer; IPL, inner plexiform layer; INL inner nuclear layer; OPL, outer plexiform layer; Ph, photoreceptors. Scale bars: 20 μm.


A
INL
Ph
OPL
RGC
IPL
B
INL
IPL
OPL
Ph
C
INL
IPL

blood vessels (see Fig. 14): these structures do not label with the PDGF-αR in situ probe. Moreover, I did 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. 9E, 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 (Fig. 11 and Fig. 12C). At P15, the PDGF-αR signal is clearly restricted to the outer (choroidal) half of the INL (Fig.12B).

C. Astrocytes in the optic nerve express PDGF-A

We previously reported that PDGF-A mRNA (Pringle et al., 1989) and activity (Raff et al., 1988) is present in the postnatal rat optic nerve, and suggested 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. 6A). Between E16 and the day of birth (E21/P0) the PDGF-A signal increases (compare Fig. 6B with 6C). PDGF-A expression persists throughout postnatal development (Fig. 6D), but declines somewhat in the adult (not shown). Similar results were obtained in the mouse optic nerve (Fig. 7A, for example). To determine the distribution of PDGF-A protein in the optic nerve sections of P5 mouse optic nerve were stained with the anti-PDGF-A antibody. Intense labelling of many cell processes was observed (Fig. 7F). 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 in astrocytes, double immunofluorescence labelling experiments were performed 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. 7D) and GFAP (Fig. 7E). Since similar patterns of labelling were obtained when either anti-GFAP (not shown) or anti-PDGF-A (Fig. 7F) 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 strongly reduced when the antibody was preincubated with excess recombinant PDGF-AA (Fig. 7G) but was unaffected by preincubating with recombinant PDGF-BB or acidic or basic fibroblast growth factors (not shown). In addition, I have performed other controls that confirm the specificity of the anti-PDGF-A antibody (see Materials and methods). Therefore, it can be concluded 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.

D. 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. 9C). 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. 9D, 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. 9H. 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

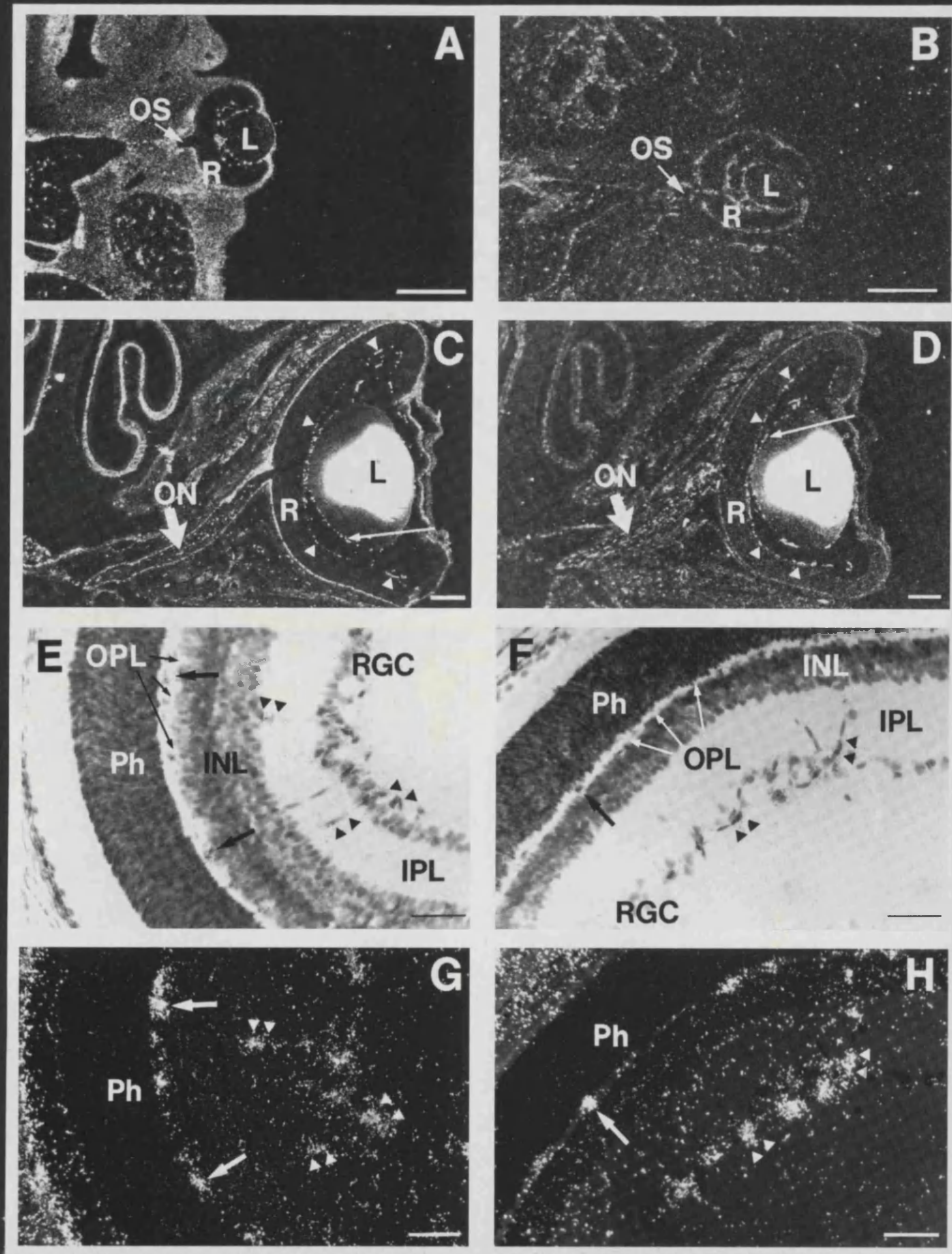
suggest previously that the PDGF-αR+ cells might correspond to oligodendrocyte precursors (Pringle et al., 1992). Oligodendrocyte precursors are thought to migrate into the developing optic nerve from germinal zones in the brain, via the optic chiasm (Small et al., 1987). The results presented here are consistent with that view.

E. PDGF-B and PDGF-ßR expression in the developing microvasculature of the retina and optic nerve.

The patterns of PDGF-B and PDGF-ßR 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-ßR 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. 13A, B). Cells in these locations continue to express PDGF-ßR and PDGF-B during postnatal development (Fig. 13C, D) and into adulthood (not shown). At P15 another zone of cells that express PDGF-ßR and PDGF-B is apparent in the retina, in the outer plexiform layer (OPL) (small arrows in Fig. 13E, G and 13F, H). At high magnification (Fig. 10), it is evident that the PDGF-ßR (Fig. 14A) and PDGF-B (Fig. 14B) signals are frequently associated with rows of cells that possess elongated nuclei. 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-ßR, suggesting that PDGF-B and PDGF-ßR may be expressed by different subsets of endothelial cells and/or other cells in the walls of blood vessels.

PDGF-B and PDGF-ßR transcripts are also present in groups of cells in the optic nerve. The distributions of cells expressing PDGF-B and PDGF-ßR 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. 14C). PDGF-ßR is most likely expressed by capillary endothelial cells in the optic nerve and

Figure 13. Distribution of PDGF-ßR 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-ßR or PDGF-B and photographed under dark-field or bright-field illumination. Panels A and B depict nearby (not adjacent) sections of the same animal, while panels C and D depict adjacent sections of the same animal. Panels E and G are corresponding bright-field and dark-field images of the same section through a P15 rat retina, hybridized with the PDGF-ßR antisense probe. Panels 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-ßR (panels A, C, E, G) and PDGF-B (panels 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-ßR and PDGF-B are also expressed in cells in the optic nerve (ON) (panels C,D); these cells sometimes appear to be arranged in parallel rows resembling blood vessels (see Fig. 14). L, lens; R, retina; OS, optic stalk; ON, optic nerve; RGC, retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer, Ph, photoreceptors. Scale bars: panels A-D, 500 µm; panels E-H, 50 µm.


A
OS
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IPL
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Ph
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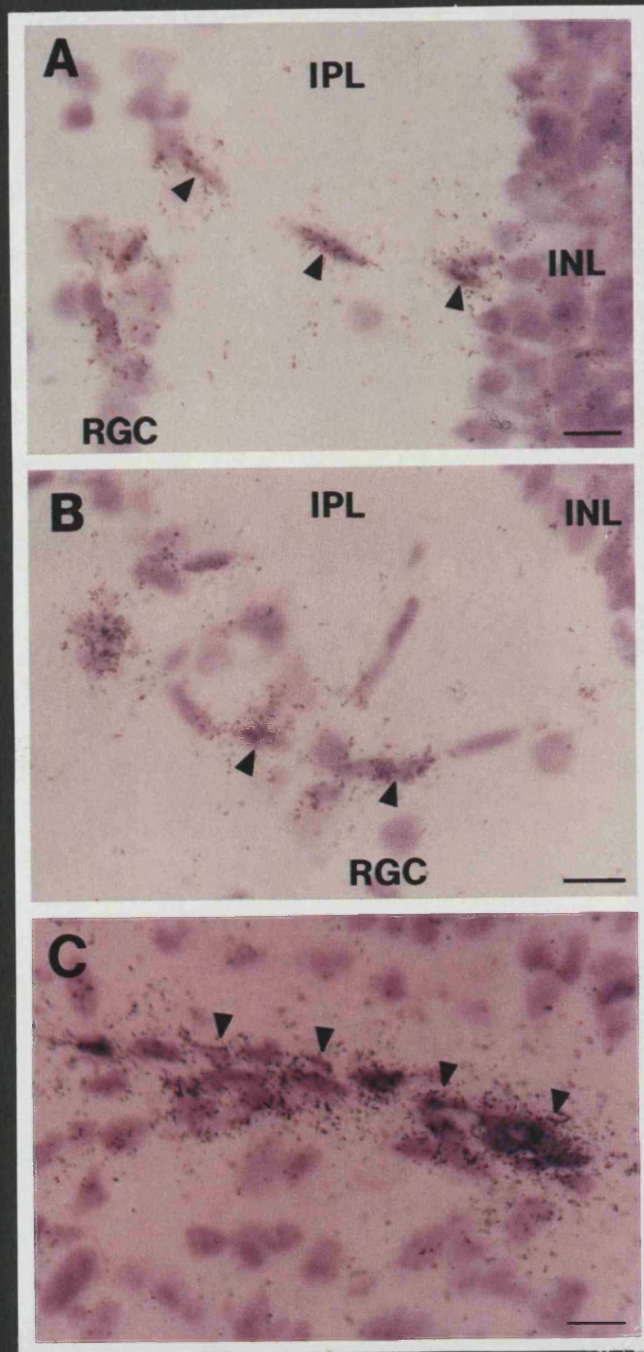
Figure 14. High magnification bright-field images of in situ autoradiographs with the PDGF-B and PDGF-ßR antisense probes on sections of P15 rat retina and optic nerve. The sections have been counterstained with haematoxylin, which visualizes the cell nuclei. Panel A shows a section through a P15 rat retina hybridized with the PDGF-ßR 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-ßR, however. This section is a higher magnification view of the one in Fig. 13F and H, which shows that cells expressing PDGF-ßR are also present in the outer plexiform layer. Panel B shows a section through a P15 rat retina hybridized with the PDGF-B antisense probe. As in panel 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-ßR-expressing cells, are probably associated with blood vessels. Panel C shows a section through a P2 rat optic nerve, hybridized with the PDGF-ßR antisense probe. As in the retina, silver grains often overly rows of cells with elongated nuclei that we interpret as blood vessels. RGC, retinal ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer. Scale bars: 10 μm.


A
IPL
INL
RGC
B
IPL
INL
RGC
C

retina, since purified endothelial cells from brain have been shown to express PDGF-ßR in vitro (Smits et al., 1989). PDGF-B might also be expressed in endothelial cells or in nearby cells such as pericytes.

3.3. Discussion

Rresults of an in situ hybridization study of PDGF and its receptors during development of the rodent retina and optic nerve have been presented. 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, I 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 retina is assembled as PDGF-AA or PDGF-BB homodimers. Likewise, cells in the retina appear to express either PDGF-αR or PDGF-ßR, but not both. In the optic nerve, because the patterns of PDGF-A and PDGF-B synthesis overlap, we cannot exclude the possibility that some cells in the nerve might synthesize PDGF-AB heterodimers. Similarly, some optic nerve cells may coexpress both receptor subunits. It seems likely from in vitro studies, however, that oligodendrocyte lineage cells in the nerve express PDGF-αR but not PDGF-ßR (Hart et al., 1989; McKinnon et al., 1990) (for a review of PDGF and its receptors, see Heldin and Westermark, 1990).

A. Neurons in the retina express PDGF-A

PDGF-A transcripts are found in the retinal ganglion cell layer after E18, and at the vitreal aspect of the INL from P10 onwards. The PDGF-A-expressing cells at these locations are retinal ganglion neurons and amacrine neurons, respectively. 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).

B. Retinal astrocytes and presumptive Müller glia 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).

Astrocytes in the retina occur only in the optic fibre layer, so the PDGF-αR+ cells that arise in the INL must be another cell type. The location of these PDGF-αR+ cells, in the interior of the INL, and the fact that not all cells in this region express PDGF-αR, suggests that they represent either bipolar neurons or Müller glia. I 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.

