GLIAL CELL LINEAGES
IN THE DEVELOPING CENTRAL NERVOUS SYSTEM

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Abstract

Glial cells far outnumber neurons in the central nervous system (CNS), yet relatively little is known about where they originate during development or how they subsequently distribute themselves throughout the CNS. In this Thesis I report the results of in situ hybridization experiments with a probe for mRNA transcripts encoding the "myelin" protein 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP); these experiments strongly suggest that the precursors of oligodendrocytes, the myelinating cells of the CNS, originate at a discrete locus in the ventral ventricular zone of the embryonic day 14 (E14) rat spinal cord. Similar experiments with a probe to proteolipid protein (PLP) mRNA, another myelin gene product, indicate that this gene is expressed by a different group of neuroepithelial precursors, not necessarily related to the oligodendrocyte lineage, in the ventral-most ventricular zone abutting the floor plate.

The notochord and floor plate, at the ventral midline of the developing spinal cord, act as organizing centres for the developing spinal cord. For example, in the absence of a notochord/floor plate complex, motor neurons (MNs) do not develop, and an extra pool of MNs can be induced in a dorsolateral position by grafting a supernumerary notochord to one side of the embryonic neural tube. Because the available markers for MNs label only the post-mitotic cells outside of the ventricular zone, it has not been possible to determine whether floor plate-derived signals act by influencing the differentiation fates of post-mitotic, pluripotent progenitor cells after they have migrated away from the ventricular zone, or whether they act by pre-specifying the future fates of neuroepithelial precursors while they still reside within the ventricular zone. I addressed this question by examining the development
of the oligodendrocyte lineage in Danforth’s Short Tail (Sd) mutant mice, which lack a notochord and floor plate in caudal regions of the spinal cord. I found that, in these mice, oligodendrocyte precursors never appeared at the ventricular surface, supporting the view that the role of the floor plate is to commit proliferating neuroepithelial cells to particular future cell fates.

I also examined the role of fibroblast growth factor (FGF) in the development of the oligodendrocyte lineage, by identifying the FGF receptor subtypes present on oligodendrocyte progenitor cells. I found that these cells express a cocktail of receptors including FGFR-1, FGFR-2 and FGFR-3 in vitro. During an in situ hybridization survey of FGFR expression in the developing CNS, I identified two foci of FGFR-3-expressing cells in the ventricular zone of the E16 spinal cord. These groups of ventricular precursors appear to generate migratory cells that spread throughout the cross section of the cord between E16 and E18. By culturing embryonic spinal cord cells in vitro and performing combined immunocytochemistry and in situ hybridization with probes for glial fibrillary acidic protein (GFAP) and FGFR-3, I showed that many of the FGFR-3-expressing cells give rise to astrocytes in vitro. Thus, two different subclasses of glia, oligodendrocytes and astrocytes, appear to originate at different loci in the ventricular zone of the neural tube. These data support the general conclusion that the ventricular zone is a mosaic of different, specialized precursor cells, each dedicated to the production of one, or a subset, of neural cell types.
ACKNOWLEDGMENTS

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Finally, I would thank my wife Su-Juan, my parents and my sister for their love and encouragement. I wish to dedicate this thesis to my daughter, Miao-Miao --she was born during my Ph.D programme at 3rd March 1993.
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ABBREVIATIONS

aFGF  acidic fibroblast growth factor
Amp  ampicillin
bFGF  basic fibroblast growth factor
BSA  bovine serum albumin
cDNA  complementary DNA
CNP  2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS  central nervous system
cyc-1  cyclops mutant (zebrafish)
DAB  3,3'-diaminobenzidine
DEPC  diethylpyrocarbonate
DMEM  dulbecco’s modified Eagles medium
DMSO  dimethylsulphoxide
DNA  deoxyribonucleic acid
DRG  dorsal root ganglia
DTT  dithiothreitol
EDTA  ethylenediaminetetraacetic acid disodium salt
E  embryonic
FCS  fetal calf serum
FGF  fibroblast growth factor
FGFR  fibroblast growth factor receptor
Fig.  figure
FITC  fluorescein-isothiocyanate
g  gravity (or gram)
GC  galactocerebroside
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>hum</td>
<td>human</td>
</tr>
<tr>
<td>l</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
</tr>
<tr>
<td>MBP</td>
<td>myelin basic protein</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
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</tr>
<tr>
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</tr>
<tr>
<td>MOPS</td>
<td>3(N-Morpholino) propanesulphonic acid monosodium salt</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
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<td>mouse</td>
</tr>
<tr>
<td>ntl</td>
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<tr>
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<td>oligodendrocyte-type 2 astrocyte</td>
</tr>
<tr>
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<td>postnatal</td>
</tr>
<tr>
<td>PBSA</td>
<td>phosphate buffered saline</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>platelet-derived growth factor receptor alpha</td>
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<tr>
<td>PDGFRβ</td>
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</tr>
<tr>
<td>PLP</td>
<td>proteolipid protein</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Sd</td>
<td>Danforth’s short tail mutant (mouse)</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
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<td>tRNA</td>
<td>transfer RNA</td>
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<td>Description</td>
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<tr>
<td>VZ</td>
<td>ventricular zone</td>
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<td>xps</td>
<td>Xenopus</td>
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<tr>
<td>zfh</td>
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Chapter One

General Introduction

The Generation of Cell lineages in the Central Nervous System of Vertebrates.
1.1 A Brief Introduction to Vertebrate Development.

1.2 Cell Diversity in the Vertebrate Central Nervous System.

1.3 Experimental Approaches to Establishing Cell Lineage Relationships.

1.4 Glial Cell Lineages in the Optic Nerve.

1.5 Cell Lineages in the Retina.

1.6 Cell Lineages in the Cerebral Cortex.

1.7 Conclusions.
1.1 A brief Introduction to Vertebrate Development.

The body of an adult multicellular organism contains trillions of cells which, in nearly all cases, are generated from a single cell—the fertilized egg, or *zygote*—which divides mitotically to produce all the diverse types of cells of the body. The process by which the fertilized egg grows and changes to generate first an embryo, then a neonate and eventually a mature animal is called *development*. Although a variety of types of animal development exist, almost all of them involve the following four stages.

First there is a series of divisions of the zygote just after fertilization, which are not accompanied by cell growth and consequently give rise to numerous relatively small cells. This process is called *cleavage*. At the end of cleavage, the small cells form a sphere, called a *blastula*.

The second stage of animal development is called *gastrulation*. During this process a variety of dramatic cell movements happen within the blastula. As a result of these cell rearrangements three cell layers are generated. The outer layer, called *ectoderm* will produce the cells of the epidermis and the nervous system during later development; the inner-most layer, called *endoderm*, will produce the lining of the digestive tube and its associated organs, e.g. pancreas, liver; the middle layer, called *mesoderm*, will give rise to several organs (heart, kidney), connective tissues (bone, muscles), the blood cells and some special structures, such as the notochord, which appears transiently during embryonic life.

After gastrulation, the cells in the three cell layers interact with each other and
rearrange themselves to produce the organs. This process is called organogenesis. Organogenesis in vertebrates is initiated when a series of cellular interactions causes the mid-dorsal ectoderm cells to form the neural tube which will give rise to brain and spinal cord, together forming the central nervous system (CNS). Most organs contain cells derived from more than one germ layer.

The fourth stage of animal development is called gametogenesis. During development, a proportion of cells give rise to germ cells which are the precursors of gametes. Gametes are sex cells which participate in fertilization to create a new individual. The development of gametes is usually not completed until the animal becomes physically mature. At maturity, the gametes may be released and undergo fertilization to begin a new life. Meanwhile, the adult organism undergoes senescence and eventually dies.

Vertebrates are distinguished from invertebrates by having a vertebral column. I will mainly concentrate on the development of vertebrates in this Introduction. During vertebrate development, one of the most important and earliest interactions between different types of cells occurs between dorsal mesoderm and its overlying ectoderm. In this event, which starts during gastrulation, mesodermal cells send signals to their overlying ectodermal cells to induce them to change shape from flattened to elongated. In doing so, the ectoderm forms a special structure, called the neural plate. The edges of the neural plate thicken and move upward to form the neural folds. A further folding of this structure creates a U-shaped groove which eventually fuses along the dorsal midline to form a hollow tube, called the neural tube, which will give rise to the brain and spinal cord. The cells at the dorsal-most portion of the neural tube become the neural crest, migrate throughout the embryo to generate several cell populations, including pigment cells and the cells of the
peripheral nervous system (PNS). This process, which creates the founders of both CNS and PNS, is called \textit{neurulation}.

