Platelet-derived growth factor and its alpha-receptor subunit in oligodendrocyte development

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Abstract

How the diverse range of cell types seen in the vertebrate central nervous system (CNS) is generated is one of the most intriguing questions in developmental neurobiology. This Thesis concentrates on the development of oligodendrocytes, the myelinating cells of the CNS. There have been several hypotheses as to where in the embryo oligodendrocyte progenitors originate. Accumulating circumstantial evidence suggests that cells expressing platelet-derived growth factor alpha-receptor (PDGFRα^+ cells) in the embryonic spinal cord are oligodendrocyte progenitors. In Chapter Three I demonstrate that these PDGFRα^+ cells differentiate into oligodendrocytes *in vitro*, providing direct evidence that oligodendrocytes develop from a discrete group of PDGFRα^+ cells in the ventral ventricular zone of the embryonic spinal cord. Further *in vitro* experiments suggest that PDGFRα^+ progenitors are the major or only source of oligodendrocytes within the developing spinal cord.

In Chapter Four I demonstrate that PDGFRα^+ cells in the embryonic brain are oligodendrocyte progenitors. I describe evidence that suggests that PDGFRα^+ oligodendrocyte progenitors originate in the ventral diencephalon and migrate throughout the brain during subsequent development. I demonstrate that dorsal forebrain cells, at an age when PDGFRα^+ cells are not present, can generate oligodendrocytes *in vitro* if treated with certain factors. However, it is not clear whether this potential is realised *in vivo*.

In Chapter Five of this Thesis I investigate the roles of the ligands for PDGFRα (PDGF-A and PDGF-B) during oligodendrocyte progenitor development *in vivo*. By examining PDGF-A and PDGF-B null mice I show that PDGF-AA but not PDGF-BB or PDGF-AB is crucial for PDGFRα^+ oligodendrocyte progenitor proliferation in the spinal cord *in vivo*. Further experiments with transgenic mice demonstrate that the amount of PDGF-AA available to each PDGFRα^+ progenitor *in vivo* controls the length of its cell cycle and; therefore, PDGF-AA availability is one factor that regulates oligodendrocyte progenitor number *in vivo*.

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I would like to dedicate this Thesis to my mum and dad; have a very happy retirement, you deserve it.

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Abbreviations

bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
BrdU	5-bromo-2'-deoxyuridine
BS medium	modified Bottenstein and Sato's medium (defined in Chapter Two)
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CNTF	ciliary-neurotrophic factor
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E	embryonic day (E11 means embryonic day eleven)
EDTA	ethylenediaminetetraacetic acid disodium salt
EGF	epidermal growth factor
FCS	foetal calf serum
g	gram
GC	galactocerebroside
GFAP	glial fibrillary acidic protein
IGF	insulin-like growth factor
IL-6	interleukin-6
l	litre
IF	leukaemia inhibitory factor
MBE	myelin basic protein
MEM-H	minimal essential medium with Hepes buffer
mg	milligram
ml	millilitre
mRNA	messenger RNA
ng	nanogram
NT-3	neurotrophin-3
O-2A	oligodendrocyte-type-2 astrocyte progenitor cell
P	postnatal (P7 means postnatal day seven)
PBS	phosphate-buffered saline
PDGF-A	platelet-derived growth factor A chain
PDGF-B	platelet-derived growth factor B chain
PDGFRα	platelet-derived growth factor alpha-receptor subunit
PDGFRα$^+$	PDGFRα expressing
PDGFRß	platelet-derived growth factor beta-receptor subunit

PLP	proteolipid protein
RNA	ribonucleic acid
SD	standard deviation
Shh	Sonic Hedgehog protein
SVZ	subventricular zone
TGFß	transforming growth factor-ß
tRNA	transfer RNA
VZ	ventricular zone
WT	wild-type
µg	microgram
µl	microlitre

Chapter One
General Introduction

1.1 A brief description of vertebrate development

Vertebrate development begins with fertilisation. The fertilised egg undergoes cleavage, a series of mitotic divisions without cell growth which creates many smaller nucleated cells. The resulting sphere of cells then undergoes gastrulation, a highly organised series of cell movements that establish the basic body plan of the developing animal. Gastrulation creates a three layered embryo consisting of an internal endoderm, an intermediate mesoderm and an external ectoderm. These layers then interact to induce the formation of the tissues and organs of the mature animal. The endoderm produces the digestive tract and its associated organs. The mesoderm generates the heart, kidney, connective tissues and cells of the blood and the cells of the ectoderm develop into the epidermis and the nervous system.

This Thesis is concerned with the development of the central nervous system (CNS) which begins to form following interactions between the dorsal mesoderm and the ectoderm. Cells at the dorsal midline of the mesoderm induce changes in the overlying ectodermal cells and neurulation begins. The midline ectodermal cells elongate and rise above the surrounding ectoderm (future epidermis) to create the neural plate. The cells at the edge of this plate then move upwards and create a neural groove. The edges of this neural groove migrate towards one another, eventually fusing to form a neural tube. When it is first formed the neural tube consists of just a single layer of neuroepithelial cells. How these neuroepithelial cells generate the diverse range of cell types seen in the mature CNS is one of the most fundamental questions in developmental neurobiology.

1.2 The development of the vertebrate central nervous system

Cells at the dorsal-most tip of the newly formed neural tube develop into neural crest and migrate away to differentiate into cells of the peripheral nervous system, epinephrine-secreting cells of the adrenal gland, pigment cells of the skin and connective tissues of the head. The cells remaining in the neural tube then begin to develop into the brain and spinal cord.

While the posterior end of the neural tube is still closing, the anterior part undergoes dramatic structural changes; it bulges and constricts to produce the three chambers that will develop into the forebrain, midbrain and hindbrain. The optic vesicles develop and protrude laterally from each side of the developing forebrain and the tube bends to create the flexures of the brain. The developing brain then becomes further subdivided.

The forebrain becomes divided into the telencephalon and the diencephalon; the telencephalon generates the cerebral cortex, basal ganglia and amygdala and the diencephalon gives rise to the thalamus and hypothalamus. The midbrain does not become subdivided before generating the tectum and tegmentum. The hindbrain becomes partitioned into regions which give rise to the cerebellum, pons and medulla. Each partitioned region surrounds a cerebro-spinal fluid-filled chamber; the telencephalon encloses the lateral ventricle, the diencephalon surrounds the third ventricle, the midbrain lies around the aqueduct and the hindbrain develops around the fourth ventricle. The neuroepithelial cells that surround the ventricles of the developing brain proliferate and generate ventricular and subventricular zones of precursor cells which differentiate into the neurons and glia (radial glia, ependymal cells, astrocytes and oligodendrocytes) of the mature brain.

The neuroepithelial cells that surround the central canal of the developing spinal cord also proliferate to generate a ventricular zone of precursor cells which differentiate into the radial glia, ependymal cells, neurons, astrocytes and oligodendrocytes of the spinal cord. Radial glia differentiate near the central canal and extend long processes to the outer pial surface of the neural tube. These cells act as structural support for the developing spinal cord and provide a surface for neuronal progenitors to migrate along. Radial glia are transient cells; they disappear once neuronal progenitor migration is complete, perhaps because they die or because they trans-differentiate into glial cells (for review see Goldman, 1996). Ependymal cells are ciliated and line the central canal of the spinal cord and the ventricles of the brain; presumably they are involved in moving and/or uptake from the cerebro-spinal fluid. Neurons conduct electrical signals throughout the nervous system. The progenitors of spinal cord neurons leave the ventricular zone as post-mitotic, migratory cells; in general the progenitors of ventral spinal cord neurons are generated before those of the dorsal spinal cord. Astrocyte and oligodendrocyte progenitors leave the ventricular zone as mitotic, migratory cells. The site(s) of origin, possible subtypes and functions of astrocytes are as yet poorly characterised. Some astrocytes extend processes which act as the limiting membrane of the CNS, some induce capillaries and venules to form tight junctions and so create the blood-brain barrier, some produce growth factors which influence surrounding cells and others appear to have roles in maintaining the correct extracellular ionic environment for neurons. Oligodendrocyte function is more thoroughly understood; these cells provide a myelin sheath around neuronal axons, so facilitating saltatory conduction of action potentials.

As the various neural progenitors are generated, the spinal cord begins to become more organised; it develops a central 'grey matter' full of densely packed neuronal cell bodies and a peripheral 'white matter' full of myelinated axons. The dorsal half of the cord receives input from sensory neurons in the periphery whilst the ventral half contains motor neurons which innervate skeletal muscles. The brain can also be divided

into a sensory dorsal half and a motor ventral half; for example, in the midbrain, the dorsal half contains optic and auditory inputs whereas the ventral half contains motor neurons that extend axons to the spinal cord and to the muscles of the face. The brain and spinal cord also contain phagocytic microglia; these cells are derived from the haemopoietic system and not the neuroepithelium.

1.3 Discovering the origin of neural progenitors

In this Thesis I will call naive cells of the neuroepithelium 'precursors' and cells which have some degree of commitment towards differentiating into a particular neural cell type 'progenitors'. If we are to understand how the CNS develops, we need to know when, where and how the progenitors for each neural cell type originate in the embryo. Information about the time and site of origin of neuronal progenitors has been obtained by using tritiated thymidine. The radioactive nucleoside is injected into rodents pregnant with embryos of various ages and it is incorporated into the DNA of any cells, including those of the neuroepithelium, that are in S phase of the cell cycle. The embryos are killed and cells that were actively synthesising DNA at the time of the injection or their progeny are identified by autoradiography. These studies have demonstrated that in the rat, ventral motor neurons differentiate from the ventral ventricular zone between embryonic day 11 (E11) and E13, intermediate grey matter neurons differentiate from intermediate ventricular zone between E12 and E15 and the neurons of the dorsal substantia gelatinosa develop from the dorsal ventricular zone between E14 and E16 (Altman and Bayer, 1984). Unfortunately tritiated thymidine-labelling does not tell us much about glial progenitor production because, unlike neuronal progenitors, glial progenitors continue to divide after leaving the neuroepithelium and therefore any radioactive label that they might contain is diluted beyond a detectable level. To date, very little is known about the origin of astrocyte progenitors. However, we are beginning to understand something about the origins of oligodendrocytes in the spinal cord and brain through studies *in situ* using oligodendrocyte lineage markers identified during *in vitro* studies of the rodent optic nerve.

1.4 The rodent optic nerve

The rodent optic nerve is a relatively simple and easily accessible region of the CNS and for these reasons has been the subject of much study. The optic nerve contains the axons of retinal ganglion cells, which project from the retina to visual processing centres in the posterior thalamus, type-1 astrocytes and cells of the oligodendrocyte lineage. Type-1 astrocyte progenitors are thought to originate within the neuroepithelium of the optic stalk and in the rat they begin to differentiate into glial

fibrillary acidic protein (GFAP) positive cells at around E15-16 (Small *et al.*, 1987). Oligodendrocyte progenitors migrate into the optic nerve from germinal zones within the brain and first appear at the chiasmal end of the nerve between E15 and E18 (Small *et al.*, 1987; Mudhar *et al.*, 1993). The first oligodendrocytes differentiate in the optic nerve on the day of birth (postnatal day 0, P0); progenitor cell proliferation and oligodendrocyte differentiation continues for several weeks after birth (Miller *et al.*, 1985). The neonatal rat optic nerve contains oligodendrocyte lineage cells at different stages of maturity: newly generated progenitors, more mature progenitors and differentiated oligodendrocytes; the optic nerve has therefore been very useful in the study of oligodendrocyte development. Oligodendrocyte progenitors in the optic nerve are referred to as O-2A progenitors because of their bipotentiality *in vitro*. If they are cultured in low serum medium O-2A progenitors differentiate into oligodendrocytes; whereas, if they are grown in medium containing at least 10% serum, they differentiate into type-2 astrocytes (Raff *et al.*, 1983). However, it is not known whether type-2 astrocytes exist *in vivo* (Fulton *et al.*, 1991).

1.5 Control of oligodendrocyte development in the optic nerve

By studying optic nerve O-2A progenitors we have learnt a lot about the cellular interactions and signalling molecules that might be important during oligodendrocyte development *in vivo*. Several factors have been shown to be mitogens for oligodendrocyte progenitors *in vitro* and/or *in vivo*. These include the A chain and B chain of platelet-derived growth factor (PDGF-A and PDGF-B), with the dimeric forms PDGF-AA and PDGF-AB inducing a maximal mitogenic response at three- to tenfold lower concentrations than PDGF-BB (Pringle *et al.*, 1989). O-2A cells express the alpha-subunit of the PDGF receptor (PDGFRα) (Hart *et al.*, 1989a). Neurotrophin-3 (NT-3) stimulates the division of optic nerve oligodendrocyte progenitors *in vitro* and appears to cooperate with PDGF to drive the division of these cells *in vitro* and *in vivo* (Barres *et al.*, 1993a, 1994a). Insulin-like growth factor-1 or 2 (IGF-1 or IGF-2) or a concentration of insulin that is high enough to activate IGF-1 receptors, is necessary for the mitogenic effect of NT-3 on O-2A cells (Barres *et al.*, 1993a). Basic fibroblast growth factor (bFGF) stimulates oligodendrocyte lineage cells to divide *in vitro* (Eccleston and Silberberg, 1985; Bögler *et al.*, 1990). If combined with PDGF, bFGF blocks the differentiation of O-2A cells into oligodendrocytes (Bögler *et al.*, 1990; McKinnon *et al.*, 1990). Since oligodendrocyte progenitors obviously do differentiate *in vivo*, this suggests that bFGF is not normally available to O-2A cells in the optic nerve.

PDGF-A is expressed by retinal ganglion cells and type-1 astrocytes of the optic nerve (Pringle *et al.*, 1989; Mudhar *et al.*, 1993). NT-3 is expressed by type-1

astrocytes and IGF-1 is produced by both glial cells and retinal ganglion cells; therefore, some or all of these factors could be important during oligodendrocyte development in the optic nerve *in vivo*.

Electrical activity in the retinal ganglion cell axons that run through the optic nerve is essential for the proliferation of oligodendrocyte progenitors in the nerve (Barres and Raff, 1993b). Electrical activity presumably results, directly or indirectly, in the release of polypeptide mitogens into the nerve, because the block of O-2A progenitor cell division that results from injecting tetrodotoxin (a sodium channel blocker) into the eye can be overcome by simultaneously delivering PDGF (Barres and Raff, 1993b). The neurons themselves might somehow release mitogens or their electrical activity might stimulate mitogen release from nearby type-1 astrocytes.

If oligodendrocyte progenitors are cultured in the presence of mitogens, they do not proliferate indefinitely; after a maximum of about eight cell divisions the cells drop out of the cell cycle and differentiate into oligodendrocytes. The progeny of an individual progenitor cell tend to stop dividing and differentiate at about the same time. If two daughter cells of a progenitor are cultured separately they often divide the same number of times before they differentiate (Temple and Raff, 1986), suggesting that an intrinsic mechanism limits their proliferation rather than timed signals from surrounding cells. Loss of PDGFRα is not the trigger for differentiation because newly differentiated oligodendrocytes express PDGFRα and respond to PDGF by transiently raising cytosolic calcium concentration and elevating the transcription of the immediate early response genes *fos* and *jun* (Hart *et al.*, 1989a, 1989b, 1992); therefore, the switch that renders O-2A progenitors unresponsive to mitogenic stimulation and triggers their exit from the cell cycle seems not to part of the early signalling apparatus that links PDGF receptor occupancy to nuclear oncogene activation. Instead, it has been proposed that oligodendrocyte progenitors contain an intrinsic 'clock' which counts and limits the number of divisions that the cells can undergo before differentiating (Raff *et al.*, 1985; Temple and Raff, 1986). As yet, very little is known about the molecular basis of this clock, it seems to consist of two components: a counting mechanism that requires mitogenic stimulation for its operation and an effector mechanism that depends on thyroid hormone or a similar hydrophobic signal in the environment (Barres *et al.*, 1994b). Recent experiments suggest that the counting mechanism counts time and not cell divisions (Gao *et al.*, 1996). The cyclin-dependent kinase inhibitor p27/Kip1 accumulates in O-2A progenitors as they proliferate and is at a high level when the cells differentiate (Durand *et al.*, 1997). p27/Kip1 could therefore be part of the progenitor's counting mechanism, cooperating with other factors to prevent re-entry into the cell cycle after the appropriate period of time. This proposal is supported by the observation that, when cultured in saturating amounts of mitogen, oligodendrocyte progenitors from p27/Kip 1 null mice tend to undergo one or two more divisions than wild-type

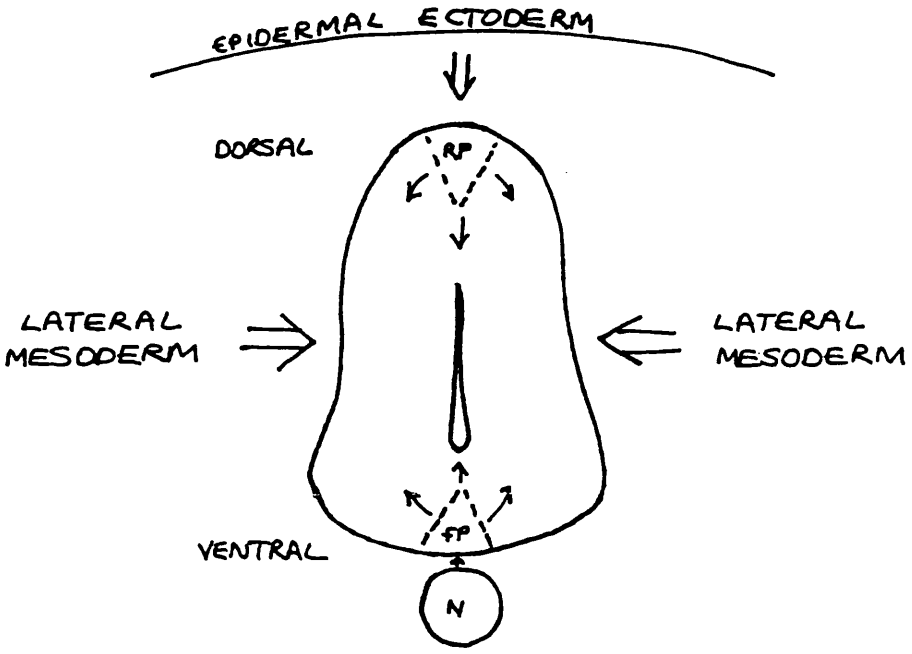
cells before they differentiate (Durand *et al.*, 1998). The clock's effector mechanism seems to involve hydrophobic signalling molecules such as thyroid hormone, glucocorticoid or retinoic acid that activate intracellular receptors (Barres *et al.*, 1994b). Without mitogens, O-2A cells will differentiate even in the absence of hydrophobic signals. In the presence of mitogens but in the absence of hydrophobic signals, O-2A cells proliferate indefinitely (Barres *et al.*, 1994b). This suggests that hydrophobic signals are not necessary for differentiation but are necessary to stop cell proliferation after an appropriate period of time in the presence of mitogens (Barres *et al.*, 1994b). Presumably, the hydrophobic signalling molecules bind to their receptors inside the O-2A cells, which then inhibit factors such as the Fos/Jun, AP-1 transcription complex, causing the cells to exit from the cell cycle into G_0 and differentiate.

As well as signals for proliferation and differentiation, both oligodendrocyte progenitors and differentiated oligodendrocytes need factors to support their survival. Every vertebrate cell that has been studied to date needs external signals to prevent it from undergoing 'programmed' cell death (apoptosis) (Raff, 1992). Oligodendrocyte progenitors need PDGF and IGFs to survive. Soon after O-2A progenitors differentiate into oligodendrocytes, they stop expressing PDGFRα and so are no longer dependent on PDGF for survival but still need IGFs to override entry into apoptosis (Barres *et al.*, 1992). Barres *et al.* (1993a) demonstrated that three classes of trophic factors can enhance the survival of mature oligodendrocytes *in vitro*: (1) insulin, IGF-1, or IGF-2, (2) neurotrophins, particularly neurotrophin-3 (NT-3) and (3) ciliary-neurotrophic factor (CNTF), leukaemia inhibitory factor (LIF) or interleukin-6 (IL-6). A single factor or combinations of factors within the same class promotes only short term survival of oligodendrocytes; at least one factor from each class is needed to enable oligodendrocytes to survive for longer periods (2-3 weeks) *in vitro*. *In vivo*, both oligodendrocyte progenitors and oligodendrocytes compete for limiting amounts of survival factors; any cell that does not succeed in obtaining an adequate dose of survival factors dies by apoptosis (Barres *et al.*, 1992; Calver *et al.*, 1998). Retinal ganglion cell axons also promote oligodendrocyte survival (Barres *et al.*, 1993c); this interaction seems to be dependent on the oligodendrocyte contacting the axonal surface and does not require electrical activity in the neuron (Barres *et al.*, 1993c), unlike the mitogenic activity of axons discussed earlier.

A small number of oligodendrocyte progenitors within the optic nerve do not differentiate, but survive as progenitors into adulthood. These 'adult' progenitors differ from neonatal O-2A cells in their morphology, slower migration rate, longer cell cycle time and in the time they take to differentiate *in vitro* (Wolswijk and Noble, 1989).
Which factors induce the formation of adult progenitors and whether these cells need different mitogens and/or survival factors than neonatal progenitors is as yet unknown.

1.6 A putative origin of oligodendrocyte progenitors in the embryonic spinal cord

The information outlined above for O-2A progenitors within the optic nerve has provided a starting point for identifying and characterising oligodendrocyte progenitors from other regions of the CNS. Since oligodendrocyte progenitors and newly differentiated oligodendrocytes are the only cells in the rat optic nerve that express PDGFRα (Hart *et al.*, 1989a), the pattern of PDGFRα expression in other regions of the CNS was examined in the hope that this would identify the origins of oligodendrocyte progenitors in the developing spinal cord and brain. Using a radioactive probe against PDGFRα mRNA for *in situ* hybridisation, Pringle and Richardson (1993) described the expression of PDGFRα in the embryonic rat spinal cord. At E14, there is a discrete group of PDGFRα$^+$ cells in the ventral ventricular zone of the spinal cord, subsequently, PDGFRα$^+$ cells increase in number and migrate throughout the spinal cord (Pringle and Richardson, 1993; Pringle *et al.*, 1998). Pringle and Richardson (1993) proposed that the PDGFRα$^+$ cells were oligodendrocyte progenitors and, consequently, that spinal cord oligodendrocytes had a tightly restricted origin in the ventral ventricular zone. Circumstantial evidence has been accumulating to support this proposal. Several molecular markers of oligodendrocyte lineage cells label cells in the same region of the ventral ventricular zone as PDGFRα; these include 2'3'-cyclic nucleotide 3'-phosphodiesterase (CNP), an enzyme found in both oligodendrocyte progenitors and mature oligodendrocytes (Yu *et al.*, 1994), antibodies against NG2, a proteoglycan known to be expressed by optic nerve oligodendrocyte progenitors (Nishiyama *et al.*, 1996) and, in chick, monoclonal antibody O4 which binds to sulphatide and other antigens present on oligodendrocyte lineage cells (Ono *et al.*, 1995). *In vitro* experiments have also demonstrated that the ability to generate oligodendrocytes is confined to the ventral half of the spinal cord in the early (E14) rat embryo (Warf *et al.*, 1991; Noll and Miller, 1993). Taken together, this evidence supports the hypothesis that spinal cord oligodendrocytes develop from a source of PDGFRα$^+$ progenitors in the ventral ventricular zone of the embryonic spinal cord (Pringle and Richardson, 1993). There have been reports that oligodendrocytes can develop from all regions of the ventricular zone (Cameron-Curry and Le Douarin, 1995; Hardy and Friedrich, 1996); however, we doubt the conclusions of these studies for reasons that are explained later in this Thesis (discussion, Chapter Three).



Patterning of the developing spinal cord Diagram of a transverse section through the embryonic spinal cord: the spinal cord receives signals from surrounding tissues that pattern it in both the dorsoventral and rostrocaudal axes. Signals from the ventral notochord (N) and floorplate (FP) ventralise the cord whereas signals from the epidermal ectoderm and roofplate (RP) initiate dorsal development. The lateral mesoderm is thought to regulate spinal cord development in the rostrocaudal axis.

1.7 Pattern formation in the developing central nervous system

At the time of their first appearance, only one or two of the hundred or so neuroepithelial cells along the dorsoventral axis of the spinal cord express PDGFRα. This restricted domain of PDGFRα expression in the ventral ventricular zone of the embryonic spinal cord demonstrates that neuroepithelial precursor cells, despite having uniform morphology, are not all identical at the level of gene expression (Pringle and Richardson, 1993; Yu *et al.*, 1994). Apart from the putative PDGFRα$^+$ oligodendrocyte progenitors (Pringle and Richardson, 1993), we have identified two small groups of cells that express fibroblast growth factor receptor-three (FGFR-3) in the midline and just above the floor plate of the E16 rat spinal cord; these might be astrocyte progenitors (Yu, 1995). The existence of these and other localised domains of gene expression suggests that the ventricular zone of the spinal cord contains an organised array of distinct subtypes of precursor cells, in other words that it is 'patterned'. It is a major challenge to determine how the ventricular zone can be patterned at such high resolution.

The spinal cord is patterned in both the dorsoventral and rostrocaudal axes. More is known about how the dorsoventral pattern of the spinal cord is established. The induction of ventral cell types in the developing spinal cord is controlled by signals from the notochord. The notochord is a slender rod of cells derived from the mesoderm that lies under the midline of the neural plate and the developing neural tube. The notochord expresses *Sonic hedgehog* (*Shh*), the vertebrate homologue of the *Drosophila* segment-polarity gene *hedgehog*. The amino-terminal portion of Sonic hedgehog protein (Shh) is secreted by the notochord and contacts the midline cells of the neural plate. Shh is believed to first induce a 'ventralised' state in the midline cells of the neural plate, involving the transcriptional repression of genes such as the 'paired-homeobox' genes *Pax 3* and *Pax 7* which become confined to the lateral neural plate (Ericson *et al.*, 1996). The midline neural plate cells that receive a high concentration of Shh develop into floor plate cells which themselves begin to express and secrete Shh (Roelink *et al.*, 1995). A concentration gradient of Shh is thought to develop as the protein diffuses away from the notochord and floor plate. Ventralised neuroepithelial cells at a particular point in this gradient of Shh are induced to develop into motor neuron progenitors (Marti *et al.*, 1995; Roelink *et al.*, 1995; Ericson *et al.*, 1996) whereas, those at other positions are induced to develop into different subclasses of interneurons (Ericson *et al.*, 1997). The small group of PDGFRα$^+$ cells in the ventral ventricular zone of the spinal cord is also induced by Shh (Pringle *et al.*, 1996).

The induction of dorsal spinal cord cell types is believed to be controlled by a contact-mediated signal from the epidermal ectoderm that flanks the neural plate (Liem *et al.*, 1995). Bone morphogenic proteins 4 and 7 (BMP4 and BMP7), members of the

transforming growth factor-beta (TGFß) family, are expressed in this region at appropriate times and appear to be responsible for inducing some dorsal interneuron subtypes (Liem *et al.*, 1995).

The little that is known about the control of rostrocaudal pattern in the spinal cord concerns the development of motor neurons. Motor neurons arise from distinct longitudinal columns of progenitors along the rostrocaudal axis. The motor neurons within each column send their axons to a common target; for example, to body wall muscles or to the muscles of a limb (Tosney *et al.*, 1995), and each column of motor neuron progenitors expresses a particular combination of LIM homeodomain transcription factors (Tsuchida *et al.*, 1994). By transplanting pieces of chick neural tube to different rostrocaudal positions, it has been demonstrated that the pattern of LIM domain expression is established by inductive signals which are themselves patterned along the rostrocaudal axis (Tsuchida *et al.*, 1994). These signals may be produced by the axial mesoderm which surrounds the spinal cord. Another class of homeodomain genes, the *Hox* genes, are expressed in restricted domains along the rostrocaudal axis and so might also play a role in motor column patterning. Thymocyte winged helix (TWH) is one transcription factor that is thought to subdivide motor columns into pools of different motor neuron subtypes (Dou *et al.*, 1997).

Patterning of the neural tube therefore begins with signals from outside the neuroepithelium acting on the neural plate to induce a 'dorsalised' and a 'ventralised' region and possibly an 'anterior' and 'posterior' region whose cells are from this point onwards competent to develop into either dorsal or ventral cell types with a particular rostrocaudal identity. Domains of homeobox gene expression and concentration gradients of gene regulatory proteins are then generated and these induce the differentiation of certain neural cell types depending on their position within the ventricular zone.

Patterning of the developing brain

The hindbrain develops from eight 'rhombomeres', which are periodic swellings in the neural tube. Each rhombomere can be seen as a 'developmental compartment' or segment, with boundaries that prevent the cells of adjacent rhombomeres from mixing (Fraser *et al.*, 1990; Guthrie and Lumsden, 1991). Prime candidates for establishing the identity of each rhombomere are the *Hox* family of homeodomain transcription factors. Expression of several *Hox* genes precedes rhombomere formation and becomes progressively restricted such that the anterior expression boundaries of individual *Hox* genes coincide with rhombomere boundaries; for example, the anterior boundary of *Hox-b2* expression maps to the junction of rhombomeres 2 and 3 (r2 and r3) and *Hox-b3* has its anterior boundary of expression at the border of r4 and r5 (Wilkinson *et al.*, 1989). It is unclear how this pattern of *Hox* genes is regulated. The

zinc finger transcription factor *Krox-20* is expressed in r3 and r5 and in *Krox-20* null mice the r3 and r5 domains of *Hox-b2* expression are lost and r3 and r5 fail to develop properly (Schneider-Maunoury *et al.*, 1993). This suggests that *Krox-20* may regulate at least one *Hox* gene that is essential for specifying the identity of r3 and r5.

The *Hox* genes are only expressed up to the hindbrain/midbrain border; the genes that are responsible for patterning the midbrain are less well understood. The midbrain, unlike the hindbrain, is not segmented; it appears to be patterned in its rostrocaudal axis by a concentration gradient of signalling molecules induced by genes expressed at the isthmic constriction which lies at the junction of the midbrain and hindbrain. Two genes that are known to be expressed at the isthmic constriction early in development are *Engrailed one* and *Engrailed two*; Engrailed-one protein ensures correct patterning of the midbrain tectum whereas Engrailed-two protein is essential for cerebellum development (for review see Joyner, 1996).

Little is known about how the forebrain is patterned. There is some controversy as to whether the forebrain is a segmented structure like the hindbrain or whether, like the midbrain, it develops in response to signals from a signalling centre. Several genes whose homologues in *Drosophila* are important for fly head development are also expressed in the mammalian forebrain; *Otx-2*, *Otx-1*, *Emx-2* and *Emx-1* have a nested pattern of expression with *Otx-2* being expressed first followed by *Otx-1*, *Emx-2* and *Emx-1*. The four genes are expressed in increasingly restricted regions of the forebrain; *Otx-2* is expressed in all dorsal regions and most ventral regions of the forebrain whereas *Emx-1* is only expressed in a small region of the dorsal telencephalon (Boncinelli *et al.*, 1993). Recent studies are starting to reveal how these crude subdivisions are refined by the establishment of local signalling centres to generate particular subclasses of neurons at appropriate positions; for example, Ye *et al.* (1998) show that Shh and FGF8 signalling in the rostral forebrain cooperate to induce forebrain dopaminergic neurons.

1.8 Conclusions and aims of this Thesis

In this brief introduction to vertebrate CNS development I state that the diverse range of neural cell types in the mature CNS develop from the neuroepithelium of the neural tube; by studying the progenitors of one particular neural cell type, the oligodendrocyte, we hope to begin to understand how this happens. The third Chapter of this Thesis provides direct proof that PDGFRα^+ progenitors within the embryonic spinal cord are oligodendrocyte progenitors and indicates that PDGFRα^+ cells are the major or only source of oligodendrocytes within the developing spinal cord. The fourth Chapter examines oligodendrocyte progenitors within the developing forebrain and provides

tentative evidence that cerebral cortical precursors do not normally give rise to oligodendrocytes, but that instead cortical oligodendrocytes develop from progenitor cells that migrate into the cortex from a source in the ventral forebrain. The fifth Chapter of my Thesis describes work which investigates the requirement for PDGF during oligodendrocyte proliferation *in vivo*. This work demonstrates that the development of the oligodendrocyte lineage in the mouse spinal cord is critically dependent on the mitogenic effects of PDGF-AA but not PDGF-AB or PDGF-BB.