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

It was found that PDGF-A and its receptor PDGF-αR are expressed by separate but adjoining layers of cells in the retina (see Fig. 15). Retinal ganglion neurons (RGCs) and amacrine neurons express PDGF-A, while cells in adjacent layers express PDGF-αR. This arrangement strongly suggests that PDGF-A and PDGF-αR mediate local, paracrine interactions between cell bodies in the retina. This may be a general property of this ligand-receptor pair, because juxtaposed layers of cells that express PDGF-A or PDGF-αR 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. 16 for a summary of our expression data). 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

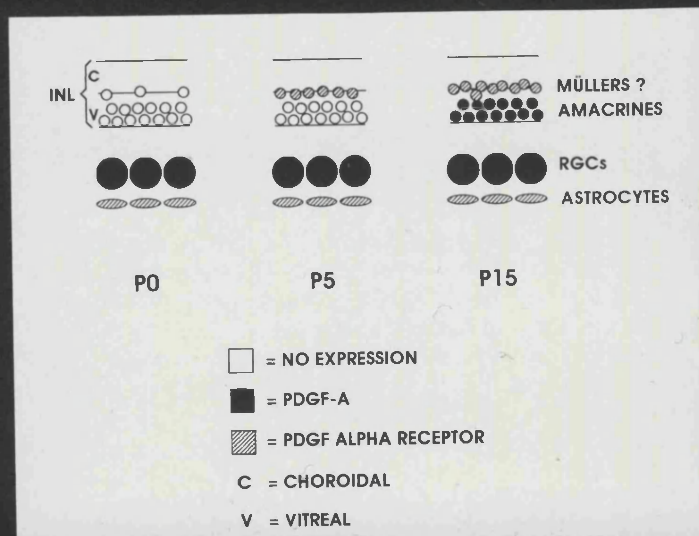


Figure 15. PDGF-αR and PDGF-A transcripts in the neural retina during development. INL, inner nuclear layer; RGCs, retinal ganglion cells. PDGF-αR and PDGF-A are expressed in distinct but neighbouring populations of cells, suggesting local paracrine interactions between neurons (RGCs, amacrines) and glial cells (astrocytes, putative Müller glia) in the retina.

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. 16).

D. 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. 16). 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). The idea that light-induced neuronal activity might regulate expression of PDGF-A in amacrine neurons can be tested experimentally.

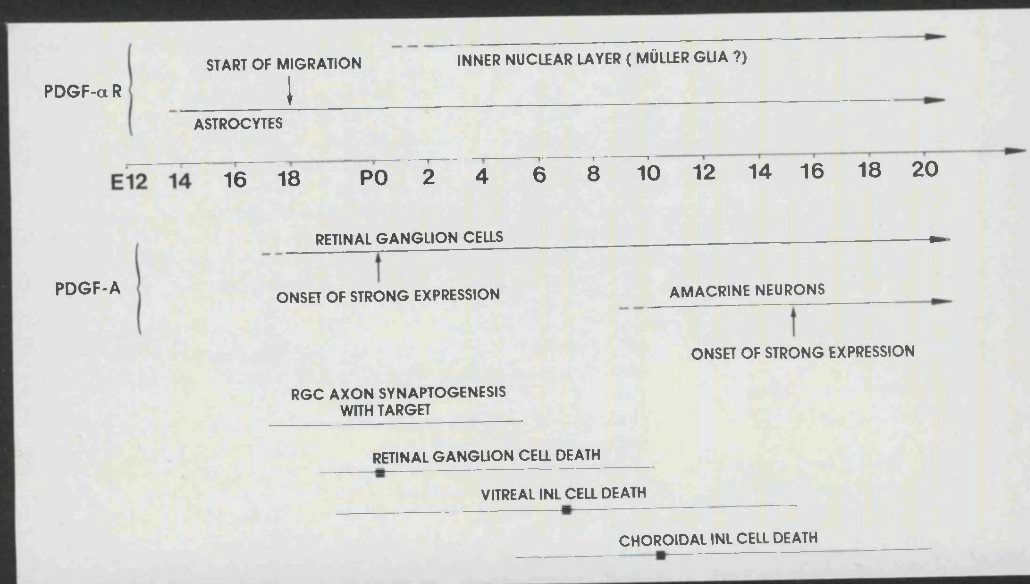


Figure 16. The dynamics of PDGF-αR and PDGF-A expression in the developing retina compared to some other key developmental events. Dotted lines represent uncertainty about the precise time of onset of gene expression. Black boxes on lines indicate the peaks of cell death in the corresponding cell layers. The lower part of the Figure is adapted from Beazley et al. (1987).

E. PDGF-A and PDGF-αR in the optic nerve

In the optic nerve, PDGF-A is expressed by cells in the meninges and throughout the interior of the nerve. Previous circumstantial evidence showed 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. I found a very close correspondence between the PDGF-A and GFAP signals, suggesting that the majority of PDGF-A in the optic nerve may be astrocyte-derived. I might have expected to see evidence of PDGF-A in the RGC axons, since axons in the optic fibre layer in the retina appear to contain PDGF-A (Figs 4B, 8A). It is possible that RGC-derived PDGF is only transported a short distance into the axons and does not reach the optic nerve. Alternatively, its concentration in optic nerve axons may be below the limit of detection with our antibody.

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., 1988; Noble et al., 1988; Pringle et al., 1989) and survival (Barres et al., 1992b). 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, is consistent with this idea (see Fig. 9C, E, H).

Since the primary function of oligodendrocytes is to interact with and myelinate axons, it would make sense if the axons themselves were a source of mitogens and/or survival factors for oligodendrocytes and their progenitors. Indeed, there is evidence that axons are required for survival and proliferation of the oligodendrocyte lineage. Transecting the mouse or rat optic nerve after birth, which results in Wallerian degeneration of the axons, causes a marked reduction in the total number of oligodendrocyte lineage cells that develop in the nerve (Privat et al., 1981; David et al., 1984; Barres and Raff, 1993a)

largely due to a reduction in proliferation of oligodendrocyte progenitors (Barres and Raff, 1993a). Blocking electrical activity in optic nerve axons by injecting tetrodotoxin (TTX) into the eye also inhibits proliferation of oligodendrocyte progenitors (Barres and Raff, 1993a). The effect of TTX can be reversed by delivering PDGF from an exogeneous source (Barres and Raff, 1993a), suggesting that blocking electrical activity somehow interrupts the endogenous supply of PDGF. This could be a direct effect of preventing transfer of PDGF from the RGC cell body into the nerve, or an indirect effect on the release of PDGF from astrocytes in the nerve (Barres and Raff, 1993a). At face value, my failure to detect PDGF immunoreactivity in optic nerve axons tends to favour the latter possibility.

F. PDGF-B and PDGF-ßR in developing and mature blood vessels

In the eye, the distribution of PDGF-B mRNA is very similar to that of PDGF-ßR mRNA. Both are found in small groups of cells at the back of the lens, at the innermost face of the retina and at the boundary between the inner nuclear and outer plexiform layers. Groups of cells that express PDGF-B or PDGF-ßR are also scattered throughout the optic nerve, sometimes occurring in parallel "tramlines" suggestive of blood vessels. Blood vessels are known to be present at the sites of PDGF-B and PDGF-ßR expression in the retina (Engerman and Meyer, 1954). Capillary endothelial cells from brain have been shown to express PDGF-ßR in culture (Smits et al., 1989), and our in situ hybridization studies of the rat brain and spinal cord (H. Mudhar and W. Richardson, unpublished) strongly suggest that the same is true in vivo, so it seems reasonable to suppose that the PDGF-ßR+ 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-ßR (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.

CHAPTER FOUR

THE ROLE OF PLATELET-DERIVED GROWTH FACTOR IN RETINAL INNER NUCLEAR LAYER DEVELOPMENT.

In the previous chapter, the identities of some of the cells expressing PDGF and its receptors were described and possible functions postulated for PDGF. In this chapter, I show that PDGF may play a role as a survival factor during development of the INL.

4.1 Introduction

Naturally occurring cell death in the developing retina proceeds as a temporal and spatial wave, commencing in the RGC layer and proceeding outwards so that the photoreceptors are the last to undergo cell death (Young, 1985; Beazley et al., 1987). In the rat retina, naturally occurring cell death in the INL occurs over two phases: the first phase, between P3 and P7 occurs in the inner, or vitreal, aspect of the INL where the amacrine cells reside. The maximal number of pyknotic cells are observed at P7. Cell death in the outer, or choroidal, part of the INL (the region in which bipolar neurons, horizontal cells and Müller glial cells reside) is maximal at P10 . Several findings suggest that the cells of the INL do not depend on efferent targets for their survival. Transecting the optic nerve, which results in rapid degeneration of the retinal ganglion cells over a period of 24 hours, does not affect the time course or the extent of cell death in the INL (Beazley et al., 1987). Secondly, studies on the rd/rd mutant mouse, where the photoreceptors completely degenerate between the end of the first postnatal week and P20, hence reducing considerably the afferent input into the INL, show no effect on the number of cells in the INL (Young, 1986). It seems likely, therefore, that the INL cells depend on one another for their survival requirements.

One of the ideas raised in Chapter three was that PDGF-A may act as a survival factor for the PDGF-αR bearing cells in the postnatal retina. In this chapter I present evidence that this might indeed be the case.

4.2 RESULTS

A. Apoptotic cells in the Inner Nuclear Layer (INL).

In order to identify apoptotic cells in the INL, frozen sections of postnatal, perfusion-fixed retinae were stained with propidium iodide (PI), to label the nuclei and the sections observed in the fluorescence microscope. The inner nuclear layer contains three types of neuronal cells, (amacrine cells, bipolar cells and horizontal cells), a glial cell type (Müller cells) and endothelial cells. With the nuclear marker propidium iodide it was easy to see that the INL consisted of two zones, differing in cell diameter. The vitreal zone, containing predominantly amacrine cells, displayed larger cell bodies whereas the choroidal half consisted mainly of densely packed, smaller cell bodies. Müller cell bodies, which were comparable in diameter to the amacrine cells, were difficult to identify but were generally seen to reside at the border of the two zones. Capillaries were often caught in cross section, displaying a lumen, lined by 2-3 curved endothelial cell nuclei.

At P10 pyknotic nuclei were seen distributed mainly in the choroidal half of the INL as previously described in the rat (Beazely et al., 1987). Pyknotic nuclei were readily recognized by their distinctive appearance. Normal nuclei were generally oval in shape, and dull red when seen through a rhodamine filter of a fluorescence microscope. Pyknotic nuclei or their fragments were much smaller, usually very dense and spherical and much brighter in staining intensity than normal nuclei (17A). When sections were viewed using phase contrast optics, pyknotic nuclei or their fragments appeared darker than normal nuclei (Fig. 17B). In animals that had received an injection of BrdU two hours before fixation, double-staining with PI and anti-BrdU antibody revealed two populations of pyknotic cells; those that were BrdU negative and those that were BrdU positive. The only cell type that undergoes proliferation in the INL in the postnatal period are the endothelial cells of the microcapillary network, which develops during the first two postnatal weeks (Engerman and Meyer, 1954). It is likely therefore, that the BrdU-positive, pyknotic cells represent dying capillary endothelial cells (Fig 17C, D). Since the majority of BrdU-negative, pyknotic cells were located in the choroidal half of the INL, these cells probably represent either bipolar cells or Muller cells. We previously showed that PDGF−αR positive cells were present in the INL, appearing initially in the centre of the layer and, in time,

occupying the whole choroidal half. Careful observation of some of the in-situ hybridization autoradiographs that had been stained with haemotoxylin showed some examples of pyknotic, PDGF-αR alpha receptor positive cells that were centrally or choroidally situated in the INL (data not shown).

B. Effect of Delivering recombinant PDGF-AA into the eye.

Although the death of the INL cells seems not to depend on afferent input or efferent targets such as retinal ganglion cells (Beazely et al., 1987), it seems likely that the death of INL cells is a consequence of competition for limiting amounts of survival factor; PDGF-AA might be one of these survival factors. To test this idea, I attempted to increase the ambient concentration of PDGF-AA. The experimental design consisted of injecting recombinant PDGF-AA (PeProtech) into the eye of at least three P7 rat pups, and perfusion fixing at P10. Similar number of control animals were injected with the vehicle only, or left uninjected. All animals received an intraperitoneal injection of BrdU 2 hours before fixation. Subsequently, 8μm frozen sections were cut and stained with propidium iodide and anti-BrdU. Five non-consecutive sections were cut through the optic nerve head from at least three retinae, from three different animals per condition. The analysis was done on three separate occasions (Table 2).

In no case did PDGF-AA injections affect the number of BrdU positive cells in the INL, as compared to retinae that had been injected with PBS (Students t-test, $P>0.05$), (Fig. 18) nor did PDGF-AA affect the number of BrdU-positive, pyknotic nuclei (Students t-test, $P>0.05$) (Fig. 19). However, significant reductions in the number of BrdU-negative, pyknotic cells, did occur. In three separate experiments there were only 57%, 40% and 58% (average 51%) of the number of BrdU-negative pyknotic nuclei compared to controls, suggesting that increasing the concentration of PDGF-AA had prevented about half of the normal cell deaths in the INL (Fig. 20).