The formation of the neural tube does not occur simultaneously throughout the ectoderm along the anterior-posterior axis of the body. Neurulation in the anterior (head) region is more advanced than the posterior (tail) region. This temporal gradient of neural tube development from anterior-to-posterior persists throughout CNS development. During neurulation, a transient structure called the \textit{notochord}, is formed from mesoderm underneath the neural tube. This longitudinal cord is believed to play a crucial role in the early definition of neural cell types within the developing neural tube.

During later development, the neural tube undergoes substantial shape changes accompanied by cell diversification and eventually forms the most complicated organ of a vertebrate, the CNS. Differentiation of the neural tube occurs simultaneously at both anatomical and cellular levels. At the anatomical level, the most anterior region of the tube expands into three primary vesicles--forebrain, midbrain, and hindbrain. Later, these three regions undergo further folding to form the brain, which includes forebrain, cerebral cortex, cerebellum and brainstem. At the cellular level, the single layer of epithelial cells of the neural tube differentiates to generate the numerous types of neurons and glial cells of the adult CNS. One of the most challenging questions in developmental neurobiology is: how do the apparently identical neuroepithelial cells in the early neural tube subsequently differentiate into the vast array of neurons and glia in the mature CNS?
1.2 Cell Diversity in the Vertebrate Central Nervous System.

The vertebrate nervous system consists of two parts, a main processing unit, the *central nervous system* (CNS) which comprises the brain and spinal cord, and the *peripheral nervous system* (PNS) which consists of nerves and their cell clusters called *ganglia*.

There are two major populations of cells in the CNS; neurons, which receive, conduct and transmit signals, and glial cells, which contact neurons and presumably support them in various ill-defined ways. Neurons can be classified into a variety of different subtypes based on their positions and biological functions. Once formed from their mitotically active precursor cells, neurons do not divide further. There are four major classes of glial cells in the CNS; oligodendrocytes, astrocytes (together called *macroglia*), microglia and ependymal cells. Of these, oligodendrocytes are the best understood. They wrap themselves around nerve axons to provide electrical insulation in the form of a myelin sheath. Microglia originate from hemopoietic tissue and function as the macrophages of the CNS. Because of their essentially non-neural nature, they will not be considered further in this Introduction. Ependymal cells are located in the internal cavities of the CNS. Astrocytes are the most plentiful and diverse of the glial cells. Although it seems that they play important roles in guiding the construction of the nervous system and in controlling the chemical and ionic environment of the neurons, the functions of astrocytes are still largely obscure. All of the macroglia and neurons share a common embryonic origin. However, unlike neurons which do not divide after they have differentiated, most glial cells remain capable of dividing over a longer period of development and even, under some circumstances, in the adult.
Differentiated cells in the CNS originate from undifferentiated precursor cells. These immature neuroepithelial precursors are initially located in the ventricular zones (VZs) surrounding the ventricles of the spinal cord and brain, where most mitotic activity is localized. Subsequently, in the brain though not in the spinal cord, the zone of mitotic activity moves away from the ventricular surface to form a deeper germinal region called the subventricular zone (SVZ). The neural precursors that reside within the VZ and SVZ stop dividing at predetermined stages of development and give rise to postmitotic, migratory progenitor cells that may or may not be predetermined to differentiate into a particular type of neuron or glial cell. These progenitor cells migrate away from the VZ or SVZ along the processes of radial glia into the developing gray matter of the brain or spinal cord. If we wish to understand how neural precursors "decide" which type of differentiated neural cell to generate, we must first work out how the different mature cell types are related to each other by genealogy, in order to know when and where specific developmental choices are made. The elucidation of cell genealogies is known as cell lineage analysis.

For cell lineage studies of vertebrate CNS, several parts of the CNS have advantages because of their structural simplicity and/or accessibility for manipulation. These include optic nerve, retina, olfactory bulb, cerebral cortex and spinal cord. In this review, I concentrate on optic nerve, retina and cerebral cortex. First, I shall introduce several general techniques used in the study of cell lineage.

1.3 Experimental Approaches to Establishing Cell Lineage Relationships.

There are three main approaches used in the study of cell lineage in the CNS: 1) identifying appropriate molecular markers that might be shared among cells of a particular lineage, 2) microculture of single neural cells and analysis of their progeny.
to study the developmental potential of a precursor in vitro and the factors involved in their proliferation and differentiation, 3) microinjection of fluorescent tracers or infection with retroviral vectors to mark the descendants of a single neural precursor in vivo or in vitro.

**Molecular markers for cell lineage.**

Specific molecular markers for a cell lineage are the most straightforward tools in the study of cell lineage development both in vivo and in vitro. Molecular lineage markers (gene products or modifications thereof) can be examined at two levels; specific mRNAs can be detected by in situ hybridization with DNA/RNA probes, or proteins or protein modifications can be detected by immunohistochemistry with specific antibodies. These approaches can be used to investigate the distribution and origin of a cell lineage in vivo. In vitro, lineage-specific antibodies can be used not only for immunolabelling, but also for other approaches, such as immunoselection of particular populations of cells from mixtures of dissociated primary cells (Barres et al 1988, Barres et al 1992, Collarini et al 1992), or removing a particular population of cells from a mixed culture by complement fixation (Raff et al 1983a, Raff et al 1983b). Several markers have been widely used in neurobiology to distinguish different populations of neural cells. For example, antibodies against some neurofilament epitopes specifically recognise certain classes of neurons, while antibodies against the glial fibrillary acidic protein (GFAP) mark several kinds of astrocytes. Oligodendrocytes are specifically labelled on their surface by antibodies to galactocerebroside (GC) (Raff et al 1978), and oligodendrocyte precursors at different stages of maturation can be recognized by several cell-surface antibodies including monoclonal A2B5 (Eisenbarth et al 1979), O4 (Sommer and Schachner 1982), anti-NG2 chondroitin sulphate proteoglycan (Levine and Stallcup 1987), and
anti-PDGFRα (Pringle et al 1992; Stallcup, personal communication; Hall and Richardson, unpublished data). These and other markers have been extremely useful for developmental studies of the oligodendrocyte lineage both in vitro (Raff et al 1983, Gard and Pfeiffer 1990) and in vivo (Reynolds and Wilkin 1988, Hardy and Reynolds 1991, Le Vine and Goldman 1988, Pringle et al 1992, Pringle and Richardson 1993).

Clonal analysis of single cells.

In this approach, single cells derived from developing neural tissue are plated in microwells and observed for extended periods of time to determine what cell types develop in the clonal progeny of the original cell. A simpler modification of this approach is to plate cells at very low density in a tissue culture dish, so that individual founder cells and their progeny can be easily distinguished from other cells in the same culture. The main difficulty with this approach is in providing the appropriate culture conditions—including factors required for proliferation, differentiation and survival of the starting cells and their progeny. Often, single cells are cultured in medium previously conditioned by other neural cells, or even on top of a monolayer of live "feeder" cells that have been made mitotically inactive by irradiation. The single cell culture approach is technically difficult and, while it has provided important information about cell lineage, especially in the hemopoietic system, suffers from the drawback that results obtained in vitro eventually need to be confirmed in vivo (Temple and Raff 1985).

Lineage tracing by dye microinjection or retroviral infection.

Another way of tracing cell lineage development is by labelling precursors of
a cell lineage with an exogeneous marker, which might be a nontoxic high-molecular-weight fluorescent dye or a heritable genetic marker, such as a genetically modified retrovirus. A variety of dyes or enzymes that can generate coloured reaction products, for example, fluorescent dextran or horseradish peroxidase (HRP), have been directly microinjected into progenitor cells and the fate of injected progenitors followed by examining the labelled progeny (Holt et al 1988). This approach is conceptually straightforward, but there are several limitations: 1) The dye needs to be microinjected into individual cells, so the target cells must be easily accessible. Cells either too small in size or buried too deep to be reached by a microelectrode can not be studied by this method. Most frequently, the method has been applied to early development of fish and frogs, of which the fertilized eggs and early developing embryos are very easily accessible. 2) Cells can be damaged by the process of microinjection. 3) During cell proliferation, the injected dyes are gradually diluted and eventually become undetectable. Consequently, long term studies of cell lineage can not be studied by this method.