Chapter Two
Materials and Methods

All cell culture reagents came from Gibco-BRL, Sigma or Boehringer Manheim and radiochemicals were purchased from Amersham Life Science. Any other sources of reagents are given in the text.

2.1 Mammalian cell culture

2.1.1 Optic nerve cell culture

Sprague-Dawley rat pups were killed by decapitation and their heads sterilised with 70% ethanol. The scalp and skull were cut away and the brain lifted up gently to expose the optic nerves and optic chiasm. The nerves were cut from the chiasm and held under gentle tension as they were cut at the level of the eye sockets. Alternatively, the optic nerves were removed by pulling the eye, with the optic nerve attached, out of the eye socket; this allowed the whole length of the optic nerve to be collected and removed at least the outer meningeal layer which reduced the number of PDGFRα^+ meningeal cells in the cultures. Any unwanted tissue still attached to the optic nerves was removed under Hepes-buffered minimal essential medium containing 4mM glutamine and 100IU/ml penicillin and streptomycin (MEM-H). The nerves were transferred to 1ml Earle's balanced salt solution without calcium or magnesium containing 100IU/ml penicillin and streptomycin (EBSS) and 0.0125% (weight to volume: w/v) trypsin (Boehringer Mannheim), chopped into pieces and incubated at 37°C in 5% CO_2 for 20 minutes. Another 1ml of EBSS/trypsin solution was added and the tissue was incubated for a further 30 minutes or until the nerve pieces started to disintegrate. The nerves were washed in Dulbecco's modified Eagle's medium with 4mM glutamine and 100IU/ml penicillin and streptomycin (DMEM) containing 10% foetal calf serum (FCS) and transferred to DMEM containing 10% FCS and 0.005% (w/v) DNase-I (Sigma). The nerves were then triturated very gently through a 23G needle several times, filtered through a 20μm pore-diameter mesh and washed again in DMEM containing 10% FCS. The cells were resuspended in DMEM containing 10% FCS and approximately 2000 cells were seeded in a 10μl droplet on a 13mm diameter poly-D-lysine-coated (20μg/ml in sterile water; Sigma) glass coverslip. The cells were incubated at 37°C for 30 minutes to allow them to attach and then 400μl of modified Bottenstein and Sato's medium (BS medium) was gently added to the culture wells. BS medium is DMEM supplemented with transferrin (0.1mg/ml), bovine serum albumin (0.1mg/ml), progesterone (60ng/ml), sodium selenite (40ng/ml), thyroxine (40ng/ml),

triiodothyronine (30ng/ml), putrescine (16μg/ml) and insulin (5μg/ml) (all from Sigma). Recombinant human PDGF-AA (10ng/ml; Peprotech) was sometimes added to the cultures before continuing their incubation.

2.1.2 Embryonic neural cell culture

Timed-mated females were killed by CO_2 asphyxiation and the embryos removed and killed by decapitation. The day of appearance of the vaginal plug was designated embryonic day zero (E0). The embryonic spinal cords or brains were dissected away from surrounding tissue in MEM-H and the meningeal membranes were peeled away using watchmakers' forceps. The tissue was transferred to 2ml EBSS containing 0.0125% (w/v) trypsin, chopped into pieces and incubated at 37°C in 5% CO_2 for 30-45 minutes or until the tissue began to disintegrate. The tissue was washed in DMEM containing 10% FCS and transferred to DMEM containing 10% FCS and 0.005% (w/v) DNase-I. The tissue was triturated gently with a Pasteur pipette. The resulting cell suspension was filtered through a 20μm pore-diameter mesh and washed by centrifugation and resuspension in DMEM containing 10% FCS. The number of live cells in a sample of the suspension was counted in a haemocytometer using trypan blue (0.4% w/v in PBS; Sigma) exclusion as the criterion of viability. PBS is phosphate-buffered saline (1 litre PBS contains: 8g NaCl, 0.2g KCl, 0.1g $CaCl_2$, 0.1g MgCl, 1.15g Na_2HPO_4, 0.25g KH_2PO_4, pH 7.4) The appropriate number of cells was then plated on poly-D-lysine coated 13mm glass coverslips in a 20-50μl droplet. For low density cultures, 5000 viable cells were seeded in a 20μl droplet; for higher density cultures 40,000-75,000 cells were seeded in a 50μl droplet. For some high density, long-term culture experiments, a 'feeder' layer of 250,000 cells was plated in a poly-D-lysine coated well containing 3-4 pieces of broken coverslip, another 75,000 cells were then seeded onto a poly-D-lysine coated coverslip which was lowered gently onto the broken coverslip pieces above the feeder layer. The cells were allowed to attach for 30 minutes at 37°C, 400μl of BS medium, with or without 10ng/ml PDGF-AA (or other growth factors), was then gently added to the cultures and incubation continued at 37°C in 5% CO_2. The medium was gently replaced every second day or, for denser cultures, every day; if necessary, fresh growth factors were added every second day.

2.1.3 Postnatal neural cell culture

The procedure for culturing postnatal brain and spinal cord cells was the same as that described above for embryonic tissue except that, following trituration, the cell suspension was washed twice in DMEM containing 10% FCS to remove much of the cell and myelin debris that is generated when postnatal neural tissue is dissociated.

2.1.4 Immunoselection

Three 60mm diameter Petri dishes were incubated overnight at 4°C with 2ml anti-Ig antibody solution - two with 10μg/ml goat-anti-mouse IgG (Jackson Immunoresearch, Pennsylvania) and one with 10μg/ml goat-anti-rabbit IgG (Jackson Immunoresearch) in 50mM Tris-HCl (pH 9). The dishes were washed three times with PBS and incubated on a level surface for several hours at room temperature with the appropriate antibodies for immunoselection (see below) diluted in MEM-H containing freshly added 0.2% (w/v) bovine serum albumin (BSA), fraction V (Sigma). This antibody solution can be stored at 4°C for several days and used again if the antibody is in short supply. The single-cell suspension of embryonic spinal cord cells (prepared as described in 2.1.2) was washed by centrifugation in DMEM containing 10% FCS and resuspended in MEM-H containing 0.5% FCS. The cell suspension was incubated for 2 x 15 minutes in an uncoated Petri dish at room temperature to remove macrophages. The cell suspension was then passed sequentially over two dishes which had been coated with a 1:10 dilution of Ran-2 hybridoma supernatant (Bartlett *et al.*, 1981) to remove astrocytes and meningeal cells, then finally over a Petri dish coated with a 1:200 dilution of polyclonal anti-PDGFRα serum (number 3979; Fretto *et al.*, 1993; Nishiyama *et al.*, 1996). Each selection step was for 30 minutes at room temperature on a level surface, with occasional gentle swirling to redisperse unattached cells. The final dish was washed very gently with MEM-H several times and then rinsed several times with EBSS to remove any unattached cells (the dish was examined regularly under the microscope to see when this point had been reached). Any cells that had attached to the final PDGFRα-coated dish were removed with a three minute incubation in 0.0125% (w/v) trypsin in EBSS at 37°C and washed and resuspended in DMEM containing 10% FCS. The cells were plated at approximately 1000 cells/3μl droplet on 6mm diameter poly-D-lysine coated coverslips in a 96-well tissue culture plate. The cells were allowed to settle for 30 minutes at 37°C before 35μl BS-medium was gently added, with or without growth factors, and the incubation continued at 37°C in 5% CO_2.

2.1.5 Antibody-mediated complement cell lysis

A suspension of E17 spinal cord cells was prepared as described in 2.1.2 except that FCS was omitted at all stages of the preparation to avoid non-specific serum-dependant complement lysis. The cell suspension was incubated simultaneously with rabbit complement (diluted 1:12 in BS-medium; Cedar Lane Laboratories) and A2B5 hybridoma supernatant (Eisenbarth *et al.*, 1979) diluted 1:5 in BS-medium, at 37°C, 5% CO_2 for 45 minutes, with gentle inversion every 15 minutes. The cells were washed twice with DMEM and then once with DMEM containing 10% FCS.

Approximately 40,000 viable cells (determined by trypan blue exclusion) were plated in a 20µl droplet on 13mm diameter poly-D-lysine coated glass coverslips. The cells were allowed to attach for 30 minutes at 37°C before 400µl of BS medium containing 0.5% FCS and PDGF-AA (10ng/ml) was gently added. On the second day *in vitro* the complement treatment was repeated. The cells were incubated in A2B5 (diluted 1:5 in BS medium) for 30 minutes at 37°C, then gently washed twice with DMEM. Rabbit complement (1:12 dilution in BS-medium) was added for 30 minutes at 37°C, then the cells were washed twice with DMEM and incubation continued at 37°C in fresh BS-medium containing 0.5% FCS and 10ng/ml PDGF-AA.

2.2 Immunocytochemistry and immunohistochemistry

2.2.1 Double-immunolabelling of cells cultured on coverslips

Cells on coverslips were lightly fixed in 2% (w/v) paraformaldehyde in PBS for 5 minutes at room temperature. The coverslips were washed three times in PBS and incubated in anti-PDGFRα rabbit serum (number 3979; Fretto *et al.*, 1993; Nishiyama *et al.*, 1996) diluted 1:100 in PBS for 30 minutes in a humid chamber at room temperature. The cells were then rinsed three times with PBS and incubated in fluorescein-conjugated goat-anti-rabbit IgG (Jackson Immunoresearch) diluted 1:100 in PBS for 30 minutes. If the second antibody was against an intracellular epitope, the cells were then permeabilised by incubating them in 0.1% Triton X-100 (iso-Octylphenoxypolyethoxyethanol; BDH) in PBS for 20 minutes at room temperature. The cells were then incubated in the second antibody for 30 minutes as above, washed three times in PBS and incubated in the appropriate class of rhodamine or Texas Red™-conjugated anti-Ig (diluted 1:100 in PBS; Jackson Immunoresearch) for 30 minutes. The cells were then washed in PBS, post-fixed in 4% (w/v) paraformaldehyde in PBS and mounted under Citifluor (City University, UK). Immunolabelled cells were viewed and photographed using a Zeiss Axioskop photomicroscope and Kodak T-Max 400 ASA film.

2.2.2 5-bromo-2'-deoxyuridine (BrdU) labelling of cells in S phase of the cell cycle

For labelling cells in culture, BrdU (Boehringer Mannheim) was diluted in DMEM and added to the cultures at a final concentration of 0.01mM. For *in vivo* labelling, pregnant female mice or postnatal pups were injected intra-peritoneally with 100µg BrdU per gram body weight, injected at 10mg/ml in PBS using an insulin (30G) syringe.

2.2.3 Anti-BrdU immunolabelling of $BrdU^+$ cells cultured on coverslips

If necessary, cells on coverslips were immunolabelled with appropriate antibodies against surface or intracellular markers as described in 2.2.1, except that they were postfixed in acid-alcohol (70% v/v ethanol, 20% v/v glacial acetic acid in water) for five minutes at room temperature to prevent the bound antibodies being removed during the following steps. The cells were then also fixed for two minutes in 4% (w/v) paraformaldehyde in PBS at room temperature and 70% (v/v) ethanol for 20 minutes at -20°C. The cells were permeabilised by incubating them in 1% (v/v) Triton X-100 in PBS for 20 minutes at room temperature. The cells were then incubated in 6M HCl in 1% (v/v) Triton X-100 in PBS for 15 minutes at room temperature followed by 0.1M $Na_2B_4O_7$ (pH 8.5) for 10 minutes at room temperature. The cells were then blocked with 50% normal goat serum in 1% Triton X-100 for 15 minutes at room temperature, and incubated overnight at 4°C in anti-BrdU (monoclonal BU209, diluted 1:5 in 1% Triton X-100 in PBS; Magaud *et al.*, 1989), washed three times in PBS and incubated in rhodamine-conjugated goat-anti-mouse IgG (diluted 1:100 in 1% Triton X-100 in PBS; Pierce). The cells were washed and post-fixed in 4% paraformaldehyde in PBS for five minutes at room temperature, mounted in Citifluor and viewed in the fluorescence microscope.

2.2.4 Hoescht staining of cultured cell nuclei

If it was necessary to identify all cell nuclei in a culture, Hoescht bisbenzimide (clone number 33258; Sigma) was used. Cells were incubated in 0.1mg/ml Hoescht bisbenzimide (prepared from a frozen 10mg/ml stock solution) in PBS for five minutes at room temperature before the final postfixation step (there is no need to permeabilise the cells with this form of Hoescht stain).

2.2.5 Anti-myelin basic protein (anti-MBP) immunolabelling of tissue sections

Tissue sections (either freshly cut or thawed from -70°C and, if necessary, rehydrated through descending alcohols) were fixed with a 15 minute incubation in 4% (w/v) paraformaldehyde in PBS at room temperature. The sections were then washed 2 x 10 minutes in 0.1% (v/v) Triton X-100 in PBS at room temperature and incubated overnight at 4°C in anti-MBP (a gift from David Colman, Mount Sinai School of Medicine, New York) diluted 1:1000 in 0.1% Triton X-100 in PBS). The sections were then washed 2 x 15 minutes in Triton X-100 in PBS and incubated for an hour at room temperature in rhodamine-conjugated goat anti-rabbit IgG diluted 1:100 in 0.1% Triton X-100 in PBS (Pierce).

The sections were washed 2 x 10 minutes in Triton X-100 in PBS and mounted under Citifluor without a postfixation step. The sections were then viewed in a fluorescence microscope or a Bio-Rad MRC 1024 confocal microscope.

2.3 Molecular Biology

2.3.1 Caesium chloride purification of DNA

Transformed bacteria containing the appropriate plasmid DNA from a thawed glycerol stock (bacteria in 15% sterile glycerol) were streaked onto an LB agar plate containing ampicillin (100μg/ml) (LB is Luria broth and contains 1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl, LB agar is LB containing 1.5% bacto-agar) and incubated at 37°C overnight. A single colony of bacteria from the plate was transferred to 2ml of LB containing 50μg/ml ampicillin and allowed to grow for 8-10 hours at 37°C in a rotating environmental shaker set at 300rpm. This was then transferred to a 1 litre flask containing 500ml LB with 50μg/ml ampicillin and incubated overnight at 37°C in a rotating environmental shaker set at 300rpm. The next day the culture was spun at 4000rpm (Beckman GS-6KR centrifuge) for 15 minutes at 4°C. The pellet of bacteria was resuspended in 100ml of ice-cold STE (0.1M NaCl, 10mM Tris-HCl pH 8, 1mM EDTA, pH 8) and spun again at 4000 rpm for 15 minutes at 4°C. The pellet was then resuspended very thoroughly in 7ml of Solution 1 (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA pH 8) containing 350μl of fresh lysozyme (100μg/ml dissolved in Solution 1). The solution was transferred to a 50ml Falcon tube and 14ml of freshly prepared Solution 2 (0.2N NaOH and 1% SDS) was added to it. SDS is the sodium salt of lauryl sulphate. The solution was gently mixed by inverting the tube slowly 5 times and left at room temperature for 10 minutes. 7ml of ice-cold Solution 3 (3M potassium acetate, 2M glacial acetic acid, pH 4.5) was added and the tube was shaken before incubation on ice for 10 minutes to lyse the bacteria. A white flocculent precipitate of chromosomal DNA and protein complexes formed. The solution was then spun at 4000rpm for 15 minutes at 4°C, the centrifuge was allowed to slow down and stop, the brake was not applied. The supernatant was transferred to a 50ml Falcon tube and thoroughly mixed with 0.6 volumes isopropanol, it was then spun again at 4000rpm for 15 minutes at 4°C. The pellet was resuspended in 6ml TE (10mM Tris-HCl, pH 7.5-8, 1mM EDTA), 2ml of 10mM ammonium acetate was added and the mixture was put on ice for 20 minutes. The mixture was then spun at 10,000rpm in a 15ml Corex™ tube for 10 minutes at 4°C. The supernatant was transferred to a 30ml Corex™ tube, 16ml of ethanol was added and the mixture was put on ice for 5 minutes before being spun again at 10,000rpm for 10 minutes at 4°C. The pellet was resuspended in 3.6ml TE and 0.4ml 3M sodium acetate (NaOAc), pH 5.2. 8ml of cold

ethanol was added and the solutions were mixed thoroughly and put on ice for 5 minutes to precipitate the DNA. The solution was then spun at 10,000 rpm for 10 minutes at 4°C and the supernatant was discarded. The DNA pellet was dried thoroughly and resuspended in 1ml TE. 1.05g of CsCl was added to the pellet and the mixture was transferred to a Quick-seal™ tube (Beckman) using a 5ml syringe and a 19G needle. 80µl ethidium bromide (EtBr) (from a 10mg/ml stock solution) was carefully added to the tube using a gel-loading tip. The tube was filled with topping solution (100ml contained 105g CsCl and 8ml EtBr at 10mg/ml in H_2O, and was filtered through a 0.22µm-diameter pore filter before use) ensuring that there was no fluid in the neck of the tube and only a very small air bubble, if any at all, at the top of the solution. The tube was then sealed and spun at 100,000rpm for at least four hours, usually overnight at 20°C, the centrifuge was allowed to slow down and stop, the brake was not applied. The top of the Quick-seal™ tube was then vented by carefully pushing a 19G needle through a small piece of paper towel into the top of the tube. A second needle was then gently pushed in, bevel side uppermost, just under the lower band of (full-length) DNA in the solution (the upper band contained nicked DNA). The band and a small volume of the surrounding solution were gently drawn off with the needle and transferred to an microcentrifuge tube. An equal volume of water-saturated butan-2-ol was then added and the tube was shaken and briefly pulse-spun in a mini centrifuge, the top layer was discarded. This extraction process was repeated until the lower aqueous phase was completely transparent and free of EtBr, the lower phase was then diluted to 4 times its volume with TE and 50ml 3M NaOAc was added. The ethanol precipitation described above was repeated and the pellet was washed twice in 70% ethanol before being resuspended in 500µl TE. The optical density (OD) of the solution was measured at 260/280nm to quantify the yield of DNA. The DNA/TE solution was stored at 4°C until needed.

2.3.2 Restriction enzyme digestion of DNA

Two or three units of the appropriate restriction enzyme (Promega) per microgram of DNA were used for digestion. Usually, 10µg of DNA was digested in 100µl reaction volume containing the appropriate restriction enzyme buffer (Promega) and the digestion was carried out at 37°C for at least an hour. Samples of the digest were run on a 1% agarose gel to check whether the digestion was complete (see 2.3.3). Digested DNA was precipitated as follows: one tenth of the volume of 3M sodium acetate (pH 5.2) and 2x the volume cold ethanol was added to the reaction solution and mixed by inverting the tube several times. The tube was then pulse-spun in a centrifuge and put at -20°C for an hour after which it was spun at 4000rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet of DNA was gently washed with 1ml of cold

70% ethanol and a 1 minute spin in a centrifuge at 4°C. The pellet of DNA was then air-dried at room temperature, resuspended in TE at 1μg/ml and stored at -20°C until needed.

2.3.3 Electrophoresis of DNA and RNA

Electrophoresis of restriction enzyme digested DNA

DNA with 1x DNA-loading buffer (10 x DNA-loading buffer contains 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF and 25% w/v Ficoll-400) was electrophoresed in a gel made from 1% (w/v) agarose in TAE (40mM Tris-acetate and 1mM EDTA, pH 7.5-7.8) at 80V for approximately 30 minutes. Gels were then stained with EtBr at 10mg/ml in H_2O for 15-30 minutes at room temperature. The stained DNA in the gel was then visualised using an ultra-violet transilluminator.

Electrophoresis of RNA (used to check the yield and hydrolysis of ^{35}S-labelled *in situ* hybridisation probes)

The samples were run in a 1% formaldehyde agarose gel. The appropriate amount of agarose was melted in 3.5 parts DEPC-treated water, cooled down and mixed with 1.1 parts MOPS running buffer (20mM 3-[N-morpholino]propanesulfhonic acid, pH 7.0, 8mM sodium acetate and 1mM EDTA, pH 8.0) and 1.1 parts formaldehyde, the mixture was poured into a gel tray and allowed to set. The RNA samples were dissolved in DEPC-treated water containing 1X RNA-loading buffer (10x buffer contains 50% glycerol, 1mM EDTA pH 8.0, 0.25% bromophenol blue and 0.25% xylene cyanol FF). The gel was run at 80V for approximately 30 minutes. Using a vacumn blotter, the RNA was then transferred onto Zeta-probe™ membrane (Bio-Rad) using 4 x SSC as the transfer buffer. The membrane was then dried and exposed overnight to autoradiography film (Amersham).

2.3.4 *In situ* hybridisation using ^{35}S-labelled RNA probes

All containers, slide racks and solutions used for *in situ* hybridisation were RNase-free unless indicated. Containers and slide racks were washed with 0.1% diethylpyrocarbonate (DEPC) and autoclaved. All solutions had 0.1% DEPC added to them, were shaken vigorously, and then autoclaved. The glass microscope slides that were used to collect tissue sections were washed with detergent, washed thoroughly with water, rinsed with distilled water and baked overnight at 200°C. The slides were then immersed for 30 seconds in the following: 2% 3-Aminopropyltriethoxysilane

(Apes) diluted in industrial methylated spirits (IMS), 9% IMS and DEPC-treated water. The coated slides were then dried at room temperature under sterile conditions and stored in dust-free containers until needed.

2.3.4.1 Preparation of frozen tissue sections

Dissected tissue or whole embryos were fixed by immersion in 4% paraformaldehyde (w/v) in PBS overnight at 4°C. After fixation the tissue was cryoprotected by immersion in 0.5M sucrose in PBS overnight at 4°C; if the tissue was large, this step was repeated. The tissue was then embedded in OCT embedding compound (Miles-Tissuetek) in aluminium foil containers and frozen on dry ice. If tissue from two strains of mice was being compared, the two pieces were aligned next to one another in a single OCT block. The embedded tissue was then sectioned or stored at -70°C until needed.

15μm thick sections of tissue were cut using a cryostat. The sections were collected on Apes-coated slides and dried at room temperature for at least an hour. A diamond scriber was used to mark the slides, both to identify the sections and to mark which side of the slide bore the sections. The sections were then fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature, washed in PBS for 5 minutes then either dehydrated through an ascending series of ethanols (30%, 60%, 80%, 95% and 100%) for one minute each and stored at -70°C or used immediately for *in situ* hybridisation.

2.3.4.2 *In vitro* transcription of RNA probes labelled with ^{35}S-UTP

The PDGFRα probe was transcribed from a 1,637 base-pair *Eco*R I cDNA fragment encoding most of the extracellular domain of mouse PDGFRα cloned into Bluescript KS (a gift from Chiayeng Wang, University of Chicago); the antisense probe was generated with T7 RNA polymerase (T7pol) and *Hin*dIII-linearised plasmid and the sense (control) probe with T3pol and *Bam*H I linearised plasmid. Proteolipid protein (PLP) is a major component of mature oligodendrocyte myelin and DM-20 is an alternative-splice isoform of PLP expressed embryonically (see Timsit *et al.*, 1992). A probe against PLP/DM-20 was transcribed from a 747 base pair cDNA fragment encompassing the entire mouse DM-20 coding sequence cloned into Bluescript KS (pBS-DM-20; Timsit *et al.*, 1992); antisense probe was generated with T3pol and *Bam*H I and sense probe with T7pol and *Eco*R I.

The appropriate plasmid DNA was linearised with appropriate restriction enzymes to generate antisense or sense (negative control) transcripts. The DNA was purified using sodium acetate and ethanol (see 2.3.2), resuspended in TE at 1μg/ml and stored until needed at -20°C. The following reaction mixture was set up, with each of the components prewarmed to room temperature and mixed in the following order: 2μl of 10x transcription buffer (400mM Tris-HCl Ph 7.9, 60mM $MgCl_2$, 20mM spermidine and 100mM DTT), 0.5μl (20 units) of RNasin (ribonuclease inhibitor; Promega), 4μl of dNTP (2.5mM each of ATP, GTP and CTP sodium salts; Pharmacia), 2.4μl of 100μM UTP, 1μl (1μg) of linearised template DNA, 5ml (50mCi) of ^{35}S-UTP (10mCi/ml; Amersham), 2μl (20 units) of the appropriate RNA polymerase (SP6, T7 or T3) to generate either an antisense or a sense (negative control) probe and, if necessary, distilled water to bring the final reaction volume to 20μl. The reaction mixture was incubated at 37°C for one hour. To remove the DNA template, 1 unit of DNase (RNase free) was added along with 20 units of RNasin and 25μg of carrier tRNA and the mixture was incubated at 37°C for a further 15 minutes. 200μl of 1mM dithiothreitol (DTT) was added and 1μl was removed and run on a formaldehyde/agarose gel to confirm that full length probes had been transcribed (see 2.3.3). The remainder of the solution was made 0.3M with the addition of 3M sodium acetate and the RNA was precipitated with 2.5 volumes of ethanol at -20°C. The RNA pellet was spun out at 4°C, briefly air-dried and resuspended in 50μl of 10mM DTT.

To ensure that the transcribed probe could penetrate the tissue sections efficiently, the probe in DTT was hydrolysed by incubation with 50μl of 100mM carbonate buffer (65mM Na_2CO_3, 35mM $NaHCO_3$, pH 10.2) for 15 minutes at 60°C. The hydrolysis was stopped by the addition of 100μl of neutralising buffer (0.2mM sodium acetate, 1% (v/v) glacial acetic acid, 10mM DTT). A 1μl sample was removed and run on a formaldehyde/agarose gel to check that the transcripts had been hydrolysed to suitably short fragments (see 2.3.3). The remaining probe was then made 0.3M with sodium acetate and precipitated with ethanol (see 2.3.2). The probe was dissolved in 25μl 10mM DTT/50% (v/v) deionised formamide containing 1μl carrier tRNA (transfer RNA, 25mg/ml; Sigma) and stored at -70°C. The probe was used within 24-48 hours of preparation to prevent possible degradation which can give high background signals on the tissue sections.

2.3.4.3 Pre-hybridisation treatment of tissue sections

Tissue sections that had been frozen were rehydrated through a descending series of ethanols (100%, 95%, 80%, 60% and 30%) for one minute each and washed briefly in PBS. These or fresh PBS-washed tissue sections were incubated in Proteinase K

buffer (50mM Tris-HCl and 5mM EDTA, pH 7.5) at room temperature for 5 minutes. EDTA is the disodium salt of ethylenediaminetetraacetic acid The sections were then treated with Proteinase K (20μg/ml) in the same buffer for 7.5 minutes at room temperature. Proteinase K activity was stopped by incubation in 0.2% (w/v) glycine in PBS for 30 seconds. The sections were washed 2 x 30 seconds in PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 15 minutes. After washing in PBS for 5 minutes, the sections were incubated in 0.1M triethanolamine (pH 8.0) for 5 minutes and then in 0.1M triethanolamine (pH 8.0) containing freshly added 0.25% (v/v) acetic anhydride for 2 x 10 minutes to reduce hydrostatic charges on the sections and so prevent non-specific binding of the probe. The slides were then washed in PBS for 3 minutes and dehydrated through an ascending series of alcohols (30%, 60%, 80%, 95% and 100%) for 1 minute each before being thoroughly air-dried under sterile conditions.

2.3.4.4 *In situ* hybridisation

Radiolabelled probes were diluted tenfold in hybridisation buffer (0.3M NaCl, 10mM Tris-HCl pH 6.8, 5mM EDTA, 10% w/v dextran sulphate, 0.1mg/ml yeast tRNA, 1x Denhardts solution, 10mM DTT and 50% v/v deionised formamide). 1 x Denhardt's solution contains: 0.02% (w/v) Ficoll-400, 0.02% (w/v) polyvinyl polypyrrolidone and 0.02% (w/v) BSA fraction V (Sigma). The solution was mixed thoroughly and centrifuged briefly to spin out any bubbles. 60-70μl of the probe was applied to each slide and the slide was gently covered with a 22 by 50 mm coverslip, taking care to avoid trapping air bubbles over the tissue sections. The slides were then placed in a sealed humid container and incubated overnight at 55°C.

2.3.4.5 Post-hybridisation washing

The slides were incubated in 4 x SSC (20x SCC: 3M NaCl and 0.3M sodium citrate, pH 7.5) until the coverslips slipped off easily. The sections were then incubated in 4 x SSC for at least an hour at room temperature, then washed in wash buffer (2x SSC, 2mM DTT and 50% v/v deionised formamide) at 65°C for 30 minutes. The sections were then incubated in RNase buffer (0.5M NaCl, 10mM Tris-HCl and 0.1mM EDTA, pH 7.5) for 2 x 5 minutes at room temperature, and treated with RNase A (20μg/ml in RNase buffer) at 37°C for 30 minutes to remove unhybridised single stranded RNA. The sections were incubated in RNase buffer for a further 15 minutes at room temperature. The slides were then washed for 30 minutes each in the following solutions: wash buffer at 65°C, 2x SSC at 45°C and finally, 0.1% SSC at 45°C. The slides were then dehydrated through an ascending series of ethanols (30%, 60%, 80%,

95% and 100%) and thoroughly air-dried before being coated with autoradiographic emulsion.

2.3.4.6 Coating slides with autoradiographic emulsion

Under safelights, an appropriate amount of autoradiographic emulsion (Ilford K5 emulsion; 5ml easily coated 12 slides) was put in a slide postal box and melted in a 40°C waterbath. An equal volume of water containing 100µl glycerol was added and mixed with the emulsion by gently inverting the slide box, avoiding the generation of bubbles. Each slide was then dipped into the emulsion mixture, if there were any bubbles over the tissue sections, the slide was dipped repeatedly until these were removed. Any excess emulsion was then drained off the slide and the back of each slide was wiped clean. The slides were laid to dry in a level light-tight box. When the slides were dry (still under safelights) the slides were put in a light-tight box containing silica gel dessicant at 4°C until they were ready to develop. Strong hybridisation signals such as that seen with anti-PLP/DM-20 probes could be detected after an exposure time of 3-4 days, anti-PDGFRα-probed slides were exposed for about a week and the PDGF-A signal was only detectable after several weeks exposure at 4°C. Test slides were developed at various times to check whether a particular batch of slides was ready to be developed.

2.3.4.7 Developing slides

The slides were allowed to reach room temperature, either in the light-tight box or under safelights and were developed by immersion in Kodak D19 developer for 2 minutes at 20°C. The developing reaction was stopped by a one minute wash in 1% (v/v) acetic acid and fixed with a five minute wash in 30% (w/v) sodium thiosulphate. The slides were then washed with distilled water for 30 minutes at room temperature, dehydrated through an ascending series of ethanols (30%, 60%, 80%, 95% and 100%) and cleared by 2 x 1 minute washes in xylene. The slides were then mounted, before the xylene evaporated, under a xylene-based mountant ('Xam'; BDH). The slides were then viewed and photographed under bright or dark field illumination in a Zeiss dissecting microscope.

2.3.5 *In situ* hybridisation using Digoxigenin (DIG) -labelled RNA probes.

(As with radioactive *in situ* hybridisation; all solutions and containers used for the prehybridisation steps should be made RNase-free by treatment with DEPC, see 2.3.4).

2.3.5.1 *In vitro* transcription of DIG-labelled RNA probes

The PDGFRα and PLP/DM-20 probe template DNAs were generated as described in 2.3.4.2. The PDGF-A probe (from Chiayeng Wang) was a 907 base pair full-length mouse cDNA cloned into the *Eco*R I site of pGEM-1 (Promega). The antisense probe was made with T7pol and *Bam*H I, the sense with SP6pol and *Pvu* II.