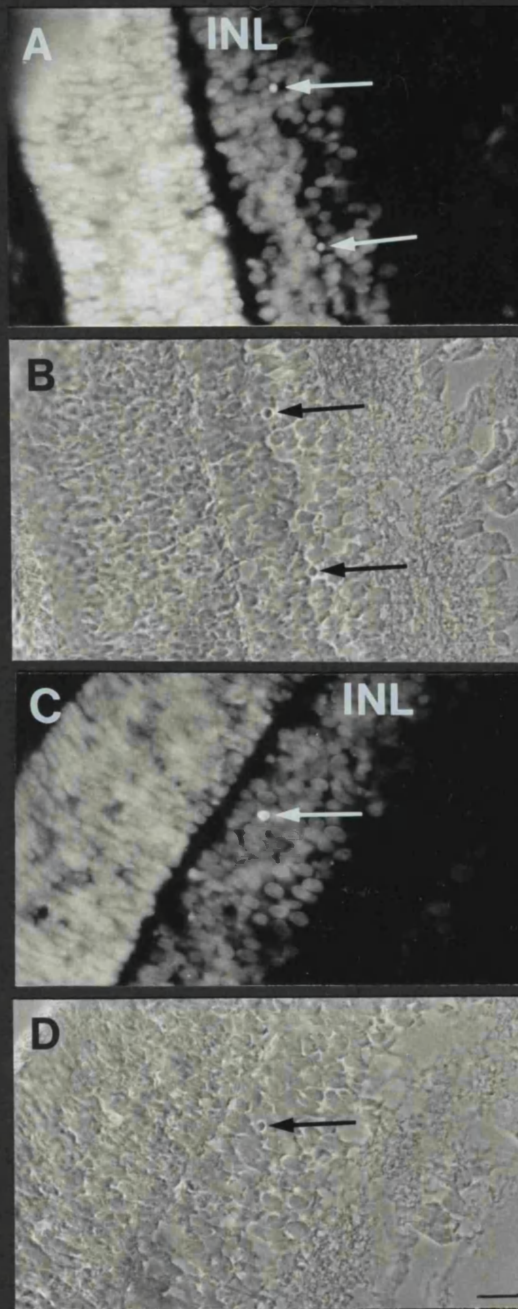


Figure 17. Distribution of pyknotic nuclei in the postnatal day 10 (P10) rat retina. Sections were labelled with propidium iodide (A) or double labelled with anti-BrdU and propidium iodide (C), and viewed under fluorescent light on a Zeiss microscope. The arrows in A show typical pyknotic nuclei in the midzone of the INL; B is the same section viewed in phase contrast, showing phase dark nuclei. C shows BrdU positive cells in the INL and D is the same section viewed under phase contrast optics showing this cell to be phase dark and therefore pyknotic. INL, inner nuclear layer. Scale bar, 50µm.

	Expt. No.	PDGF	PBS	Uninjected
	1	125 ± 26	130 ± 14	126 ±15
BrdU + cells	2	64 ± 7	59 ± 6	64 ± 10
	3	75 ± 3	80 ± 6	72 ± 2
	1	15 ± 3	18 ± 5	13 ± 4
BrdU+ pyknotic nuclei	2	9 ± 3	8 ± 2	10 ± 3
	3	11 ± 3	9 ± 2	13 ± 4
	1	14 ± 4	33 ± 6	35 ± 6
BrdU- pyknotic nuclei	2	16 ± 2	27 ± 3	30 ± 3
	3	14 ± 2	34 ± 6	41 ± 6

Table 2. Showing the number of BrdU+ and BrdU- pyknotic nuclei under the experimental conditions. Each value represents the mean of 15 sections, that is, 5 sections counted from each of three retinae from 3 separate rat pups per condition. Standard deviations have been rounded up to the nearest whole number. Expt. No.= Experiment number. PDGF= eyeballs injected with PDGF-AA; PBS= eyeballs injected with PBS, and uninjected. The experiment was repeated 3 times (1, 2, 3).

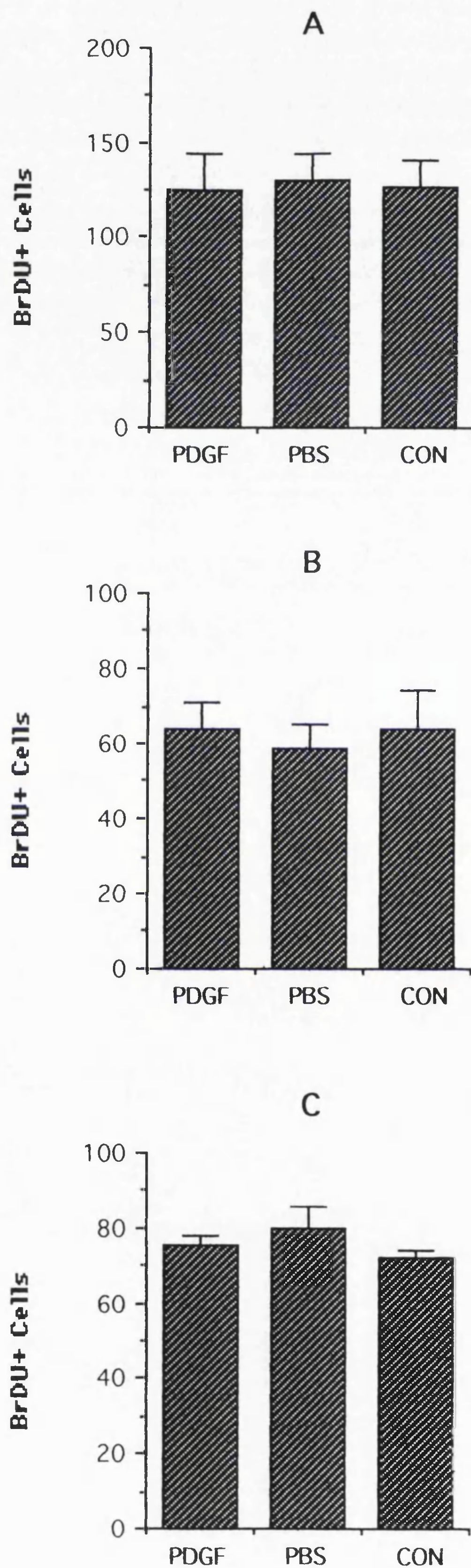


Figure 18. Number of BrdU positive cells in the INL of the rat retina at P10, of retinae sectioned from eyeballs under experimental and control conditions. A, B and C represent three separate experiments. Each bar represents the mean of 15 sections, that is, 5 sections from each of 3 retinae from 3 rat pups, per condition. PDGF- eyeballs injected with recombinant PDGF-AA; PBS- eyeballs injected with PBS; the third bar represents uninjected eyeballs.

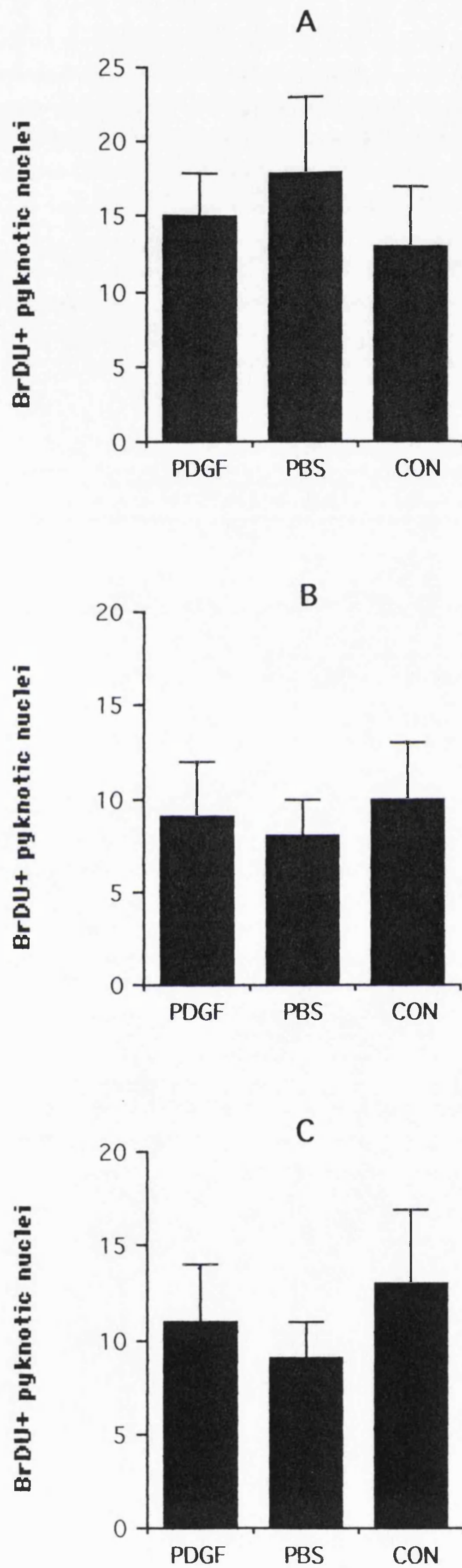
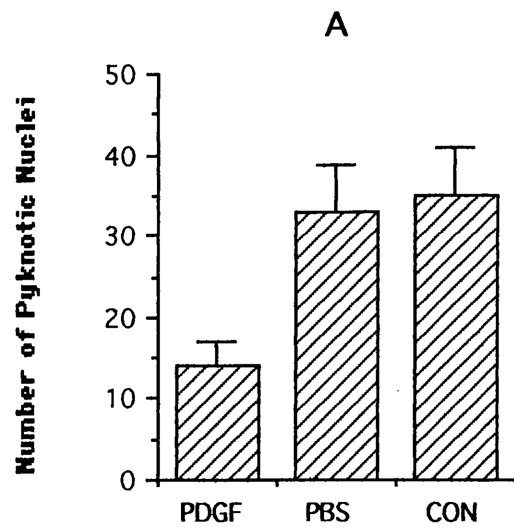
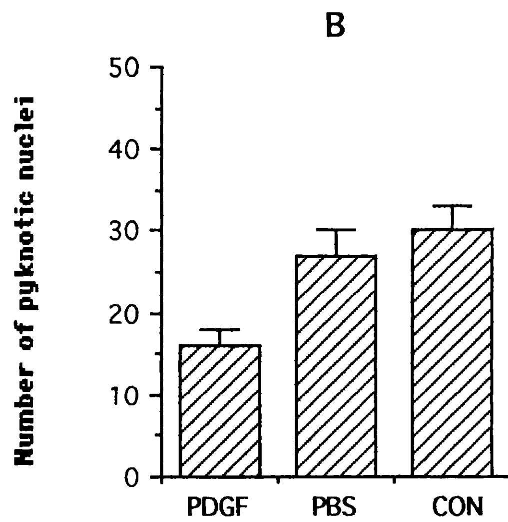


Figure 19. Number of BrdU positive, pyknotic nuclei in the INL at P10 in the rat retina, under experimental and control conditions. For details of abbreviations see legend for figure 18.





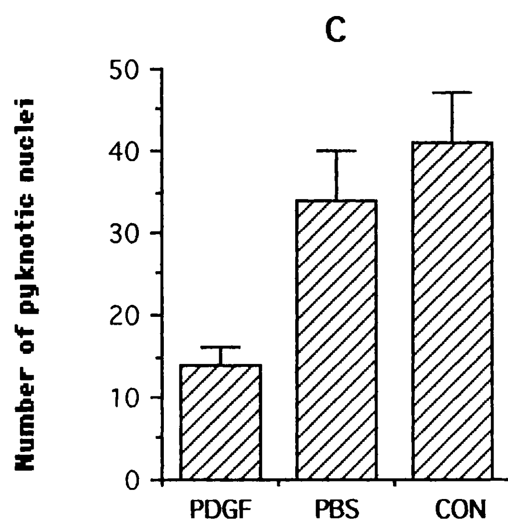


Figure 20. Number of pyknotic nuclei in the INL at P10 in the rat retina, under experimental and control conditions. For details of abbreviations see legend for figure 18.

4.3. Discussion

Neuronal cell death is a key event that takes place in the sculpting of the CNS. Many studies have shown, that this results at least in part from a competition for limiting amounts of neurotrophic factors that are secreted from the target cells they innervate (Barde, 1989; Cowan et al., 1984; Hamburger and Levi-Montalcini, 1949; Oppenheim, 1991; Purves, 1988). The same seems to hold true for glial cells in the developing rat optic nerve, where up to 50% of newly formed oligodendrocytes die as a consequence of competing for limiting amounts of survival factors, which appear to emanate from or to be controlled by the axons (Barres et al., 1992a; Barres et al., 1993c).