In order to overcome some of these difficulties, particularly the problem of dye dilution, recombinant retroviral vectors have been developed that allow permanent, heritable marking of infected cells and their progeny with a reporter gene product such as β-galactosidase (Price 1987, Cepko 1988, Sanes 1989). The genetically modified retrovirus can be targetted by microinjection into particular regions of the developing CNS, such as the central canal of the embryonic mouse spinal cord or the ventricles of the brain. When a retrovirus infects a dividing cell, its genome integrates into a chromosome of the infected cell and is inherited by the subsequent progeny of that cell. Since some of the viral genes that are required for viral packaging have been replaced by the reporter gene, these integrated retroviruses can not produce new infectious viruses, and can not be horizontally transmitted to neighbouring cells.
There are several advantages to the retroviral approach: 1) As they are infectious, the retroviruses need only to be implanted in the general position of the cells under study, avoiding the difficulty of intracellular injection. 2) As the integrated retroviral genome is heritable the gene remains stable and undiluted in daughter cells, which enables a long term studies of cell lineage development in vivo. There are unfortunately, also several disadvantages to this method: 1) The identification of a clone is based on the assumption that clustered cells that express the lacZ gene are clonally related. This assumption has been supported by two observations: First, the number of lacZ-expressing clusters are decreased with the dilution of the injected virus, whereas the number of lacZ-expressing cells in each cluster is not. Second, by injecting a mixture of viruses that containing different reporter genes, it was found that cells in most individual clusters only express one of the reporter genes. 2) Although all the progeny of an infected progenitor presumably carry the retroviral genome, only cells that express lacZ are detected. Since levels of lacZ expression might vary with cell type and site of integration, "false negatives" could always be a problem. 3) As with other retrospective methods, cells that die cannot be visualized and therefore are omitted from the analysis.

1.4 Glial Cell Lineages in the Optic Nerve.

The optic nerve is one of the simplest parts of the vertebrate CNS. It develops from an extension of the neural tube, in the region of the diencephalon, called the optic stalk. In the optic nerve there are no neuronal cell bodies, only the axons of retinal ganglion neurons and a variety of glial cells. Therefore, the optic nerve is an ideal place to study glial cell lineages. Two major types of glial cells and their precursors have been identified in the neonatal rat optic nerve (excepting the microglial cells, which originate from hemopoietic tissue and are not discussed here).
The first glial cells to appear in the nerve during development are astrocytes, which first appear around embryonic day 16 (E16), while oligodendrocytes first appear on the day of birth (Miller et al 1985).

**Optic nerve astrocytes and oligodendrocytes are generated from two separate cell lineages.**

In cultures of dissociated rat optic nerve cells, Raff et al (1983) identified a glial progenitor cell that could give rise to either oligodendrocytes or astrocytes (type-2), according to the composition of the culture medium. In defined medium containing less than 0.5% fetal calf serum (FCS) these bipotential glial progenitors differentiated into GC-positive oligodendrocytes, whereas in 10% FCS they generated type-2 astrocytes. The progenitors were therefore named O-2A progenitors, a name that has been preserved even though subsequent attempts to identify type-2 astrocytes in the optic nerve in vivo have not been successful (Fulton et al 1992). The other identified class of glial cells in optic nerve cultures are so-called type-1 astrocytes, which develop from their own dedicated progenitor cells and which presumably correspond to those astrocytes that populate the optic nerve in vivo. These various glial cells and their progenitors can be distinguished by reactivity with antibodies; O-2A progenitors are A2B5+/PDGFRα+/GFAP+/GC−, oligodendrocytes are GC+, type-2 astrocytes are A2B5+/GFAP+ and type-1 astrocytes are A2B5−/GFAP−.

O-2A progenitor cells from rat optic nerve stop dividing and differentiate into oligodendrocytes within two days when cultured in defined medium containing low (< 0.5%) FCS (Raff et al 1983, Temple and Raff 1985). Moreover, when a single O-2A progenitor is cultured on its own in a microwell in low-serum medium, it differentiates into an oligodendrocyte, suggesting that oligodendrocyte differentiation
is the default pathway for O-2A progenitors when they are deprived of signals from other cells. O-2A progenitors first appear in the rat optic nerve at E16 (Small et al 1987), but differentiated oligodendrocytes do not appear in the nerve until the day of birth, about 5 days later (Miller et al 1987). The difference in the timing of oligodendrocyte development in vitro and in vivo suggests that the embryonic optic nerve contains a factor(s) that can keep O-2A progenitor cells dividing and inhibit their differentiation.

We now know that one factor that promotes O-2A progenitor cell proliferation and inhibits oligodendrocyte differentiation in vivo is PDGF. The evidence can be summarised as follows: First, pure PDGF is a potent mitogen for O-2A progenitors in vitro (Richardson et al 1988, Raff et al 1988, Noble et al 1988) and O-2A progenitors possess high affinity receptors for PDGF (Hart et al 1989, McKinnon et al 1990, Pringle et al 1992). Second, PDGF, in the presence of IGF-1 or insulin, can reconstitute the normal timing of oligodendrocyte development in embryonic optic nerve cell cultures (Raff et al 1985, Raff et al 1988). Third, PDGF mRNA (Pringle et al 1989, Mudhar et al 1993) and protein (Mudhar et al 1993) can be detected in the perinatal optic nerve at the time O-2A progenitors are actively proliferating, and protein extracts of optic nerve contain mitogenic activity for O-2A progenitors, most of which can be neutralized by anti-PDGF antibodies (Raff et al 1988). Fourth, transgenic "knockout" mice that lack a functional PDGF-A gene are severely compromised in the number of oligodendrocytes and oligodendrocyte progenitors that develop in the spinal cord (unpublished results from this laboratory).

There is evidence that PDGF in the developing optic nerve is derived from type-1 astrocytes. 1) Astrocytes derived from rat cerebral cortex, which resemble optic nerve astrocytes in culture, make and secrete PDGF in vitro (Richardson et al
1988). 2) PDGF can be detected in astrocyte cell bodies and processes in the optic nerve by immunohistochemistry (Mudhar et al 1993). Taken together, the evidence strongly suggests that proliferation of O-2A progenitors and their timely differentiation into oligodendrocytes in the developing optic nerve is controlled by astrocyte-derived PDGF. However, there is also evidence that the axons of retinal ganglion neurons (RGCs) that pass along the nerve are also required for proliferation of O-2A progenitors (Barres and Raff 1993). Moreover, electrical activity in these axons seems to be necessary for proliferation (Barres and Raff 1993). Retinal ganglion neurons also make PDGF (Mudhar et al 1993), and could conceivably supply PDGF to the optic nerve. However, although PDGF immunoreactivity can be detected in and around the cell bodies of RGCs in the retina, PDGF can not be detected in their distal axons in the optic nerve. One possibility is that electrical activity in RGC axons controls the synthesis or release of PDGF from astrocytes in the nerve (Barres and Raff 1993).

In addition to PDGF, FGF and neurotrophin-3 might also play roles in oligodendrocyte development. FGF is a mitogen for O-2A progenitors in vitro (Eccleston and Silberberg 1985, Saneto and DeVellis 1985, Bögler et al 1990, McKinnon et al 1990). The combination of bFGF and PDGF has a striking cooperative effect, stimulating prolonged proliferation of O-2A progenitors and preventing oligodendrocyte differentiation in vitro (Bögler et al 1990). Although it has been shown that bFGF is present in the developing and mature CNS (Gospodarowicz 1984, Gonzalez et al 1990) and it seems that O-2A progenitors possess FGF receptors (Appendix I of this Thesis), it does not seem likely that bFGF and PDGF act together on O-2A progenitors in the postnatal optic nerve or other developing white matter tracks, otherwise oligodendrocyte differentiation presumably would be inhibited. The involvement of FGF in O-2A progenitor development in vivo
is still not clear.

Neurotrophin-3 by itself can stimulate a small percentage of O-2A progenitor cells to incorporate BrdU in vitro. However, in combination with PDGF, neurotrophin-3 can both significantly expand the size of O-2A progenitor cell clones and drive the intrinsic clock that controls oligodendrocyte development in vitro (Barres et al 1994). There is evidence that neurotrophin-3 might be released by type-1 astrocytes in the optic nerve and that O-2A progenitors possess the neurotrophin-3 receptor, Trk C (Barres et al 1994). Thus, neurotrophin-3 might participate in regulating the normal process of O-2A progenitor development in vivo. In addition, insulin-like growth factors have been shown to serve as survival factors but not mitogens for O-2A lineage cells (Barres et al 1992).

After O-2A progenitors have differentiated into oligodendrocytes, they lose their PDGF receptors and become reliant on other factors for their long-term survival. These survival factors include representatives of the CNTF/LIF family, the neurotrophin family, and IGF-1 (Barres et al 1992).