In an RNase-free microcentrifuge tube the following were mixed at room temperature, in the order shown: 1μg of linearised template DNA, 5μl of 5x transcription buffer (Stratagene), 7.5μl 0.1M DTT, 2.5μl 10x DIG-NTP mix (10mM ATP, CTP, GTP, 6.5mM UTP and 3.5mM DIG-UTP; Boehringer Mannheim), 1μl RNasin, 20 units of the appropriate RNA polymerase and if necessary DEPC-treated water to bring the reaction volume up to 25μl. The reaction mixture was incubated at 37°C for 2 hours. 55μl DEPC-treated water, 20μl 10M ammonium acetate and 300μl ethanol were then added and the mixture was put at -20°C for 2 hours. The tube was then spun at 4°C for 15 minutes and the pellet of RNA was washed with 70% ethanol and a 1 minute spin in a microcentrifuge. All traces of ethanol were removed from the pellet and it was resuspended in 100μl 10mM DTT. The amount of transcript was quantified by running a sample on a formaldehyde/agarose gel against a sample of known concentration. The RNA/DTT solution was stored at -20°C until needed, it will keep for years.

Many thanks to Andy Calver for generating the DIG-labelled probes used in Chapter five.

2.3.5.2 *In situ* hybridisation

The tissue was embedded and 15μm sections were cut using a cryostat and collected on Apes-coated slides as described in 2.3.4.1. The tissue sections were allowed to dry at room temperature under sterile conditions for at least an hour and were then stored at -70°C in a sealed box containing silica gel dessicant until use. When needed, the slides in their box were allowed to reach room temperature. The probe was diluted to an optimum level (determined empirically; usually 200-1000 fold, i.e. 0.1μg-1μg/ml) in hybridisation buffer (1x salt, 50% deionised formamide v/v, 10% dextran sulphate w/v, 1mg/ml rRNA, 1x Denhardt's solution in distilled water). One litre of 10x salt contains: 114g NaCl, 14.04g Tris-HCl, 1.34g Tris base, 7.8g $NaH_2PO_4 2H_2O$, 7.1g

Na_2HPO_4 and 100ml 0.5M EDTA, pH 7.5 in distilled water. The probe and hybridisation buffer were mixed thoroughly and were briefly centrifuged to spin out any bubbles. The probe was then denatured by an incubation at 70°C for 5-10 minutes. 75µl of the probe mix was added to each slide and the tissue sections were covered with a 22mm x 50mm coverslip, avoiding the trapping of air bubbles over sections. The tissue sections were then placed in a sealed box lined with chromatography paper (Whatman) moistened with 1x salt and 50% (v/v) deionised formamide and incubated overnight at 65°C.

2.3.5.3 Post-hybridisation washes

The slides were incubated in prewarmed wash buffer (1x salt, 50% v/v deionised formamide and 0.1% v/v Tween-20) at 65°C until the coverslips slipped easily off the slides. Tween-20 is monolaurate polyoxyethylenesorbitan (Sigma). The slides were then incubated for 2 x 30 minutes in wash buffer at 65°C and 2 x 30 minute washes in MABT (100mM maleic acid, 150 mM NaCl, 0.1% v/v Tween-20, pH 7.5) at room temperature.

2.3.5.4 Blocking and immunolabelling with alkaline phosphatase-conjugated anti-DIG antibody

A wax pen was used to draw a boundary line at the edge of the sections to reduce the volume of blocking and antibody solutions needed. Non-specific staining was blocked by incubating the sections in MABT containing 2% blocking reagent (Boehringer Mannheim blocking agent BM 1096 176, made up in MABT as 10% stocks and frozen) and 20% (v/v) heat-inactivated sheep serum (Sigma) for at least an hour at room temperature. The alkaline phospatase-conjugated-anti-DIG (anti-DIG-AP Fab fragments; Boehringer Mannheim catalogue number 1093 274) was diluted 1:1500 in blocking solution. 200µl of the antibody solution was applied to each slide and the slides were incubated in a humid box at 4°C overnight.

2.3.5.5 Post-immunolabelling washes and detection of DIG-labelled RNA hybrids

The slides were washed at room temperature 5 x 20 minutes in MABT and then incubated for 2 x 10 minutes in staining buffer 1 (100mM NaCl, 5mM MgCl, 100mM Tris-HCl, pH 9.5) at room temperature. The alkaline phosphatase reaction buffer was prepared as follows; 50% of the final volume of staining buffer 1 containing 0.2mM BCIP (5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide; Boehringer Manheim, catalogue number 1383 221) and 0.2mM NBT (nitroblue tetrazolium salt in

dimethylformamide; Boehringer Mannheim, catalogue number 1383 213) was prepared and mixed thoroughly. This was then added to 50% of the final volume of polyvinyl alcohol (PVA) solution (10% v/v PVA in 100mM Tris-HCl, pH 9 containing 100mM NaCl, this needed to be at 90°C for many hours to drive the PVA into solution). The two solutions were mixed very thoroughly and briefly centrifuged to remove bubbles, the slides were then incubated in this solution in vertical 'Coplin' staining jars at 37°C (one Coplin jar holds 30ml staining solution and 10 slides). Periodically, the slides were examined for the appearance of the blue BCIP/NBT alkaline phosphatase reaction product; if a signal had not yet developed the slides were returned to the staining solution and incubated further. Once a satisfactory hybridisation signal was obtained the slides were washed 2 x 15 minutes in distilled water, dehydrated through an ascending series of alcohols (30%, 60%, 80%, 95% and 100%), cleared 2 x 1 minute in xylene and mounted under a xylene-based mountant ('Xam'; BDH). The stained sections were viewed and photographed under phase illumination in a fluorescence microscope using Kodak 25ASA Technical Pan film.

Chapter Three

Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors

In the neonatal rat optic nerve oligodendrocytes differentiate from progenitor cells known as O-2A progenitors (for reviews see Raff, 1989; Richardson *et al.*, 1990 and Chapter One of this Thesis). Cells similar to O-2A progenitors have been found in other regions of the developing central nervous system including the cerebral cortex (LeVine and Goldman, 1988a, 1988b), the cerebellum (Reynolds and Wilkin, 1988) and the spinal cord (Warf *et al.*, 1991). Where the O-2A progenitors in all these regions originate has until recently been obscure. Oligodendrocytes are known to be generated after neurons; therefore, many have assumed that the dividing cells present in the ventricular zone after neurogenesis is complete include oligodendrocyte progenitors (Altman, 1966). Choi *et al.* (1983) and Hirano and Goldman (1988) suggest that spinal cord oligodendrocytes might develop directly from trans-differentiating radial glia whose nuclei are close to or in the ventricular zone. Oligodendrocytes are found throughout the CNS; therefore, it was assumed that their progenitors would arise throughout the ventricular zone. Warf *et al.* (1991) tested this assumption; they dissociated and cultured cells from the dorsal and ventral halves of spinal cord at various ages and tested whether the cells could generate oligodendrocytes *in vitro*. At E14, ventral but not dorsal spinal cord cells could give rise to oligodendrocytes whereas, later in development, both halves of the cord could produce oligodendrocytes. This result could be due to an oligodendrocyte inducing factor being produced in the ventral cord and diffusing dorsally during development; however, if dorsal cells were cultured next to ventral cells, they still did not generate oligodendrocytes. This result led Warf *et al.* (1991) to propose that oligodendrocyte progenitors are confined to the ventral half of the spinal cord at E14 and migrate dorsally during subsequent development.

Noll and Miller (1993) carried out experiments to investigate where in the ventral half of the spinal cord oligodendrocyte progenitors originate. In the rat spinal cord neurogenesis is largely over by E16 whereas, most glial cell production has yet to occur (Nornes and Das, 1974; Altman and Bayer, 1984); therefore, Noll and Miller (1993) injected the thymidine analogue bromodeoxyuridine (BrdU) into rats pregnant with E16.5 embryos to identify proliferating glial progenitors. At E16.5, $BrdU^+$ cells were present in the ventral ventricular zone of the spinal cord. If the BrdU-labelled cells were cultured, many differentiated into oligodendrocytes. These experiments confirmed that spinal cord oligodendrocyte progenitors develop from the ventral ventricular zone.

Oligodendrocyte progenitors and newly differentiated oligodendrocytes within the optic nerve express the platelet-derived growth factor alpha-receptor subunit

(PDGFRα). Since cells of the oligodendrocyte lineage are the only cell types in the optic nerve that express PDGFRα (Hart *et al.*, 1989a), Pringle and Richardson (1993) investigated whether PDGFRα might be a useful marker of oligodendrocyte progenitors in other regions of the CNS, including the spinal cord. Using a radioactive probe against PDGFRα mRNA and *in situ* hybridisation, Pringle and Richardson (1993) demonstrated that PDGFRα is expressed by a longitudinal row of cells in the ventral ventricular zone of the E14 rat spinal cord. Subsequently, these PDGFRα expressing cells (PDGFRα$^+$ cells) increase in number and migrate away from the ventricular zone; by the day of birth, PDGFRα$^+$ cells are seen throughout the spinal cord (Pringle and Richardson, 1993).

Several facts suggested that the PDGFRα$^+$ cells identified by Pringle and Richardson were glial cells including: their nuclear morphology, their distribution in white matter tracts and their presence in the optic nerve, which lacks neuronal cell bodies. The number of PDGFRα$^+$ cells in various regions of the CNS agreed with previous studies of the distribution of oligodendrocyte progenitors (see Pringle and Richardson, 1993). Evidence has since accumulated that is consistent with the PDGFRα$^+$ cells being oligodendrocyte progenitors; for example, the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) which is expressed by oligodendrocyte progenitors and myelinating oligodendrocytes is coexpressed by the PDGFRα$^+$ cells (Yu *et al.*, 1994). However, all of this evidence is circumstantial in nature, being based on co-localisation of PDGFRα with other putative markers of the oligodendrocyte lineage (see Chapter One). Furthermore, there have been reports that PDGFRα is expressed by neurons in the embryonic spinal cord (Vignais *et al.*, 1995). For these reasons it was important to attempt to obtain more direct evidence that PDGFRα$^+$ cells in the spinal cord are oligodendrocyte progenitors. The experiments described in this chapter were carried out for this purpose. The first half of this chapter describes experiments which demonstrate directly that PDGFRα$^+$ cells from the embryonic spinal cord are oligodendrocyte progenitors and the second half describes work which suggests that PDGFRα$^+$ cells are the major source of oligodendrocytes within the developing spinal cord.

Results

1. Characterisation of a polyclonal anti-PDGFRα: the identification of PDGFRα expressing cells in postnatal rat optic nerve cultures

In order to immunoselect living PDGFRα$^+$ cells from dissociated embryonic spinal cord so that I could test their differentiation potential *in vitro*, I needed an antibody specific to the extracellular domain of PDGFRα. Hart *et al.* (1989a) have demonstrated that the vast majority of cells in optic nerve cell cultures that express PDGFRα are oligodendrocyte progenitors and newly differentiated oligodendrocytes. I therefore tested the specificity of anti-PDGFRα sera by co-labelling optic nerve cultures with anti-PDGFRα and a panel of oligodendrocyte lineage marker antibodies. The antibodies I used were: A2B5, O4 and anti-galactocerebroside (anti-GC). Monoclonal antibody A2B5 recognises a subset of gangliosides expressed on oligodendrocyte progenitors as well as many neuronal progenitors (Eisenbarth *et al.*, 1979; Raff *et al.*, 1983). Monoclonal antibody 04 binds to sulphatide and other antigens expressed by later-stage oligodendrocyte progenitors and early oligodendrocytes (Sommer and Schachner, 1981; Bansal and Pfeiffer, 1992). Anti-GC binds to galactocerebroside and an unidentified antigen expressed by oligodendrocytes (Raff *et al.*, 1978; Ranscht *et al.*, 1982; Bansal and Pfeiffer, 1992). Oligodendrocyte progenitors from rat optic nerve are bipolar, A2B5$^+$, mitotic cells that develop into more highly branched (A2B5$^+$, 04$^+$) cells and finally differentiate into multipolar, post-mitotic GC$^+$ oligodendrocytes.

Optic nerve cells were cultured in modified Bottenstein and Sato's medium (BS medium, Bottenstein and Sato, 1979) with or without PDGF-AA, fixed after various culture periods and immunolabelled. Cultured optic nerve cells immunolabelled with anti-PDGFRα (number 3979, Fretto *et al.*, 1993; Nishiyama *et al.*, 1996) and either A2B5 or O4 are shown in Figure 3.1. The vast majority of PDGFRα$^+$ cells in these cultures had small cell bodies and a few slender processes, with punctate PDGFRα labelling all over their surface including the processes. All PDGFRα$^+$ cells with this morphology co-labelled with A2B5, and all A2B5$^+$ cells were PDGFRα$^+$ confirming them as oligodendrocyte progenitors (Raff *et al.*, 1983). Therefore, in rat optic nerve cultures, all oligodendrocyte progenitors express PDGFRα and all PDGFRα$^+$ process-bearing cells are oligodendrocyte progenitors. Of the O4$^+$ cells in these cultures, 64±3% co-labelled with anti-PDGFRα. The (O4$^+$, PDGFRα$^+$) cells were of two types: those that had an immature morphology with only a few processes and were brightly PDGFRα$^+$ and those with a more mature, oligodendrocyte-like morphology that showed weak PDGFRα immunoreactivity. Only a small number of GC$^+$

oligodendrocytes (6±4%) were PDGFRα$^+$, with weak labelling on their cell bodies and processes; therefore, PDGFRα immunoreactivity seems to be rapidly lost from oligodendrocyte progenitors after they differentiate into GC$^+$ oligodendrocytes.

A few large, flat cells with a fibroblast-like morphology in these cultures also bound the anti-PDGFRα in a bright, punctate pattern all over their surface. These cells did not bind A2B5 and are almost certainly meningeal cells, which often contaminate optic nerve cultures, due to the difficulty of completely removing the optic nerve meninges. Unfortunately, a meningeal specific antibody is not yet available to confirm the identity of the flat PDGFRα$^+$ cells; however, there is little doubt that they are meningeal cells, because pure cultures of dissociated meningeal membranes from optic nerve, brain and spinal cord are full of PDGFRα$^+$ cells with the same morphology and staining pattern as those seen in mixed optic nerve cultures. It is also known from *in situ* hybridisation studies that meningeal cells express PDGFRα (Pringle *et al.*, 1992 and Chapter Five of this Thesis).

2. PDGFRα$^+$ cells immunoselected from embryonic spinal cord develop into oligodendrocytes *in vitro*

Once the specificity of anti-PDGFRα (number 3979) had been established, it could be used to immunoselect PDGFRα$^+$ cells from embryonic rat spinal cord to test their differentiation potential *in vitro*. A suspension of E17 spinal cord cells was incubated, first on an uncoated Petri dish to remove macrophages, then on a dish coated with monoclonal anti-Ran2 that binds astrocytes (Bartlett *et al.*, 1981) and finally on a dish coated with anti-PDGFRα to bind PDGFRα$^+$ cells. Any cells that adhered to this final dish were removed using trypsin and cultured in defined BS medium. The great majority (97-100%) of the immunoselected cells were PDGFRα$^+$, process-bearing cells. The immunoselected PDGFRα$^+$ process-bearing cells were all A2B5$^+$, with a rounded cell body and a punctate pattern of anti-PDGFRα$^+$ staining all over their surface (Figure 3.2A). These cells were all (NG2$^+$, 04$^-$, GC$^-$), and did not bind anti-glial fibrillary acidic protein (anti-GFAP), which labels astrocytes (Bignami *et al.*, 1972); therefore, PDGFRα$^+$ cells immunoselected from the embryonic spinal cord have the same morphology and antigenic phenotype as oligodendrocyte progenitors from the neonatal optic nerve (Raff *et al.*, 1983 and see above). Rare contaminating cells in these cultures were PDGFRα$^+$ meningeal cells and macrophages, both of which were easily recognised by their morphology and inability to bind A2B5.

I tested whether PDGF-AA and bFGF were mitogenic for the immunoselected cells, as they are known to be for optic nerve oligodendrocyte progenitors (Eccleston *et al.*, 1985; Noble *et al.*, 1988; Pringle *et al.*, 1989; McKinnon *et al.*, 1990). I incubated the selected cells in defined (BS) medium overnight, and then switched them to BS medium containing BrdU (0.01 mM) with or without PDGF-AA (10ng/ml) or bFGF (10ng/ml) for 24 hours. Of those cells grown with PDGF-AA, 50±3% had incorporated BrdU during the 24 hour culture period, and of those grown with bFGF, 55±6% were $BrdU^+$. No cells in the growth factor-free medium incorporated BrdU; this demonstrates that PDGF-AA and bFGF are mitogenic for $PDGFR\alpha^+$ cells in the embryonic spinal cord.

In order to discover their differentiation potential *in vitro*, the immunoselected $PDGFR\alpha^+$ cells were cultured overnight in BS medium containing 0.5% FCS and PDGF-AA (10ng/ml), gently washed, and the medium replaced by BS medium containing 0.5% FCS alone. After 36 hours in this medium, the cells began to develop a more highly branched morphology, and a subset started to express the O4 antigen, a characteristic of late oligodendrocyte progenitors (Sommer and Schachner, 1981; Bansal and Pfeiffer, 1992). At no time did the immunoselected cells express GFAP or the 200 kDa neurofilament protein (recognised by antibody N4142; Sigma), indicating that they had not differentiated into astrocytes or neurons. After 48 hours *in vitro* without growth factors, at least 95% of the immunoselected cells were GC^+, demonstrating that they had differentiated into oligodendrocytes (Figure 3.2B).

In some experiments the immunoselected cells were cultured in medium containing 10% FCS. After 12 hours in this high-serum medium, the cells had begun to flatten and lose their bipolar morphology. After three days, the majority of these cells (>97%) expressed GFAP and A2B5, indicating that they had differentiated into type-2 astrocytes (Figure 3.2C and 3.2D). This experiment demonstrates that $PDGFR\alpha^+$ cells from embryonic spinal cord are bipotential *in vitro*, with their fate depending on the serum concentration of the culture medium, just like $PDGFR\alpha^+$ oligodendrocyte progenitors from the optic nerve (Raff *et al.*, 1983).

Controls for the specificity of these immunoselection experiments included: coating the final selection dish with goat-anti-rabbit IgG without anti-PDGFRα, or with preimmune serum from the rabbit that produced the anti-PDGFRα, or with an anti-rhodopsin antiserum (rabbit IgG) that can be used to immunoselect photoreceptor cells from retinae (Neophytou *et al.*, 1997). In all these control experiments, fewer than 20 cells adhered to the final selection dish, whereas at least 5000 cells adhered to the anti-

PDGFRα coated dish. It should also be noted that very few cells (fewer than 2 per thousand cells plated) died in either the low or high serum media.

3. The distribution of (PDGFRα$^+$, A2B5$^+$) cells in the developing rat spinal cord; oligodendrogenic capacity originates in the ventral spinal cord, spreads dorsally and correlates with the presence of PDGFRα$^+$ cells

The immunoselection experiments described above demonstrate that PDGFRα$^+$ cells in the embryonic rat spinal cord are oligodendrocyte progenitors. *In situ* hybridisation studies have demonstrated that at E14, PDGFRα$^+$ oligodendrocyte progenitors are only present in the ventral half of the spinal cord; subsequently, these cells increase in number and are found throughout the dorsal and ventral halves of the cord (Pringle and Richardson, 1993). Warf *et al.* (1991) found that at E14, only the ventral half of the spinal cord gave rise to oligodendrocytes in culture, whereas later in development both the dorsal and ventral halves of the cord were able to produce oligodendrocytes. Taken together, these studies strongly suggest that spinal cord oligodendrocytes develop from ventrally derived PDGFRα$^+$ cells. I tested this suggestion by culturing dorsal and ventral halves of spinal cord at different embryonic ages to find out whether the ability to give rise to oligodendrocytes *in vitro* correlated strictly with the presence of PDGFRα$^+$ cells. I dissected the spinal cords from E14, E16 and E18 rats, removed the meninges, dissociated the cells, and cultured 5000 viable cells on glass coverslips in BS medium containing 0.5% FCS. The day after plating, I immunolabelled at least two coverslips from each culture with anti-PDGFRα and A2B5 to identify oligodendrocyte progenitors. At the equivalent of the day of birth, that is E14 + seven days *in vitro* (7 DIV), E16 + 5 DIV, etc., I stained at least two coverslips with anti-GC to identify oligodendrocytes. The results of these culture experiments are shown in Table 1 and Figure 3.3. At E14, dorsal spinal cord lacks (PDGFRα$^+$, A2B5$^+$) cells and, in agreement with Warf *et al.* (1991), does not give rise to oligodendrocytes *in vitro*. At E16, dorsal spinal cord does contain a small number of (PDGFRα$^+$, A2B5$^+$) cells, and does give rise to oligodendrocytes. At E18, there are many (PDGFRα$^+$, A2B5$^+$) cells in both the ventral and dorsal half of the cord, and both halves are capable of producing many oligodendrocytes. These experiments demonstrate that the ability of spinal cord tissue to give rise to oligodendrocytes *in vitro* first develops in the ventral spinal cord, progresses to the dorsal half and correlates with the presence of PDGFRα$^+$ progenitor cells.

The results shown in Table 1 and Figure 3.3 were obtained from low density cultures that were only cultured until the equivalent of the day of birth (postnatal day 0, P0). I was concerned that there might be other oligodendrocyte lineages, whose progenitors are not (PDGFRα$^+$, A2B5$^+$), or that need survival factors other than those present in BS medium containing 0.5% serum, or that generate oligodendrocytes after the first wave of oligodendrocyte differentiation which starts around the day of birth (Jordan *et al.*, 1989; Warf *et al.*, 1991). If such oligodendrocyte lineage cells exist, they would not have been recorded in the above experiments. To address this issue, I cultured E14 dorsal and ventral spinal cord cells at a high density (75,000 cells/coverslip) above an even denser 'feeder' layer of spinal cord cells for 14 days, until the equivalent of P7 *in vivo*. At this higher density, the medium should become more richly conditioned with survival factors that might keep other as-yet-unidentified oligodendrocyte progenitors alive and enable them to differentiate *in vitro*. However, even in high density culture and after two weeks *in vitro*, no oligodendrocytes developed from E14 dorsal spinal cord cells (Figures 3.4 and 3.5). This is in contrast to the large number of oligodendrocytes that developed from E14 ventral spinal cord cultures (Figures 3.4 and 3.5). The small number of PDGFRα$^+$ progenitors sometimes seen in the dorsal cultures were present in small clusters, as though they had arisen from single isolated cells in the starting population, possibly due to contamination of the starting culture with ventral spinal cord cells.

If bFGF (10ng/ml), which might play a role in oligodendrocyte progenitor induction (see Chapter Four) or purified recombinant Sonic hedgehog protein (7×10^{-9} M), which is known to induce oligodendrocyte progenitors in intermediate avian spinal cord tissue (Pringle *et al.*, 1996), was added to E14 dorsal spinal cord cultures, no GC$^+$ oligodendrocytes developed after 9 days *in vitro*. Therefore, neural precursor cells in the E14 dorsal spinal cord are not normally specified as oligodendrocyte progenitors nor are they capable of being re-specified as such by factors known to have oligodendrocyte-inducing activity for precursor cells in the E14 cerebral cortex (see Chapter Four).

4. Selectively removing (PDGFRα$^+$, A2B5$^+$) cells from embryonic spinal cord tissue dramatically reduces oligodendrocyte production *in vitro*

The culture experiments described above strongly suggest that the ability of spinal cord tissue to give rise to oligodendrocytes depends on the presence of (PDGFRα$^+$, A2B5$^+$) progenitor cells and that these cells are the only type of oligodendrocyte progenitors in the embryonic spinal cord. To test this suggestion, I selectively killed

PDGFRα$^+$ cells in a spinal cord cell suspension and investigated whether the remaining cells could generate oligodendrocytes. I wanted to use complement and anti-PDGFRα serum to selectively lyse PDGFRα$^+$ cells in dissociated embryonic spinal cord cell suspensions. Unfortunately, cells labelled with anti-PDGFRα were not lysed by complement, perhaps because the PDGFRα/anti-PDGFRα complex protrudes too far away from the cell surface for the complement proteins to interact with both the antibody and the cell membrane. At E17, there is almost 100% co-expression of PDGFRα$^+$ and A2B5 by cells of the spinal cord, presumably because the PDGFRα$^-$ /A2B5$^+$ cells that are present in the earlier spinal cord have either differentiated or died; of 2000 A2B5$^+$ cells examined, only 13 were PDGFRα negative. I therefore used rabbit complement and A2B5 to selectively lyse (PDGFRα$^+$, A2B5$^+$) cells in a suspension of E17 spinal cord cells. I repeated the treatment after 24 hours *in vitro* and cultured the remaining cells until the equivalent of P0, when I immunolabelled them with anti-GC. The results of these experiments are shown in Figures 3.6 and 3.7. Treatment with complement and A2B5 reduced the number of (PDGFRα$^+$, A2B5$^+$) oligodendrocyte progenitors in these cultures by 30-fold and the number of oligodendrocytes that subsequently developed was at least 25-fold less than in the control cultures (Figure 3.6); therefore, the reduction in the number of oligodendrocytes approximately matched the reduction in the number of (PDGFRα$^+$, A2B5$^+$) progenitors in the starting population.

Controls for this experiment included: incubating the cells in complement alone, A2B5 alone, or complement and anti-ß-galactosidase which, like A2B5, is a monoclonal IgM. Neither of these treatments killed significant numbers of cells. These complement lysis experiments add support to the hypothesis that most or all oligodendrocytes in the embryonic rat spinal cord develop from ventrally derived PDGFRα$^+$ progenitors.

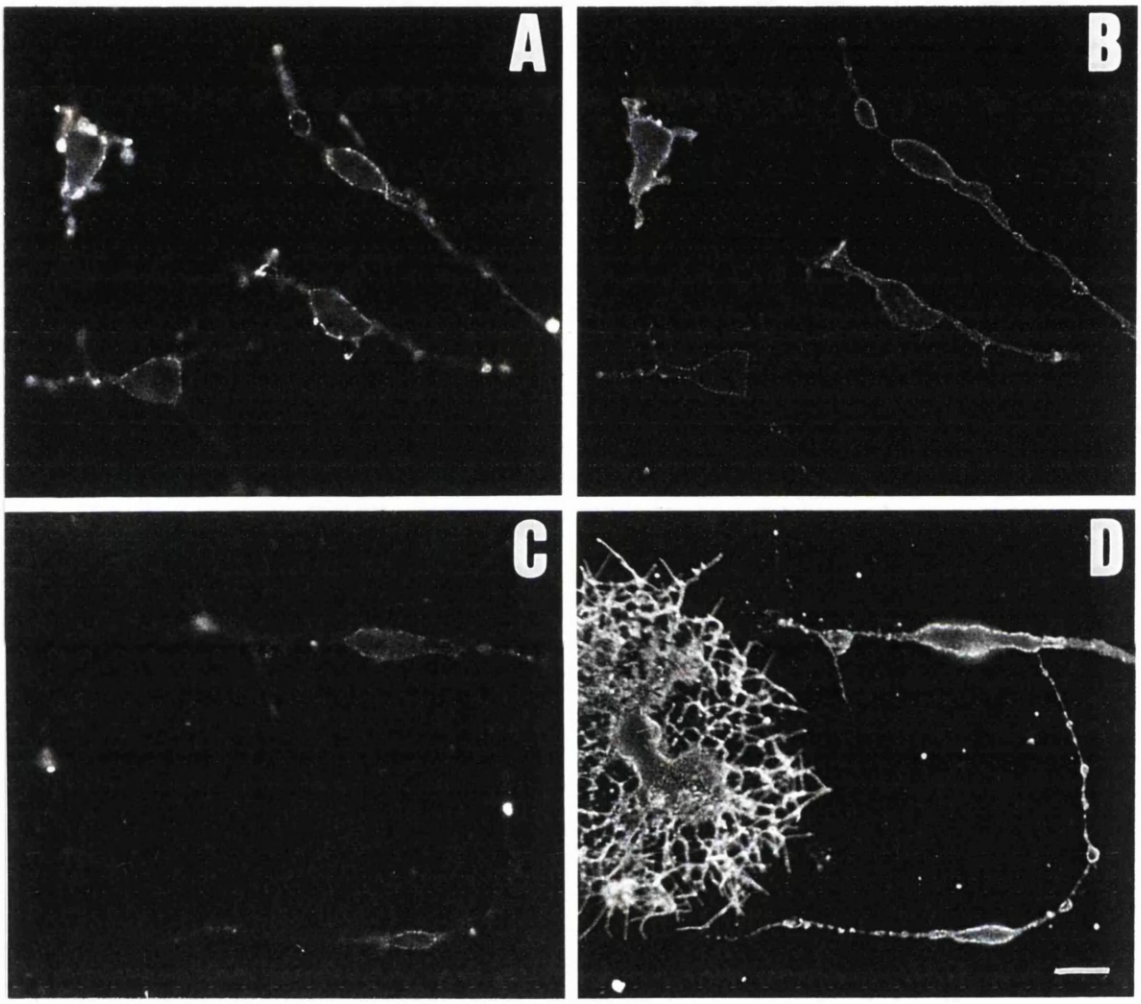


Figure 3.1 Characterisation of PDGFRα$^+$ cells in rat optic nerve cell cultures P7 rat optic nerves were dissociated and cultured on glass coverslips in BS medium (see Materials and methods) containing 0.5% FCS and 10ng/ml PDGF-AA for 16 hours, then fixed and stained with rabbit anti-human PDGFRα (A,C) and either monoclonal A2B5 (B) or monoclonal O4 (D). All PDGFRα$^+$, process-bearing cells also labelled with A2B5, and vice versa (A,B). A proportion (approximately 64%) of O4$^+$ cells also labelled with anti-PDGFRα (C,D). The (PDGFRα$^-$, O4$^+$) cells were almost certainly (O4$^+$, GC$^+$) oligodendrocytes (e.g. the large, multiple-process-bearing cell in D). Scale bar, 50μm.