The postnatal rat retina is also subject to cell death, that passes as a wave from the RGC layer towards the ONL, such that the deaths in different layers are well separated in time and space (Beazley et al., 1987). In the case of the RGCs, for example, it is known that about 100 000 cells, which constitutes 50% of the total population, die, within the first postnatal week (Perry et al., 1983). This death, reflects a competition between the RGC growth cones, for limiting amounts of survival factors released from their targets in the superior colliculus. The INL cells also undergo massive death and it has been estimated that up to 66% of these cells die (Beazley et al., 1987). This population consists of amacrine cells, bipolar cells, horizontal cells and Müller cells; all of the processes of these cells are intrinsic to the retina, unlike the RGCs that have long projection axons and a distant target to innervate. Why should death occur in this population? Perhaps cell death is required to match the numbers of INL cells to each other in order to achieve and maintain an appropriate interconnected structure. I showed in Chapter 3 that these cells in the choroidal half of the INL express PDGF-αR, at a time when there is no cell proliferation among this cell population, suggesting that PDGF might perform some role other than as a mitogen for PDGF-αR-expressing cells. In this Chapter I have tested the notion that PDGF-AA functions as a survival factor for INL cells, by artificially increasing the amount of PDGF-AA in the developing eye. This treatment prevented about half of the normally occurring cell deaths in the choroidal half of the INL, supporting the idea that PDGF-AA normally acts as a survival factor for these cells and that a limiting amount of PDGF-AA is normally available to these cells during development. What is the identity of the PDGF-AA

responsive cell? The timing and spatial distribution of the cells that express PDGF-αR mRNA suggests that they might be Müller cells. However, definite identification of these cells requires further experimentation.

The second population of cells in the INL that were identified as pyknotic were cells in blood vessels. This population is proliferating, hence the appearance of BrDU+, pyknotic nuclei, which indicates that the cells die soon after S phase. Earlier studies have clearly shown that there is an overproduction of capillaries which, over the first two postnatal weeks, regress to form pericapillary spaces (Engerman and Meyer, 1954). Endothelial cells express PDGF-ßR receptor (Mudhar et al., 1993) and neighbouring endothelial cells express PDGF-BB. Since beta receptors bind PDGF-BB with high affinity but do not bind PDGF-AA, one would expect them not to be affected by the introduction of excess PDGF-AA. Indeed, this was the case as the final number of BrdU+ pyknotic nuclei were unaffected by exogenous PDGF-AA.

I have assumed that a reduction in the number of pyknotic nuclei in the INL is caused by the survival-enhancing effects of PDGF-AA. An alternative but, I think, unlikely explanation might be that PDGF-AA increases the clearance rate of dead cells. Another reservation about the conclusions reached here is that PDGF might be a survival factor when supplied exogenously, but might nonetheless not normally perform this role in vivo. Formal confirmation that endogenous PDGF-AA is a survival factor for INL cells would require a demonstration that inhibiting PDGF signalling in the intact, living retina resulted in an increase in cell death. Although not attempted in the series of experiments described here, we have a means of approaching this (see Chapter 5).

CHAPTER FIVE

PDGF IS REQUIRED FOR FORMATION OF THE RETINAL ASTROCYTE NETWORK.

5.1 INTRODUCTION

It was demonstrated in Chapter 3 that retinal astrocytes express PDGF-αR. This identification was made possible from three pieces of evidence: 1.The distribution of PDGF-αR correlated well with the distribution of GFAP immunoreactivity on consecutive retinal sections. 2. The progress of the PDGF-αR expression from the optic nerve head towards the periphery of the retina coincided with the rate of migration of astrocytes (Watanabe and Raff, 1988) 3. combination of GFAP immunohistochemistry and in-situ hybridization with a probe to PDGF-αR on the same retinal section revealed GFAP-positive cells with overlying silver grains (Mudhar et al., 1993).

Astrocytes migrate into the retina from the optic nerve (Ling and Stone, 1988; Watanabe and Raff, 1988) and their distribution appears to be strongly determined by the vasculature of the retina (Stone and Dreher, 1987). In the rat retina, on the day of birth, the astrocytes cover the area around the optic nerve head and slightly beyond. By the eighth postnatal day, they reach the peripheral edge of the retina, and from this point onwards cover the entire inner surface of the retina. As the astrocytes spread across the inner surface, they precede the formation of patent blood capillaries by a small but distinct margin, suggesting that the astrocytes might be responsible for inducing the immature blood vessels, which are already in place before the start of astrocyte migration, to differentiate with the formation of a lumen. The immature precursors of blood vessels along which the astrocytes presumably migrate, are cords of so-called spindle cells, which are mesenchymal in origin (Ling and Stone 1988; Chan-Ling and Stone, 1991).

Very little is known about the molecules that are responsible for setting up the astrocyte/capillary network. In order to address whether PDGF-A plays a role in their development, we have attempted to inhibit PDGF activity in the developing retina, by implanting Cos cells that have been transfected with an expression vector encoding a soluble, ligand-binding domain of the PDGF-αR that can sequester endogenous PDGF in the interstitial fluid.

5.2 RESULTS

The experimental design consisted of injecting one eye of each newborn rat pup with Cos cells that had been transfected with the soluble PDGF-αR construct. The pups were left to develop for five days and the retinae whole-mounted for GFAP immunohistochemistry. A similar number of control rats received injections of mock-transfected Cos cells, or no injections. At least five to six pups were used per condition and the experiment was repeated three times. The reason for selecting this number of pups per condition was that not all of the injections were accurate enough for the retina to be used for analysis. If after five days, there was blood in the vitreous, or the Cos cells had infiltrated the retina, or in fact, there were no Cos cells to be seen in the eyeball on dissecting it (caused by Cos cells leaking out along the needle tract at the start of the experiment), these retinae were not included in the experimental analysis. Only eyeballs, which were blood free and in which a distinct bolus of cells was observed at the back of the lens, not infiltrating the neural retina, were used for immunohistochemistry.

A. EXPRESSION OF THE EXTRACELLULAR DOMAIN OF PDGF-αR IN COS CELLS.

A full-length cDNA encoding rat PDGF-αR (Lee et al., 1990) was truncated at a *Not*I site just outside the membrane-spanning domain and subcloned into an expression vector containing the adenovirus major late promoter, herpesvirus thymidine kinase polyadenylation site and the simian virus 40 (SV40) replication origin. This vector can replicate autonomously in Cos cells under the influence of the endogenous SV40 large-T antigen. The vector also contains a Myc epitope tag, recognized by monoclonal antibody 9E10 (Evan et al., 1985), in-frame with the truncated PDGF-αR at its extreme carboxy-terminus. The final construct was named pRα17, and the encided polypeptide Rα17.

The pRα17 vector was tested form its ability to express truncated PDGF-αR in Cos cells. DNA was electroporated into Cos cells, which were fixed 72 h later and labelled with monoclonal antibody 9E10 or an anti-PDGF-

αR rabbit serum. Both antibodies labelled a proportion of the cells, giving intense intracellular labelling of the secretory apparatus (Fig. 23 A shows 9E10 labelled Cos cells, in vitro).

Cos cells transfected with the pRα17 were metabolically labelled with ^{35}S-amino acids and the cell lysate and supernatent were immunopreciptated with monoclonal 9E10, followed by polyacrylamide gel electrophoresis and autoradiography.The immunoprecipitates from both cell supernatent and cell lysate contained high-molecular-weight polypeptides that were absent from control immunoprecipitations (Fig. 21)

B. BIOLOGICAL ACTIVITY OF THE SOLUBLE EXTRACELLULAR DOMAIN OF PDGF-αR

To test the PDGF-binding ability of the secreted Rα17 polypeptide, pRα17 and a plasmid encoding PDGF-A (Pollock and Richardson, 1992) were electroporated separately into cultured Cos cells, which were subsequently incubated with ^{35}S-amino acids. The cell culture media were collected and co-incubated overnight at 4ºC to allow PDGF-AA and the truncated pRα17 receptor to come together. The supernatents were then immunoprecipitated with either an antiserum raised against PDGF-AA (Mercola et al., 1990; Wang et al., 1992) or an antiserum against the recombinant extracellular domain of human PDGF-αR (Fretto et al., 1993). A proportion of the PDGF-AA and the Rα17 truncated receptor co-precipitated in this experiment, regardless of which primary antiserum was used, demonstrating that the Rα17 polypeptide is capable of binding to PDGF-AA homodimers in solution (see Kruger et al., 1994).

To assess the ability of the Rα17 truncated receptor to inhibit the mitogenic effect of PDGF, NIH 3T3 cells were cultured in the presence of saturating amounts of PDGF-AA, PDGF-BB or PDGF-AB or basic fibroblast growth factor (bFGF) together with different concentrations of conditioned medium from Cos cells expressing pRα17. DNA synthetic activity was measured by ^{3}H-thymidine incorporation. DNA synthesis induced by all three

isoforms of PDGF was inhibited, in a dose-dependent manner, by Cos cell medium containing Rα17 but not by control cell medium (Fig. 22). The mitogenic effect of bFGF was unaffected by Rα17. These results are consistent with the known ligand-binding properties of intact PDGF-αR, which can bind, and be activated by, all three dimeric isoforms of PDGF (Heldin et al., 1988; Gronwald et al., 1988; Hart et al., 1988). The inhibitory effect of Rα17 on ^{3}H-thymidine incorporation could in turn be overcome by increasing the concentration of PDGF in the medium (see Kruger et al., 1994), demonstrating that the PDGF-neutralizing effect of Rα17 is saturable. Under the conditions of this experiment, Rα17-conditioned medium neutralized between 1 and 3ng/ml PDGF-AA. (The above work was carried out by W. Kruger).

C. NORMAL APPEARANCE OF ASTROCYTES IN RETINAL WHOLEMOUNTS AT POSTNATAL DAY 5.

Uninjected retinae presented a classic picture of astrocyte distribution at postnatal day 5. At this age, the astrocytes are approximately 2.1 mm from the optic nerve head and about 0.5 mm from the peripheral margin of the retina (Watanabe and Raff, 1988) (Fig. 24A). The processes of the astrocytes traced out the major blood vessels and the capillaries, forming a complex network, with many more astrocytes in the central region of the retina than at the periphery. Those astrocytes at the migration front differed slightly in morphology from the others in possessing long, tapering processes which often pointed out towards the retinal margin. The astrocyte somas were rather difficult to visualize, and in only a few cases could cytoplasmic GFAP filaments be seen to trace out the cell body. Careful observation also revealed a natural asymmetry in the distribution of astrocytes across the inner surface of the retina, in that in some areas, the astrocytes were less advanced in their migration than other regions; it was difficult to ascertain however, whether this asymmetry was fixed with regard to the nasal/temporal axis or whether it had no consistent orientation.

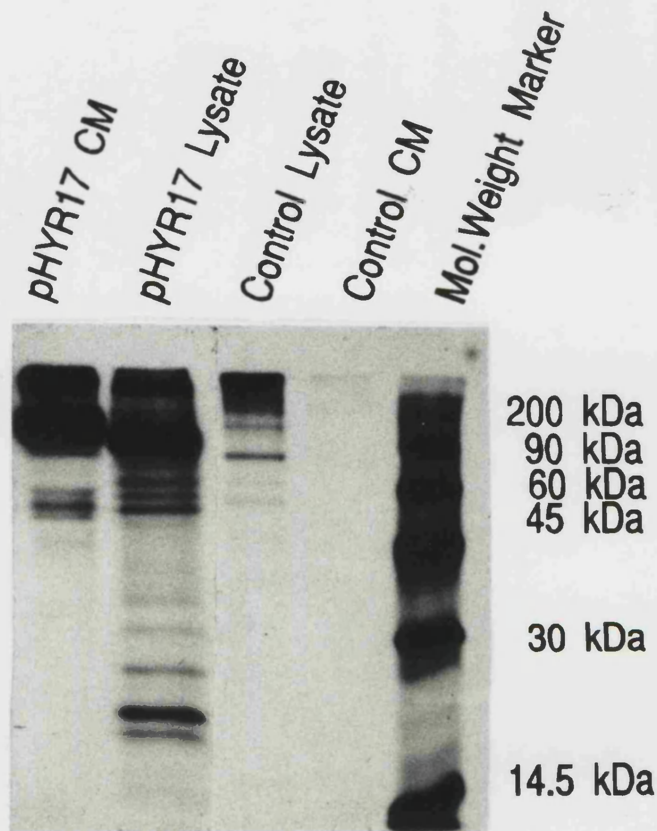


Figure 21. Immunoprecipitation of Rα17 truncated receptor. Cos cells were electroporated with pRα17 and metabolically labelled with a ^{35}S-methionine/cysteine mixture. Cell culture supernatents were collected and immunoprecipitated at 4°C with 9E10 (anti-c-Myc) antibody. The precipitates were run on a polyacrylamide gel and visualized by fluorography. Lane 1: Rα17-conditioned medium (CM) precipitated with anti-c-Myc. Lane 2: Rα17 Cos cell lysate precipitated with anti-c-Myc. Both lanes show the presence of high-molecular-weight polypeptides. The other two lanes represent lysate and CM from mock transfected Cos cells respectively, immunoprecipitated with anti-c-Myc, showing the specific high-molecular-weight polypeptides to be absent..