**O-2A progenitors are migratory cells during development.**

Time-lapse microcinematographic studies of neonatal optic nerve cells in culture have shown that the O-2A progenitor cells migrate until they differentiate into oligodendrocytes, when they stop dividing and migrating (Small et al 1987). There are also several lines of evidence that O-2A progenitors are migratory cells in vivo. First, O-2A progenitors accumulate in the embryonic optic nerve in a wave progressing from the brain end of the nerve towards the eye (Small et al 1987, Mudhar et al 1993) suggesting that they migrate into the nerve from the brain.
Second, oligodendrocytes or their progenitors, O-2A cells, have been shown to migrate substantial distances into normal CNS tissue from both CNS transplants in vivo (Lachapelle et al 1984, Gansmuller et al 1986, Baulac et al 1987) and CNS explants in vitro (Wolf et al 1986). Third, when a segment of quail spinal cord is transplanted into a chick spinal cord at open neural plate stage of development, the graft can be seamlessly incorporated into the host to form a chimeric spinal cord and the bird can develop to hatching; when the area of the graft is examined subsequently, quail oligodendrocytes are found up to 2mm from the graft, demonstrating long-range migration during "normal" spinal cord development (unpublished results from this laboratory).

Unlike the O-2A cell lineage, evidence suggests that type-1 astrocytes are generated, directly from the non-migratory neuroepithelial cells of the optic stalk (Temple and Raff 1986, Small et al 1987).

**Oligodendrocyte precursors in adult optic nerve.**

Unlike neurons, glial cells can be stimulated to proliferate in the adult CNS. For example, astrocytes proliferate in areas subjected to mechanical injury, and new oligodendrocytes can be generated to remyelinate demyelinated axons. Are these newly generated oligodendrocytes generated from the same O-2A progenitor cells that populate the CNS during development? This seems unlikely, since it has been shown that perinatal O-2A progenitor cells can undergo a limited number of cell divisions before differentiating into oligodendrocytes in vitro (Raff et al 1985). The fact that oligodendrocytes can be generated throughout the life time of an animal suggests that there may be a different mechanism, perhaps a different population of progenitors, to maintain the oligodendrocyte population in the adult CNS.
Indeed, slowly dividing oligodendrocyte precursor cells can be isolated from adult optic nerves (Wolswijk and Noble 1989). Comparative studies of 0-2A progenitors from adult and perinatal rat optic nerve reveal that these cells differ from one another in several ways (Wolswijk and Noble 1989), and to reflect this fact the two types of precursors have been named O-2A$^{\text{adult}}$ and O-2A$^{\text{perinatal}}$ progenitors, respectively. The two types of progenitor differ as follows: 1) In culture, O-2A$^{\text{adult}}$ progenitors have an unipolar morphology (Wolswijk and Noble 1989), whereas O-2A$^{\text{perinatal}}$ progenitors are often bipolar (Temple and Raff 1986). 2) O-2A$^{\text{adult}}$ progenitors have a long average cell cycle time of 65 hours in vitro (Wolswijk and Noble 1989) than O-2A$^{\text{perinatal}}$ progenitors for which the average cell cycle time is 18 hours (Raff et al 1983, Noble et al 1988). 3) O-2A$^{\text{adult}}$ progenitors migrate more slowly in vitro and take longer to differentiate into oligodendrocytes than O-2A$^{\text{perinatal}}$ progenitors do (Wolswijk and Noble 1989, Temple and Raff 1986). 4) O-2A$^{\text{adult}}$ progenitors can be labelled with antibodies against vimentin, whereas O-2A$^{\text{perinatal}}$ progenitors can not.

O-2A$^{\text{adult}}$ progenitors first appear in the optic nerve in small numbers during the second postnatal week and gradually replace the O-2A$^{\text{perinatal}}$ progenitors as the dominant progenitor population during the first postnatal month (Wolswijk et al 1990). In culture, single O-2A$^{\text{perinatal}}$ progenitor cells grown in the presence of type-1 astrocytes divide a limited number of times and all of their daughter cells differentiate into oligodendrocytes almost simultaneously (Temple and Raff 1986). However, O-2A$^{\text{adult}}$ progenitor cells divide asymmetrically and give rise to both oligodendrocytes and O-2A$^{\text{adult}}$ progenitors (Noble et al 1989, Noble et al 1990). Thus, an O-2A$^{\text{adult}}$ progenitor cell might be capable to self-renewal and thus might be a kind of stem cell, capable of generating new oligodendrocytes continuously in vivo. There is evidence that O-2A$^{\text{adult}}$ progenitors develop from a subpopulation of O-2A$^{\text{perinatal}}$ progenitors.
1.5 Cell Lineages in the Retina.

The mature retina is a layered structure containing six major classes of neurons and two types of glial cells, Müller cells and retinal astrocytes. The six major classes of retinal neurons are photoreceptor cells, horizontal cells, bipolar cells, amacrine cells, interplexiform cells and ganglion cells. Some of these can also be classified into different subtypes; for example, photoreceptor neurons can be subdivided into rod and cone photoreceptors according to their different morphologies. All the retinal cells are located in different layers and work together to process and transduce visible signals into the brain via the optic nerve. Apart from retinal astrocytes, which appear to migrate into the retina from the optic nerve head (Watanabe and Raff 1987), all the other types of cell are generated locally from pluripotent retinal precursor cells that reside near the outer surface of the retina (see below).

During early development of the CNS, a ventrolateral portion of diencephalic wall of each side of the brain extends, grows out and finally contacts the inner surface of the ectoderm to form a bilayered structure called optic cup. The cells of the outer layer produce pigment and ultimately become the pigmented retina, while the cells of the inner layer proliferate rapidly and generate the cells of the neural retina (Young 1985, Carter Dawson and LaVail 1979, Blanks and Bok 1977). How are these different populations of cells in the neural retina generated from the proliferating neuroepithelium, the ventricular zone of the developing retina?

By infecting the newborn rat retina at different ages (P0 to P7) with a recombinant retroviral vector and examining the clonal progeny of infected precursors at a later age (usually 4-6 weeks postnatally), it has been found that although most retinal precursors produced only one of the four postnatally produced cell types, a
significant proportion of cell clones contained two or even three different types of retinal cells (Turner and Cepko 1987). Two-cell-clones were frequent (3%-18% of total) and included clones consisting of a rod photoreceptor cell and a bipolar cell, a rod photoreceptor and an amacrine cell, or a rod photoreceptor and a Müller glial cell. Three-cell-clones appeared less-frequently (0.2%-0.6% of all clones); they included clones containing a rod photoreceptor, a bipolar cell and an amacrine cell, or a rod photoreceptor, a bipolar cell and a Müller glial cell. These results suggest that the postnatal rat retina possesses a common precursor that can give rise to several retinal cell types. Unlike precursors in other parts of the CNS, for example the cerebral cortex (see below), in which it seems that the precursors diversify at an early stage of development (E16) before they leave the ventricular zone, retinal precursors appear to be largely uncommitted even after birth.

When a recombinant retroviral vector is injected into an embryonic, rather than postnatal, mouse retina, labelled clones are usually larger and contain more cell types, sometimes even all six major types of cells in the retina (Turner et al 1990). Similar results have been obtained by microinjecting individual developing Xenopus retinal precursors with fluorescent dextran or horseradish peroxidase (Wetts and Fraser 1988, Wetts et al 1989, Holt et al 1988). These investigations have also demonstrated that single progenitor cells can give rise to all major cell types in the frog retina.

The appearance of large clones following embryonic infections implies that the production of retinal cells is not governed by a stem cell mode of division. In this scheme, a daughter cell and another stem cell should be generated from a mother stem cell in each cell cycle. Thus, after a given period of cell division, the number of daughter cells should be more-or-less equivalent to the number of cell divisions. However, in the retina the cell number in each clone is actually much greater than the
number of cell cycles, suggesting that retinal progenitor cells undergo symmetrical divisions and at least some of the resulting daughter cells are mitotic and are themselves able to generate new cells.

Taken together it seems that, in the vertebrate retina, precursor cell specification is made at a late stage of development. Moreover, in the postnatal studies, it was shown that some clones only contain two cells and that these two cells belong to two different cell types (Turner and Cepko 1987). This result suggests that cell fate determination of retinal progenitors may be made at or after the final division. However, interpretation of the data might be complicated if significant numbers of retinal cells are lost by programmed cell death. It is known, for example, that around 50% of ganglion neurons die during development (Young 1984) presumably as a result of competition for limiting quantities of survival factors derived from their targets in the superior colliculus. The appearance of a common retinal precursor that can give rise to several distinct cell types during late development implies that the determination of cell fate in the retina might rely largely on the environment where the precursors begin to differentiate rather than an intrinsic mechanism operating within the cells. However, there is also some evidence that retinal precursors might be committed to give rise to particular cell types before they leave the ventricular zone. First, the existence of characteristic birth dates for different cell types implies that retinal precursors do not remain uncommitted for long periods after becoming postmitotic. Second, all the retinal precursors in the ventricular zone do not seem to be identical antigenically. For example, a subset of precursors express an amacrine cell-specific antigen during late embryogenesis in the rat (Barnstable et al 1985). Similarly, a ganglion cell-specific antigen is expressed in some ventricular cells in the developing chicken retina (McLoon and Barnes 1989). This suggests that some precursors might become pre-specified and begin to
differentiate before leaving the retinal ventricular zone. Some in vitro studies also support the idea that cell type determination can occur in postmitotic chicken retinal progenitor cells (Adler and Hatlee 1989). Although some models of retinal precursor differentiation have been proposed (Reh and Tully 1986, Reh 1987, Wetts and Fraser 1988, Wetts et al 1989, Turner et al 1990), whether the future cell fate of retinal precursors is specified before the precursors leave the ventricular zone or is determined by the microenvironment where they begin to differentiate is still uncertain and needs further investigation.