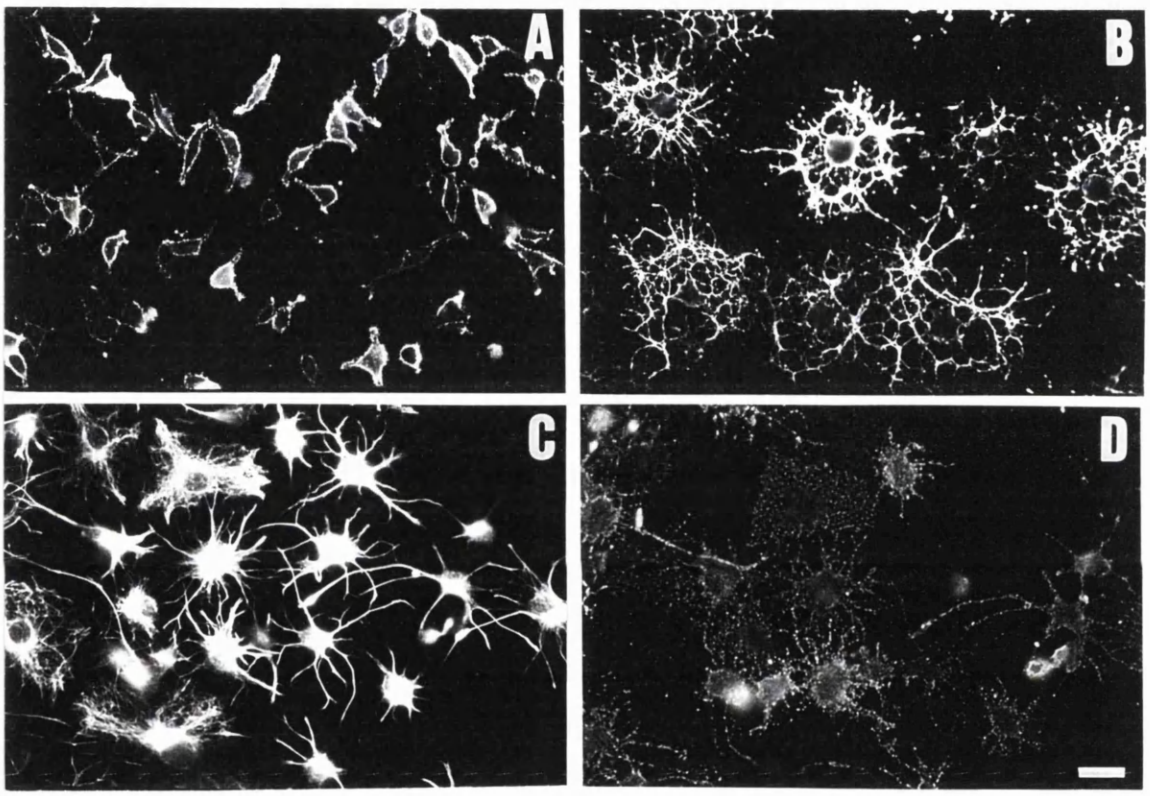


Figure 3.2 PDGFRα$^+$cells, immunoselected from embryonic rat spinal cords with an anti-PDGFRα antibody, differentiate into GC$^+$ oligodendrocytes or (GFAP$^+$, A2B5$^+$) astrocytes Cell suspensions of E17 spinal cords were immunoselected as described in Materials and methods, and cultured on glass coverslips in BS medium containing 0.5% FCS and 10ng/ml PDGF-AA. After 24 hours, the cells were fixed and immunolabelled with anti-PDGFRα, A2B5, anti-NG2, O4 or anti-GC. More than 99% of the process-bearing cells were (PDGFRα$^+$, A2B5$^+$, NG2$^+$, O4$^-$, GC$^-$). Panel A shows these cells labelled with anti-PDGFRα. Some coverslips were transferred after 24 hours in culture into BS medium containing 0.5% FCS without PDGF, or into BS medium with 10% FCS. After culturing for a further 1-3 days, the cells were fixed and immunolabelled with anti-GC or double-labelled with anti-GFAP and A2B5. In the presence of 0.5% FCS, more than 99% of the cells became GC$^+$ oligodendrocytes (B). In the presence of 10% FCS, more than 99% of the cells became GFAP$^+$ astrocytes (C), the majority of which also labelled with A2B5 (D). Scale bar, 50μm.

AGE	(PDGFRα⁺, A2B5⁺) cells per coverslip after 16 hrs *in vitro*		GC⁺ oligodendrocytes per coverslip at the equivalent of P0	
	dorsal	ventral	dorsal	ventral
E14	0	44± 9	0	1387±245
E16	46±3	212±18	368±71	908±204
E18	1360±90	1865±516	262±170	323±55

Table 1 (PDGFRα⁺, A2B5⁺) progenitors and GC⁺ oligodendrocytes in cultures of rat spinal cord cells Rat spinal cords were dissected into dorsal and ventral halves and 5000 cells from each were cultured separately on glass coverslips as described in Materials and methods. After 16 hours the cells were fixed and labelled with anti-PDGFRα and A2B5 to visualise presumptive oligodendrocyte progenitors. Parallel coverslips were cultured longer, until the equivalent of the day of birth (i.e. E14 + 7 DIV, E16 + 5 DIV, E18 + 3 DIV; DIV means days *in vitro*), then fixed and labelled with anti-GC to visualise oligodendrocytes. PDGFRα⁺ cells were present initially and oligodendrocytes developed subsequently, in all cultures except cultures of E14 dorsal cells. Tabulated are mean numbers of cells and standard deviations of three independent experiments conducted in duplicate or triplicate. There was not a strict correlation between the number of PDGFRα⁺ cells in the starting population and the number of oligodendrocytes that developed in culture. For example, fewer oligodendrocytes developed in the E18 cultures than in the E16 cultures, despite the fact that there were more PDGFRα⁺ cells initially present in the E18 cultures. Part of the reason for this is presumably that the E18 cells were cultured for a shorter time than the E16 cells (3 days rather than 5 days *in vitro*). However, my impression was that there was more cell damage and death caused during the dissociation of E18 than E16 spinal cords, probably reflecting increased mechanical damage to neurons. Reduction in the number of neurons in the cultures might have affected the rate at which oligodendrocytes differentiated or the proportion that survived in cultures.

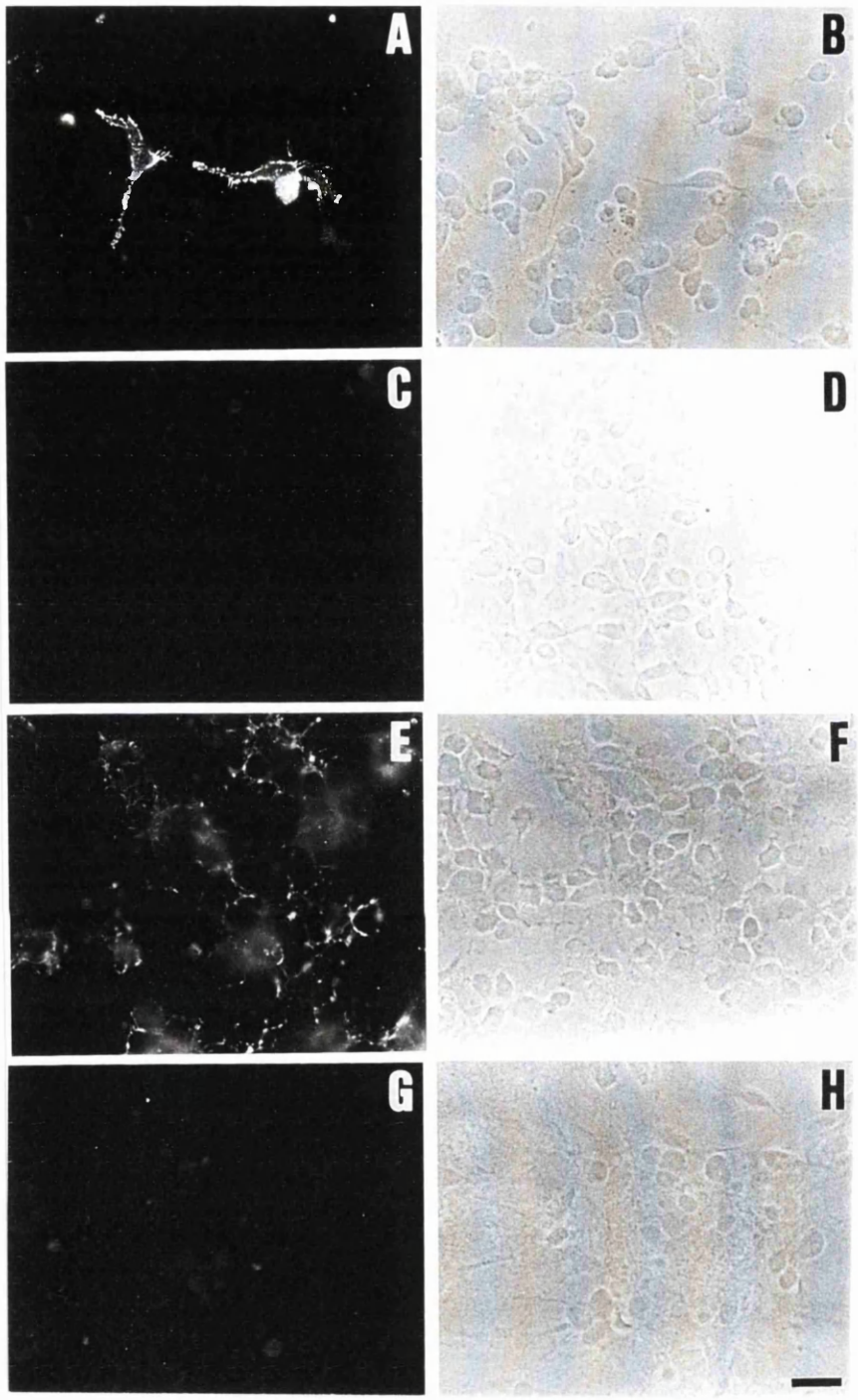


Figure 3.3 PDGFRα⁺ progenitors and oligodendrocytes in cultures of E14 rat ventral and dorsal spinal cord cells E14 spinal cords were divided longitudinally into ventral and dorsal halves, dissociated and cultured on glass coverslips in BS medium containing 0.5% FCS with or without 10ng/ml PDGF-AA. Cultures were immunolabelled with anti-PDGFRα (A-D) or anti-GC (E-H) and photographed under fluorescence or phase contrast optics. After 16 hours *in vitro*, ventral cultures contained small numbers of PDGFRα⁺ cells (A,B) but dorsal cultures did not (C,D). After 7 days in culture, many GC⁺ oligodendrocytes had developed in ventral cultures (E,F) but few or none developed in dorsal cultures (G,H). Scale bar, 50μm.

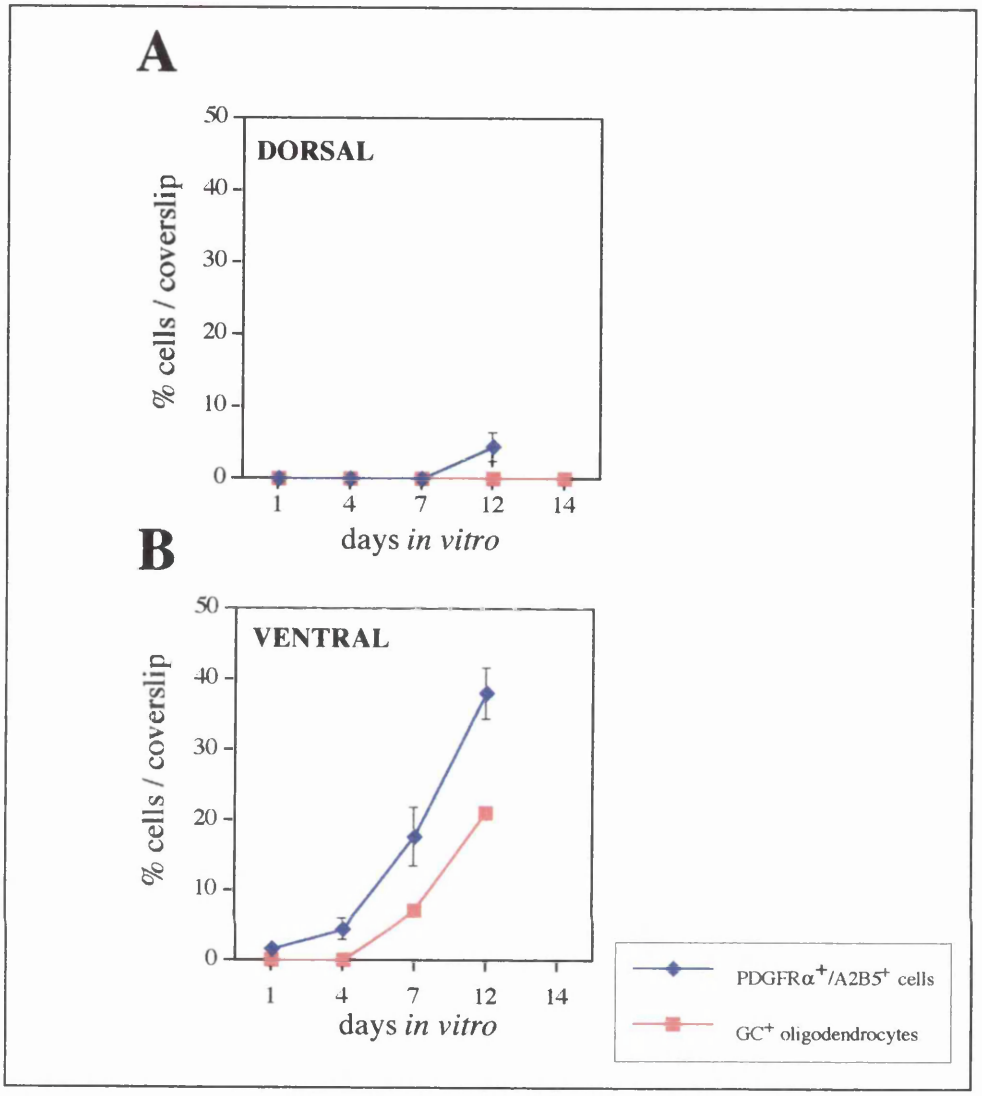


Figure 3.4 Time course of appearance of PDGFRα$^+$ progenitors and GC$^+$ oligodendrocytes in cultures of E14 dorsal and ventral spinal cord cultures E14 spinal cords were divided into dorsal and ventral halves, dissociated and plated at high density on glass coverslips (75,000 cells per coverslip) above a dense 'feeder' layer of spinal cord cells in BS medium containing 0.5% FCS and PDGF-AA (10ng/ml). After various culture periods, the cells were fixed and immunolabelled with anti-PDGFRα together with A2B5, or anti-GC antibodies. The cells were given a final wash in Hoescht 33258 (Sigma) to label all cell nuclei. The numbers of (PDGFRα$^+$, A2B5$^+$) process-bearing cells and GC$^+$ oligodendrocytes were counted (triplicate coverslips from at least two independent experiments) and expressed as a percentage of the total number of cells in 50 random fields. Small numbers of PDGFRα$^+$ cells were present in ventral cultures at early times after plating, and these increased in number throughout the culture period. GC$^+$ oligodendrocytes first developed in ventral cultures between four and seven days after plating and increased in number thereafter. In dorsal cultures, no (PDGFRα$^+$, A2B5$^+$) cells were detected early on, although a few were sometimes detected at 7 days and later. These always appeared in tight clusters as though clonally derived from one or two cells in the starting population. GC$^+$ oligodendrocytes did not develop in the dorsal cultures even after 14 days *in vitro*. The whole dorsal culture coverslip was scanned to confirm that no (PDGFRα$^+$, A2B5$^+$) progenitors or oligodendrocytes were present.

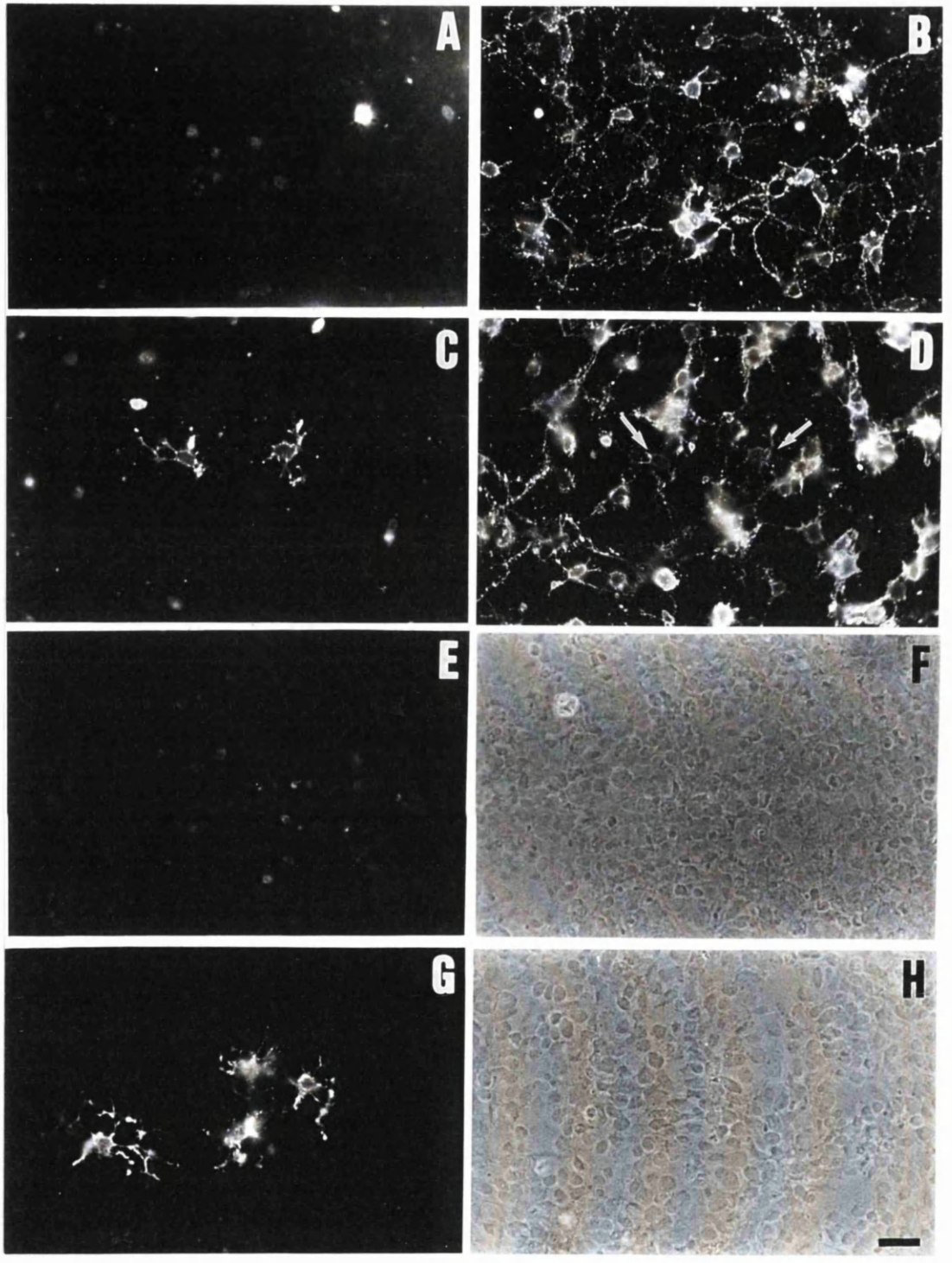


Figure 3.5 PDGFRα⁺ progenitors and GC⁺ oligodendrocytes in long-term, high density cultures of E14 rat dorsal and ventral spinal cord cells E14 spinal cords were divided longitudinally into dorsal and ventral halves, dissociated and cultured in BS medium containing 0.5% FCS and PDGF-AA (10ng/ml). After one day *in vitro*, the cells were fixed and immunolabelled with anti-PDGFRα (A,C) and A2B5 (B,D). No PDGFRα⁺ progenitors were present in the dorsal cultures (A,B) but a small number was present in ventral cultures (C,D, arrows). After 14 days *in vitro*, no GC⁺ oligodendrocytes had developed in dorsal cell cultures (E,F) but there were many in ventral cell cultures (a small group is shown in G and H). Scale bar, 50μm.

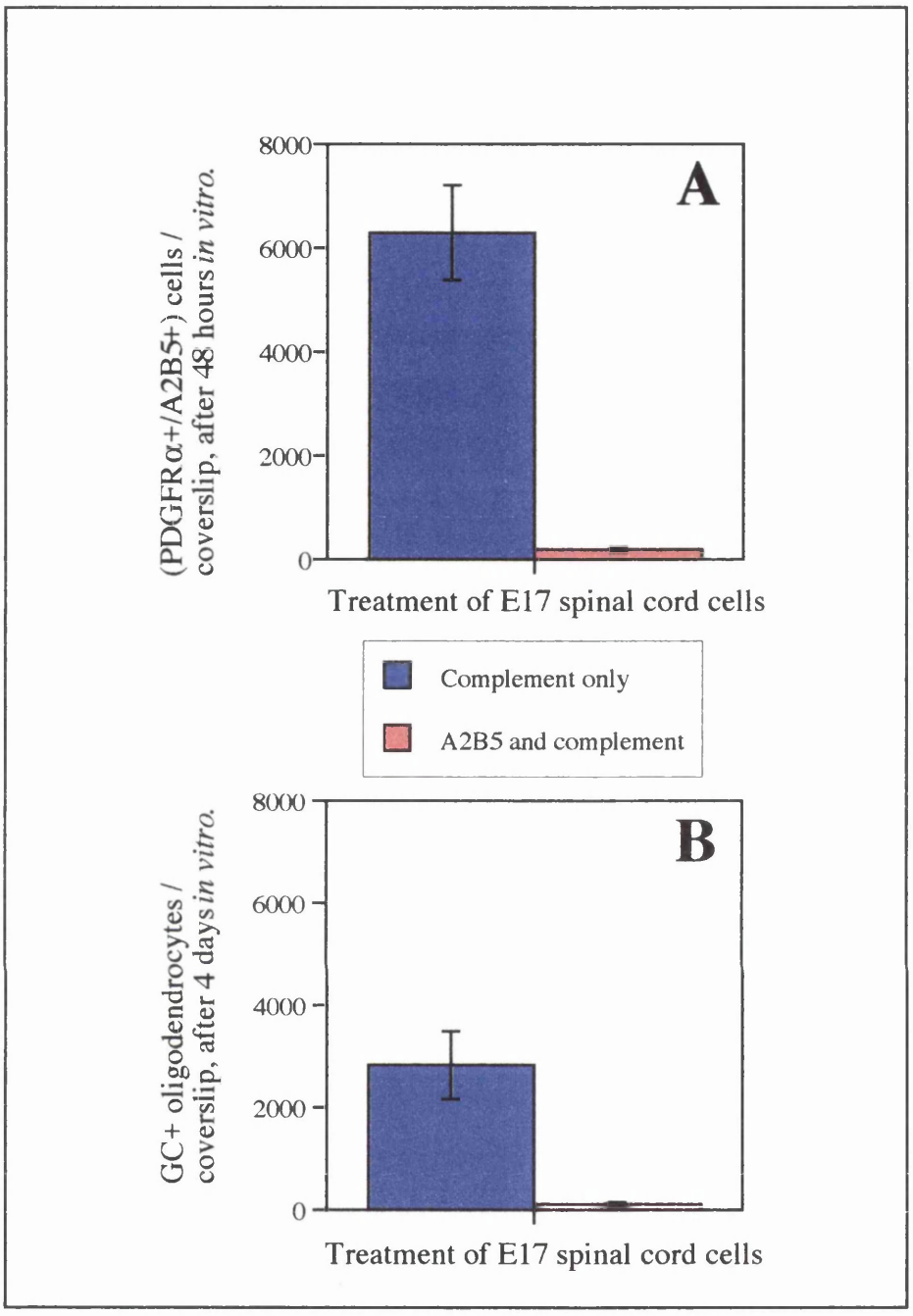


Figure 3.6 Antibody-mediated complement lysis of (A2B5$^+$, PDGFRα$^+$) cells greatly reduces the ability of embryonic rat spinal cord cultures to generate oligodendrocytes *in vitro* E17 rat spinal cord cells were dissociated and the cells subjected to two rounds of incubation with antibody A2B5 plus complement, or with complement alone (see Materials and methods). The cultures were maintained in BS medium containing 0.5% FCS and 10ng/ml PDGF-AA; 24 hours after the second round of complement treatment, the cells were fixed and immunolabelled with anti-PDGFRα and A2B5. Parallel coverslips were cultured for longer, until the equivalent of the day of birth (a total of 4 days *in vitro*), then fixed and immunolabelled with anti-GC to visualise oligodendrocytes. Shown are the numbers of (A2B5$^+$, PDGFRα$^+$) process-bearing cells (A) or GC$^+$ oligodendrocytes (B) in the cultures, means and standard deviations of at least three independent experiments conducted in duplicate or triplicate. Depleting the spinal cord cultures of (A2B5$^+$, PDGFRα$^+$) cells severely diminished the ability of the cultures to generate oligodendrocytes.

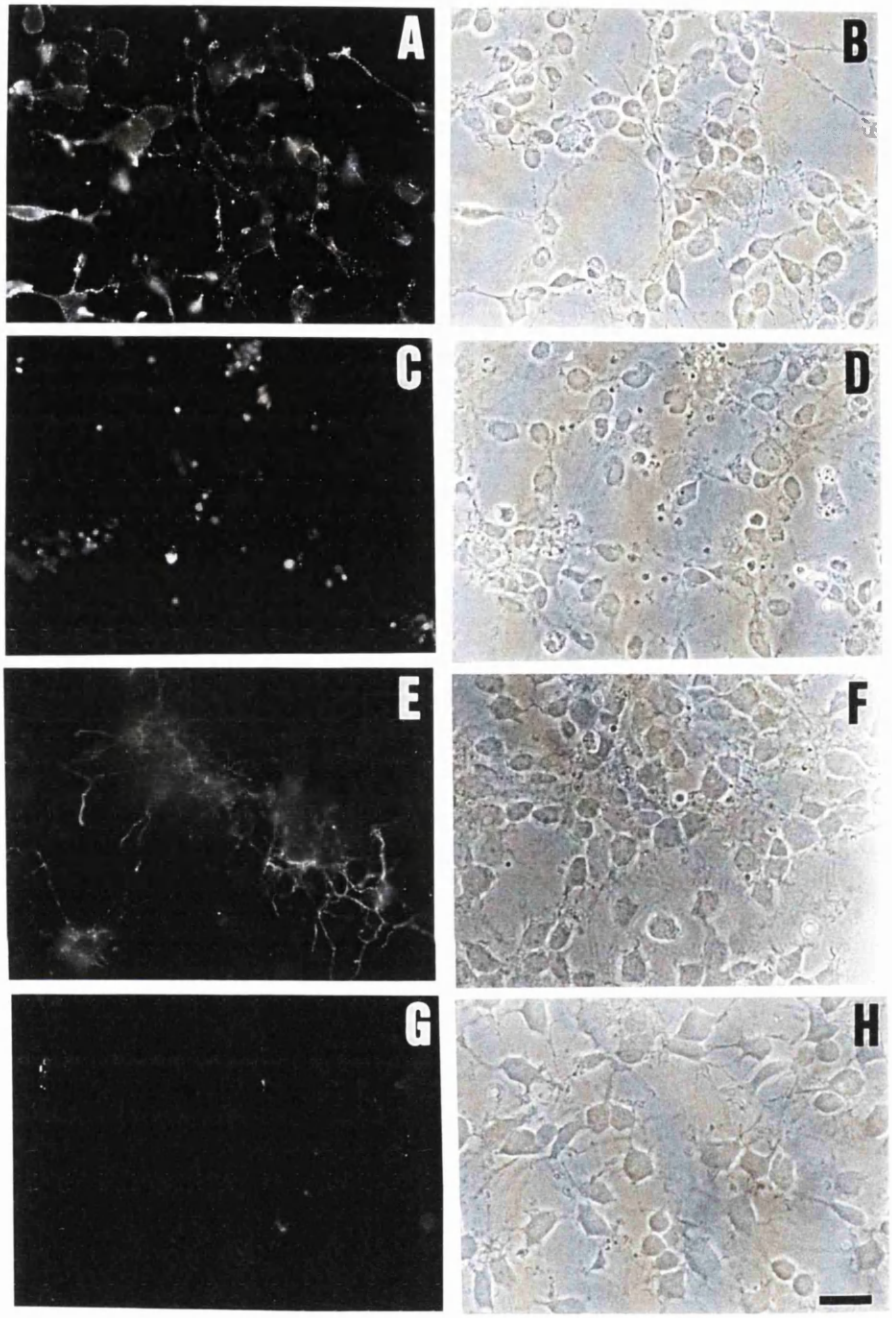


Figure 3.7 Oligodendrocytes develop from (A2B5$^+$, PDGFRα$^+$) progenitor cells in mixed cell cultures of embryonic rat spinal cord Cell suspensions from E17 rat spinal cords were treated with complement alone (A,B) or monoclonal antibody A2B5 and complement (C,D) as described in Materials and methods. At E17 almost all (at least 97%) A2B5$^+$ process-bearing cells in the spinal cord cultures are also PDGFRα$^+$, and vice versa. After 24 hours in culture the cells were fixed, immunolabelled with A2B5 and anti-PDGFRα and photographed in fluorescence and phase contrast optics. The complement-killing procedure removed the great majority (at least 96%) of (A2B5$^+$, PDGFRα$^+$) cells from the cultures (C,D). Parallel coverslips were cultured for 4 days, until the equivalent of the day of birth, then the cells were fixed and immunolabelled with anti-GC. Very few GC$^+$ oligodendrocytes developed in the cultures depleted of (A2B5$^+$, PDGFRα$^+$) cells (G,H) compared to control cultures treated with complement alone (E,F) (see figure 3.6 for further information). Scale bar, 50μm.

Discussion

1. Ventrally derived PDGFRα$^+$ cells are the major or only source of oligodendrocytes in the embryonic spinal cord

The experiments described in this chapter have demonstrated that PDGFRα$^+$ cells in the embryonic spinal cord are oligodendrocyte progenitors and have tested the contribution that these cells make to oligodendrogenesis within the spinal cord. PDGFRα$^+$ cells immunoselected from E17 spinal cord differentiated into oligodendrocytes when cultured in defined low serum medium and into type-2 astrocytes when cultured in medium containing 10% serum; therefore, PDGFRα$^+$ cells from the embryonic spinal cord are oligodendrocyte progenitors with the same antigenic phenotype and differentiation potential as oligodendrocyte progenitors from the perinatal optic nerve (Raff *et al.*, 1983). I have confirmed and extended the data of Warf *et al.* (1991) who demonstrated that at E14, only ventral spinal cord cells can give rise to oligodendrocytes, and that dorsal spinal cord cells acquire this ability only at later ages. I considered the possibility that there may be a population of oligodendrocyte progenitors in the dorsal spinal cord that do not survive under the culture conditions described here. Therefore, I cultured dorsal spinal cord cells at high density and for prolonged periods, to increase the likelihood that the medium would become highly conditioned by survival factors, and would more closely mimic the conditions found *in vivo*. However, even under these conditions dorsal spinal cord cells did not give rise to oligodendrocytes. One could argue that local cell-cell interactions or a gradient of a morphogen secreted by cells of the roof plate, both of which will be disrupted by dissociating the dorsal tissue might be necessary for induction of dorsal oligodendrocyte progenitors. However, explant cultures of dorsal avian tissue, which preserve local cell-cell interactions and contain the roof plate, do not generate oligodendrocytes *in vitro* (Trousse *et al.*, 1995; Pringle *et al.*, 1996). One could further argue that PDGFRα$^+$ oligodendrocyte progenitors in the dorsal ventricular zone arise late and mingle undetected with ventrally derived progenitors. However, Sonic hedgehog protein, which induces PDGFRα$^+$ oligodendrocyte progenitors in intermediate spinal cord explants (Pringle *et al.*, 1996), does not induce PDGFRα$^+$ cells or oligodendrocytes within cultures of E14 dorsal spinal cord cells, suggesting that dorsal cells are not competent to develop into PDGFRα$^+$ progenitors. Therefore, none of the evidence quoted lends any support to the idea that PDGFRα$^+$ oligodendrocyte progenitors are normally generated by dorsal neuroepithelium.

If PDGFRα$^+$ cells are removed by complement lysis from an embryonic spinal cord suspension, very few oligodendrocytes develop. Taken together, my experiments suggest that ventrally derived PDGFRα$^+$ progenitor cells are the only source of oligodendrocytes in the developing spinal cord.

There have been reports that disagree with our proposal that spinal cord oligodendrocytes develop from a ventral source of progenitors and which suggest instead that oligodendrocytes develop from all regions of the neuroepithelium. Hardy and Friedrich (1996) dissected tissue fragments from E10.5 and E12.5 hindbrain, spinal cord and dorsal telencephalon which we know do not contain PDGFRα$^+$ progenitors at these ages (Pringle *et al.*, 1992; Pringle and Richardson, 1993 and this Thesis). The tissue fragments were transplanted into the subventricular zones of the cerebral hemispheres of postnatal mice. Some of the transplanted cells differentiated into oligodendrocytes and Hardy and Friedrich (1996) state that this demonstrates that oligodendrocytes normally develop from all regions of the neuroepithelium. I disagree with this conclusion; I think it more likely that the transplanted tissue fragments contained naive, uncommitted precursor cells that could be re-specified to develop into oligodendrocytes by signals they encountered at their new positions within the host. This is not the same as saying that the donor cells would have generated oligodendrocytes if left undisturbed at their original position in the embryo. There are many reports which demonstrate that the actual fates of neuroepithelial progenitors are only a subset of the full range of fates that the cells can adopt if placed in novel neural environments (see for example: Yamada *et al.*, 1991; Brustle *et al.*, 1995; Fishell, 1995), this issue is discussed more thoroughly in Chapter Four.