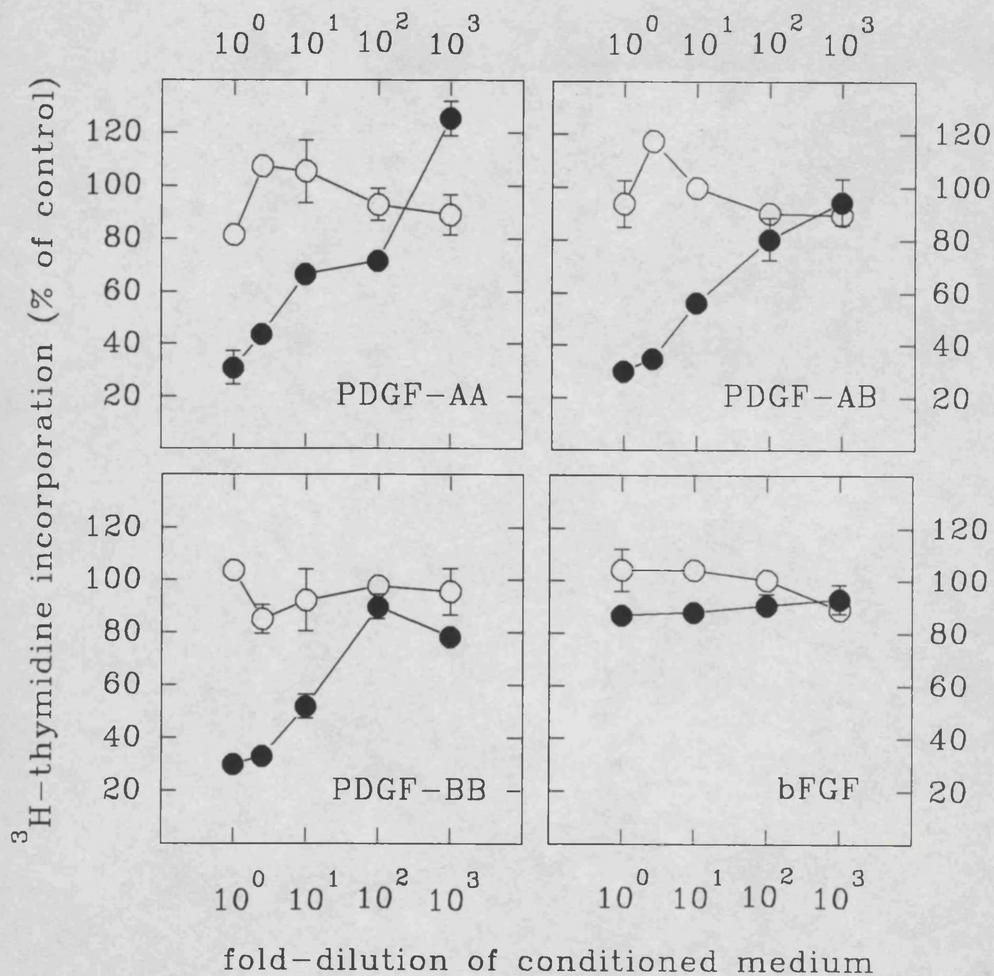


Figure 22. Neutralization of PDGF isoforms with Rα17 truncated receptor. Subconfluent cultures of NIH 3T3 cells were growth-arrested by serum deprivation. Purified growth factors were added to a fixed concentration, sufficient to stimulate half-maximal mitogenic response, together with different dilutions of conditioned medium from Cos cells that had ben transfected with plasmid pRα17 (filled symbols) or with the vector backbone alone (open symbols). After overnight incubation at 37ºC, ^{3}H-thymidine was added to the cultures for 4h before solubilizing the cells and determiningthe amount of TCA-precipitable radioactivity by scintillation counting. Assays were performed in triplicate. The results are expressed as a percentage of the incorporation obtained in response to the same concentration of growth factor alone. Conditioned medium containing R α17 truncated receptor was able to neutralize all three dimeric isoforms of PDGF, ut not bFGF, in a dose- dependent fashion.

D. NEUTRALIZING ENDOGENOUS PDGF IN THE RETINA ALTERS THE DISTRIBUTION OF RETINAL ASTROCYTES.

The injected Cos cells formed a large plug of cells covering the posterior pole of the lens after 5 days (Fig.23 B, C) . The mass of cells was not invasive, as determined by inspection so they did not alter the morphology of the developing retina.The Cos cell mass was usually associated with the vascular network that covers the back of the lens, and could easily be removed in one piece with a pair of forceps. Mock- transfected Cos cells had little effect on the distribution of retinal astrocytes. However, Cos cells transfected with the PDGF-αR17 exerted a local but striking effect on the distribution of astrocytes (Fig. 24B). This distribution differed in several ways from control retinae (Fig, 24A): The most striking feature was the reduced advancement of the astrocytes from the optic nerve head towards the periphery of the retina. This effect was presumabely restricted to the side where the Cos cell mass accumulated, presumably reflecting a local accumulation of R17 protein in the neighbourhood of the Cos cells.

In order to quantify this effect,the retinal whole-mounts were conceptually rotated so that the most affected sectors of each preparation were aligned, and each set of superimposed sectors analyzed separately. Using a graticule in one of the eye pieces of a Zeiss fluorescence microscope, each wholemount was measured in two ways: the distance from the centre of the optic nerve head to the edge of each sector and the distance from the optic nerve head to the furthermost GFAP-positive astrocyte in each sector. Cos cells did not inhibit overall growth of the eye.

Using this method, it was observed that uninjected retinae displayed a natural asymmetry of astrocyte distribution across the inner surface, but not as marked as in the cat retina (Stone et. al., 1987) (Fig. 25B, D). It was difficult to determine whether the asymmetry was in a particular plane ; marking one edge of the retina with a small cut to define orientation became problematic during the staining procedure, resulting in poor retinal integrity.

In the experimental groups, namely those injected with mock transfected and transfected Cos cells, several observations were made. First, mock- transfected Cos cells did exert a slight effect on the distribution of the astrocytes, in that those that were adjacent to the Cos cells appeared to be

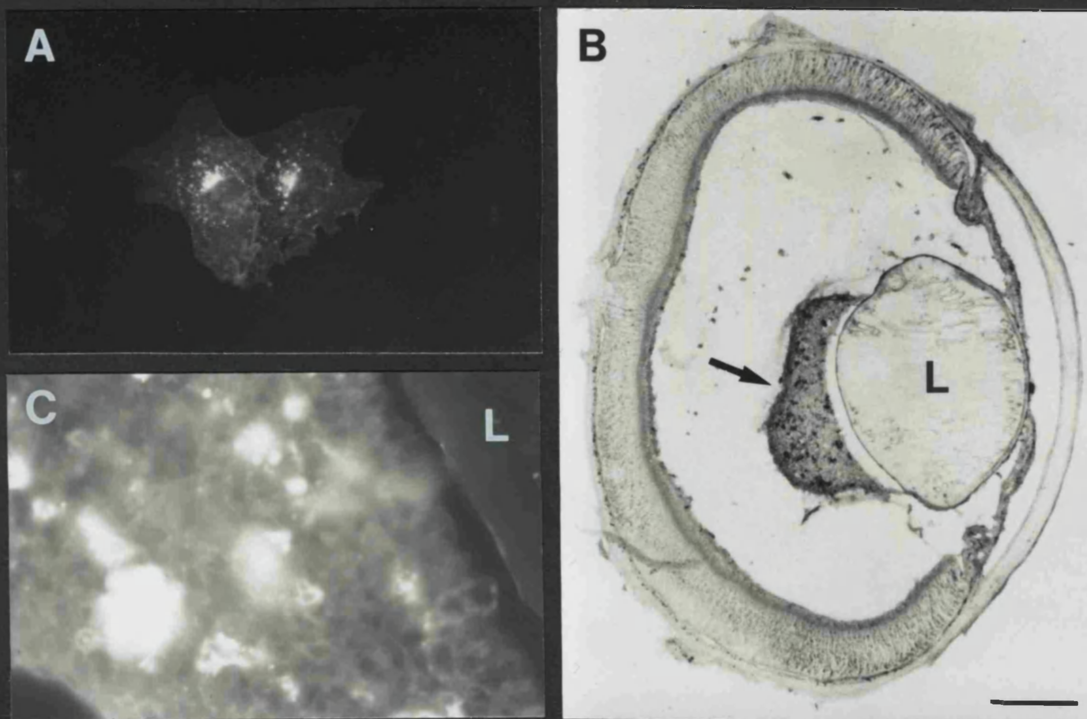
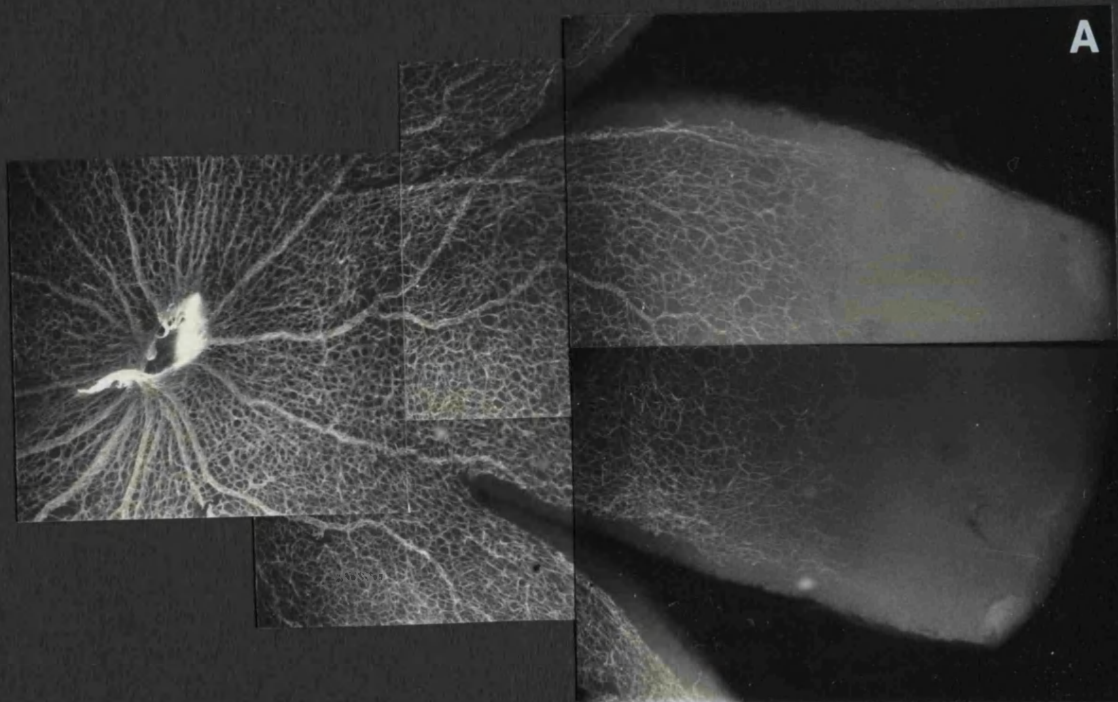


Figure 23. Expression of R α17 in Cos cells in vitro and in vivo. Panel A; immunofluorescence micrograph of a couple of Cos cells transfected with pR α17, labelled with monoclonal 9E10 and fluorescent secondary antibodies. Panel B; micrograph of a cryosection through an eye containing an implant of Cos cells that were transfected with pR α17. The cells were injected at P0 and the eye processed for micography on P5. The section was labelled with antibody 9E10 followed by immunoperoxidase detection system. Arrow points to the bolus of Cos cells adhering to the back of the lens (L). The dark granules within the Cos cell mass are R α17-expressing cells visualized by peroxidase reaction product. Panel C; high magnification micrograph of a Cos cell mass like that in B, labelled with antibody 9E10 followed by fluorescent secondary antibodies. Isolated cells or clusters of cells within the cell mass label strongly for R α17 truncated receptor. L=lens. Scale bar in B=500μm.