Another feature of retinal cell development is that the progeny of a single retinal precursor cell are always arranged radially in the retina and rarely migrate away from the sector in which they were generated. This might result from the cells leaving the ventricular zone by migrating along the radially-oriented process of Müller glia.

1.6 Cell Lineages in the Cerebral cortex.

The cerebral cortex, one of the largest structures in the CNS, consists of a sheet of interconnected neurons and a large number of associated glial cells. That part of the cortex that lies on the surface of the brain is called neocortex. This accounts for more than 90% of the total cortical area, while the remainder is made up of paleocortex and archicortex. Paleocortex consists of some restricted parts of the base of the telencephalon, and archicortex comprises the hippocampal formation. All neocortical areas go through a period of development during which they have a six-layered structure. This layered appearance persists in only some areas of the adult brain but, in view of its uniform early development, the neocortex is also referred to as homogenetic cortex or isocortex. In contrast, paleocortex and archicortex never
undergo such a six-layered stage and are therefore referred to collectively as heterogenetic cortex or allocortex. The hippocampal formation is a component of the limbic system, and the paleocortex, which develops in conjunction with the olfactory system, is closely interconnected with limbic structures.

As mentioned above, most portions of the neocortex have six-layered structures as viewed by several different methods of staining during development. From outside in, the six layers are referred as the molecular layer, the external granular layer, the external pyramidal layer, the internal granular layer, the internal pyramidal layer and the multiform layer. Both vertically and horizontally, the neocortex can be divided into anatomically and functionally distinct areas.

There are two principal neuronal cell types in the neocortex, stellate (or granule) neurons and pyramidal neurons. In addition to neurons, there are also a large number of oligodendrocytes and astrocytes. All the cortical neurons and glial cells are originally derived from the embryonic ventricular zone. A lot of attention has focussed on the genesis of these cortical cell types during development. For example, how many types of precursors are there in the ventricular zone? i.e. is there, as in the retina, a single pluripotent precursor cell that is able to generate all the cortical cell types, or is each type of mature cortical cell generated by its own dedicated class of precursor?

By using a retroviral vector that expresses β-galactosidase to trace the development of single precursors (Price 1987), several groups have shown that separate precursors for cortical neurons, oligodendrocytes and astrocytes have been set aside before they leave the ventricular or subventricular zone of the cortex (Luskin et al 1988, Price and Thurlow 1988, Walsh and Cepko 1988, Luskin et al 1993). By
injecting the recombinant retrovirus into E12-E14 mouse brain ventricles to mark
telencephalic precursor cells, Luskin et al (1988) found that nearly all individual
clones contained either neurons or glial cells, but not both. This result indicated that
separate populations of neuroblasts and glioblasts are present in the ventricular zone
in E12-E14 mouse, and implies that neuronal and glial lineages have diverged by this
time. This result was also confirmed by in vitro studies in which clones consisting
solely of either neurons or glial cells were derived from distinct retrovirus-infected
precursor cells (Luskin et al 1988).

A similar result has been obtained from a cell lineage study of rat cerebral
cortex (Price and Thurlow 1988, Grove et al 1993), in which the recombinant
retrovirus vector was injected into the ventricular zone of E16 rat brain and the
labelled progeny examined at P14. Four distinct types of cell clones were identified:
First, there was a type of clone which usually contained 20-30 cells and spread
throughout the cortical grey matter. As initially identified by morphology and by their
frequent association with blood vessels or the pial surface (Price and Thurlow 1988)
and later by GFAP staining (Grove et al 1993), these cells were identified as
astrocytes. The second type of clone contained a small number of cells (usually one
to four and rarely more than six cells) which were identified as neurons by
morphology (Price and Thurlow 1988) and by immunohistochemical staining (Grove
et al 1993). Some but not all of these cells had the typical shape of pyramidal
neurons and most possessed a major vertically-directed dendrocyte, which is typical
of cortical neurons. The third type of clone was confined to the white matter and
consisted of cells with small round cell bodies and few processes. Although it was
difficult to identify these by morphology, they were thought to be undifferentiated
astrocyte or oligodendrocyte precursors because of the time and place that they
appeared (Price and Thurlow 1988). A later investigation (Grove et al 1993) revealed
that these undifferentiated white matter cells differentiate either into oligodendrocytes or astrocytes by P28/29. However, there has been no evidence that both oligodendrocytes and astrocytes can appear in the same cluster. Since O-2A progenitor cells in the optic nerve and elsewhere can generate both oligodendrocytes and astrocytes, it was suggested that the undifferentiated white matter cells referred to above could be the cortical counterparts of optic nerve O-2A progenitor cells. In addition, a fourth type of clone has been identified. The progenitor that gave rise to these clones are capable of giving rise to both neurons and white matter cells. A later in vitro investigation has suggested that the white matter cells in this group are oligodendrocytes (Grove et al 1993) and the progenitors cell have therefore been called N-O cells.

Most of these studies suggest that the precursors for distinct cell types in the cerebral cortex have diversified before giving rise to postmitotic progeny that migrate away the ventricular zone, and thus imply that the ventricular and subventricular zones of the brain at this stage of development (E12/14 in mice and E16 in rat) actually comprise a variety of distinct neural precursors. However, the presence of N-O clones implies the existence of a small population of precursors that still remain bipotential or multipotential at this age. A similar type of bipotential precursor has also been reported in the embryonic telencephalon (Williams et al 1991) and in the hippocampal formation (Grove et al 1992). During neurogenesis, the developmental potential of cells in the ventricular zone undergoes a transition from more general to more restricted. The presence of a small percentage of bipotential precursors at E16 and the absence of these cells at the E18 suggests that the final stages of cell lineage restriction are occurring during this period in the rat cortex.
A further detailed study has demonstrated that neuronal clones can be subdivided into clones containing exclusively pyramidal neurons or nonpyramidal neurons, but not both (Parnavelas et al 1991).Taken together, it seems that the cells that make up the cerebral cortex are generated from precursors in the ventricular zone that, by the onset of neurogenesis, have already diversified and become dedicated to the production of particular subpopulations of neurons, oligodendrocytes or astrocytes. This result implies that, in the cortex, cell lineage rather than the microenvironment where progenitors begin to differentiate might be the crucial factor in determining the fate of cells.

1.7 Conclusions.

One of the most challenging topics in neurobiology is to understand how the single cell layer of neuroepithelial cells in the embryonic neural tube gives rise to the huge variety of neuronal and glial cell types during neurogenesis. Although it is far from clear how the developmental fate of a cell is determined, it seems that at least two crucial factors might be involved—the intrinsic, heritable characteristics of the cell and the extrinsic microenvironment at the site where cell differentiation occurs. The relative contributions of these two influences might vary from site to site in the developing CNS.
Chapter Two

Materials and Methods
Unless otherwise stated all chemicals and reagents were purchased from BDH Chemicals Ltd., which are Analar grade, or Sigma Chemical Company, which are Molecular Biology Reagent grade.

All restriction endonucleases and other enzymes were purchased from New England Biolabs, Inc., or Promega Ltd.

All radio-chemicals were purchased from Amersham International.

All specialised bacterial media components were purchased from Difco Laboratories Ltd.

All tissue culture media components were obtained from GIBCO BRL Life Technologies Company.

Solutions were autoclaved at 15 lb/sq.in. for 20 minutes where necessary, and store at room temperature unless otherwise stated.
2.1 Bacteriology.

All methods used in this section were taken from Sambrook et al (1989).

2.1.1 Bacterial strains.

For general cloning of PCR products and sub-cloning of recombinant plasmids, the following *Escherichia coli* (*E. coli*) strains were used.