Cameron-Curry and Le Douarin (1995) grafted dorsal quail neural tube onto the ventral neural tube of a chick host and demonstrated that some of the oligodendrocytes that subsequently developed in the resultant chimeric animal were of dorsal (quail) origin. Cameron-Curry and Le Douarin (1995) concluded from these experiments that oligodendrocyte progenitors originate in both the dorsal and ventral halves of the spinal cord. We believe that this is a misleading conclusion due to an error of interpretation on the part of Cameron-Curry and Le Douarin and the nature of the grafting experiments. Cameron-Curry and Le Douarin (1995) define their grafts as 'dorsal' with reference to the amount of ependymal zone that the graft contributes to at the end of the experiment; however, much of the ependymal zone regresses during development during a process known as obliteration (Bohme *et al.*, 1988), leaving only the ventral-most-one-fifth in the chicken at E12 (see Richardson *et al.*, 1997). Cameron-Curry and Le Douarin (1995) define a graft that occupies the top half of the remaining ependymal zone at E12 as 'dorsal', whereas it is in fact very ventral tissue. Grafts that descend to this level at E12 would have included the ventral foci of donor quail PDGFRα$^+$ progenitors; that

they produce oligodendrocytes is therefore no evidence for an alternative dorsal oligodendrocyte lineage. The interpretation of these experiments is also complicated by the fact that oligodendrocyte progenitors can migrate considerable distances along the longitudinal axis of the spinal cord *in vivo* (Pringle *et al.*, 1998). This can mean that oligodendrocytes derived from a length of the graft that includes the ventral origin of oligodendrocytes can move into areas of the spinal cord where the graft contains only dorsal tissue, giving the incorrect impression that they were derived from dorsal spinal cord. Pringle *et al.* (1998) have carried out experiments similar to those of Cameron-Curry and Le Douarin (1995), ensuring that they analyse only grafts which remain strictly dorsal along their whole length. These grafts do not give rise to oligodendrocytes although they do generate dorsal white-matter astrocytes (Pringle *et al.*, 1998), providing further evidence that oligodendrocytes do not normally develop from dorsal progenitors. Recently, Chandran *et al.* (1998) have reported that E14 dorsal spinal cord cells can produce A2B5$^+$ presumptive oligodendrocyte progenitors and subsequently, oligodendrocytes, if they are exposed to EGF and bFGF *in vitro*. It would be interesting to repeat this experiment and test whether the progenitors are PDGFRα^+. I believe this type of experiment reveals the plasticity of early neuroepithelial cells, rather than their actual fate *in vivo* (a point discussed further in Chapter four).

2. Are PDGFRα^+ progenitors dedicated solely to the production of oligodendrocytes?

The experiments described above suggest that all oligodendrocytes in the spinal cord develop from PDGFRα^+ progenitors; however, they do not absolutely rule out the possibility that PDGFRα^+ progenitors might give rise *in vivo* to cells other than oligodendrocytes. Perhaps, if I had immunoselected the earliest PDGFRα^+ cells from E14 spinal cord, I would have seen them differentiate into cells other than oligodendrocytes or type-2 astrocytes *in vitro*. In general, the fate adopted by progenitor cells depends on the signals that they encounter in their environment; it remains possible that PDGFRα^+ progenitors *in vivo* are exposed to signalling molecules other than those present in my culture system. Therefore, I can not state unequivocally that PDGFRα^+ progenitors do not generate cells other than oligodendrocytes *in vivo*, although I believe this to be unlikely. BS medium does support the short term survival of neurons (N. Pringle, personal communication and my observations) and astrocytes (Raff *et al.*, 1983 and my observations); therefore, I would expect to detect any neurons or astrocytes that could differentiate from E17 PDGFRα^+ cells under these conditions.

3. Are oligodendrocytes a heterogenous class of cells?

Mature oligodendrocytes differ in their morphology and biochemistry. Oligodendrocytes tend to myelinate either one or a small number of large-diameter axons or a greater number of smaller axons (Bjartmar *et al.*, 1994). Oligodendrocytes which ensheath larger axons do not express carbonic anhydrase, whereas those that myelinate many small axons do express this enzyme (Butt *et al.*, 1995). This heterogeneity in morphology and biochemistry could be because there are different subtypes of oligodendrocytes, each derived from a different class of progenitor or, alternatively, it could reflect different environmental influences on a single oligodendrocyte lineage. Our experiments support the latter possibility. This view is also supported by the observation that oligodendrocyte progenitors taken from rat optic nerves, which contain only small-bore axons, can myelinate both large and small-bore axons following transplantation into the rat spinal cord (Fanarraga *et al.*, 1998). This suggests that oligodendrocytes are not intrinsically 'programmed' to myelinate a particular axon type. The morphological and biochemical differences between oligodendrocytes are of potential clinical importance as oligodendrocytes that myelinate larger axons seem more resistant to certain disorders of myelin lipid metabolism (Bjartmar *et al.*, 1994).

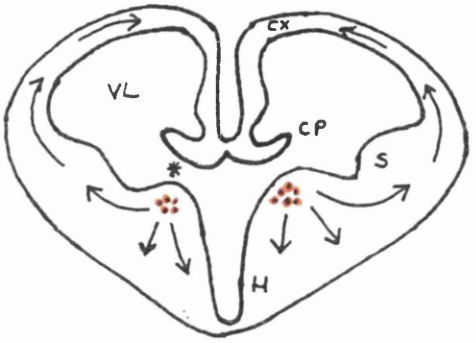
Chapter Four

PDGFRα expressing cells in the embryonic brain

Chapter Three has described work which demonstrates that PDGFRα$^+$ cells in the embryonic spinal cord are oligodendrocyte progenitors and suggests that these cells are the major or only source of oligodendrocytes in the developing spinal cord. This chapter describes similar work that investigates oligodendrocyte development in the embryonic brain. The adult brain has a much more complex topology than the spinal cord and differs in the types of neurons (and possibly glia) that it contains; nevertheless, the brain develops from the same embryonic neural tube that gives rise to the spinal cord, so it is quite possible that its development has fundamental similarities to that of the spinal cord.

There are several theories as to the source of brain oligodendrocyte progenitors. One hypothesis is that oligodendrocyte progenitors originate from cells present throughout the germinal subventricular zones (SVZ) that surround the ventricles of the developing brain. This proposal results from immunohistochemical studies of sections of brain tissue and clonal analysis of brain cells *in vitro*. LeVine and Goldman (1988b) carried out immunohistochemical studies of the embryonic brain at sequential developmental stages using anti-GD3, anti-carbonic anhydrase and anti-GC. They concluded that oligodendrocyte progenitors in the forebrain and cerebellum arise within the SVZ that surrounds the lateral and fourth ventricles. Reynolds and Wilkin (1988) also used anti-GD3 and anti-GC to study the development of the oligodendrocyte lineage within the cerebellum and they too concluded that these cells originate within the SVZ of the fourth ventricle. There are two reasons to view these immunohistochemical studies with a degree of caution; first, the studies were carried out on late embryonic brains and so might not have identified the earliest oligodendrocyte progenitors and their site or sites of origin. Second, we now know that not all the antibodies used are specific to cells of the oligodendrocyte lineage. Anti-GD3 also labels microglia and subpopulations of both developing and mature neurons and astrocytes (Goldman and Reynolds, 1996). Anti-carbonic anhydrase labels astrocytes as well as cells of the oligodendrocyte lineage (Cammer and Tansey, 1988).

In vitro clonal analyses of embryonic SVZ cells suggest that stem cells within the embryonic brain SVZ give rise to oligodendrocyte progenitors. For example, Davis and Temple (1994) cultured single E12 and E14 cerebral cortical cells and demonstrated that a small number can behave like stem cells *in vitro*, giving rise to clones containing neurons, astrocytes, oligodendrocytes and copies of themselves. As with all *in vitro* studies, the stem cell behaviour of some cortical cells *in vitro* needs to be demonstrated *in vivo* before it can be accepted as a true reflection of what happens in the developing brain.



Key

CX, cerebral cortex
VL, lateral ventricle
CP, choroid plexus
S, striatum
H, hypothalamus

The origin and accumulation of PDGFRα⁺ cells within the developing brain Diagram of a coronal section through the rat forebrain: PDGFRα⁺ cells are first seen in the rat brain at E14 (red dots), present as a small cluster close to the foramen of Monro (*), at the junction of the lateral and third ventricles. Subsequently, PDGFRα⁺ cells are seen in the hypothalamus and striatum and, by E18, in the lateral-most tips of the sub cortical white matter. By P0, PDGFRα⁺ cells are seen throughout the brain, including the cerebral cortex. We propose that the changing distribution of PDGFRα⁺ cells is due to the cells migrating away from their ventral origin and proliferating to supply much or all of the developing brain with oligodendrocyte progenitors (see also Pringle *et al.*, 1992).

Pringle and Richardson (1993) propose that oligodendrocytes develop from PDGFRα$^+$ cells within the embryonic brain. Pringle and Richardson (1993) describe how columns of PDGFRα$^+$ cells extend from the spinal cord into the hindbrain and midbrain, stopping at the mesencephalic/diencephalic boundary. Pringle and Richardson (1993) also show that, in the E14 rat forebrain, there is a discrete group of PDGFRα$^+$ cells in a small region of the SVZ of the ventral diencephalon close to the foramen of Monro, at the junction of the lateral and third ventricles, defined as hypothalamic epithelium region H1a by Altman and Bayer (1995). As development progresses these PDGFRα$^+$ cells increase in number and are found in other regions of the developing forebrain; for example, at E16, there are many PDGFRα$^+$ cells in the thalamus and hypothalamus but almost none in the cerebral cortex. At E18, there are PDGFRα$^+$ cells at the lateral edges of the cerebral cortex and on the day of birth, PDGFRα$^+$ cells are found throughout the forebrain, including the most medial parts of the cerebral cortex (Pringle *et al.*, 1992; Pringle *et al.*, 1992). The changing distribution of PDGFRα$^+$ cells suggests that, as in the spinal cord, these cells originate in the ventral half of the forebrain and migrate throughout the forebrain, including the most dorsal parts of the cerebral cortex.

This chapter investigates whether the PDGFRα$^+$ cells in the embryonic forebrain are oligodendrocyte progenitors and, if so, whether they are the major source of oligodendrocytes within the developing forebrain or whether other precursor cell types, in other regions of the forebrain SVZ, also generate oligodendrocytes. First, I describe experiments that demonstrate that, as in the spinal cord, PDGFRα$^+$ cells in the embryonic brain all differentiate into oligodendrocytes when cultured under appropriate conditions *in vitro*. We conclude that there is a common population of PDGFRα$^+$ oligodendrocyte progenitors along the entire length of the neural tube.

In the second part of this chapter I describe preliminary experiments that investigate whether PDGFRα$^+$ progenitors are the only source of oligodendrocytes in the developing forebrain. I demonstrate that only forebrain tissue that contains PDGFRα$^+$ progenitors can give rise to oligodendrocytes *in vitro*; for example, E15 cerebral cortex, which does not contain PDGFRα$^+$ cells, does not give rise to oligodendrocytes *in vitro*, whereas E17 cerebral cortex, which does contain PDGFRα$^+$ cells, does generate oligodendrocytes. This supports the idea that forebrain oligodendrocytes develop from a ventral source of PDGFRα$^+$ progenitors (Pringle and Richardson, 1993). This proposal is controversial as there have been reports of E14 cerebral cortex, which does not contain PDGFRα$^+$ progenitors, generating oligodendrocytes *in vitro* if exposed to certain factors such as medium conditioned by astrocytes and meningeal cells or containing basic fibroblast growth factor (Davis and Temple, 1994; Ghosh and Greenberg, 1995; Qian *et al.*, 1997).

I have confirmed and extended these *in vitro* studies by showing that bFGF and Sonic hedgehog protein induce the development of PDGFRα$^+$ progenitors in E14 cerebral cortical cultures and the subsequent development of oligodendrocytes. Therefore, it is clear that some E14 cortical cells differentiate into oligodendrocytes of the PDGFRα$^+$ lineage *in vitro* if exposed to appropriate inducing signals, although it is unclear whether this oligodendrogenic potential is actually realised *in vivo*.

Results

1. PDGFRα$^+$ cells in the embryonic brain are oligodendrocyte progenitors

I used polyclonal anti-PDGFRα serum (number 3979, Fretto *et al.*, 1993; Nishiyama *et al.*, 1996) to immunoselect PDGFRα$^+$ cells from E17 and E19 whole brain and E19 cerebral cortex in order to test their differentiation potential *in vitro*. In each of these three experiments, 98-100% of the immunoselected cells were process-bearing PDGFRα$^+$ cells; sometimes the cultures were contaminated with small numbers of macrophages. The process-bearing PDGFRα$^+$ cells were bipolar with small cell bodies, and were (A2B5$^+$, NG2$^+$, O4$^-$, GC$^-$ and GFAP$^-$). PDGFRα$^+$ cells immunoselected from E19 whole brain and immunolabelled with anti-NG2 are shown in Figure 4.1A. The immunoselected cells were cultured overnight in defined BS medium containing 0.5% FCS and PDGF-AA (10ng/ml), gently washed and the medium replaced by defined medium containing 0.5% FCS alone. After 36 hours in growth factor-free medium, the cells developed a more highly branched morphology and many were O4$^+$, a characteristic of late oligodendrocyte progenitors (Sommer and Schachner, 1981; Bansal and Pfeiffer, 1992). After 48 hours *in vitro*, almost all (>98%) of the immunoselected cells had differentiated into GC$^+$ oligodendrocytes (Figure 4.1B). If the immunoselected cells were instead cultured in defined medium containing 10% FCS, the cells differentiated within three days into flat, stellate (GFAP$^+$, A2B5$^+$) type-2 astrocytes (Figures 4.1C and 4.1D).

These experiments demonstrate that PDGFRα$^+$ cells from the embryonic brain are oligodendrocyte progenitors, and that they have the same antigenic phenotype and differentiation potential *in vitro* as oligodendrocyte progenitors from the perinatal optic nerve (Raff *et al.*, 1983) and embryonic spinal cord (Hall *et al.*, 1996 and Chapter Three of this Thesis).

2. The presence of (PDGFRα$^+$, A2B5$^+$) cells in the cerebral cortex and ventral diencephalon predicts the ability of these regions to give rise to oligodendrocytes *in vitro*

In situ hybridisation studies have demonstrated that at E15, PDGFRα$^+$ oligodendrocyte progenitors are present in the ventral diencephalon of the rat brain beneath the foramen of Monro, at the junction between the lateral and third ventricles (Pringle and Richardson, 1993). At E15, PDGFRα$^+$ cells are not present in the cerebral cortex; from E17 onwards, increasing numbers of PDGFRα$^+$ cells are present in the cerebral cortex, first at its lateral edges and later in progressively more medial regions (Pringle *et al.*, 1992). By P0, PDGFRα$^+$ cells are numerous and uniformly distributed throughout the cerebral cortex and other regions of the brain (Pringle *et al.*, 1992). To test whether the oligodendrogenic capacity of different forebrain regions reflects the presence of PDGFRα$^+$ progenitor cells as revealed by *in situ* hybridisation, I dissociated and cultured cortical and ventral diencephalic tissue at different embryonic ages and identified (PDGFRα$^+$, A2B5$^+$) oligodendrocyte progenitors by immunolabelling after 16 hours *in vitro*. Other *in vitro* experiments (not shown) had demonstrated that GC$^+$ oligodendrocytes begin to appear in significant numbers in the cortex and diencephalon at P1. Therefore, in parallel, I maintained forebrain cell cultures until at least the equivalent of P1, when I immunolabelled them with anti-GC to identify oligodendrocytes. The results of these experiments are presented in Figure 4.2 and 4.3. At E15, small numbers of PDGFRα$^+$ cells were present in the cultures of ventral diencephalon, and oligodendrocytes developed in these cultures. However, PDGFRα$^+$ progenitors were absent from E15 cortical tissue, which did not give rise to oligodendrocytes during the eight days of culture. At E17 and later ages, PDGFRα$^+$ cells were present in the cerebral cortex and this tissue was now able to produce oligodendrocytes *in vitro*; therefore, the ability of embryonic cortical or ventral diencephalic tissue to give rise to oligodendrocytes correlates with the presence of PDGFRα$^+$ progenitors, supporting the hypothesis that brain oligodendrocytes develop from ventrally derived PDGFRα$^+$ progenitor cells.

Unfortunately, I could not use A2B5 and complement to lyse the PDGFRα$^+$ cells to test whether there are other, PDGFRα-negative oligodendrocyte lineages in the embryonic brain, as I did with embryonic spinal cord cells (see Chapter Three). This is because (A2B5$^+$, PDGFRα$^-$) cells, presumably undifferentiated neurons (Eisenbarth *et al.*, 1979), persist in the brain until at least E19; therefore, complement and A2B5 would lyse cells other than PDGFRα$^+$ progenitors and so invalidate the experiment.

3. PDGFRα$^+$ oligodendrocyte progenitors are induced in E14 cerebral cortical cell cultures exposed to basic fibroblast growth factor (bFGF)

The experiments above suggest that if a region of embryonic forebrain lacks PDGFRα$^+$ cells at the time it is isolated, it does not generate oligodendrocytes *in vitro*. However, there have been reports that E14 cerebral cortex, which we have shown does not contain PDGFRα$^+$ cells (Pringle and Richardson, 1993 and my observations), can nevertheless generate oligodendrocytes if exposed to bFGF *in vitro* (Ghosh and Greenberg, 1995; Qian *et al.*, 1997). To confirm this result and to investigate the oligodendrocyte progenitor type involved, I dissociated and cultured E14 cortical cells in BS medium containing 0.5% serum with and without PDGF-AA (10ng/ml) or bFGF (40ng/ml). I immunolabelled the cultures after 16 hours *in vitro* with anti-PDGFRα and A2B5 to identify PDGFRα$^+$ oligodendrocyte progenitors. None of the cultures contained PDGFRα$^+$ progenitors; the majority of the cells were (A2B5$^+$, PDGFRα$^-$), these were presumably neurons and their progenitors (Eisenbarth *et al.*, 1979). I immunolabelled parallel cultures at regular intervals to test for the development of (PDGFRα$^+$, A2B5$^+$) oligodendrocyte progenitors *in vitro*, these never developed in the growth factor-free cultures rarely, one or two were seen in PDGF-AA treated cultures. However, in the cultures containing bFGF, many clusters of (PDGFRα$^+$, A2B5$^+$) cells were seen after three days *in vitro* (Figure 4.4). These (PDGFRα$^+$, A2B5$^+$) cells subsequently increased greatly in number. Not all the A2B5$^+$ cells were induced to express PDGFRα$^+$ in the presence of bFGF; (A2B5$^+$, PDGFRα$^-$) cells were still present in the bFGF-treated cultures after three days *in vitro*, presumably these were neuronal progenitors (Eisenbarth *et al.*, 1979). I immunolabelled all the cultures after 8 or 10 days *in vitro* (the equivalent of P1 and P3 *in vivo*) with anti-GC to identify oligodendrocytes. No oligodendrocytes developed from E14 cortical cells cultured in medium containing 0.5% serum alone or with PDGF-AA (10ng/ml), but hundreds developed in cultures exposed to bFGF (40ng/ml) (Figure 4.5G). These experiments confirm the findings of Ghosh and Greenberg (1995) and Qian *et al.* (1997), and strongly suggest that the oligodendrocytes that develop from E14 cortical cells exposed to bFGF *in vitro* are derived from PDGFRα$^+$ progenitors.

4. Basic fibroblast growth factor appears to stimulate the proliferation of an oligodendrocyte 'pre-progenitor' cell *in vitro*

As the (PDGFRα$^+$, A2B5$^+$) cells appeared on the third day *in vitro* in bFGF-treated cortical cultures and were present in clusters, we propose that they developed from a 'pre-progenitor' cell that had undergone several mitotic divisions before giving rise to dedicated oligodendrocyte progenitors. Basic FGF could be an inducing factor, a survival factor or a mitogen for this 'pre-progenitor' cell or it could have an indirect effect through its influence on some other cell type in the culture. As a preliminary step towards distinguishing among these possibilities, I quantified the mitogenic effect of bFGF on E14 cortical cells as follows. I treated cultures of E14 cortical cells with bFGF, PDGF-AA, Shh or no added factors for various times after plating, then added BrdU to the cultures for 8 hours before fixing the cells and labelling for BrdU. bFGF had a dramatic mitogenic effect on many cells in the E14 cortical cultures; cell number in bFGF-treated cultures was still increasing after 60 hours *in vitro*, whereas it had plateaued in cultures treated with PDGF-AA, Shh or 0.5% serum alone (Figure 4.5). Only some of the dividing cells in the bFGF treated cultures were PDGFRα$^+$. After six days *in vitro* the bFGF-treated cells had formed a confluent culture, several cell layers thick, covered with a lawn of (PDGFRα$^+$, A2B5$^+$) cells (Figure 4.5B). After eight days *in vitro*, many GC$^+$ oligodendrocytes had developed in the bFGF treated cultures (Figure 4.6). This experiment suggests that bFGF could be a mitogen for the oligodendrocyte 'pre-progenitor' cell as well as the committed oligodendrocyte progenitor; alternatively, bFGF could be a mitogen for another cell type that is essential for the survival and division of the 'pre-progenitor' cell.

5. Epidermal growth factor (EGF) and Neu differentiation factor (NDF) do not stimulate oligodendrocyte development from E14 cerebral cortical cells *in vitro*

Reynolds and Weiss (1996) have reported that EGF can stimulate the development of neurons, astrocytes and oligodendrocytes from a multipotential stem cell isolated from the embryonic striatum. I tested whether such an EGF-responsive cell, capable of generating oligodendrocytes, exists in the E14 cerebral cortex. No PDGFRα$^+$ progenitors or GC$^+$ oligodendrocytes developed when E14 cortical cells were cultured in the presence of EGF (20ng/ml) until the equivalent of P2 *in vivo*. This experiment suggests that at E14, EGF-responsive stem cells that can generate oligodendrocytes are only present in the ventral, striatal region of the developing forebrain and not in the cerebral cortex.

Neu differentiation factor (NDF) is a factor known to induce Schwann cell (Dong *et al.*, 1995) and oligodendrocyte differentiation (Canoll *et al.*, 1996). I found that PDGFRα$^+$ progenitors or oligodendrocytes did not develop from E14 cortical cultures that had been treated with NDF (NDFß3, 10ng/ml; a gift from Rhona Mirsky, University College London).

6. Sonic hedgehog protein can stimulate PDGFRα$^+$ oligodendrocyte progenitors to develop from E14 cerebral cortical cells *in vitro*

Sonic hedgehog protein (Shh) induces ventral cell types, including oligodendrocytes, in the embryonic spinal cord (Yamada *et al.*, 1991; Pringle *et al.*, 1996). Sonic hedgehog secreted by the ventral midline cells of the brain also induces ventral neurons in the hindbrain, midbrain and diencephalon and might act at a distance to induce ventral telencephalic neurons (Ericson *et al.*, 1995). Whether Shh induces oligodendrocytes in the brain is as yet unknown. I tested whether dorsal telencephalic (cerebral cortex) cells at E14 are able to generate oligodendrocytes in response to Shh. Cortical cell cultures exposed at the beginning of the culture period to 7 x 10^{-9} M recombinant Shh protein did generate (PDGFRα$^+$, A2B5$^+$) cells after three days *in vitro*. (Figures 4.6 and 4.7). There were fewer, smaller clusters of PDGFRα$^+$ cells in these cultures than were seen in the presence of bFGF, suggesting that Shh might be acting on a different, rarer cell type. GC$^+$ oligodendrocytes subsequently developed in the Shh-treated cultures (Figures 4.6 and 4.7). This experiment suggests that at E14, some cerebral cortical cells have not yet committed to a dorsal forebrain fate. This is in contrast to cells of the dorsal spinal cord at E14, which were unable to generate oligodendrocytes *in vitro* in response to Shh and so are restricted in their possible fates (see Chapter Three).

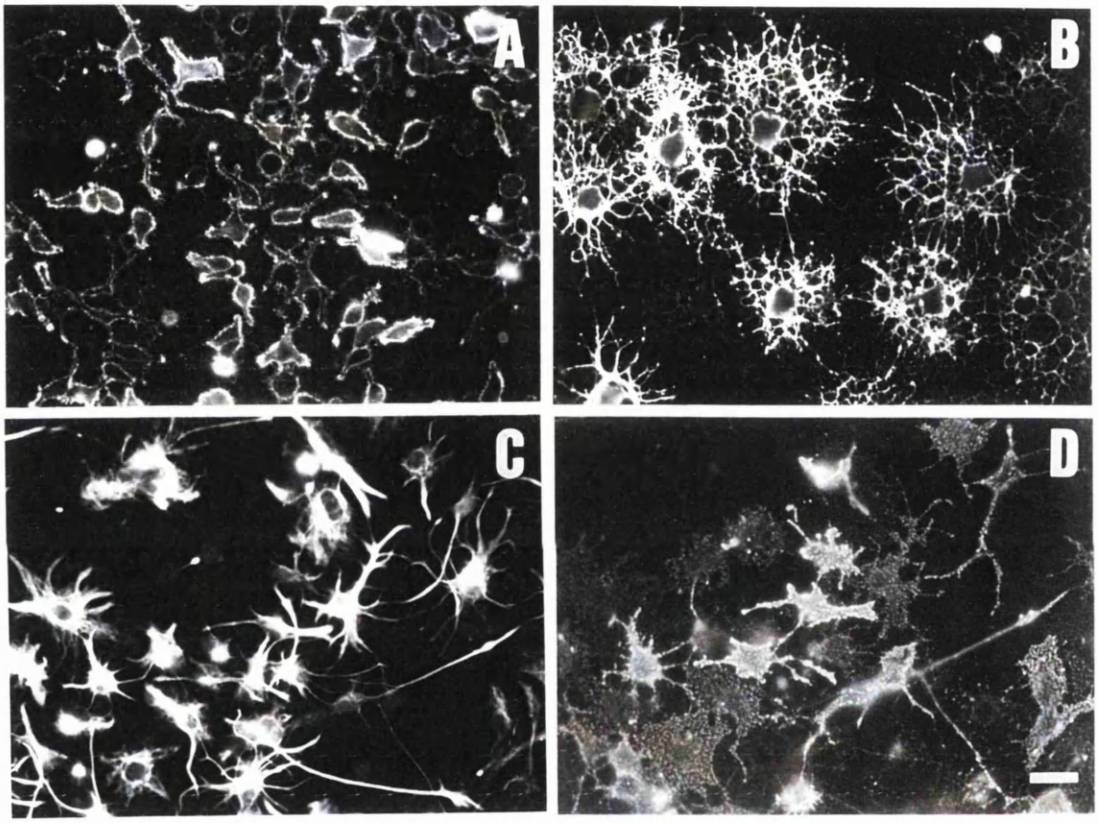
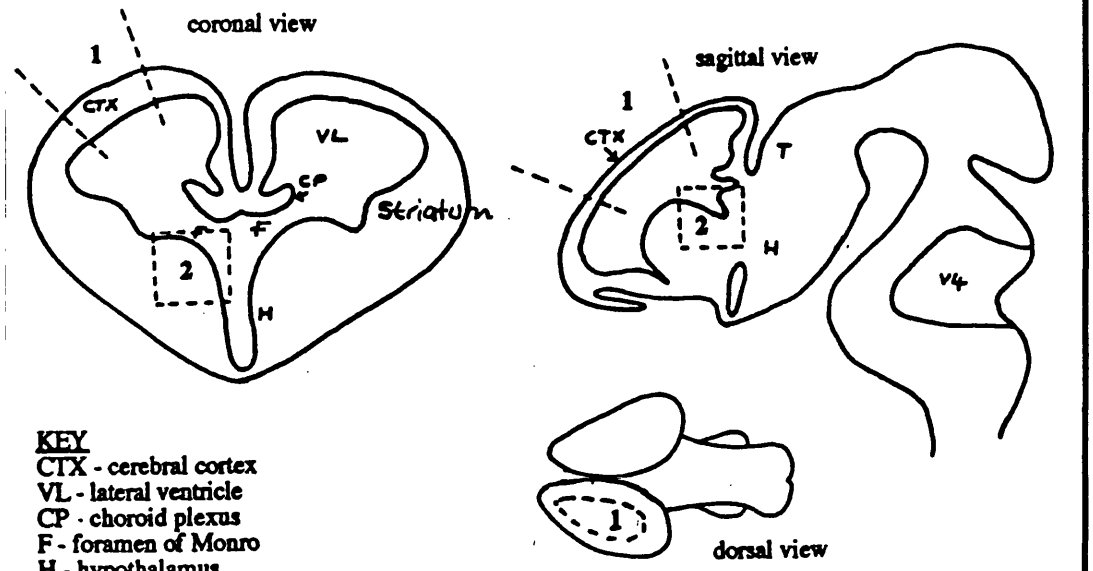


Figure 4.1 PDGFRα⁺ cells, immunoselected from embryonic rat brain with an anti-PDGFRα antibody, differentiate into GC⁺ oligodendrocytes or (GFAP⁺, A2B5⁺) astrocytes Cell suspensions of E19 brain were immunoselected as described in Materials and methods and cultured on glass coverslips in BS medium containing 0.5% FCS and 10ng/ml PDGF-AA. After 24 hours, the cells were fixed and immunolabelled with anti-PDGFRα, A2B5, anti-NG2, O4 or anti-GC. More than 99% of the process-bearing cells were (PDGFRα⁺, A2B5⁺, NG2⁺, O4⁻, GC⁻). Panel A shows these cells labelled with anti-NG2. Some coverslips were transferred after 24 hours in culture into BS medium containing 0.5% FCS without PDGF-AA, or into BS medium with 10% FCS. After culturing for a further 2-3 days, the cells were fixed and immunolabelled with anti-GC or double-labelled with anti-GFAP and A2B5. Without PDGF-AA but in the presence of 0.5% FCS, more than 99% of the cells became GC⁺ oligodendrocytes (B). In the presence of 10% FCS, more than 99% of the cells became GFAP⁺ astrocytes (C), the majority of which also labelled with A2B5 (D). Scale bar, 50 μm.

A



KEY
CTX - cerebral cortex
VL - lateral ventricle
CP - choroid plexus
F - foramen of Monro
H - hypothalamus
T -thalamus
1 - region dissected as 'cerebral cortex'
2 - region dissected as 'ventral diencephalon'

B

AGE	(PDGFRα⁺, A2B5⁺) cells per coverslip after 16 hrs *in vitro*		GC⁺ oligodendrocytes per coverslip at the equivalent of P1	
	cerebral cortex	ventral diencephalon	cerebral cortex	ventral diencephalon
E15	0	24±10	0	546±128
E17	56±51	270±107	56±33	309±55
E19	187±36	361±48	44±17	85±11

Figure 4.2 The number of (A2B5⁺, PDGFRα⁺) oligodendrocyte progenitors and oligodendrocytes in the embryonic rat forebrain Panel A shows the regions of the cerebral cortex and ventral diencephalon that were dissected, dissociated and cultured during this experiment. The cells (75,000 per coverslip) were cultured in BS medium containing 0.5% FCS and 10ng/ml PDGF-AA as described in Materials and methods. The table in panel B shows the number of (A2B5⁺, PDGFRα⁺) cells within these regions from animals of different ages and the number of GC⁺ oligodendrocytes present at the equivalent of P1 (mean ± SD of 3 experiments, each conducted in triplicate). E15 cortex which does not contain (A2B5⁺, PDGFRα⁺) cells does not give rise to oligodendrocytes whereas E15 ventral diencephalon which does contain a small number of (A2B5⁺, PDGFRα⁺) cells, does. At later ages, both regions of the forebrain contain (A2B5⁺, PDGFRα⁺) cells and both can now give rise to oligodendrocytes.