Figure 24. Composite immunofluorescence micrographs of whole-mount retinae, labelled with anti-GFAP and fluorescein-conjugated secondary antibodies to visualize the retinal astrocyte network. Panel A; part of a normal P5 retina. Panrl B; part of a retina injected at P0 with Cos cells expressing R α17 and processed for micography at P5. The extent of the astrocyte network, measured from the centre of the optic nerve head (point of confluence of the major vessels, towards the left of the Figure) to the tip of the furthest astrocyte process, is much reduced in B. Scale bar=250μm.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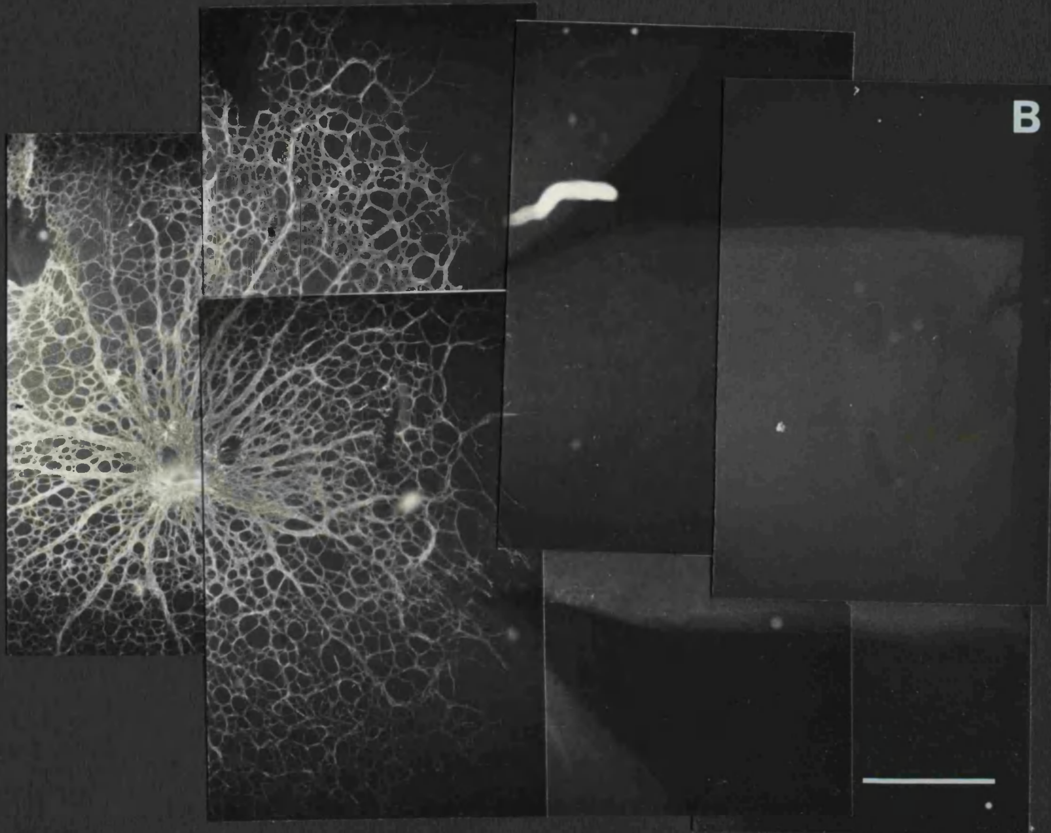
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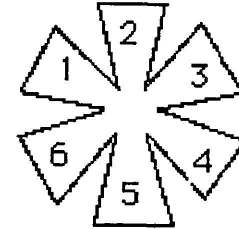


Transfected Cos cells

A

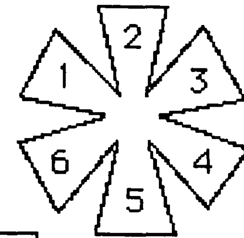
	Sector number						Length of Wholemount
	1	2	3	4	5	6	
1	12.5	11	12	15.5	15	16.5	24
	14	14.5	15	19.5	20	18	26
	13	12	17	21	22.5	20	26
2	13	12	17.5	21	22	18.5	25
	10	9	11.5	18	18	17.5	25
	15	13.5	18.5	20.5	21	15	26
3	11	10	13	10.5	19.5	20.5	24
	14	12	18.5	18	19.5	20	24
	16	15	18.5	20	22	22.5	26
Mean	13.1	12.1	15.7	18.2	19.9	18.6	25.5
S.D.	1.87	1.98	2.91	3.4	2.36	2.28	0.93

Cos control-contralateral eye

B

	Sector number						Length of Wholemount
	1	2	3	4	5	6	
1	19.5	18	20.5	19.5	20	18	25.5
	18.5	18	19	22.5	21	20	25
	20	19	19.5	21	22	21.5	26
2	19	19	20	23	23	20	26
	18.5	19	19	20	22.5	21	25.5
	19	18.5	19	20	22.5	19	25.5
3	19	20.5	20	21	23	22.5	26
	19.5	19.5	20	20.5	21	19	25
	21	20.5	20	20.5	22	22	26
Mean	19.3	19.1	19.7	20.9	21.9	20.3	25.4
S.D.	0.79	0.92	0.55	1.16	1.02	1.52	0.41

Table 3. Distance of astrocytes from optic nerve head measured in units, where 1 unit= 0.1 mm, at postnatal day 5 (P5), in the presence (A) and absence (B) of Cos cells transfected with pRalpha17. Each figure represents an individual wholemount from a rat pup and numbers 1,2 and 3 are three separate experiments with three pups per experiment. SD=standard deviation



Mock transfected Cos cells

C

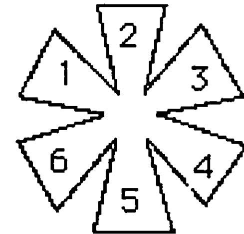
	Sector number 1	2	3	4	5	6	Length of Wholemount
1	18	16.5	16.5	20	21.5	20	26
	18.5	17	16	20.5	20	21.5	26
	18.5	16	17.5	18.5	19	17	24
2	19	18	18.5	20.5	21	21.5	25
	19	19	20.5	21.5	22	18.5	26
	16	17	19.5	21	20.5	19.5	24
3	18	19.5	20	21.5	20.5	21	24
	19.5	19	18.5	21	22	20.5	25
	18	17.5	17.5	19	20.5	18	25
Mean	18.2	17.6	18.2	20.3	20.7	19.6	25
S.D.	1.0	1.22	1.54	1.05	0.97	1.6	1.27

Mock control-contralateral eye

D

	Sector number 1	2	3	4	5	6	Length of Wholemount
1	18.5	19	21.5	21.5	21	20	26
	20	19	21.5	20	22.5	19.5	26
	21	19.5	19	22	23	21.5	26
2	18	19	21.5	22	22	20	25
	20.5	20	21.5	22	23	22	26
	19	19	20	21	22.5	20	26
3	20	19	19.5	20	21	19	25
	19	18.5	21	20	21.5	20	25
	18	18.5	19	20	21	20.5	26
Mean	19.3	19	20.4	21	21.9	20.3	25.6
S.D.	1.08	0.46	1.11	0.95	0.84	0.94	0.5

Table 4. Distance of astrocytes from optic nerve head measured in units, where 1 unit= 0.1 mm, at postnatal day 5 (P5), in the presence (C) and absence (D) of mock transfected cos cells. Each figure represents an individual wholemount from a rat pup and numbers 1,2 and 3 are three separate experiments with three pups per experiment. SD=standard deviation.

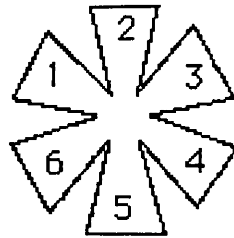


	Sector number					
	1	2	3	4	5	6
1	8 7.5 7.5	7 7 8	7.5 7.5 8	8 8.5 7	8.5 8 8	8 8 7.5
2	8 7.5 8	7.5 8 8	7 8.5 7.5	7 8 8.5	7 8 7	7 7 7.5
3	7 8 8.5	7 7.5 8	8 8 7.5	7 8 7.5	7 8 7	7.5 7 7.5

Table 5. Distance of astrocytes from the centre of the optic nerve head in each sector at P0. 1unit=0.1 mm.Each number is an individual wholemount.
Numbers 1,2 and 3 represent three separate experiments, each one done in triplicate. The mean distance from the optic nerve head=8 units s.d. 0.125

		Sector Number					
		1	2	3	4	5	6
A	Transfeted Cos cells	5.1	4.1	7.7	10.2	11.9	11.6
B	Transfected Cos cell control-contralateral eye	11.3	11.1	11.7	12.9	13.9	12.3
C	Mock transfected Cos cells	10.2	9.6	10.2	12.3	12.7	11.6
D	Mock transfected Cos cell control-contralateral eye	11.2	11	12.4	13.4	13.9	12.3

Table 6. Mean distances travelled by astrocytes in the 5 day experimental period in the presence of pRalpha 17transfected cos cells (A) and mock transfected cos cells (C). The distances, in units, where 1 unit=0.1 mm, have been calculated by subtracting 8 units, the mean distance of the astrocytes from the optic nerve head at P0, from the mean values in tables 3 and 4



A

B

15 10 5 0 5 10 15

15 10 5 0 5 10 15

15 10 5 0 5 10 15

15 10 5 0 5 10 15

C

D

Figure 25.Graphical representation of the astrocyte migration data (Table 6) for retinae exposed to Cos cells expressing Ralpha17(A), their contralateral uninjected controls (B), retinae exposed to mock-transfected Cos cells (C) and their contralateral controls (D).In each sector, the symbol represents the mean and standard deviation, of the furthest distance migrated within that sector by an astrocyte during the course of the experiment. 1 unit=0.1mm.

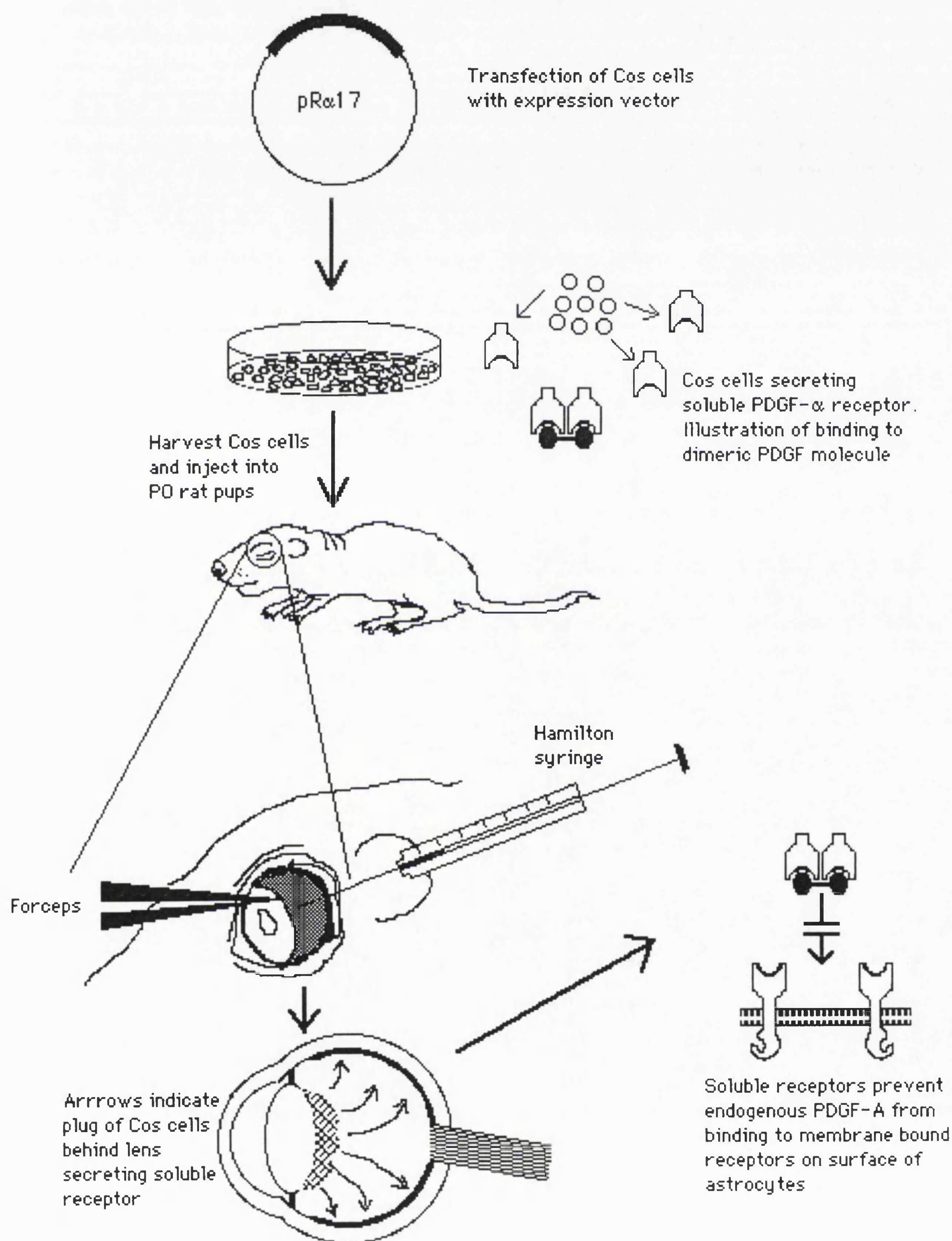


Figure 26. Experimental set up to study the effect of Ralpha 17 soluble alpha receptor upon the development of rat retinal astrocytes.

slightly less advanced across the optic fibre layer, than those in the uninjected controls (Table 4; Fig 25C, D). However, this effect was very small compared to retinae that received an injection of PDGF-αR17-transfected Cos cells. On average, the side that was most affected by the Rα17 protein domain had advanced only about one half, of the distance of the astrocytes on the opposite side of the same retina, or in the contralateral, uninjected retina (Table 3 A, B). The effect extended to 2-3 sectors on the affected side, although there was some variation from experiment to experiment in the extent of the effect (Table 3A).

There were other ways that distribution of astrocytes was altered by R α17. First the spaces between the astrocyte processes were enlarged (Fig.24B) and second, some of the astrocyte processes had undergone fasciculation to form bundles that radiated out from the optic nerve head (Fig.24B). Fasciculation was variable from retina to retina and was not always observed.

The data shown in tables 3 and 4 are absolute values that do not take into account the extent of astrocyte migration at P0, before the introduction of the Cos cells. Table 6 shows the same experimental datas shown in Tables 3 and 4 corrected for astrocyte migration prior to the start of the experiment. I found that, on average, the astrocytes were 0.8mm from the optic nerve head in P0 retinae and the asymmetry observed at P5 was not apparent. This corrected data shows that astrocytes migrate 0.4-0.7 mm in the presence of Cos cells secreting PDGF-αR17, compared to 1.1 mm in the absence of Rα17. The PDGF-αR17 therefore exerts a 2 to 3-fold retarding effect on the migration of the astrocytes as compared to controls, and causes them to fasciculate. Figure 25 shows a graphical representation of the data in table 6.