- **JM101**: $(supE thi\Delta (lac-proAB) F'[traD36 proAB* lac I\^ lacZ\Delta M15])$
- **DH5α**: $(supE44 \Delta lacU169 (\phi80 lac\Delta M15) hsdT17 recA1 endA1$
  $gyrA96 thi-1 relA1)$
- **XL1-Blue**: $(supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac, F'[proAB*$
  $lac\Delta lacZ\Delta M15 Tn10(tec)\])$

2.1.2 Growth media and agar plates.

Bacteria were grown in liquid Luria Broth (LB) (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl), or LB plates (LB containing 1.5% bacto-agar). LB was made to 200ml aliquot per bottle, autoclaved at 15 lb/sq.in. for 20min, and stored at room temperature for indefinite period. Where appropriate, ampicillin was added to LB media or LB agar to a final concentration of 100μg/ml when cooled down to 55°C. LB agar plates were made by pouring LB agar below 55°C into Petri dishes (Falcon), cooled down at room temperature, and dried at 37°C for about 30min before use. All strains of *E. coli* used were grown at 37°C. Liquid cultures were continuously agitated in a rotating environmental shaker.
2.1.3 Storage of bacteria.

For short-term storage bacteria were stored on LB agar plates at 4°C for about two weeks. For long-term storage (over one month) bacteria were cultured to OD_{260nm} to 0.4-0.8 in LB media. 75% sterile glycerol was added to a final concentration of 15%. This mixture can be stored at -20°C for a few years.

2.1.4 Preparation of competent bacteria.

2.1.4.1 Calcium chloride method.

A single colony from an LB agar plate or 10µl of bacteria in glycerol stored at -20°C were cultured in 5ml of LB media without ampicillin at 37°C overnight. 1 ml of this overnight culture was added to 50ml of LB media without ampicillin in a 500ml of flask. It was shaken vigorously at 37°C until the OD_{260nm} reached between 0.2-0.4 (it takes about 2-4 hours.) and cooled down on ice for 30min. Bacteria were collected by spinning at 3500rpm, 4°C for 10min, resuspended in 20ml of ice cold 100mM CaCl$_2$ (sterilised through a 0.22µm filter), and left on ice for 30min. Bacteria were spun again, the pellet was resuspended in 2ml of ice cold 100mM CaCl$_2$ and left at 4°C overnight.

2.1.4.2 TFB method for fresh competent bacteria.

TFB (10mM MES (2-[N-morpholino]ethanesulfonic acid), 45mM MnCl$_2$, 10mM CaCl$_2$, 100mM KCl, and 3mM hexamminecobalt chloride, pH 6.0) was made by mixing 1M MES pH 6.3 with the other chemicals. The pH of 1M MES was adjusted with 5M KOH. TFB was sterilized by filtration through a 0.45µm filter (Millipore).
50ml of bacteria at OD$_{260nm}$ 0.2-0.4 were collected by spinning at 3500rpm, 4°C for 10min. The pellet was resuspended in 20ml of ice cold TFB. Cells were spun again and the pellet was resuspended in 1.6ml of ice cold TFB. 56μl of DMSO (dimethyl sulfoxide) was added. Cells were left on ice for 5min, then 56μl of 2.2M DTT (dithiothreitol, in 10mM potassium acetate pH 7.5) was added. Cells were left on ice for 10min and another 56μl of DMSO was added. The competent bacteria were left on ice until ready to use.

2.1.4.3 Preparation of frozen competent bacteria by FSB method.

FSB (10mM potassium acetate, 45mM MnCl$_2$, 10mM CaCl$_2$, 100mM KCl, 3mM hexamminecobalt chloride, and 10% glycerol, pH 6.1) was made by mixing 1M potassium acetate pH 7.5 with the other chemicals. The final pH value should be between 6.1 and 6.2.

The procedure was the same as the previous one, except FSB was used instead of TFB. Instead of 1.6ml of TFB, 2ml of ice cold FSB was used to resuspend the pellet, and then 70μl of DMSO was added to the cells which were left on ice for 15min. Another 70μl of DMSO was added. Cells were quickly dispensed to chilled eppendorf tubes (50μl each) and immersed in liquid nitrogen. These frozen competent bacteria were stored at -70°C.

2.1.5 Transformation of recombinant plasmids.

2.1.5.1 Transformation.

For each transformation 50μl of fresh or frozen competent cells and 2μl (no more than 100ng) of recombinant plasmid DNA were used. If frozen competent cells
were used, they are thawed between the fingers, then quickly put on ice. The competent cells were mixed with recombinant plasmid DNA and left on ice for 30 min. After addition of 0.8 ml of LB media without antibiotics, the cells were heat-shocked at 42°C for exactly 90 sec, then incubated at 37°C in a shaker at 225 rpm for 1 hour.

### 2.1.5.2 Preparation of X-gal plates and spreading of transformed bacteria.

To the freshly made LB plates of 82 mm diameter with available antibiotics, 35 μl of 50 mg/ml X-gal in dimethylformamide and 10 μl of 100 mM IPTG (isopropyl β-D-thiogalactopyranoside) were spread. Before use the dishes were left at room temperature for 30 min to dry.

### 2.1.5.3 Spreading the transformed cells on the LB plates.

100 μl of transformed cells were transferred to an available plate. The cells were spread with a sterile glass rod. The plates were then left at room temperature for 30 min. After the liquid had been absorbed, plates were inverted and incubated at 37°C incubator overnight.

### 2.2 Molecular Biology.

#### 2.2.1.1 DNA/RNA participation with ethanol.

0.1 volume of 3M NaOAc pH 5.2 was added to the DNA/RNA samples to a final concentration of 0.3M and 2.5 volumes of cold ethanol was added. Samples were shaken and kept on dry ice for 15 min, then centrifuged at 12,000 g, 4°C for
15 min. The supernatant was aspirated and the pellet was washed with 70% ethanol, air-dried for 10 min, and redissolved in suitable volume of water or TE.

2.2.1.2 DNA/RNA phenol/chloroform extraction.

a) Preparation of phenol and chloroform.

Phenol from BDH or BRL was melted in a 68°C water bath. After melt, 8-hydroxyquinoline was added to a final concentration of 0.1%. The phenol was then extracted with an equal volume of 0.5M Tris.Cl pH 8.0, left at room temperature until two phases were clearly separated and the upper water phase was aspirated. Then the phenol was extracted with an equal volume of 0.1M Tris.Cl pH 8.0 several times until the pH of phenolic phase was > 7.5 when checked with pH paper. The water phase was aspirated, 0.1 volume of 0.1M Tris.Cl pH8.0 was added and dispensed to 40ml aliquots stored at -20°C. One aliquot was kept at 4°C for routine use.

Chloroform was made by mixing 24 parts of chloroform with 1 part of isoamyl alcohol, and kept in a dark bottle at room temperature.

Water-saturated phenol was made by adding 0.1% 8-hydroxyquinoline, then extracting the phenol with DEPC-treated water several times, finally layered with 0.1 volume of DEPC-treated water, and kept at 4°C. The water-saturated phenol was used for RNA extraction.

b) Extraction of nucleic acids with phenol and chloroform.

To the nucleic acids dissolved in water or TE half volume of phenol and half volume of chloroform were added, mixed with a vortex for 15 sec until an emulsion
formed, the centrifuged at 12,000g for 30sec. The upper aqueous phase was transferred to a new tube, and another extraction with phenol and chloroform was repeated if necessary. After the final extraction with phenol and chloroform, an equal volume of chloroform was added to the aqueous phase, mixed, and centrifuged at 12,000g for 30sec. The upper aqueous phase was transferred to a new tube.

2.2.1.3 Plasmid mini-prep.

Bacterial colonies were grown in 5ml of media containing 100 μg/ml ampicillin (Amp) at 37°C overnight with continuous shaking. 1.5ml were centrifuged at 10,000g for 15sec at room temperature. The pellets were washed with STE (0.1M NaCl, 10mM Tris.Cl pH 8.0 and 1mM EDTA pH8.0) once, resuspended in 100μl of Solution I (50mM glucose, 25mM Tris.Cl pH8.0 and 10mM EDTA pH8.0) and left at room temperature for 5min. 200μl of Solution II (0.2N NaOH and 1% SDS, freshly made) were added and mixed by gentle inversion. The mixture was left on ice for a further 10min to lyse the bacteria. 150μl Solution III (3M KOAc and 2M HOAc pH4.5) was added and mixed by gentle inversion. After incubation on ice for another 15min. The mixture was centrifuged at 10,000g, 4°C for 15min. The supernatant was extracted with phenol/chloroform twice, chloroform once, and then precipitated with ethanol. The pellet was washed once with 70% ethanol and dissolved in 30μl of water. At this stage, the plasmid DNA was ready for subsequent restriction digestion and sequencing.