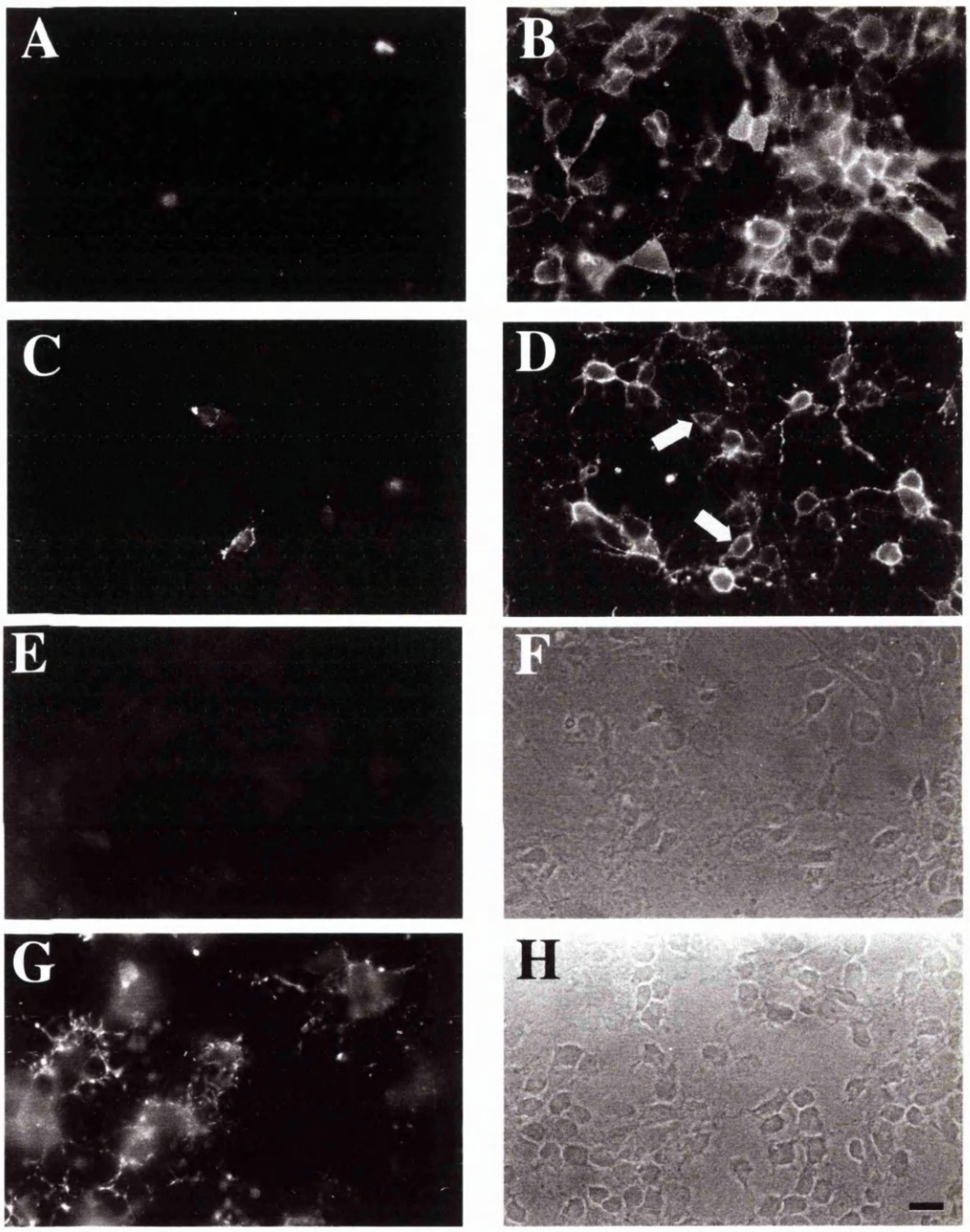


Figure 4.3 (A2B5$^+$, PDGFRα$^+$) oligodendrocyte progenitors and oligodendrocytes in the embryonic rat forebrain E15 cerebral cortex or ventral diencephalon was dissected and dissociated as described in Materials and methods. 75,000 viable cells per coverslip were cultured in BS medium containing 0.5% FCS and 10ng/ml PDGF-AA. After overnight culture, the cells were immunolabelled with anti-PDGFRα (A,C) and A2B5 (B,D). After eight days in culture, the cells were immunolabelled with anti-GC to identify oligodendrocytes (E,G). (A2B5$^+$, PDGFRα$^+$) cells were not present in cerebral cortical cultures (A,B) and no oligodendrocytes developed subsequently in these cultures (E,F). Small numbers of (A2B5$^+$, PDGFRα$^+$) cells were present in ventral diencephalon cultures (C,D, arrows), and oligodendrocytes did develop in these cultures (G,H). Scale bar, 25μm.

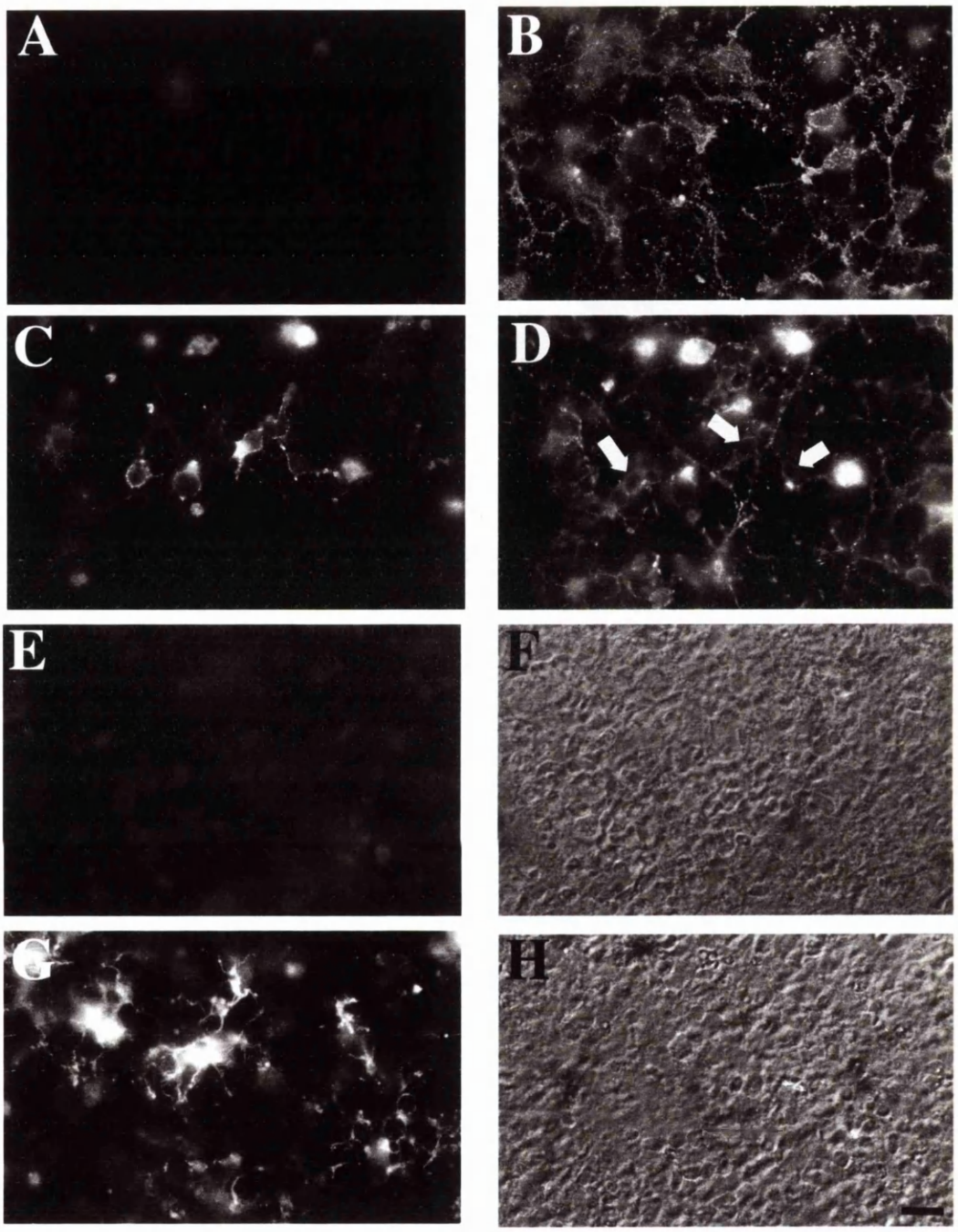
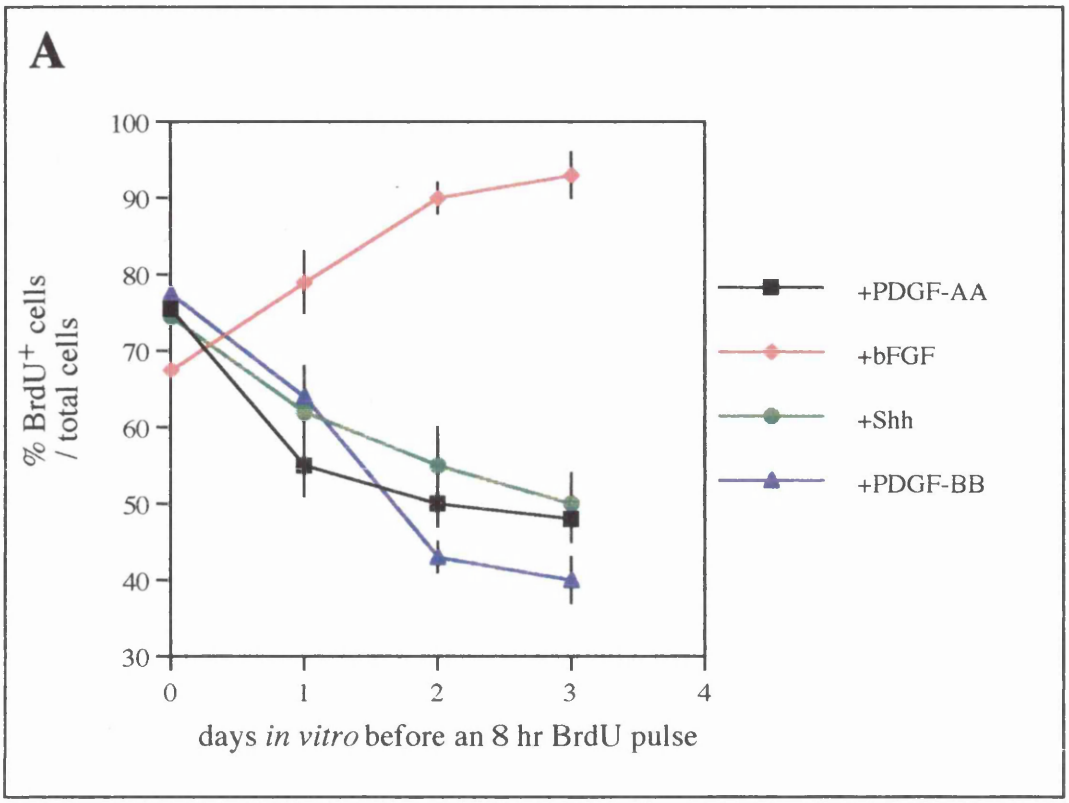


Figure 4.4 PDGFRα⁺ progenitors and GC⁺ oligodendrocytes are induced in E14 rat cerebral cortical cell cultures exposed to basic fibroblast growth factor (bFGF) 50,000 viable cells were plated on poly-D-lysine coated coverslips and cultured in BS medium with 0.5% FCS and bFGF (40ng/ml), the medium was replaced every two days. Each day, cultures were immunolabelled with anti-PDGFRα (A,C) and A2B5 (B,D). Groups of (PDGFRα⁺, A2B5⁺) cells appeared after 3 days *in vitro* in cultures treated with bFGF (C,D, arrows) but were not seen in cultures treated with PDGF-AA (10ng/ml, A,B) or 0.5% FCS alone (data not shown). After 8 days *in vitro*, the cultures were stained with anti-GC; GC⁺ oligodendrocytes had developed in cultures treated with bFGF (G,H) but were not present in cultures treated with PDGF-AA (E,F) or serum alone (data not shown). Scale bar, 25μm.



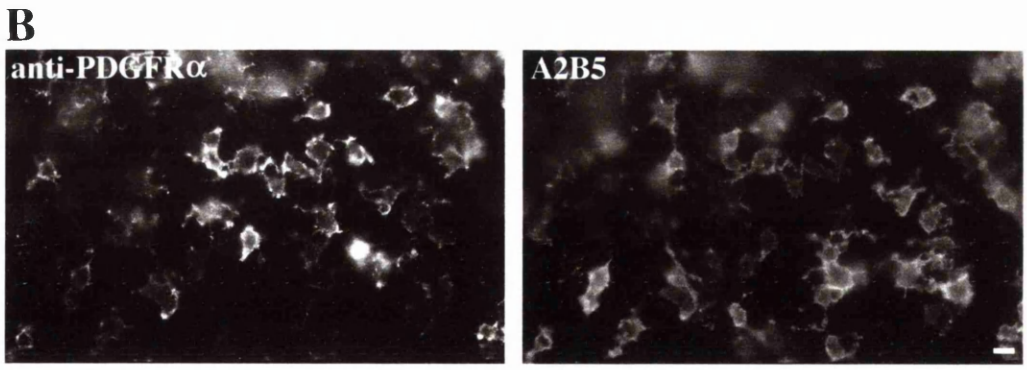


Figure 4.5 Basic fibroblast growth factor (bFGF) is a mitogen for many cells in E14 cerebral cortical cultures 50,000 viable cortical cells were cultured in BS medium containing 0.5% FCS and one of the following factors: PDGF-AA (10ng/ml), bFGF (40ng/ml), PDGF-BB (30ng/ml) or purified recombinant Sonic hedgehog protein (7 x 10^{-9} M). The medium plus factors was replaced every second day. The cultures were maintained for 1, 2 or 3 days *in vitro* before being given an 8 hour pulse of BrdU (0.01mM). The cells were then fixed and immunolabelled with anti-BrdU. The results shown in panel A are from two experiments with two coverslips examined at each timepoint. Cells were still dividing after 3 days *in vitro* in the bFGF treated cultures; many of these were (PDGFRα$^+$, A2B5$^+$) cells. Panel B shows the lawn of (PDGFRα$^+$, A2B5$^+$) cells on the surface of bFGF treated cultures after 6 days *in vitro*. Scale bar, 25μm.

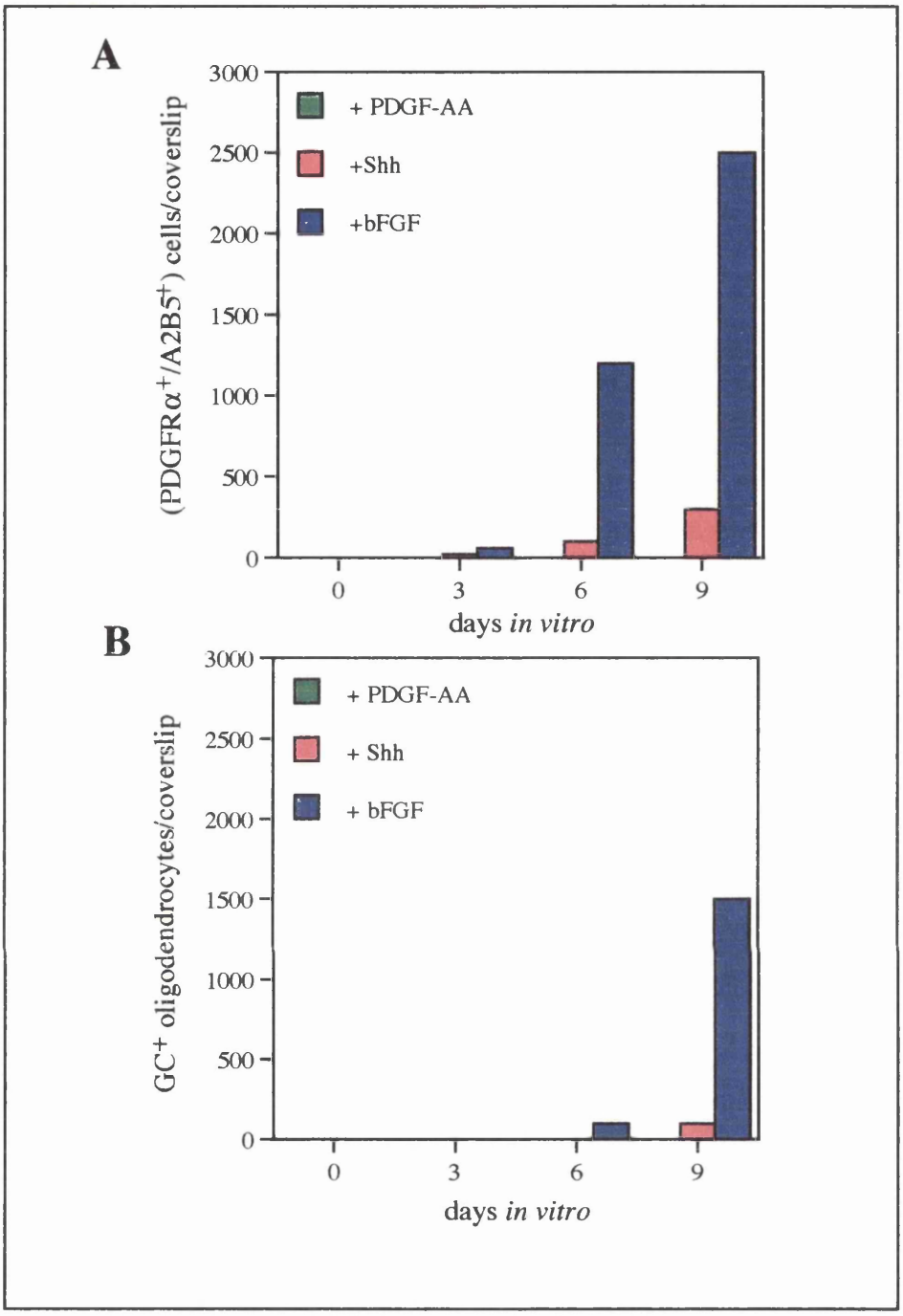


Figure 4.6 PDGFRα⁺ progenitors and GC⁺ oligodendrocytes develop in cultures of E14 cerebral cortical cells treated with recombinant Sonic hedgehog protein 50,000 viable cells were plated on poly-D-lysine coated coverslips and cultured in BS medium with 0.5% FCS and purified recombinant Sonic hedgehog protein (Shh, 7 x 10^{-9} M) or bFGF (40ng/ml) or PDGF-AA (10ng/ml). Each day, cultures were immunolabelled with anti-PDGFRα and A2B5 (A) or anti-GC to label oligodendrocytes (B). Small groups of (PDGFRα⁺, A2B5⁺) cells were present after 3 days *in vitro* in the presence of Shh (A); after nine days *in vitro*, GC⁺ oligodendrocytes developed in these cultures (B). Fewer (PDGFRα⁺, A2B5⁺) cells and oligodendrocytes developed in Shh treated cultures than in cultures treated with bFGF.

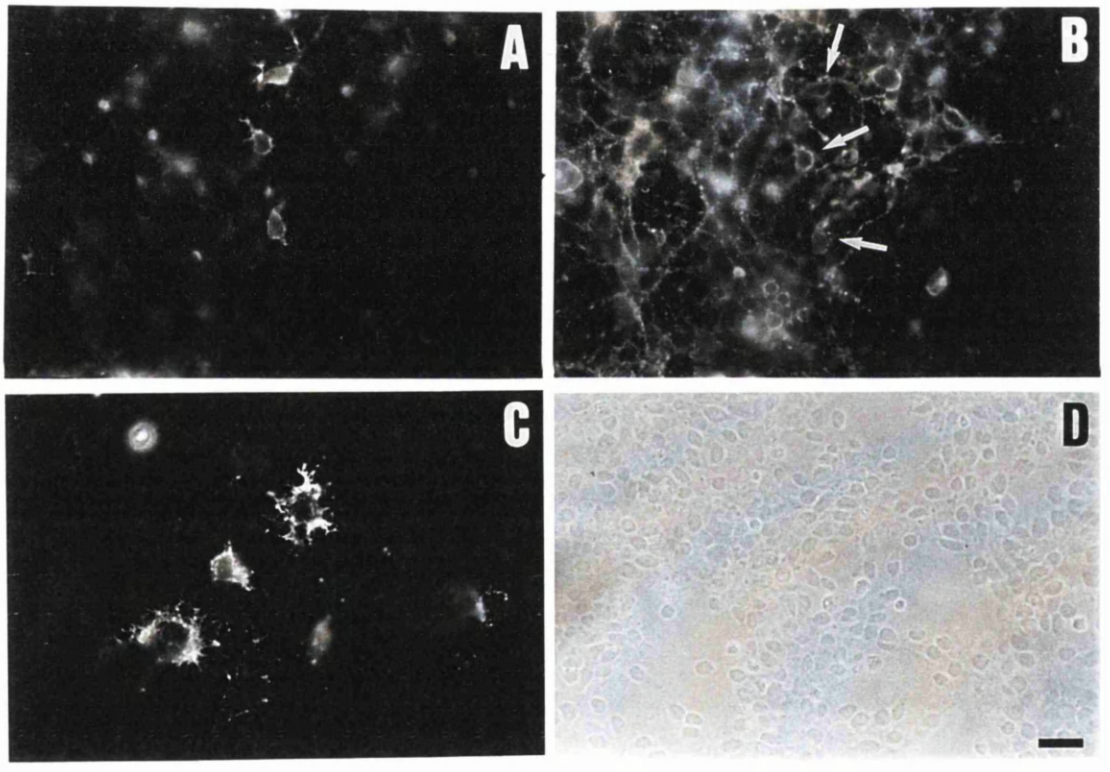


Figure 4.7 Recombinant Sonic hedgehog protein induces PDGFRα⁺ progenitors and the subsequent development of GC⁺ oligodendrocytes from E14 cortical cells *in vitro* 50,000 viable cells were plated on poly-D-lysine coated coverslips and cultured in BS medium with 0.5% FCS and purified recombinant Sonic hedgehog protein (Shh, 7 x 10^{-9} M). Each day, cultures were immunolabelled with anti-PDGFRα (A) and A2B5 (B). Small groups of (PDGFRα⁺, A2B5⁺) cells first appeared after 3 days *in vitro* (A,B, arrows); after eight days *in vitro*, GC⁺ oligodendrocytes developed in these cultures (C,D). Scale bar, 50μm.

Discussion

1. PDGFRα$^+$ cells in the embryonic brain are oligodendrocyte progenitors

The immunoselection experiments described in this chapter provide direct evidence that PDGFRα$^+$ cells in the embryonic brain are oligodendrocyte progenitors; therefore, the brain and spinal cord both contain a population of PDGFRα$^+$ oligodendrocyte progenitors (Hall *et al.*, 1996 and Chapter Three of this Thesis). The *in situ* hybridisation studies of PDGFRα$^+$ progenitors carried out by Pringle and Richardson (1993) and Pringle *et al.* (1992), suggest that there is a source of migratory PDGFRα$^+$ oligodendrocyte progenitors in the E14 ventral diencephalon. We therefore propose that the development of oligodendrocytes in the embryonic forebrain is similar to that in the embryonic spinal cord (Pringle and Richardson, 1993; Hall *et al.*, 1996).

2. The role of Sonic hedgehog protein in brain oligodendrogenesis

In the spinal cord, Sonic hedgehog protein (Shh) produced by the notochord induces floor plate cells. The notochord and floor plate then both secrete Shh which induces motor neurons (Roelink *et al.*, 1994) and oligodendrocytes (Pringle *et al.*, 1996). Shh has a role in patterning the embryonic brain as well as the spinal cord. In the brain, Shh is expressed by cells of the ventral midline from the hindbrain to the diencephalon/telencephalon border (Roelink *et al.*, 1994). Shh induces ventral neuronal types in the diencephalon directly and can act at a distance to induce telencephalic neurons (Ericson *et al.*, 1995). I have demonstrated that PDGFRα$^+$ oligodendrocyte progenitors can be induced from E14 cortical (dorsal telencephalic) cells *in vitro* by Shh. I think it is unlikely that the cerebral cortex is exposed to Shh *in vivo* due to the distance between the ventral midline and the cortex. It is more likely that some cortical cells at E14 have not yet become irreversibly committed to a particular cell fate and are still plastic and able to respond to Shh *in vitro*. However, Shh expressed by ventral midline cells could be responsible for inducing PDGFRα$^+$ cells in the ventral diencephalon, just as Shh induces PDGFRα$^+$ oligodendrocyte progenitors in the ventral spinal cord (Pringle *et al.*, 1996).

3. Do cortical oligodendrocyte progenitors develop from the cortical neuroepithelium or from a source in the ventral diencephalon *in vivo*?

In situ hybridisation studies have demonstrated that PDGFRα$^+$ oligodendrocyte progenitors first appear at the lateral-most edges of the cerebral cortex at E17 and are subsequently found in progressively more medial regions of the cerebral cortex as well as in the developing hippocampus (Pringle *et al.*, 1992). Pringle and Richardson (1993) proposed that these cells migrate into the cerebral cortex from their origin in the ventral diencephalon. However, in this chapter I have demonstrated that E14 cortical cells, in the presence of bFGF or Shh for three days *in vitro* that is, at the equivalent of E17 *in vivo*, can give rise to PDGFRα$^+$ progenitor cells and subsequently, oligodendrocytes. This raises the possibility that the PDGFRα$^+$ cells seen by *in situ* hybridisation in the E17 cerebral cortex could develop from cortical SVZ cells rather than from cells that migrate into the cortex from elsewhere. To resolve this issue, we need to know whether my own and other published *in vitro* studies of cortical cell behaviour truly reflect what happens *in vivo*. I will therefore briefly review what is known about the developmental potential of embryonic forebrain cells *in vitro* and *in vivo*.

Birling and Price (1998) report that E13 rat cerebral cortical cells did not give rise to oligodendrocytes *in vitro* if cultured alone whereas, E13 striatal cells did. If the E13 cortical cells were exposed to both EGF and striatal cells, a number of oligodendrocytes did develop from them; it was as if these conditions mimicked 'oligodendrogenic' conditions *in vivo*. Davis and Temple (1994) cultured single E12 and E14 cortical cells and demonstrated that a small number behaved like stem cells; producing clones that contained neurons, astrocytes, oligodendrocytes and copies of themselves. Davis and Temple (1994) used a culture medium that had been conditioned by astrocytes and meningeal cells; therefore, the cortical cells would have been exposed to a cocktail of inducing factors, survival factors and mitogens which they might not have encountered *in vivo*. One signalling molecule that is known to induce oligodendrocyte development from cortical cells *in vitro* is basic fibroblast growth factor (bFGF) (Ghosh and Greenberg, 1995; Qian *et al.*, 1997 and this Thesis); how it does so is as yet unknown. I have shown that bFGF is a potent mitogen for many cells within the developing cortex; could mitogens *in vitro* push the progenitors through more divisions than they would normally undergo *in vivo* and so alter their fate?

In order to confirm or discard our hypothesis that oligodendrocyte progenitors in the brain originate in the ventral diencephalon and migrate into the cerebral cortex, we need to know whether the *in vitro* oligodendrogenic potential of cortical cells is realised *in vivo*. Two techniques that have been used to identify which cell types embryonic forebrain cells differentiate into *in vivo* are retroviral lineage tracing and cell

transplantation. In the former approach, replication deficient retroviruses carrying a marker gene such as beta-galactosidase are injected into the lateral ventricles of embryonic rodents *in utero*. The virus infects a few scattered neuroepithelial cells and is inherited by the progeny of each of these cells. The age of the embryo at the time of injection and the selection of clones for analysis are critical to the interpretation of these experiments. Retroviral injections have demonstrated that at a particular age, some cortical progenitors are committed to generating a single cell type (for example, Parnavelas *et al.*, 1991) and that others can migrate considerable distances and generate several different cell types (Reid *et al.*, 1995). Unfortunately for us, retroviral analysis has to date been used to investigate whether the fate of cortical cells is restricted rather than whether cortical cells generate oligodendrocytes *in vivo*; very few of the published retroviral lineage analyses state whether labelled clones contain oligodendrocytes. Price and Thurlow (1988) describe a rare type of clone obtained after injecting retrovirus into the lateral ventricles of the E16 rat that contained neurons and white matter glia. Subsequent *in vitro* analysis suggested that these clones were comprised of neurons and oligodendrocytes (Williams *et al.*, 1991). This neuron/oligodendrocyte clone was very rare and only developed *in vitro* in low density cultures (J. Price, personal communication) where conditions are presumably very different to those found *in vivo*. As yet, retroviral studies have not provided convincing evidence that cortical SVZ cells give rise to oligodendrocytes *in vivo*. This may be due to limitations of the technique or due to labelled clones not being examined for the presence of oligodendrocytes or it may be a true reflection of the fact that the cerebral cortex does not generate oligodendrocytes *in vivo*.

Another technique that has been used to investigate forebrain progenitor cell fate *in vivo* is cell transplantation. SVZ cells from one animal that carries a marker transgene or SVZ cells that are labelled with dye are injected into the brain of another embryo and the cell types that they differentiate into are identified. Brustle *et al.* (1995) injected marked cells from E14 mouse dorsal and ventral forebrain into the telencephalic vesicles of E16-E18 rat embryos. Some of the injected cells integrated into the host brain and differentiated into neurons and astrocytes appropriate to the region of the brain that they encountered. Brustle *et al.* (1995) state that the injected cells did generate a few oligodendrocytes but do not say whether the injected cells came from the dorsal or ventral forebrain or in which part of the host brain they differentiated. The injected cells might have included PDGFRα^+ oligodendrocyte progenitors or they might have differentiated into oligodendrocytes in regions of the ventral forebrain that receive oligodendrocyte-inducing signals. Hardy and Friedrich (1996) injected tissue fragments from E10.5, E12.5 and E14.5 dorsal telencephalon, which we know does not contain PDGFRα^+ cells at these ages, (Pringle and Richardson, 1993 and this Thesis) into the SVZ of the cerebral hemispheres of early postnatal mice and found that some of the

donor cells developed into oligodendrocytes. Hardy and Friedrich (1996) conclude from these experiments that oligodendrocytes develop from all regions of the SVZ in the developing brain. Before this can be taken as proof that the cerebral cortex normally generates oligodendrocytes *in vivo* it is necessary to mention some of the evidence which demonstrates that SVZ cells can alter their fate if placed in novel neural environments. For example, Fishell (1995) injected striatal progenitors from E15 mouse into the cortical SVZ of the E16.5 rat; despite the transplanted cells being derived from a mouse and already expressing several striatal specific genes such as DlX-1 and MASH-1, the cells were able to integrate into the rat cortex and develop the morphology and projections appropriate to cortical neurons. This experiment demonstrates that the fate of SVZ cells is determined by local signals and can change if the cell is placed in a different part of the brain where it will receive different signals. McConnell and Kaznowski (1991) demonstrate that cortical neuronal progenitors remain uncommitted to a particular neuronal fate until the end of the S-phase of their final cell division cycle. Perhaps forebrain SVZ cells can also respond to oligodendrocyte-inducing molecules until this relatively late stage in their development.

Rather than demonstrating that oligodendrocytes develop from all regions of the SVZ, the cell transplantation experiments of Hardy and Friedrich (1996) and the *in vitro* clonal analyses of other groups demonstrate the plasticity of SVZ cells in the early embryonic brain; that is, if the cells are exposed to novel signals *in vivo* or *in vitro*, they can adopt, amongst others, an oligodendrocyte fate. These experiments are therefore not very useful in determining whether SVZ cells of the cerebral cortex generate oligodendrocytes *in vivo*. It is still open to debate therefore, whether cortical oligodendrocytes develop from a ventral source of migratory progenitors as proposed by Pringle and Richardson (1993) or whether cortical SVZ cells *in vivo* generate oligodendrocytes.