5.3. DISCUSSION

Using a soluble, extracellular, ligand-binding fragment of rat PDGF-αR to sequester endogenous PDGF in the developing retina, I have found evidence that PDGF is important for the proper formation of the retinal astrocyte/capillary network. The truncated PDGF-αR was delivered into the vitreous of the eye by implanting Cos cells that were transfected with an

expression vector encoding the reagent. The Cos cells remained viable because of the suitable environment provided by the vitreous and proliferated over the 5 day duration of the experiment.

To my knowledge, this is the first time a truncated growth factor receptor of this type has been successfully delivered in vivo. This approach might be of general use; other truncated receptors analogous to our PDGF-αR17 have been shown to bind their cognate ligand(s) and , in some cases, efficiently sequester its activity in vitro, for example, PDGF-ßR (Duan et al., 1990). The truncated PDGF-αR inhibited the migration of retinal astrocytes from their generative zone in the optic nerve across the surface of the developing rat retina. I have previously shown that migrating retinal astrocytes express mRNA encoding PDGF-αR (Mudhar et al., 1993 and Chapter 3) and that the optic fibre layer over which they migrate is immunoreactive for PDGF-A. I also showed that cells in immature blood vessels in the optic fibre layer and elsewhere express mRNA encoding PDGF-B and PDGF-ßR. These observations suggested that the PDGF signalling pathway might be important for astrocyte migration across the optic fibre layer. The results from these sets of studies support this idea, although they do not distinguish between a direct or an indirect effect of PDGF on astrocyte migration. Retinal astrocytes are thought to migrate along cords of spindle cells, the precursors of blood vessels, that are laid down in the developing retina prior to astrocyte migration (Shakib et al., 1968; Stone and Dreher, 1987; Chan-Ling and Stone, 1991). The spindle cells become capillaries as the astrocytes migrate out over the inner surface of the retina. PDGF might act through PDGF-αR on astrocytes to stimulate astrocyte motility directly, or might act indirectly through PDGF-ßR on spindle cells to enhance their property as a substrate for astrocyte migration. Since the spindle cells are in place prior to astrocyte migration, the experimental regimen might interfere with postnatal development of the prevasculature, thus affecting the astrocytes.

PDGF might also enhance the survival of astrocytes, act as a mitogen for astrocyte precursors or for astrocytes directly. The latter I think is unlikely since preliminary BrdU incorporation experiments showed that astrocytes were not actively engaged in DNA synthesis in normal retinae as

well as in those that had been injected with Cos cells (data not shown). This suggests that astrocytes do not proliferate as they migrate. Further studies with the truncated PDGF-αR and specific blocking agents to PDGF and its receptors are required to clarify these issues.

CHAPTER SIX

6.1 GENERAL DISCUSSION

Growth factors play key roles during animal development. The evidence for this assertion is of three types. Many studies have documented the expression of growth factors and their receptors during development, suggesting that these factors might fulfil some function(s) during development.The second line of evidence comes from in vitro studies which attempt to recreate in culture a faithful representation of the cellular context in vivo; often, purified growth factors are found to have a profound effect on 'development' in vitro. The third type of evidence is from analysis of animal mutants with lesions in genes encoding growth factors and their receptors, which often display developmental defects. This latter type of evidence provides the only formal proof of the involvement of a growth factor in some aspect of development. In the absence of naturally-occurring or manufactured mutants, however, some other approach must be taken to interfere with the production of the growth factor in question, and assess its role in development.

Many roles have been proposed for PDGF and some of these have been tested in vitro, but few in vivo experiments have been done to establish a role for PDGF during development. The work described in this Thesis was carried out with the intention of determining the functional role(s) of PDGF in the development of the retina, a relatively simple region of the CNS. I mapped in detail the sources and targets for PDGF in the retina throughout development and in the adult, used this information to formulate ideas about possible functions of PDGF, and tested some of these ideas by artificially increasing or decreasing ambient levels of PDGF in the eye during the postnatal period of retinal development. I found evidence that PDGF acts as a survival factor for cells in the INL, which so far remain unidentified but which may well be Muller glia, and in addition I found evidence that PDGF is an important factor for the development of the retinal astrocyte network.

A. Distribution of PDGF and its receptors.

A direct immunohistochemical and in-situ hybridization approach was taken to localize PDGF and its receptors in the developing retina and optic nerve. In-situ hybridization is a very useful technique for visualising cells that express mRNA encoding a particular growth factor.Generally, it should always be done in parallel with antibody staining as it is often difficult to

precisely identify the cell type that expresses the mRNA species of interest, especially if one is employing radioactive probes, because of the relatively low resolution of the in situ technique and the poor preservation of the cellular detail because of the harsh tissue treatments employed.Therefore, I used a polyclonal antiserum to mouse PDGF-A (Mercola et al., 1990; Wang et al., 1992), to identify the retinal ganglion cells and a small subset of amacrine neurons as the cells that express PDGF-A chain mRNA and protein. Unfortunately, the same strategy could not be applied to the cells expressing PDGFα-receptor, as a good anti-PDGF-αR antibody was not available. A combined in-situ, antibody-labelling protocol was employed to identify GFAP-positive retinal astrocytes as expressing PDGF-α receptor. In situ hybridization showed that a second cell type, possibly Muller glia, in the interior of the INL also expresses PDGF-αR mRNA during development, but this second cell type could not be unambiguously identified.

An improved method of visualizing these cells and identifying these latter cells would have been to adopt a non-radioactive in-situ approach. For example digoxygenin labelled probes , which give very good cellular localization, could have been used in combination with cell-type- specific antibodies and might have allowed the identification of the INL cell type expressing PDGF-αR. However, it remains a fact that very few studies have successfully combined in situ hybridization and immunocytochemistry on the same tissue slices (Watts and Swanson,1989).

B. Significance of retinal neurons expressing PDGF

Previous studies using in situ hybridization and immunohistochemistry, together with expression studies in transgenic mice, have shown that CNS neurons express PDGF (Sasahara et al., 1991; Yeh et al., 1991). RGCs and a subset of amacrine cells express PDGF during times when paracrine interactions may be taking place between the various layers of the retina or in the optic nerve (Mudhar et al., 1993).

There are two questions that are raised by my retinal study: what regulates neuronal PDGF expression and what is the role of neuron-derived PDGF ? The RGCs and amacrine cells have been in existence for several days before they begin to express detectable levels of PDGF mRNA and protein and

they up-regulate the expression some time later. The expression of the PDGF-A gene could be controlled either at the transcriptional level or post-transcriptionally, for example at the level of mRNA stability. It is possible that the PDGF-expressing neurons are intrinsically programmed to express the gene at the appropriate point in time or, alternatively, external factors in their environment might be responsible for inducing expression. It is known that, at the time when up-regulation of PDGF-A is occurring in RGCs , their growth cones are encountering and making contacts with secondary neurons in the superior colliculus (Lund and Bunt, 1976), the primary site of termination of the RGC axons in the rat. Thus it is possible that target-derived signals might influence PDGF-A expression in the RGCs. What form might these putative signals take? In the case of RGCs, there are two mechanisms that can be easily envisaged. The first is the onset of electrical activity; it is known that electrical activity can alter the expression of mRNA encoding the neurotrophins (Zaffra et al., 1990; Zaffra et al., 1991; Lu et al., 1991) and also of FGF (Riva et al., 1992), from studies employing injections of excitotoxins into rat brains. It seems reasonable to suggest that this might also be the case for PDGF-A. Alternatively, molecular signals from the target, retrogradely transported to the RGC cell bodies, could be responsible for the induction of PDGF-A expression and its subsequent up-regulation and maintenance. Evidence for this mode of regulation comes from studies of the innervation of sweat glands, where a diffusible factor, probably related to LIF, is known to influence the neurotransmitter phenotype of the sympathetic neurons (reviewed in Landis, 1990). In addition, there is evidence that target-derived NGF can up-regulate substance P expression in sensory neurons in vitro (Lindsay and Harmar, 1989). Further studies using the retino-tectal system might begin to shed further light on this problem.

The other neuronal population to express PDGF-A is a small subset of amacrine cells in the INL. Previous studies of these cells have focused exclusively on neurotransmitters and neuropeptide expression and function (reviewed in Morgan, 1993). Amacrine cells constitute a diverse chemical and neuroanatomical cell type and most recent estimates suggest up to 50 different chemical types in the vertebrate retina. Why should amacrine cells express PDGF-A at the time that they do? Amacrines begin to express PDGF-A at P-10 but up-regulate its expression about four days later, around the

time of eye opening. This event is important for the anatomical and synaptic maturation of the INL, which involves the formation of several types of synapse between bipolars, amacrines and RGCs (Weidman and Kuwabara, 1968) and it is towards the end of the second postnatal week that the pupillary constriction reflex peaks (Ratto et al., 1991; Radel et al., 1992). Electrical activity generated as a consequence of eye opening could up-regulate PDGF-A in amacrines although, since expression commences just before eye opening, it is perhaps more likely that post-synaptic targets release diffusible factors that induce, up-regulate and maintain amacrine PDGF-A expression in the amacrine neurons that innervate them.

C. Roles for Neuron-derived PDGF

In the retina, we have found that PDGF-A and its receptor PDGF-αR are expressed by juxtaposed layers; neurons express PDGF-A and glial cells express the receptor. This in itself strongly suggests that paracrine (or 'juxtacrine') interactions are occurring between cells in adjacent layers during postnatal retinal development (Mudhar et al., 1993; Orr-Urtreger and Lonai, 1992). Fig 26 which illustrates three possible sites at which PDGF-A could act, and the functional significance of these local interactions. PDGF-A released from the RGC somas might interact with PDGF-αR on retinal astrocytes and/or their precursors, resulting in their proliferation , migration or long term survival. There is, however, one piece of evidence that suggests otherwise; transecting the optic nerve of neonatal rats in such a way that the blood supply is left intact, but resulting in death of all of the RGCs in the space of 24 hours, does not affect the number or distribution of the astrocytes (Baker and Perry, 1985). While this suggests that RGC-derived PDGF-A is unimportant for the development of the astrocyte network, it remains a possibility that PDGF-A, synthesized and deposited in the extracellular matrix before death of the RGCs, is sufficient to fuel normal development in this experimental system.

Our previous studies (Pringle et al., 1992; Mudhar at al., 1993) show there to be PDGF-αR expressing cells in the developing rat optic nerve; these almost certainly represent oligodendrocyte precursor cells. Could axon-derived PDGF-A be responsible for the migration of these cells into the optic nerve, and/or a mitogen or survival factor for these cells or their newly-formed

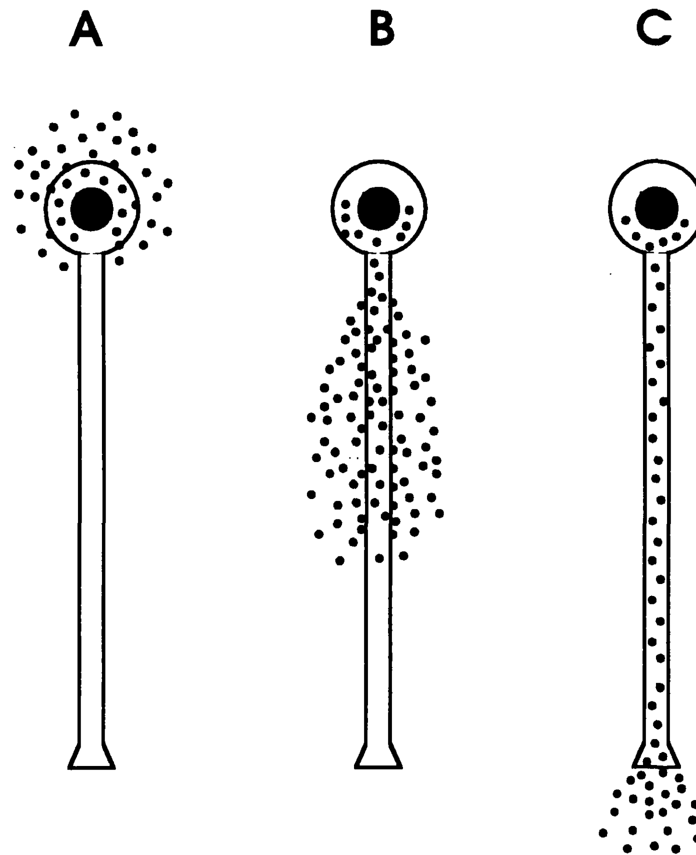


Figure 27. Some scenarios regarding the synthesis and release of PDGF from neurons. In A, PDGF is released from the soma and is acting locally in the retina . In B PDGF may be anterogradely transported down the axon and exit into the optic nerve. C PDGF may be anterogradely transported along the entire length of the axon and be released at the target. It is possible that in reality, combinations of all three scenarios operate. ▪ = PDGF molecules

progeny? There are two possible sources of PDGF-A in the optic nerve, the RGC axons or the astrocytes which I have shown to contain PDGF-A protein in the work presented here. Since we were unable to detect visible levels of PDGF-A protein associated with the axons in the optic nerve and since astrocytes expressed PDGF-A protein strongly, it is likely that astrocytes are the source of PDGF-A for the oligodendrocyte precursors (Richardson et al., 1988; Noble et al., 1988). This does not exclude axons playing a role in optic nerve development however. It is now known that proliferation of oligodendrocyte precursors in the optic nerve depends on electrical activity in axons, and that this activity-dependence can be circumvented by experimentally increasing the concentration of PDGF in the nerve. Injecting Tetrodotoxin (TTX, a sodium channel blocker), into the eyes of rats results in an 80% reduction in the number of BrdU positive oligodendrocyte-lineage cells, but does not alter the proliferation of other optic nerve cells (Barres and Raff, 1993). Since axons have not as yet been shown to secrete signalling molecules but astrocytes have, a distinct possibility is that electrical activity in axons might regulate the release of PDGF from astrocytes, which in turn might affect the oligodendrocyte precursors.