2.2.1.4 Plasmid maxi-prep.

This method was based on the QIAGEN Plasmid Kit. 100ml of an overnight bacterial culture were collected by spinning at 3,500rpm (Beckman GS-6KR
centrifuge), 4°C for 20min. The pellet was suspended in 10ml of P1 buffer (100μg/ml RNase A, 50mM Tris.Cl and 10mM EDTA, pH8.0). An equal volume of P2 buffer (0.2N NaOH and 1%SDS, freshly made) was added and mixed by gentle inversion, followed by an incubation at room temperature for 5min. Buffer P3 (3M KOAc, pH5.5) was added, mixed by gentle inversion, incubated on ice for 20min, and centrifuged at 10,000g for 30min at 4°C. The supernatant was applied to a pre-equilibrated (0.75M NaCl, 50 mM MOPS, 15% ethanol and 0.15% Triton X-100, pH7.0) QIAGEN resin column. The column was then washed with 30ml of buffer QC (1.0M NaCl, 50mM MOPS and 15% ethanol, pH7.0). DNA was eluted from the column with 15ml of buffer QF (1.25M NaCl, 50mM Tris.Cl and 15% ethanol, pH8.5). DNA was precipitated with 0.7 volumes of isopropanol and centrifuged at 15,000g for 30min at 4°C. The pellet was washed with 70% ethanol once and resuspended in 200μl water. At this stage, the plasmid DNA was ready for restriction digests, sequencing and transfection.

2.2.1.5 RNA extraction.

Tissue was homogenized in 5 volumes or cultured cells (10 cm plates) were scraped into 2ml of GTC buffer (4M guanidinium thiocyanate, 25mM sodium citrate pH7.0, 0.5% sarcosyl and 0.72% 6-mercaptoethanol (added just before using)). The following reagents were then added in order: 0.1 volume of 2M NaOAc pH4.0, 1 volume of water-saturated phenol, and 0.2 volume of chloroform. The solution was mixed by gently inverting between each addition and a vigorous vortexing after the final addition of chloroform. Then aqueous phase was collected by centrifuging at 10,000g for 20min at 4°C. RNA was precipitated by the addition of equal volume of isopropanol to the aqueous phase. Samples were left at -20°C for at least one hour, then centrifuged at 10,000g for 20 min at 4°C. The pellet was dissolved in DEPC-
treated water and extracted with water-saturated phenol/chloroform several times (until no interphase was seen). The aqueous phase was then extracted with chloroform alone and precipitated with ethanol. The RNA pellet was dissolved in DEPC-treated water and quantified by determination of OD_{260nm}. The value of OD_{260nm}/OD_{280nm} should be 1.8-2.0.

2.2.1.6 Restriction digestion of DNA.

Two or three units of appropriate restriction enzyme per microgram of DNA were used for digestion. Four different buffers, supplied by New England Biolabs Inc., were used for different enzymes. NEB buffer1 contains 10mM Bis Tris Propane-HCl, 10mM MgCl₂, 1mM DTT (pH 7.0 at 25°C). NEB buffer2 contains 10 mM Tris-HCl, 10mM MgCl₂, 50mM NaCl, 1mM DTT (pH 7.9 at 25°C). NEB buffer3 contains 50mM Tris-HCl, 10mM MgCl₂, 100mM NaCl, 1mM DTT (pH 7.9 at 25°C). NEB buffer4 contains 20mM Tris-acetate, 10mM magnesium acetate, 50mM potassium acetate, 1mM DTT (pH 7.9 at 25°C). Most of restriction digests were carried out at 37°C for over 1 hour. Heating at 65°C for 5min or extraction with phenol:chloroform were used for inactivating the enzyme after digestion if necessary.

2.2.1.7 Ligation of DNA.

DNA was ligated to an appropriate vector in 1x ligation buffer (50mM Tris-HCl (pH 7.8), 10mM MgCl₂, 10mM dithiothreitol, 1mM ATP, 25µg/ml bovine serum albumin) with T4 ligase at 16°C for 3 hours to overnight. After it was finished, the T4 ligase was inactivated by heating the reaction at 65°C for 10min. The usual concentrations of DNA and vector used were 50ng/10µl of reaction. After inactivation, 2µl of the reaction was used for transformation. The rest of the reaction
was stored at -20°C.

2.2.1.8 GeneClean purification of DNA.

To DNA in either aqueous solution or agarose gel, three volumes of 4M NaI solution were added. The tubes were incubated in 55°C until all the agarose was dissolved. 5μl of "glass beads" from the manufacturer Bio 101 per ml DNA solution were added and incubated on ice for 20min. The tubes were centrifuged for 10sec, the glass bead pellet was washed with "new wash" (0.1M NaCl, 10mM Tris.Cl pH 7.5 and 1mM EDTA) three times (0.5ml each) and finally resuspended in 10μl of water. DNA was recovered by incubating at 55°C for 3min, then spinning and removing the supernatant. At this stage the DNA can be used for any subsequent treatment directly.

2.2.1.9 DNA labelling by random priming with 32P-dATP.

Insert template was digested with appropriate restriction enzymes and purified by the GeneClean method. In a 50μl of reaction, the following reagents were added in order: 10μl 5x OLB buffer (250mM Tris.Cl pH8.0, 25mM MgCl₂, 10mM DTT, 1M HEPES pH6.6 and 26 A₂₆₀ units/ml random hexadeoxyribonucleotides), 2μl of 25xdNTP (0.5mM of each dCTP, dGTP and dTTP), 2μl of 10mg/ml BSA, 50-100ng of template DNA (denatured at 95°C for 5min and cooled down on ice water just before added into the reaction mixture), 5μl of 32P-dATP (3000Ci/mmole) and 5 units of Klenow large fragment (DNA polymerase). The volume was adjusted to 50μl with water. The reaction was allowed to proceed at room temperature for 2-3 hours. A Sephadex G50 column was prepared by spinning an 1ml syringe filled with Sephadex G50 in TE for 30sec at 3,000rpm (about 1,800g). The reaction mixture was diluted with 200μl of TE and applied on the prepared Sephadex column. The labelled DNA
passes through the column, while the free nucleotides are retained in the column. The labelled DNA was heated at 95°C for 5 min before adding to the hybridization buffer.

### 2.2.1.10 Southern/Northern hybridization of DNA/RNA.

DNA/RNA were transferred from an agarose gel onto the nylon membrane (Bio-Rad Zeta Probe membrane) using a vacuum blotting device with 10xSSC (1xSSC: 0.15M NaCl and 0.015M sodium citrate) for 1.5 hours. The membrane was baked at 80°C for 1 hr. The efficiency of transfer was checked by staining the remaining DNA/RNA in the gel with ethidium bromide. In most cases, the transfer was nearly complete, with little visible DNA/RNA left. For Northern blots, the efficiency of transfer was double-checked by rinsing in 1xSSC, then staining the membrane with 0.02% methylene blue in 0.3M NaOAc until rRNA bands were clearly visible. The membrane was briefly washed with several changes of water, and photographed next to a ruler. Before hybridization, the membrane was destained with 0.2xSSC and 1% SDS until no visible rRNA bands remained. Prehybridization was carried out in an appropriate volume (usually 10ml) of prehybridization buffer (6xSSC, 10% dextran sulphate, 5xDenhardt's solution (100xDenhardt's: 2% ficoll MW 400,000, 2% polyvinylpyrrolidone and 2% bovine serum albumin Fraction V), 1% SDS, 100µg/ml denatured, fragmented salmon sperm DNA and 50% deionized formamide) at 42°C for at least one hour. Then, the denatured radioactive-labelled probe was added and the hybridization carried out overnight. The membrane was first washed with 2xSSC and 0.5% SDS at room temperature for one hour, and subsequently washed either at high stringency (0.5xSSC and 0.5% SDS at 65°C) or low stringency (2xSSC and 0.5% SDS at 42°C) depending on conditions.
2.2.1.11 Electrophoresis of DNA/RNA.

DNA was electrophoresed in agarose in TAE (40mM Tris-acetate and 1mM EDTA, pH 7.5-7.8). The concentrations of agarose ranged from 0.8 to 2.0% depending on the size of DNA being analysed. The gel was made in 1xTAE, ethidium bromide was added to a final concentration of 0.5μg/ml. 10X DNA loading buffer contained 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF and 25% w/v Ficoll-400. The maximum running voltage was 5V/cm. DNA bands were visualized with an ultra-violet (UV) transilluminator.