Chapter Five

PDGF-AA, and not PDGF-BB or PDGF-AB, is crucial to the development of oligodendrocyte progenitors *in vivo*.

Chapters Three and Four have presented evidence which suggests that the major population of oligodendrocyte progenitors in the embryonic spinal cord and brain are cells that express PDGFRα. PDGFRα can bind and be activated by all three dimeric forms of PDGF (PDGF-AA, -AB, -BB) *in vitro* (Heldin *et al.*, 1988). PDGF has been reported to have several effects on PDGFRα$^+$ cells *in vitro* and *in vivo* but whether PDGF is necessary for the development of oligodendrocytes *in vivo* is unknown; this chapter investigates the roles of PDGF-A and PDGF-B in the development of the PDGFRα$^+$ oligodendrocyte lineage *in vivo*.

PDGF was first identified as being important to the development of oligodendrocytes *in vitro* following the observation that purified PDGF could act as a mitogen for rat optic nerve oligodendrocyte progenitors (Richardson *et al.*, 1988; Noble *et al.*, 1988), stimulating them to proliferate and differentiate *in vitro* on the same time schedule as they do *in vivo* (Raff *et al.*, 1988). For example, if E18 optic nerve O-2A cells were cultured in low serum medium containing PDGF, they divided several times and then differentiated on the third day *in vitro*, the equivalent of the day of birth *in vivo* (Raff *et al*, 1988), which is when significant numbers of oligodendrocyte progenitors start to differentiate in the intact optic nerve (Miller *et al.*, 1985). Pringle *et al.* (1989) demonstrated that all three dimeric forms of PDGF can stimulate DNA synthesis in optic nerve oligodendrocyte progenitors, although an approximately three- to tenfold lower concentration of PDGF-AA or PDGF-AB than PDGF-BB is required to stimulate the maximum mitogenic response.

Barres *et al.* (1992) demonstrated that PDGF-AA can act as a survival factor for PDGFRα$^+$ cells in the optic nerve. COS cells transiently transfected with a plasmid vector designed to express c-Myc epitope-tagged PDGF-A were transplanted into the subarachnoid space of neonatal rats. The c-Myc epitope-tagged PDGF-A diffused into the optic nerve. When examined four days after the transplantation, the optic nerves of PDGF-A treated animals had 20% more O-2A cells and two-fold more GC$^+$ oligodendrocytes than control animals (Barres *et al.*, 1992). The number of mitotic cells was similar in the treated and control optic nerves, demonstrating that the exogenous PDGF-A had acted as a survival factor for PDGFRα$^+$ cells in the nerve. Presumably the oligodendrocytes that were rescued from apoptosis by the exogenous PDGF-A were newly differentiated oligodendrocytes, which still express PDGFRα (Hart *et al.*, 1989a; Hall *et al.*, 1996).

There have also been reports that PDGF can act as a chemoattractant for oligodendrocyte progenitors in a microchemotaxis chamber (Armstrong *et al.*, 1991) or in a droplet of agarose (Milner *et al.*, 1997).

To understand how PDGF-A and PDGF-B might influence the development of PDGFRα$^+$ oligodendrocyte lineage cells *in vivo*, it is necessary to examine where these growth factors are expressed in the developing CNS. Mudhar (1994) and Lindahl *et al.* (1997) have demonstrated that PDGF-B is expressed in the embryonic mouse by cells, presumably capillary endothelial cells, in the walls of blood vessels. Sasahara *et al.* (1991) carried out immunohistochemical studies of the mature macaque monkey using an antibody that recognises PDGF-AB and PDGF-BB and showed that many neurons in the mature CNS express one or both of these isoforms. The protein was mainly localised to neuronal cell bodies but it was also seen in some nerve fibre tracts and presumptive nerve terminals (Sasahara *et al.*, 1991). Sasahara *et al.* (1991) also generated transgenic mice expressing bacterial chloramphenicol acetyltransferase (CAT) under the transcriptional control of the human PDGF-B promoter. PDGF-B expression appears to be confined to neurons in the mature animal as CAT activity was not found within the optic nerve, an area of the CNS that does not contain neuronal cell bodies.

The first part of this chapter describes a study of PDGF-A expression in the developing mouse spinal cord. This demonstrates that PDGF-A is first expressed at E11 in the mouse spinal cord, by cells of the floor plate. From E15, PDGF-A is expressed strongly by motor neurons and, at a lower level, by other neurons in the grey matter of the spinal cord. Postnatally, PDGF-A is expressed both by neurons and by glial cells, presumably astrocytes, in the white matter of the spinal cord.

The second part of this chapter describes work that investigates whether PDGF-A and/or PDGF-B are important during the development of the PDGFRα$^+$ oligodendrocyte lineage *in vivo*. I describe studies of the number and distribution of PDGFRα$^+$ oligodendrocyte progenitors and differentiated oligodendrocytes within the spinal cords of mice that have had either the PDGF-A or PDGF-B gene 'knocked-out' by homologous recombination in embryonic stem cells. These studies demonstrate that PDGF-AA, but not PDGF-AB or PDGF-BB, is essential for the proliferation of PDGFRα$^+$ oligodendrocyte progenitors in the spinal cord *in vivo*. A preliminary study of the role of PDGF-A in brain oligodendrocyte development is also presented.

The final part of this chapter describes experiments carried out to attempt to explain why PDGFRα$^+$ progenitors do not proliferate indefinitely in response to PDGF-AA in the developing spinal cord, but instead stop proliferating several days before birth. I describe experiments which demonstrate that progenitor proliferation in the embryonic spinal cord slows down during development because the cell cycle time of the progenitors lengthens. Other experiments carried out using cells from a transgenic

mouse that overexpresses PDGF-A have led us to conclude that PDGFRα$^+$ progenitor proliferation slows down during development because of competition for available PDGF-AA within the spinal cord. We propose that competition for limiting amounts of mitogens might be a general mechanism for limiting progenitor proliferation throughout the developing embryo.

Results.

1. PDGF-A expression in the developing mouse spinal cord

The results of a study of PDGF-A expression in the developing mouse spinal cord using a Digoxigenin-labelled PDGF-A mRNA probe and *in situ* hybridisation are shown in Figure 5.1. PDGF-A is first expressed in the mouse spinal cord at E11, by cells of the floor plate (Figure 5.1A); this confirms the findings of Orr-Urtreger and Lonai (1992). At E13, there is a low level of PDGF-A expression in the lateral horns of the spinal cord (within the cell bodies of newly differentiated motor neurons) as well as in ventricular zone cells (Figure 5.1B). A population of cells in the dorsal half of the spinal cord, which appear to be migrating away from the ventricular zone, is also PDGF-A$^+$ at E13 (Figure 5.1B). This dorsal PDGF-A expression is probably very transient, as it is not seen in every E13 embryo examined. At E15 (Figure 5.1C), PDGF-A is expressed at a higher level by motor neurons and by many other neurons throughout the grey matter of the spinal cord and this level of expression is maintained until at least P0 (Figure 5.1D). At P19, the oldest age that I examined, PDGF-A is expressed by neurons and by many radially-oriented cells, presumably astrocytes, throughout the spinal cord white matter (Figure 5.1E and 5.1F). White-matter astrocytes are known to express PDGF-A postnatally (Richardson *et al.*, 1988; Pringle *et al.*, 1989; Yeh *et al.*, 1991).

2. PDGF-B is not essential for the development of the PDGFRα$^+$ oligodendrocyte lineage *in vivo*

PDGF-B chain-containing dimers (i.e., PDGF-BB and PDGF-AB) can bind to and activate PDGFRα (Heldin *et al.*, 1988). In the embryonic spinal cord, PDGF-B is expressed by capillary endothelial cells (Mudhar, 1994 and Lindahl *et al.*, 1997). In postnatal animals, PDGF-B is expressed by neurons throughout the CNS (Sasahara *et al.*, 1991). I investigated whether PDGF-B has a role in the development of the

PDGFRα$^+$ oligodendrocyte lineage in the spinal cord *in vivo*. Leveén *et al.* (1994) have generated mice deficient for active PDGF-B by homologous recombination in embryonic stem cells. Part of exon 3 and all of exon 4 of the PDGF-B gene were deleted, these regions include the cysteine residues that are essential for the dimerisation of the PDGF-B chains; therefore, these PDGF-B null mice cannot produce PDGF-BB or PDGF-AB dimers (Leveén *et al.*, 1994). We obtained PDGF-B heterozygous knockout mice from C. Betsholtz (University of Goterborg) and established a breeding colony at University College London. Heterozygous PDGF-B +/- mice are fertile and have no detectable developmental defects. Homozygous PDGF-B null mice are easily recognised as they have large patches of subcutaneous haemorrhaging on their head, neck and along their back due to the absence of pericytes around their blood vessels (Lindahl *et al.*, 1997). I used a Digoxigenin-labelled probe against PDGFRα mRNA and *in situ* hybridisation to identify PDGFRα$^+$ oligodendrocyte progenitor cells within the spinal cords of two newborn PDGF-B null mice. The PDGF-B null mice had the same number and distribution of PDGFRα$^+$ cells in their spinal cord as their wild-type littermates (Figure 5.2A and C). Using a Digoxigenin-labelled probe which recognises proteolipid protein (and its alternatively spliced isoform DM-20; see Timsit *et al.*, 1992), I demonstrated that P0 PDGF-B null mice develop the same number of PLP$^+$ oligodendrocytes within their spinal cord as wild-type mice (Figure 5.2B and D). These experiments demonstrate that PDGF-B (and therefore the dimers PDGF-BB and PDGF-AB) is not essential for the specification, proliferation, migration or differentiation of PDGFRα$^+$ oligodendrocyte progenitors within the embryonic spinal cord *in vivo*.

3. PDGF-AA is essential for the development of the PDGFRα$^+$ oligodendrocyte lineage *in vivo*

Having established that PDGF-B is not essential for the development of PDGFRα$^+$ oligodendrocyte progenitors *in vivo*, I investigated the role of PDGF-A. Böstrom *et al.* (1996) have generated PDGF-A null mice by homologous recombination in embryonic stem cells. Exon 4 of the PDGF-A gene was deleted, this deletes six of the eight cysteine residues that are essential for PDGF-A chain dimerisation and also introduces a frame shift, which deletes the carboxy terminal of the PDGF-A protein; therefore, these mice cannot produce PDGF-AA or AB dimers (Böstrom *et al.*, 1996). Approximately 50% of PDGF-A null mice die of unknown causes before E1C. The remaining 50% survive until birth; many of these die shortly after birth but, rarely, some can survive for as long as four weeks. PDGF-A null mice lack alveolar myofibroblasts in their

lungs, without these cells alveolar septa fail to form, leaving large air-filled spaces throughout the lung and causing fatal breathing problems (Böstrom *et al.*, 1996). We obtained tissue from PDGF-A null mice and analysed the number of PDGFRα$^+$ oligodendrocyte progenitors in 15μm frozen sections of their spinal cords using Digoxigenin-labelled *in situ* hybridisation probes against PDGFRα and PLP/DM20 mRNA.

The youngest PDGF-A null mouse that I examined was E17. There was a small number of PDGFRα$^+$ progenitors within the spinal cord of these animals, demonstrating that PDGF-A is not crucial for the specification of the PDGFRα$^+$ lineage (see also Fruttiger *et al.*, 1999). The number of PDGFRα$^+$ oligodendrocyte progenitors in E17 PDGF-A null mice is only 5% of that seen in wild-type animals. E17 PDGF-A null mice had 12±8 PDGFRα$^+$ oligodendrocyte progenitors per 15μm frozen section of spinal cord (seven sections from 2 animals examined), whereas wild-type animals had 236±24 PDGFRα$^+$ cells per frozen section (six sections from 2 animals examined) (Figure 5.3A and B). This difference is not due to a down-regulation of the PDGFRα$^+$ mRNA in the absence of PDGF-A as normal levels of PDGFRα$^+$ mRNA were seen in tissues other than the spinal cord. PDGFRα$^+$ cells within the PDGF-A null spinal cord do not differentiate prematurely in the absence of PDGF-A as they do *in vitro* (Raff *et al.*, 1988); the same (small) number of PLP$^+$ oligodendrocytes are present in their spinal cord as are present in wild-type animals (Figure 5.3C and D). A preliminary observation was that some of the PDGFRα$^+$ cells and oligodendrocytes present in PDGF-A null mice are less widely distributed throughout the spinal cord than those in wild-type animals (Figure 5.4). This suggests that, without PDGF-A, PDGFRα$^+$ oligodendrocyte progenitors cannot migrate as quickly and/or as far as normal before differentiating.

The small number of PDGFRα$^+$ progenitors present in the null mouse do proliferate to some extent as there is an increase in their number between E17 and P9 (P0 and P9 data not shown) but their number is never more than 20% of that found in wild-type animals. It is still not known whether this is because the progenitors are dividing very slowly or because they die in the absence of PDGF-A.

4. The PDGF-A null phenotype does not recover; PDGF-A null mice have a reduced number of PDGFRα$^+$ progenitors and oligodendrocytes throughout their life. The severity of the phenotype is PDGF-A dose dependent.

P0 and P9 PDGF-A null mice have a reduced number of PDGFRα$^+$ progenitors and PLP$^+$ oligodendrocytes (data not shown). The oldest PDGF-A null mouse that I examined was a severely runted P19 animal. This mouse had no PDGFRα$^+$ progenitors within its spinal cord (Figure 5.4E). Perhaps PDGF-A is necessary for the maintenance of adult O-2A progenitors. I also examined a P19 heterozygous PDGF-A (+/-) mouse. This animal had half the wild-type number of PDGFRα$^+$ progenitors in its spinal cord (Figure 5.4C); that PDGFRα$^+$ progenitor number is proportional to the expression level of PDGF-A is discussed further in Calver *et al.* (1998).

5. PDGF-AA is also important for the development of oligodendrocytes within the brain

I examined the brain of a P9 PDGF-A null mouse; in all regions it had far fewer PDGFRα$^+$ oligodendrocyte progenitors than its wild-type littermate. The PDGFRα$^+$ progenitors that were present in the PDGF-A null mouse were clustered around the foramen of Monro (a putative origin of PDGFRα$^+$ oligodendrocyte progenitors in the brain, see Chapter Four) and the brainstem. The number of PLP$^+$ oligodendrocytes in the knock-out mouse was also much smaller than wild-type in all regions of the brain except the brainstem (Figure 5.5). This suggests that either, other growth factors can slightly compensate for the lack of PDGF-A in the brainstem and stimulate PDGFRα$^+$ progenitor proliferation or, that the brainstem contains a different, non-PDGF-A dependent, oligodendrocyte lineage.

6. PDGFRα$^+$ oligodendrocyte progenitors in the spinal cord of wild-type mice stop proliferating after E15

Figure 5.6, panel A, shows that the number of PDGFRα$^+$ progenitor cells in the wild-type mouse spinal cord increases rapidly between E12.5 and E15 and plateaus between E15 and E19. Many thanks to Andy Calver for supplying this data. Since very few oligodendrocyte progenitors differentiate before birth in the mouse, the number of oligodendrocyte progenitors must be held constant before birth, either because the cells

stop dividing or because the rate of cell division equals the rate of cell death. Very little is known about what regulates progenitor number in the developing embryo and we chose to investigate what controls PDGFRα$^+$ progenitor number in the spinal cord in order to try to understand this process. I injected pregnant female mice and young postnatal animals with the thymidine analogue bromodeoxyuridine (BrdU) (100μg/g body weight in PBS). Two hours after the injection, I culled the animals and dissociated and cultured cells from their spinal cords. The next day I immunolabelled the cultures with anti-NG2, which is co-expressed with PDGFRα$^+$ on oligodendrocyte progenitors (Nishiyama *et al.*, 1996 and my observations), and anti-BrdU to identify any progenitors that had been in S phase of the cell cycle during the BrdU pulse. Figure 5.6 shows the results of these experiments. At E13, 75% of progenitors were BrdU$^+$, this percentage decreased steeply with age; at P3, only 20% of the progenitors were BrdU$^+$. This decrease could be due to several reasons: some of the progenitors could stop dividing, the progenitors could separate into different subpopulations, with some dividing rapidly and others more slowly or, the progenitor cell cycle length could increase in older animals. To distinguish between these possibilities I tested whether, with increased exposure to BrdU, all the O-2A progenitors in the spinal cord could incorporate BrdU. Figures 5.6B and 5.6C show the results of giving repeated BrdU injections, at four hourly intervals, to E14 and E17 embryos. At both these ages we were able to label essentially all the progenitors in these animals, indicating that the whole population of progenitors was actively dividing. The BrdU labelling index increased linearly, suggesting that the whole progenitor population was cycling together at the same rate. These experiments show that the plateau in progenitor number between E15 and E19 is mainly due to the cell cycle time of the PDGFRα$^+$ progenitors increasing with age; a point confirmed by the fact that it took longer to label all the progenitors at E17 than at E14 (compare Figure 5.6B and C). From E17 onwards, a small number of PDGFRα$^+$ progenitors within the spinal cord do die by apoptosis (Calver *et al.*, 1998) and this explains why there is no net increase in progenitor number during the period of slow progenitor proliferation.

7. Competition for PDGF-AA slows the cell cycle and limits the number of PDGFRα$^+$ oligodendrocyte progenitors in the spinal cord *in vivo*

We wondered why the progenitor cell cycle lengthens with age. It could be because mitogens that drive the cells quickly through division become limiting. We could test this by increasing the mitogen concentration available to the progenitors during development. Andrew Calver has generated a transgenic mouse that over-expresses

PDGF-A under the control of the neuron specific enolase promoter (NSE-PDGF-A mice) (Fruttiger *et al.*, 1996). Spinal cord neurons of these mice over-produce PDGF-A from E11 until adulthood (Calver *et al.*, 1998). I have repeated the BrdU pulse-chase experiments described above with NSE-PDGF-A mice; the results of these experiments are shown in Figure 5.7. At E13, the number of $BrdU^+$ progenitors is the same in wild-type and NSE-PDGF-A mouse spinal cords; however, between E13 and E15, $PDGFR\alpha^+$ progenitors proliferate more rapidly in the NSE-PDGF-A animal than in wild-type mice. This demonstrates that the concentration of PDGF-A in the spinal cord is normally limiting for oligodendrocyte progenitor proliferation beyond E13. However, even in the NSE-PDGF-A mouse, the rate of progenitor proliferation slows down with age until, at P3, it again matches wild-type (Figure 5.7 and see Calver *et al.*, 1998). We propose that this is because progenitor number eventually increases to the extent where even the increased amount of PDGF-A in the transgenic spinal cord becomes limiting.

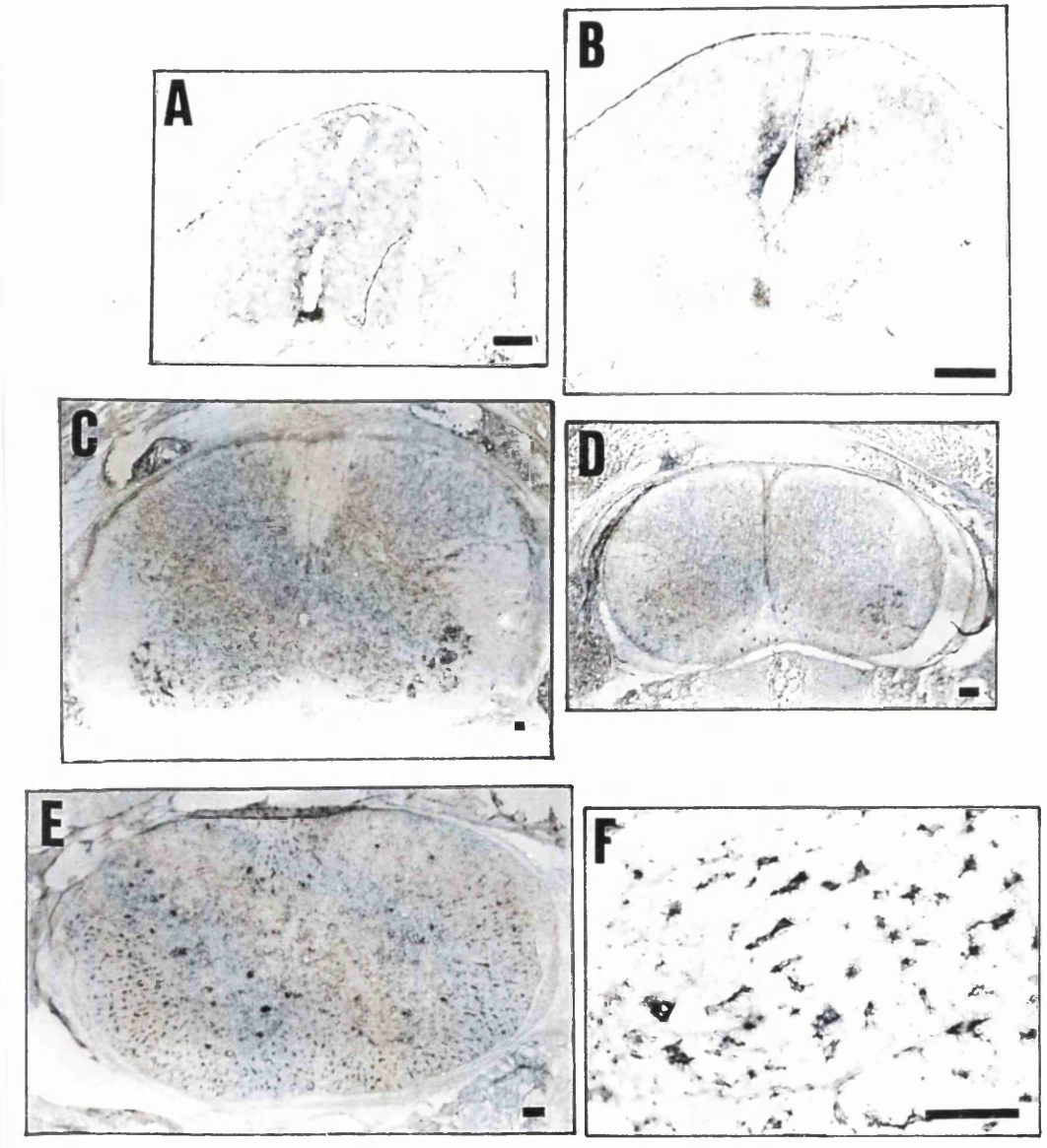


Figure 5.1 PDGF-A transcripts in the developing mouse spinal cord
Transverse sections of upper thoracic spinal cord were collected and subjected to *in situ* hybridisation with a Digoxygenin-labelled probe for mouse PDGF-A mRNA. PDGF-A is first expressed at E11, in the floorplate (A). At E13, PDGF-A is also expressed by cells of the ventricular zone and transiently by a group of cells in the dorsal cord (B). At E15, PDGF-A is expressed at a low level by neurons throughout the cord and at a higher level by motor neurons in the ventral horns of the cord (C). This pattern of expression continues until PO (D). Postnatally, PDGF-A is expressed by neurons throughout the cord and by white matter astrocytes (P9 spinal cord, E). Panel F is an enlargement of a region of P9 white matter showing PDGF-A$^+$ cells, presumably, astrocytes. Scale bars, 200μm

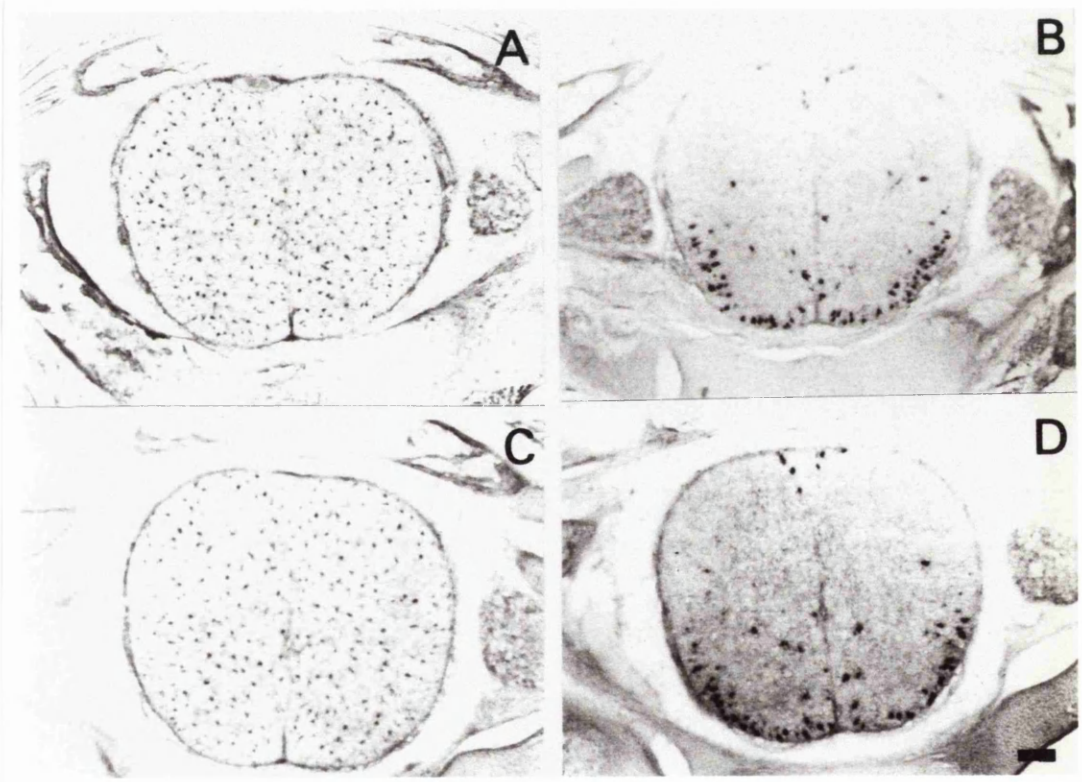


Figure 5.2 PDGF-B null mice have a wild-type number of PDGFRα$^+$ oligodendrocyte progenitors and PLP$^+$ oligodendrocytes in their spinal cord at birth Transverse sections through the upper thoracic spinal cord of PO wild-type (C,D) and PDGF-B null (A,B) mice were collected and subjected to *in situ* hybridisation using a Digoxygenin-labelled probe for mouse PDGFRα (A,C) to identify oligodendrocyte progenitors or PLP/DM-20 (B,D) to label oligodendrocytes. PDGF-B null mice have a wild-type number of PDGFRα$^+$ oligodendrocyte progenitors (A) and PLP$^+$ oligodendrocytes (B) in their spinal cord at birth. Scale bar, 100μm.

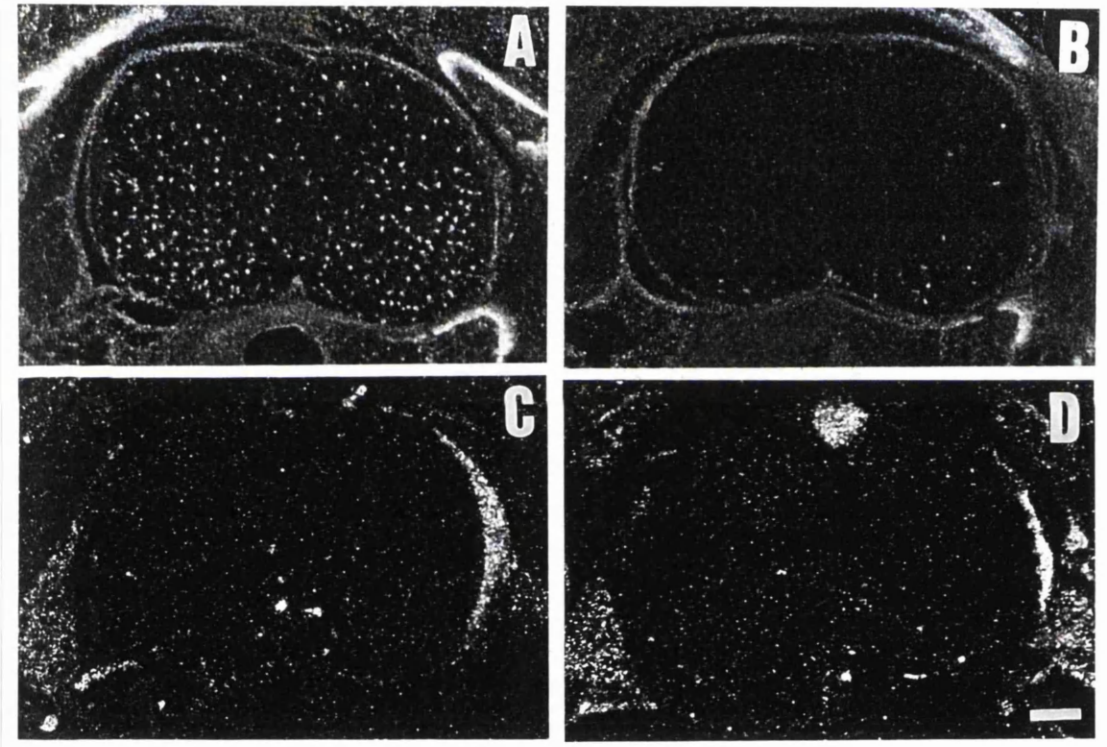


Figure 5.3 PDGF-AA is the form of PDGF important for the development of the PDGFRα⁺ oligodendrocyte lineage *in vivo* Transverse sections through the upper thoracic spinal cord of E17 wild-type (A,C) and PDGF-A null (B,D) mice were collected and subjected to *in situ* hybridisation with a ^{35}S-labelled probe against mouse PDGFRα (A,B) or PLP/DM-20 (C,D), autoradiographed and photographed under dark-field illumination. PDGF-null mice have less than 5% wild-type number of PDGFRα⁺ progenitors (B). The lack of progenitor cells in the PDGF-A null mouse is not caused by premature differentiation into oligodendrocytes as there are very few PLP⁺ oligodendrocytes in either wild-type (C) or PDGF-A null (D) mice. The bright structure above the dorsal side of the PDGF-A mouse (D) is a blood vessel whose red blood cells scatter light and give a false positive signal. Scale bar, 100 μm.