The third model depicted in Fig27suggests that RGC-derived PDGF has no role to play in the retina or optic nerve but is required at the target. Previous studies have shown that enucleation in frogs and mice profoundly inhibits gliogenesis in the optic tectum (De Long and Sidman, 1962; Currie and Cowan, 1972). Moreover, inhibiting anterograde transport in RGC axons by injecting colchicine into the eyes of rats quickly results in death of cells in the superior colliculus (Catiscas et al., 1992). Since PDGF is a mitogen for oligodendrocyte precursors, it is possible that it is released from the terminal boutons , under the influence of synaptic activity, and is required by the cells in the target for survival and proliferation. Indeed, supporting evidence from in-situ hybridisation studies show there to be PDGF-αR positive cells in the superior colliculus which could, therefore, respond to terminal bouton- derived PDGF from RGCs (Richardson et al., 1988; Pringle et al., 1992).

D. Significance of different isoforms of PDGF in paracrine interactions

The mRNA for the PDGF A chain generates two transcripts; a long version and a short version, which differ by the presence or absence of a single short exon (Tong et al., 1987; Rorsman et al., 1988; Bonthron et al., 1988). The alternative splicing generates two polypeptides that differ in length by 15 amino acids, with the longer form (PDGF-A_L) possessing a highly basic carboxy-terminal that retains this form of PDGF at the cell surface after secretion, most likely associated with heparin-like components of the extracellular matrix. The short form, (PDGF-A_S) is secreted into the extracellular medium as a freely diffusible molecule (LaRochelle et al., 1991; Pollock and Richardson, 1992). The fact that two isoforms exist, each with different spatial distributions outside the cell, has implications for the cell-cell interactions taking place in the retina. Our in-situ hybridization probes were unable to distinguish between the two forms of PDGF-A, although the antibody staining did show two types of distribution in the retina; expression by RGCs was localized to the RGC cell bodies and associated processes. In contrast staining was observed, not only in amacrine neuron cell bodies, but also diffusely throughout the INL, around most cells. Could RGCs be making PDGF-A_L and amacrine cells PDGF-A_S ? It is possible that in situ hybridization with an oligonucleotide probe directed against the alternative exon might resolve this question. At any rate, it is clear that various mechanisms including alternative splicing (Flanagan et al., 1991), post-translational modification (LaRochelle et al., 1991) and affinity for proteoglycans (Klagsbrun and Baird, 1991; Massague, 1991), can influence the extracellular distribution of a growth factor and hence its ability to interact with other nearby cells. The appositional pattern of ligand and receptor expression described above is a common feature in the PDGF/PDGF-receptor system as well as in other ligand/receptor systems. Studies of early mouse embryos has shown PDGF to be expressed by epithelial sheets and the receptor in mesenchyme, suggesting key epithelial-mesenchyme interactions (Orr-Urtreger and Lonai, 1992) which are responsible for sculpting many organs. Similar transcriptional localizations have been reported for the PDGF-B/ß receptor system in development of the human placental circulation (Holmgren et al., 1991). PDGF-B was expressed in cells situated in the central part of the blood vessel endothelium, whereas PDGF-ßR was expressed by

fibroblast-like and smooth muscle cells surrounding the blood vessels. This distribution feature is also characteristic for other members of the PDGF-receptor sub-group of receptor tyrosine kinases (Ullrich and Schlessinger, 1990). For instance c-Kit/W and its ligand SCF/Steel are expressed in many sites in the mouse (Orr-Urtreger et al., 1990). Comparisons of ligand and receptor revealed that in several organs including visceral endoderm, primitive ectoderm, adult gonads, olfactory bulb, cerebral cortex, hippocampus and cerebellum, c-Kit and SCF are transcribed in separate but adjacent sheets of cells (Motro et al., 1991; Keshet et al., 1991). Thirdly, CSF-1, which is a macrophage-granulocyte growth factor , is present in uterine epithelium while its receptor, c-Fms, is expressed in the decidua and then in the spongiotrophoblast, suggesting foeto-maternal interactions (Regenstreif and Rossant, 1989).

E. Significance of appositional ligand/receptor expression in the retina: testing the hypothesis.

Since the retina is accessible and subject to easy experimental manipulation, I designed experiments to test ideas about the function of PDGF-A in retinal development and the significance of the appositional arrangement of ligand and receptor referred to above.Two experiments were conducted. One involved the introduction of exogenous PDGF into the retina and the other involved introducing genetically engineered soluble PDGF-α receptors, to compete with and inhibit the interactions of endogenous PDGF with normal PDGF-αR.

Exogenous PDGF has been introduced into living tissues before. For example, introducing PDGF into the skin by injection stimulates connective tissue proliferation (Grotendorst et al., 1985; Sprugel et al., 1987) and, when perfused into minimally injured blood vessels, the smooth muscle cells migrate and proliferate just as they would if disturbed by injury (Jawien et al., 1992). Another way of introducing exogenous PDGF into a system is to implant cells that express PDGF into the area of interest. This approach has been used in studies that have addressed the roles of PDGF, CNTF and IGF-1 in the developing optic nerve (Barres et al., 1992a, 1993b, 1993c). By injecting Cos cells that had been transfected with a PDGF plasmid expression vector into

the subarachnoid space, Barres et al., showed that it decreased the naturally occurring cell death that takes place in the newly formed oligodendrocyte population by up to 90%, and thereby doubled the number of oligodendrocytes in 4 days (Barres et al., 1992a). In further studies it was shown, using the same experimental Cos cell design,that CNTF and IGF-I prevented the death of mature oligodendrocytes in transected optic nerves (Barres et al., 1993c).

The approach taken in this thesis was to inject recombinant PDGF-AA into the vitreous of the developing rat eye, in order to analyze its effect on cell survival in the INL of the developing rat retina. I have shown that by so doing, the number of pyknotic cells in this layer is reduced by 50% over a period of 3 days. Furthermore the effect was specific as the proliferating endothelial cell population, which express PDGF-ß receptors, was not affected by the exogenous PDGF-AA. It was not possible to identify which cells were being saved by the treatment, although it is a strong possibility that they are Muller glia. Injections of growth factors is becoming a popular way of addressing their roles in retinal development and in pathological studies (Faktorovich et al., 1990; Lewis et al., 1992; LaVail et al., 1992). One criticism that can be levelled at this kind of approach is that, it does not demonstrate that PDGF normally performs a function in vivo. What is required, therefore, is some way of neutralizing endogenous PDGF in the living animal in order to analyze the extent of its contribution to a particular process in vivo.

Neutralizing antibodies to NGF have been successfully used to dissect its role in the development of the sensory and sympathetic neurons (Cohen, 1960; Levi-Montalcini and Booker, 1960; Rohrer et al., 1988). Recently, a neutralizing monoclonal antibody to NT-3 has been used to investigate its role in the development of the rat optic nerve (Barres et al., 1994). This showed that NT-3 is a major mitogen for oligodendrocyte precursors, as treatment with this antibody resulted in smaller optic nerves and a large decrease in the proliferation of these precursors. Since PDGF is poorly antigenic, it has been difficult to produce effective neutralising monoclonals, although there has been some success using neutralising antisera. For example, injecting anti-PDGF antiserum in the region of an injured carotid artery, reduces the migration of vascular smooth muscle cells, suggesting and confirming original suspicions that PDGF is an important component of the proliferative response to injury (Ferns et al., 1991).

A second way of neutralising endogenous PDGF is to use truncated

forms of the PDGF receptors. A soluble, extracellular region of the PDGF ß-receptor has been generated and shown to antagonize PDGF-stimulated responses in vitro (Duan et al., 1991). We generated a soluble PDGF-α receptor (Krüeger et al., 1994 submitted), which antagonizes the actions of PDGF in vitro and, as shown in this thesis, in vivo also. In using such a soluble extracellular receptor domain (XR fragment) one assumes that it has the same affinity for PDGF as does the normal, transmembrane form of receptor. To prove this, it is important to do several tests before proceeding to use the construct in vivo. The XR fragment must be shown to bind the ligand, which should ideally be shown on a gel and an apparent dissociation constant calculated. Secondly, in the case of PDGF, a biological assay is required, to show that the XR fragment antagonises the effects of exogenously added PDGF. These criteria were fulfilled by the Rα17 fragment and so it was then used in the in vivo assay (Krueger et al., 1994 submitted). To our knowledge, this is the first time that an XR fragment has been delivered in this way to antagonize the effect of a growth factor in vivo. The retina is ideal for this kind of study since the XR fragments are enclosed in a relatively small volume, such that a high concentration of the fragment can accumulate in the vitreous. This delivery system therefore gets around the problem of repeated injections of antibodies which would very likely damage the delicate retina. We found that the results of the astrocyte assay raised several interesting questions. The astrocytes have two potential sources of PDGF; the RGCs which make PDGF-A and the endothelial cells which make PDGF-B (Both A and B forms of PDGF are capable of binding to the PDGF-αR). It is known that if the optic nerve is transected, which causes death of the RGCs, the astrocyte pattern is not affected (Baker and Perry, 1985; Chan-Ling and Stone 1991). This implies therefore that the astrocytes derive their PDGF from an alternative source, perhaps the endothelial cells. The interpretation of the lesioning experiment must be cautious, as it is quite possible that there may be sufficient levels of PDGF-A laid down in the extracellular matrix, to sustain the development of the astrocytes even after the RGCs die, thereby implicating RGCs in a possible paracrine interaction. However, since the XR fragment binds both PDGF-A and PDGF-B, it is not possible to say from which source the PDGF is coming. Perhaps astrocytes require PDGF-A and PDGF-B at different stages of their development. Another possibility is that

PDGF acts primarily on blood vessels and that astrocytes follow blood vessels, with which they will form tight associations. What can be concluded is that the astrocytes require PDGF for their development, as antagonizing endogenous PDGF results in a decrease in their migration across the inner surface of the retina .

F. Future investigations

Clearly the investigations conducted in this thesis have raised many questions that need further work to be answered. One thing that is required is the definitive identification of the INL cell that expresses the PDGF-αR, with the use of a suitable anti-PDGF-αR antibody. Given the identification of this cell type, 'panning' experiments would need to be done to isolate them, possibly with the use of the same anti-receptor antibody, in order to assay the cells' survival requirements in vitro. The key experiment is to neutralize endogenous PDGF, using the approach that has been used in the studies conducted here. Time constraints did not allow this obvious study to be conducted, namely, using the XR fragment to study the role of PDGF in cell survival in the INL.

To test the idea that electrical activity might be responsible for PDGF expression by amacrine cells and RGCs, simple studies such as raising rat pups in the dark, or preventing normal activity with TTX may be conducted. Such dark- rearing experiments were attempted but the results were not definitive and could not be repeated due to time constraints. Similar dark-rearing studies have shown that light regulates the expression of BDNF mRNA in rat visual cortex (Castren et al., 1992).

Tests to show that target tissues regulate the expression of PDGF in RGCs are conceptually simple, yet my early attempts were fraught with technical difficulties. The approach taken was to co-culture immunoselected RGCs with superior collicular tissue, which was placed proud of the RGCs , using a Sigma co-culture chamber. The chambers were then removed from the wells in which the RGCs were cultured and total RNA made from the RGCs. A semi-quantitative PCR analysis was then conducted and although control experiments displayed incremental increases, technical difficulties with the PCR prevented proper interpretation of the data from the co-culture experiments. Refining the PCR should in future hold the answer to this potentially very interesting question.

Lastly, chapter 5 dealt with the effects of the soluble PDGF-αR on the development of the astrocytes. Despite the successful nature of this assay, several questions still remain unresolved. What, if any, is the effect of the XR fragment on the blood vessels; simple Bandeira lectin staining is required to identify the endothelial cells on the same wholemounts that are stained for GFAP. In addition to resolving the question of whether the change in the distribution of the astrocytes is due to a change in the pattern of blood vessels, is it possible to disturb blood vessel development without altering astrocyte distribution, or are the two inextricably linked? Using an XR fragment of the PDGF-ß receptor , which would only bind PDGF-B and therefore would be likely to affect only the retinal endothelial cells, should help resolve this fascinating problem.

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