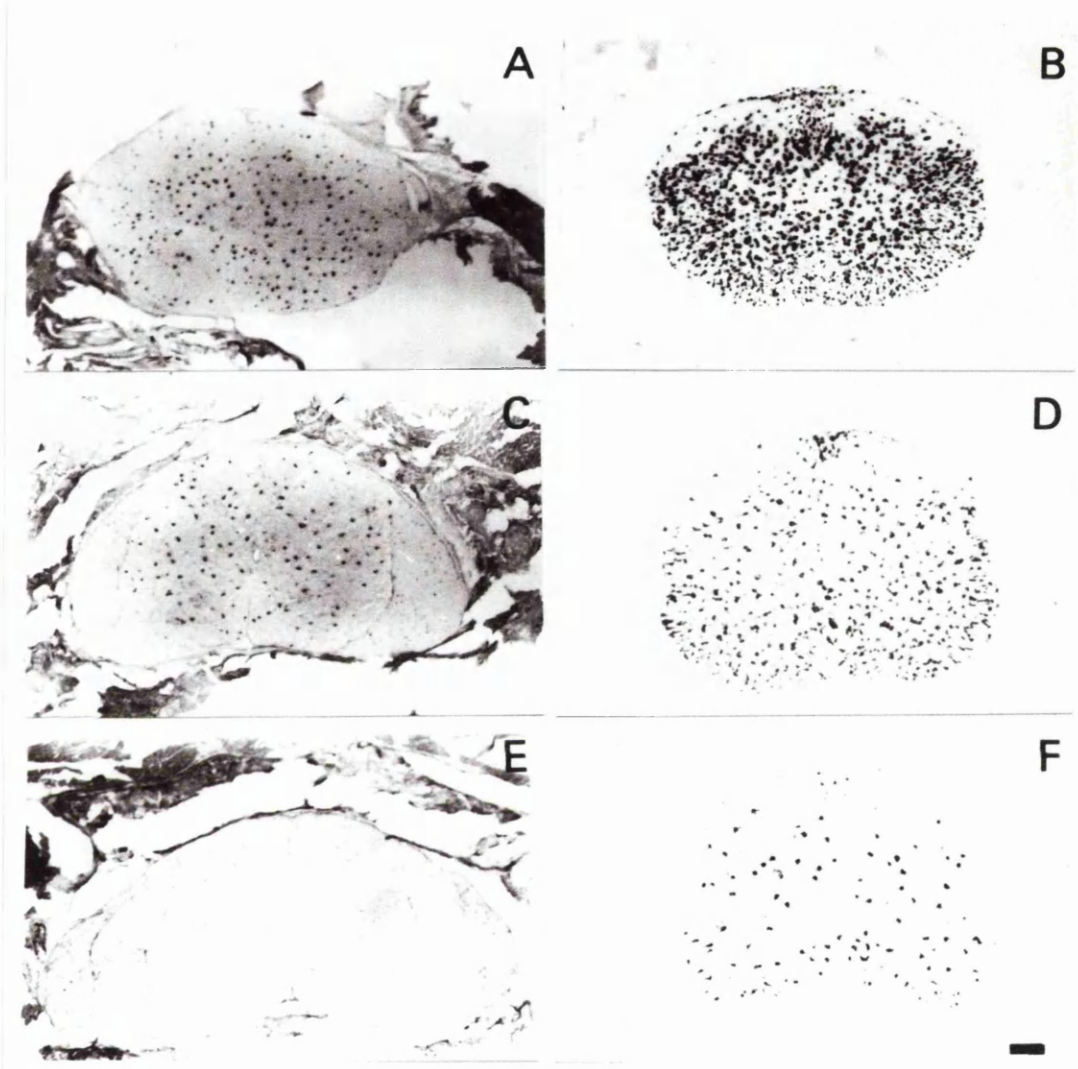


Figure 5.4 The PDGF-A null phenotype does not recover; PDGF-A null mice have a reduced number of PDGFRα$^+$ progenitors and oligodendrocytes throughout their life. The severity of the phenotype is PDGF-AA dose dependent. Transverse sections through the upper thoracic spinal cord of postnatal day nineteen (P19) wild-type (A,B), heterozygous (i.e. PDGF-A +/-; C,D) and homozygous PDGF-A null (E,F) mice were collected and subjected to *in situ* hybridisation with a Digoxygenin-labelled probe against mouse PDGFRα (A,C,E) or PLP/DM-20 (B,D,F). P19, PDGF-A null mice have no oligodendrocyte progenitors (E) and a greatly reduced number of PLP$^+$ oligodendrocytes (F). Heterozygous mice have a number of oligodendrocyte progenitors (C) and mature oligodendrocytes (D) which is intermediate between that of wild-type (A,B) and homozygous PDGF-A null animals (E,F). Scale bar, 150μm.

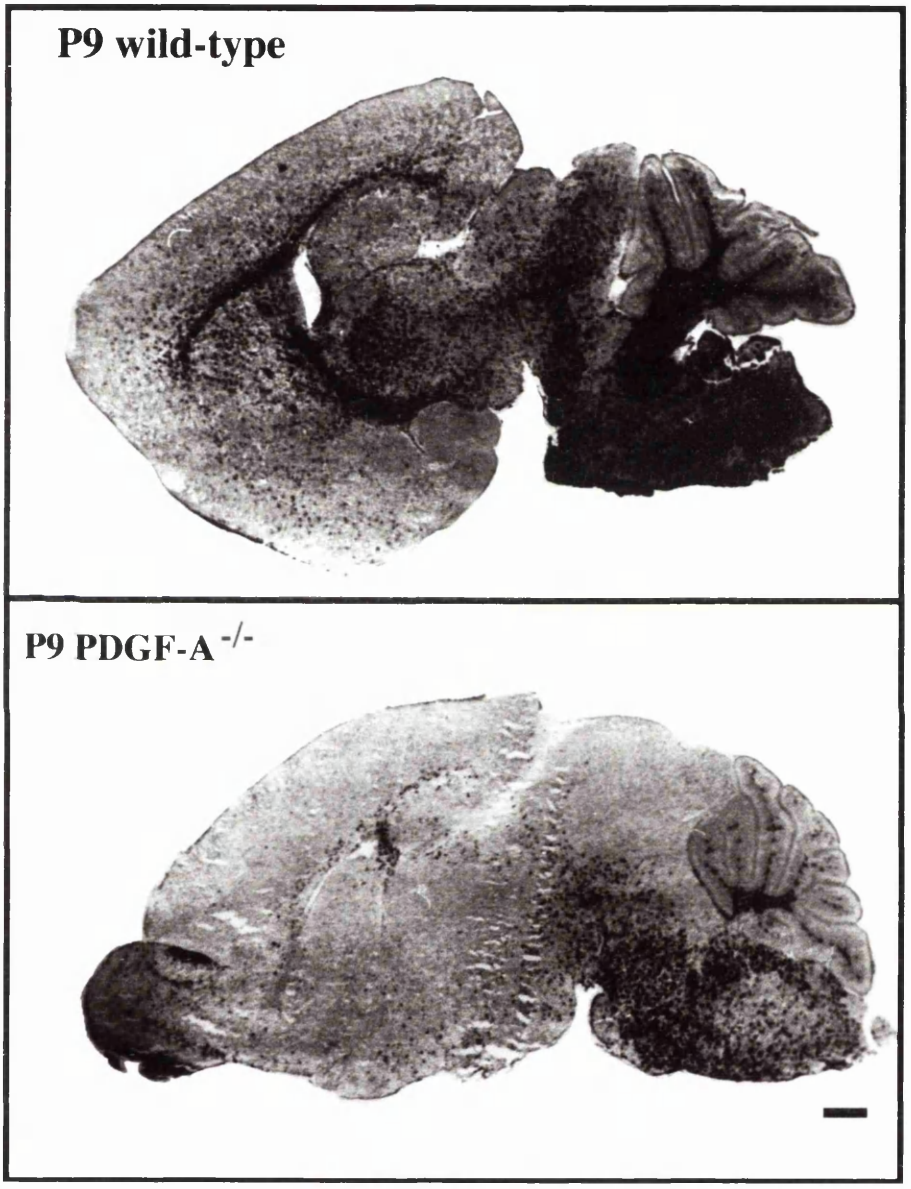


Figure 5.5 The brains of postnatal PDGF-A null mice have a reduced number of PLP$^+$ oligodendrocytes Sagittal sections through the brains of P9 wild-type or PDGF-A null mice were collected and subjected to *in situ* hybridisation using a Digoxygenin-labelled probe for mouse PLP/DM-20 to label oligodendrocytes. PDGF-A null mice have a severely reduced number of oligodendrocytes in all regions of the brain, except the brainstem, which has only a mild phenotype. Scale bar, 200μm.

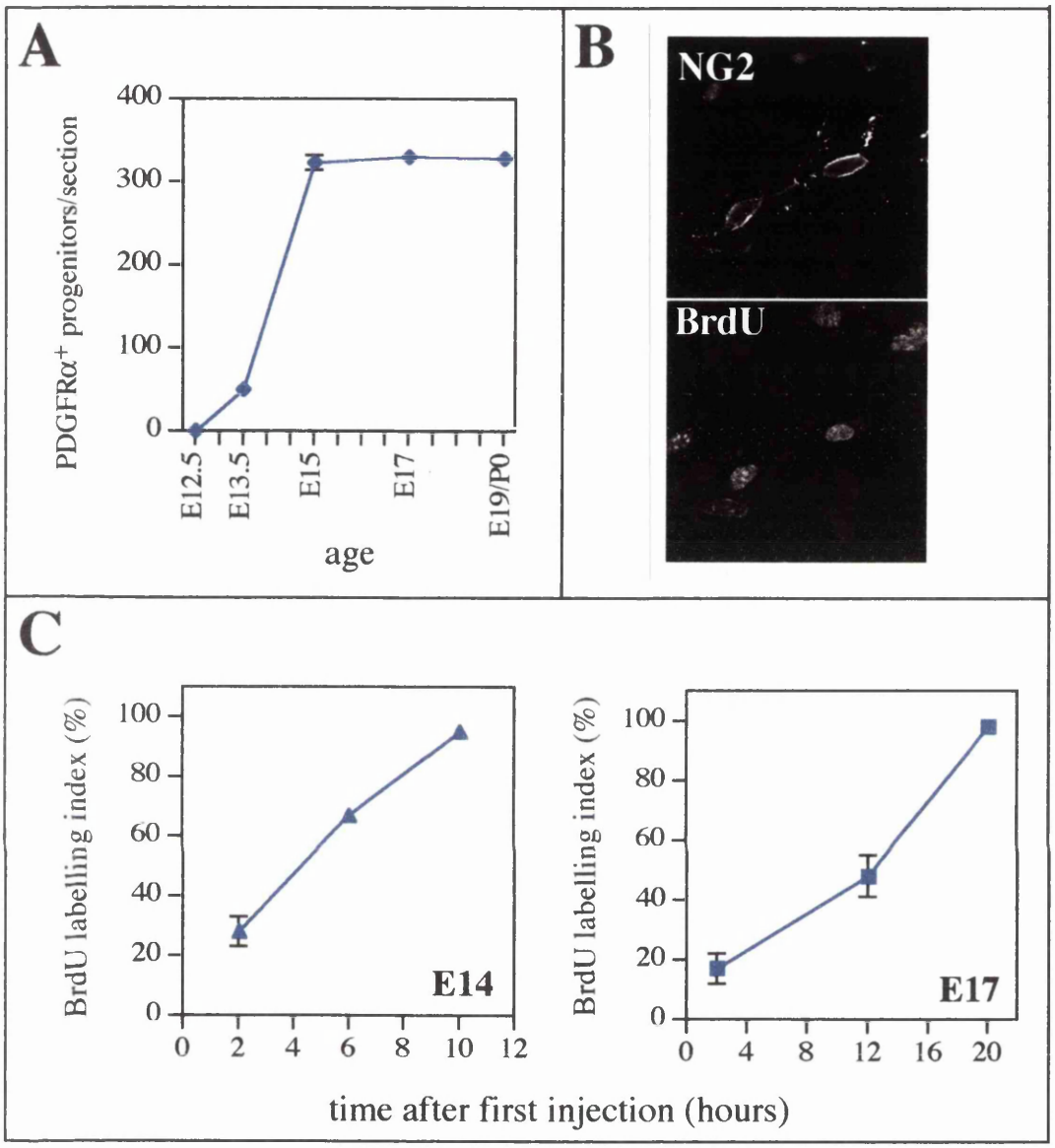


Figure 5.6 PDGFRα⁺ oligodendrocyte progenitors in the spinal cord of wild-type mice do not increase in number after E15. This is due to lengthening of the cell cycle and not to the cells exiting the cell cycle. Panel A shows the number of PDGFRα⁺ cells in sections of wild-type mouse spinal cord (mean ± SD of counts from 3 or 4 sections from 2 animals of each age). Oligodendrocyte progenitor number does not increase beyond E15. Panel B shows NG2⁺/BrdU⁺ oligodendrocyte progenitors from labelled spinal cord. Panel C: pregnant wild-type females were given BrdU injections at 4 hourly intervals starting at E14 or E17 and the BrdU labelling index of NG2⁺ (PDGFRα⁺) oligodendrocyte progenitors was determined. Almost 100% of progenitors can be labelled with BrdU at both E14 and E17, indicating that all the progenitors are dividing at these ages. It takes longer to label all the progenitors at E17 than E14, indicating that the progenitor cell cycle slows down after E14.

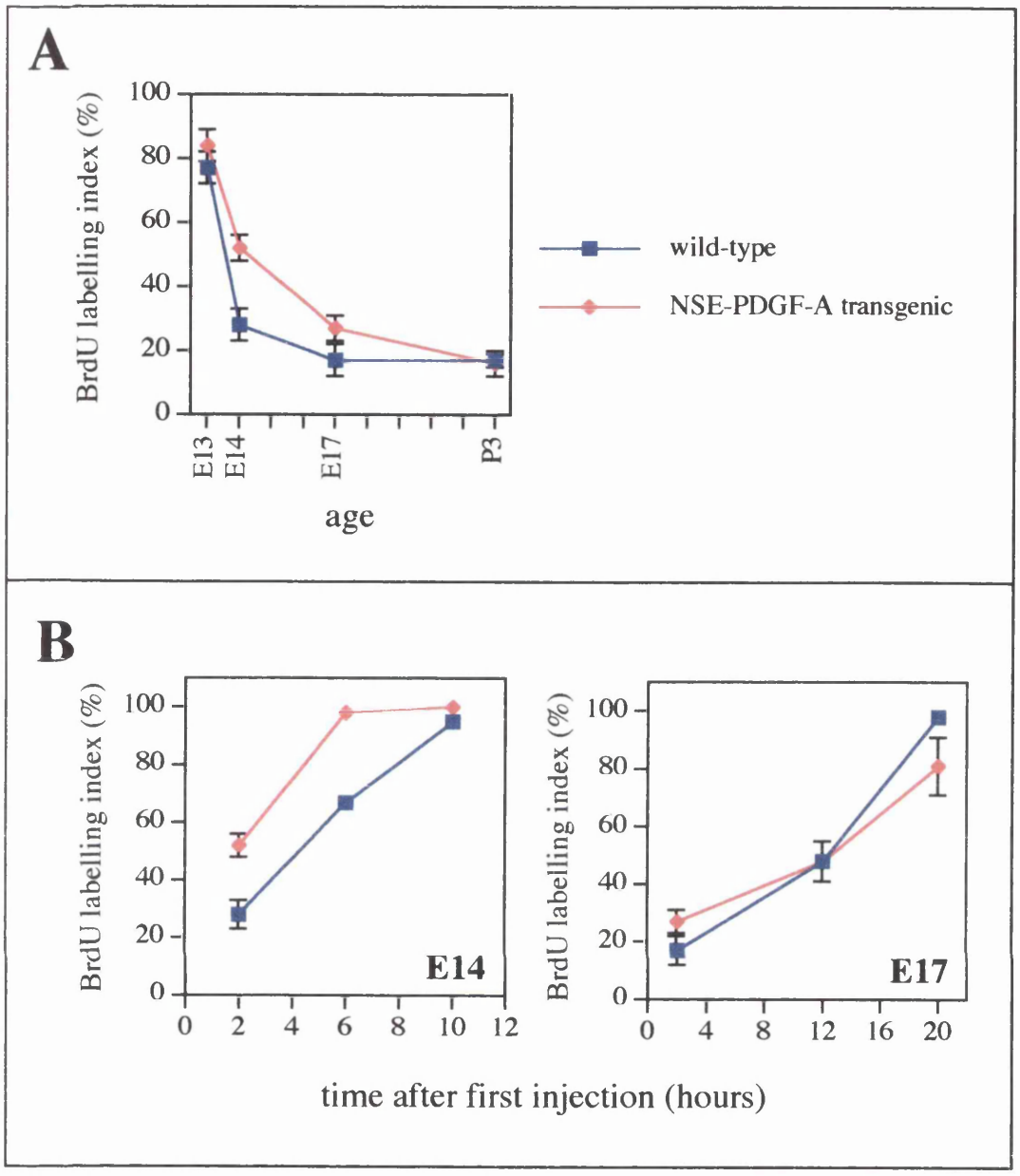


Figure 5.7 PDGFRα⁺ oligodendrocyte progenitors in the spinal cords of mice overexpressing PDGF-A divide more quickly than wild-type cells at E13 and E14 but, like wild-type cells, their cell cycle also slows with age. Panel A: Pregnant females or neonates were injected with BrdU, two hours later, they were killed, their spinal cords were then dissociated and labelled with anti-NG2 and anti-BrdU to identify proliferating oligodendrocyte progenitors. At E13, the BrdU labelling index was the same in wild-type and NSE-PDGF-A mice. However, the decrease in labelling index was more gradual in transgenics than in wild types; therefore, between E13 and E17, progenitors divide more rapidly in NSE-PDGF-A mice than in wild types. Panel B: Pregnant females were given sequential BrdU injections at four hourly intervals. Almost 100% of progenitors can be BrdU labelled in both wild-type and NSE-PDGF-A mice at E14 and E17; therefore, all progenitors are cycling at these ages. The time taken for all progenitors to label with BrdU at E14 was longer in wild-type embryos than transgenic animals, indicating that the cell cycle is shorter in the transgenic mice at this age. The time taken for all progenitors to label at E17 was longer than at E14 for both genotypes, indicating that the cell cycle slows in both sets of animals. The rate of BrdU incorporation and hence the rate of cell division is the same in wild-type and transgenic at E17. The above is the mean ± SD from 2-10 animals (each in triplicate) from one or two litters.

Discussion

1. PDGF-AA, and not PDGF-AB or PDGF-BB, is essential for the development of the PDGFRα$^+$ oligodendrocyte lineage in the spinal cord *in vivo*

In this chapter I have investigated which form of PDGF is essential to the development of the PDGFRα$^+$ oligodendrocyte lineage *in vivo*. Newborn PDGF-B null mice have a normal number and distribution of PDGFRα$^+$ oligodendrocyte progenitors in their spinal cord. PDGF-BB or PDGF-AB is therefore not essential for the induction, proliferation or migration of oligodendrocyte progenitors *in vivo*. However, we cannot assess the role of PDGF-B postnatally because the animals die shortly after birth. PDGF-B is expressed by capillary endothelial cells in the embryonic spinal cord (Mudhar, 1994 and Lindahl *et al.*, 1997). *In vitro*, PDGF-B is retained by the cells that make it, either as dimers within their cytoplasm, or tightly bound to the extracellular matrix (LaRochelle *et al.*, 1991; Östman *et al.*, 1991). Perhaps, in the embryonic spinal cord, PDGF-B is retained in or close to the endothelial cells of capillaries and so is unable to influence PDGFRα$^+$ oligodendrocyte progenitors. With advances in transgenic technology, such as the Cre-lox recombinase system, the postnatal role of PDGF-B in the CNS could be investigated. The severe reduction in the number of PDGFRα$^+$ progenitors and mature oligodendrocytes in the spinal cord of PDGF-A null mice demonstrate that PDGF-AA is the form of PDGF that is important to the development of the PDGFRα$^+$ oligodendrocyte lineage *in vivo*. The fact that the great reduction in PDGFRα$^+$ cells correlates with a great reduction in oligodendrocytes supports the hypothesis that most CNS oligodendrocytes develop from PDGFRα$^+$ progenitors.

Although PDGFRα$^+$ cells do migrate away from the ventricular zone in the spinal cords of PDGF-A null mice, they do not become as widely dispersed throughout the spinal cord as PDGFRα$^+$ cells in the spinal cord of wild-type animals and regions of the CNS far from the ventricular zone seem most severely affected in the PDGF-A null mouse. Perhaps PDGF-A acts as a chemoattractant within the developing CNS. Alternatively, or in addition, a reduced rate of proliferation could prevent the progenitors from migrating as quickly and/or as far before they differentiate. An increase in proliferation due to an excess of PDGF-A prevents migration of PDGFRα$^+$ astrocytes within the developing retinae of NSE-PDGF-A mice (Fruttiger *et al.*, 1996); perhaps, a decrease in the rate of proliferation in PDGF-A null mice can also hinder PDGFRα$^+$ cell migration.

It is not surprising that oligodendrocytes can survive in PDGF-A null mice, as oligodendrocyte progenitors lose PDGFRα soon after they differentiate and so become dependent on other factors for survival. These factors will presumably be present in normal amounts in the spinal cords of PDGF-A null mice.

2. Oligodendrogenesis in the brain.

There is also a reduction in the number of PDGFRα$^+$ progenitors within the brain of the PDGF-A null mouse, and a reduction in the number of oligodendrocytes that develop postnatally; this supports the hypothesis that brain oligodendrocytes develop from PDGFRα$^+$ progenitors (see Chapter Four). It is interesting that one group of PDGFRα$^+$ progenitors that remains in the PDGF-A null mouse lies around the foramen of Monro, a putative origin of brain oligodendrocytes (Pringle and Richardson, 1993). There is another cluster of PDGFRα$^+$ cells within the brainstem, perhaps this represents the source of hindbrain oligodendrocyte progenitors. That there is a much less dramatic reduction in the number of PDGFRα$^+$ cells in the brain of the PDGF-A null mouse than in the spinal cord suggests that there may be other factors involved in stimulating brain oligodendrocyte progenitor proliferation. Perhaps, PDGF-B plays a role in oligodendrocyte development within the developing brain; the expression pattern of PDGF-B in the developing brain is not known and needs to be investigated. Recently, a population of PLP/DM-20$^+$ cells within the E9.5 mouse brain has been described (Spassky *et al.*, 1998). These cells lie in the basal plate of the diencephalon and caudal hypothalamus and do not express several known neuronal progenitor markers or PDGFRα, at least at a level detectable by *in situ* hybridisation. Spassky *et al.* propose that these cells are the progenitors of a second, PDGFRα negative, oligodendrocyte lineage in the developing brain. Perhaps this lineage gives rise to some of the oligodendrocytes seen in the PDGF-A null mouse brain.

3. The supply of PDGF-AA *in vivo* regulates PDGFRα$^+$ progenitor cell cycle length and is one factor which can influence the final number of progenitors

I have demonstrated that the rate of PDGFRα$^+$ progenitor proliferation in the spinal cord is high at E13 and declines with age. This decline in progenitor proliferation rate is due to a lengthening of the cell cycle and not to cells exiting from the cell cycle. We propose that the decline in proliferation rate is due to PDGFRα$^+$ progenitors competing

for limiting amounts of PDGF-A within the spinal cord. At E13, there are very few PDGFRα$^+$ cells in the spinal cord and each of these can bind sufficient PDGF-AA to be driven quickly past restriction points in the G_1 phase of the cell cycle. At later ages, there are more PDGFRα$^+$ cells present in the spinal cord, competing for the same supply of PDGF-AA; therefore, each progenitor can bind less PDGF-AA and so passes through the cell cycle less quickly. We were able to test this proposal by examining progenitor proliferation in the spinal cords of mice that over-express PDGF-A (NSE-PDGF-A mice). Hemizygous NSE-PDGF-A mice have three times the number of PDGFRα$^+$ progenitors in their spinal cord at E15 as wild-type animals (Calver *et al.*, 1998). This demonstrates that the concentration of PDGF-A in the spinal cord normally limits progenitor proliferation, a point strengthened by the fact that homozygous NSE-PDGF-A mice have seven times the wild-type number of progenitors at E15 (Calver *et al.*, 1998). Later in development, the cell cycle time of progenitors in NSE-PDGF-A mice also lengthens. We propose that this is because the number of progenitors in these animals eventually increases to a level where even the extra PDGF-A present cannot support their rapid proliferation.

We have demonstrated that the final number of oligodendrocyte progenitors in the spinal cord can be regulated by competition for PDGF-A and the balance between PDGF-A supply and the number of progenitors 'demanding' to bind it. If each cell can bind only a little PDGF-A then, presumably, some threshold concentration of a factor needed to push the cell through a restriction point in G_1 of the cell cycle is reached very slowly and the time taken for the cell to enter the cell cycle is increased.

We have also demonstrated that PDGFRα$^+$ progenitors compete for PDGF-A within the spinal cord *in vivo* and that the concentration of this growth factor can limit the number of progenitors by slowing their entry into the cell cycle. This is not the only method of regulating progenitor number within the spinal cord *in vivo*. At E17, 20% of progenitors are still dividing, and yet the number of these cells does not increase (Figure 5.8 and Calver *et al.*, 1998); as progenitors are not differentiating at this age, some must be dying. Small numbers of apoptotic PDGFRα$^+$ progenitors are present in the E17 spinal cord (Calver *et al.*, 1998). When there is a large number of progenitors within the spinal cord, competition for survival factors may become so intense that some progenitors cannot bind sufficient amounts to prevent themselves from undergoing apoptosis. External factors are not all that determines progenitor cell number. There are cell intrinsic mechanisms that limit the number of times a progenitor can divide before differentiating, even if exposed to an excess of mitogens, at least *in vitro* (Raff *et al.*, 1985). The number of oligodendrocyte progenitors in the spinal cord *in vivo* could therefore be determined by a combination of: competition for mitogenic

PDGF-AA, competition for survival factors that might also include PDGF-AA and an intrinsic mechanism within the progenitor cells that counts and limits cell divisions.

Chapter Six
General discussion

Oligodendrocyte lineage cells within the developing optic nerve have been extensively studied and characterised. Optic nerve oligodendrocyte progenitors are known as O-2A progenitors because of their bipotentiality *in vitro*. When cultured in low serum, O-2A cells differentiate into oligodendrocytes whereas, in high serum medium, they differentiate into type-2 astrocytes. O-2A progenitors have been shown to express PDGFRα and to survive and divide in response to PDGF. PDGFRα$^+$ cells have been identified in other regions of the developing CNS; this Thesis aimed to discover whether these cells in the developing spinal cord and brain are oligodendrocyte progenitors and to find out more about what controls their development.

Pringle and Richardson (1993) identified a small group of PDGFRα$^+$ cells in the ventral half of the embryonic spinal cord. In Chapter Three I have provided direct evidence that these cells are oligodendrocyte progenitors with the same developmental potential *in vitro* as oligodendrocyte progenitors from the optic nerve. I then showed that the ability of embryonic spinal cord cells to give rise to oligodendrocytes *in vitro* correlates with the presence of PDGFRα$^+$ cells in the cultures; if these are removed by complement lysis, few, if any, oligodendrocytes can develop *in vitro*. These experiments support the hypothesis that there is one ventral, PDGFRα$^+$, oligodendrocyte lineage within the developing spinal cord. Of course *in vitro* experiments may not reveal the full *in vivo* developmental potential of the PDGFRα$^+$ cells. It is possible that if I had exposed the E17 PDGFRα$^+$ cells to appropriate factors, they would have differentiated into cells other than oligodendrocytes and type-2 astrocytes. We still do not know whether spinal cord oligodendrocyte progenitors ever develop into type-2 astrocytes *in vivo*. Once the complete promoter region of the PDGFRα gene is cloned, a transgenic mouse bearing a permanent marker gene under the control of the PDGFRα promoter could be generated to identify all the progeny of PDGFRα$^+$ cells in the spinal cord.

Chapter Three provides more evidence to support the proposal that spinal cord oligodendrocytes arise from progenitors in the ventral half of the cord; why is this? Why can't dorsal spinal cord cells give rise to oligodendrocytes? Culturing dorsal versus ventral spinal cord cells from younger animals may tell us whether dorsal cells ever have the capacity to generate oligodendrocytes. If they lose this ability at a certain age, we could then investigate why this is so. Perhaps patterning genes responsible for 'ventralising' the spinal cord are essential for the initiation of oligodendrocyte differentiation and/or 'dorsalising' genes act to prevent oligodendrogenesis.

At the time of its first appearance, the focus of PDGFRα expression in the E14 spinal cord is remarkably small; only one cell on either side of the spinal cord central canal initially expresses PDGFRα. How gene expression can be spatially regulated like this is a general question in developmental biology. Perhaps, as the expression patterns of more neural tube patterning genes are elucidated, and mutations in them studied, the mechanism of neural tube patterning will become clear and we may understand how the tiny region of PDGFRα expression is achieved. As more markers for different neural progenitors emerge, we can ask whether every neural cell type develops from a small group of ventricular zone cells or whether the neural tube is organised in some other way. There is still much to be discovered about for example, astrocyte or dorsal neuronal development.

Now that PDGFRα has been shown to be a marker for spinal cord oligodendrocyte progenitors we can use it as a marker to discover more about the early stages of oligodendrocyte development; for example, explants of E14 spinal cord could be cultured to investigate the migration of the progenitors away from the ventricular zone. Do the cells disperse in a random fashion or do they migrate along particular cell types and which factors are important in this process?

Pringle and Richardson (1993) identified groups of PDGFRα$^+$ cells in the developing brain; Chapter Four of this Thesis investigates these cells. Immunoselection experiments provide direct evidence that PDGFRα$^+$ cells within the late embryonic brain are oligodendrocyte progenitors; therefore, the entire length of the neural tube contains a PDGFRα$^+$ oligodendrocyte lineage. Other *in vitro* experiments suggest that all oligodendrocytes within the developing brain are of the PDGFRα$^+$ lineage. I have not resolved the question of whether PDGFRα$^+$ cells of the cerebral cortex arise from the cortical ventricular zone or whether, as proposed by Pringle and Richardson, they migrate into the cortex from a source in the ventral diencephalon. More thorough *in situ* hybridisation studies at different ages may answer this question and could reveal the migration routes taken by oligodendrocyte progenitors as they move throughout the developing brain. *In vitro* experiments described in Chapter Four show that bFGF and Shh can induce the development of PDGFRα$^+$ cells and the subsequent development of oligodendrocytes from E14 cortical cells. I have assumed that the oligodendrocytes arise from the PDGFRα$^+$ cells but this should be demonstrated directly. More experiments are needed to establish whether bFGF and Shh play a role in brain oligodendrocyte development *in vivo*. bFGF is a notoriously difficult factor to study; to date, there are fifteen ligands, with no known secretory signal, and four receptor types. A bFGF null mouse has been generated recently; this mouse showed neuronal defects, but the glia in its brain were not described (Dono *et al.*, 1998), it would be interesting

to examine them. Although I think it unlikely that Shh induces oligodendrocytes from cortical cells *in vivo*, it would be interesting to examine the cortex of a mouse which ectopically expresses Gli-1, the mediator of Shh function, in the dorsal midbrain and hindbrain (Hynes *et al.*, 1997) to see whether early and/or ectopic brain oligodendrocyte progenitors are induced in this animal. As we know that Shh induces oligodendrocyte progenitors in the ventral spinal cord (Pringle *et al.*, 1996), it might also be responsible for inducing the group of PDGFRα$^+$ oligodendrocyte progenitors in the ventral diencephalon of the early brain. At E14, cortical cells can be induced to generate oligodendrocytes by treatment with bFGF whereas, at the same age, dorsal spinal cord cells cannot, why is this so? Clearly there are differences in the developmental potential of brain and spinal cord progenitors that are worth investigating. Further *in vitro* studies might reveal differences between brain and spinal cord oligodendrocyte progenitors and their differentiated progeny.

Chapter Five of this Thesis aimed to investigate the relative roles of PDGF-A and PDGF-B, both of which can bind and activate PDGFRα, during the development of the PDGFRα$^+$ oligodendrocyte lineage *in vivo*. Studies of PDGF-A and PDGF-B null mice reveal that it is PDGF-AA which is crucial for the development of the oligodendrocyte lineage *in vivo*. The expression pattern of PDGF-A in the developing spinal cord prompts several questions. What is PDGF-A doing in the early floorplate? How is the PDGF-A produced by neurons able to signal to surrounding oligodendrocyte progenitors? We would also like to know the function of astrocyte-derived PDGF-A which reaches high levels later in development. Chapter Five describes experiments which demonstrate that the cell cycle of spinal cord PDGFRα$^+$ oligodendrocyte progenitors lengthens during development and we propose that this is due to increased competition for mitogenic PDGF-A within the developing spinal cord. This proposal implies that it is largely external factors which control the number of progenitors within the cord and yet there is a large body of literature which suggests that, at least *in vitro*, there are cell intrinsic limits as to how many divisions an oligodendrocyte progenitor can undergo. Culturing purified spinal cord and brain PDGFRα$^+$ progenitors in different concentrations of PDGF-A might tell us more about cell intrinsic changes to PDGF-A signalling. For example; do progenitors maintain the same sensitivity to PDGF-A right up to the time of their differentiation or does this sensitivity change? Perhaps there are changes in the number of PDGFRα on the progenitor's surface or perhaps there are changes to the intracellular signalling pathways between the receptor and nucleus. Experiments similar to the very elegant studies of Dyson and Gurdon (1998), using radioactively-labelled PDGF-A and purified progenitor cells, might tell us more about how many PDGFRα receptors have to be occupied to drive oligodendrocyte progenitor division and/or prevent differentiation and whether this changes as the progenitors age and/or go through

division cycles. Investigations such as these might bring us closer to a synthesis of how cell number during development is controlled through a combination of external factors and cell intrinsic changes.

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