RNA was electrophoresed in a formaldehyde agarose gel in MOPS running buffer (20mM MOPS (3-[N-morpholino]propanesulfonic acid) pH 7.0, 8mM sodium acetate and 1mM EDTA pH 8.0). The appropriate amount of agarose was melted in 3.5 parts of DEPC-treated water, cooled down to 60°C, then 1.1 parts of MOPS running buffer and 1 part of 12.3M formaldehyde solution (37%) was added and the gel poured into a tray. The RNA samples were dissolved in RNA sample buffer (0.5X MOPS running buffer, 6.5% formaldehyde and 50% formamide) and denatured at 65-70°C for 5-10min. 10x RNA loading buffer contains 50% glycerol, 1mM EDTA (pH 8.0), 0.25% bromophenol blue, and 0.25% xylene cyanol FF. The gel was run at maximum voltage of 3-4 V/cm for a few hours or at a low voltage of 0.5-1 V/cm overnight with the buffer recirculating in the opposite direction to the RNA.

2.2.2 Polymerase chain reaction.

2.2.2.1 Strategy for designing the PCR primers.

When designing a set of PCR primers (especially the degeneracy primers), the following considerations were always taken: 1) Primers should have a length of 17-35
bases and consist of a more or less balanced nucleotide composition. 2) The 3'-end nucleotide should ideally be G or C. 3) For the degeneracy primers, an array of amino acids containing most of the following if possible: W(trp), M(met), N(asn), D(asp), C(cys), Q(gln), E(glu), H(his), K(lys), F(phe), and Y(tyr); R(arg), L(leu) and S(ser) should be avoided. 4) If a restriction site is included at the 5'-end of a primer, an extra three unrelated nucleotides should be added upstream. 5) Sequence similarities between the two primers in a set or within a primer should be compared; no more than five nucleotides should cross-hybridize within them. The follows are primers used in the described work.

\[ \text{P}^\text{PFR-A} : \text{atttcggtcga} \text{cGA(T/C)AA(A/G)GGNAA(T/C)ACNTG} 3' \]
AA sequence: DKGNYTC, underlined: restriction sites for XhoI and Sall

\[ \text{P}^\text{PFR-B} : \text{attgagctctgcagCA(A/G)CCNCA(T/C)AT(T/C/A)CA(A/G)TGG} 3' \]
AA sequence: QPHIQW, underlined: restriction sites for SacI and PstI

\[ \text{P}^\text{downFR-C} : \text{tgcgcgctcagCAT(T/C)TTCATCAT(T/C)TCCAT(T/C)TC} 3' \]
AA sequence: EMEMMMK, underlined: restriction sites for NotI and XbaI

\[ \text{P}^\text{downFR-D} : \text{GGNCC(A/G)(T/C)C(T/C)TGNGT(A/G)CANGC} 3' \]
AA sequence: ACTQDGP

\[ \text{P}^\text{PBEK} : \text{tttcgtcagGN(GA)NGNA(A/G)AA(A/G)GA(A/G)AT(T/C/A)ACNGC} 3' \]
AA sequence: GREKEITA, underlined: restriction site for PstI

\[ \text{P}^\text{downBEK} : \text{taattctctagCACGGAACCGCTCCTTGG} 3' \]
AA sequence: VTVAEKP, underlined: restriction site for XbaI
P^upFR4-A : 5' CAGGCCTTCCACGGGGAGAA 3'
AA sequence: QAFHGEN

P^upFR4-B : 5' AGCATTCGCTACAGCTATCT 3'
AA sequence: SIRYSYL

P^downFR4-C : 5' GCTGGTTTGGTCTGGCCGGGAGG 3'
AA sequence: SSRPDQTS

P^downFR4-D : 5' GGCCCATCAGGGCTGAGATC 3'
AA sequence: DLSPDGP

P^upFR-INTR : 5' GAGAAGGACCTGTGGATCTG 3'
AA sequence: EKDLSDL

P^downFR-INTR : 5' GTCGCGCATCATCATGTACAG 3'
AA sequence: LYMMMRD

P^downFR3LINKER : 5' CAGGCATCGAAGGAGTAATCC 3'
AA sequence: MDYSFDAC

P^upAR-CHI : 5' CAATGAGGTGTTGACCTGGAGATGAC 3'

P^downAR-CHI : 5' CTGCTAGATCTCTAGGACTATTACCC 3'

P^upAR-ZFA : 5' ATGTCTGA(A/G)CTGAAGAT(T/C/A)ATGAC 3'
2.2.2.2 First cDNA strand synthesis from RNA by reverse transcription.

10μg of total RNA was heated at 70°C with 2.5μg of random hexadeoxyribonucleotides primers in a volume of 12μl for 10min and cooled down quickly on ice water. The reaction was set by adding the following reagents in order: 4μl of 5xRT buffer (0.25M Tris.Cl pH8.3, 0.375M KCl and 15mM MgCl₂), 2μl of 0.1M DTT, 1μl of dNTP(10mM each of dATP, dTTP, dGTP and dCTP) and 1μl of Moloney murine leukemia virus reverse transcriptase (from BRL) (200 units). The reaction was allowed to proceed at 37°C for one hour and stopped by a subsequent heating at 95°C for 5min. 0.5μl-1μl of the synthesized cDNA were used for each PCR reaction (see below).

2.2.2.3 PCR reaction.

The PCR reaction mixture contained 5μl of 10xPCR buffer (500mM KCl, 100mM Tris.Cl pH8.3, 15mM MgCl₂ and 100μg/ml BSA), 4μl of dNTP (2.5mM of each dATP, dCTP, dGTP and dTTP), 5μl of each of the two primers (10pmole/μl), and 1μl of template DNA and water to 50μl. The mixture was overlaided with 50μl of paraffin oil and denatured at 95°C for 5min. After one unit of Taq polymerase (from Promega) was added, the reaction was heated 95°C for another 2min before
starting the PCR reaction cycles. A PCR cycle consists of 95°C for 30sec, 55°C for 2min and 72°C for 2min. Usually 30 cycles were used in a PCR reaction. After the last cycle was finished, elongation of the PCR products was achieved by incubation for 10min at 72°C. PCR products were stored at -20°C.

### 2.2.2.4 PCR products purification and cloning.

An equal volume of chloroform was added to remove the paraffin oil. The aqueous phase (which contains the PCR products) was mixed with three volumes of NaI solution (GeneClean) and purified by the GeneClean method. Then the PCR products were cloned by direct ligation with a TA vector (a vector that has a T overhang at the 3' end). 25-50ng of purified PCR products were added to 25ng of commercial TA vector with ligase at 16°C overnight. 10ng of the ligated PCR products were used for transformation and other subsequent cloning procedures.

### 2.2.3 cDNA library amplification and screening.

The cDNA library used was a zebrafish cDNA library constructed from total RNA of a post-somitogenesis (20-28 hours) embryo. The vector was lambda ZAP II. The 3'-end restriction site was XhoI, and the 5'-end restriction site was EcoRI. The primer used for the cDNA synthesis was poly d(A)\textsubscript{12-18}. The size of the library before amplification was $1.0\times10^7$ of independent plaques, and the mean size of inserts was 1.5kb (the range of inserts checked by PCR was 0.7-2.2kb).

### 2.2.3.1 cDNA library amplification and storage.

$2.4\times10^6$ phages were amplified on 15 NZCYM agar (1% NZ amine, 0.5%
NaCl, 0.5% bacto-yeast extract, 0.1% casamino acids, 0.2% MgSO₄·7H₂O and 1.5% agar) plates (15cm) with host bacterium XL1-Blue. Each plate contained 1.6x10⁸ plaques. The infected cultures were incubated at 42°C overnight to prevent the formation of lysogens. The amplified phages were harvested into SM (0.1M NaCl, 50mM Tris.Cl pH7.5, 0.2% MgSO₄·7H₂O and 0.01% gelatin) and stored in SM contained 7% dimethyl sulfoxide and 2-3% chloroform at -80°C.

2.2.3.2 cDNA library screening.

After the amplification, 100µl of the harvested phages in SM were boiled for 10min and centrifuged for 30sec in a microcentrifuge. 2µl of the supernatant were amplified by PCR with the primers P_{up}^{AR-ZF} and P_{down}^{AR-ZF} (see 2.2.2.1). The plate which had the strongest expected PDGFαR band, presumably because it had more PDGFαR positive clones, was selected for the screening.

A total of 1.2x10⁸ phages (half of the total individual phages of the positive plate) were plated onto two 15cm petri dishes (60,000 phages on each dish). Two duplicate filters were made from each of the plates. The filters were subsequently hybridized with the zebrafish PDGFαR probe (the PCR product). A total of eight positive clones (four on each plate) were found. For each of these positive individual clones, a 2mm diameter agar spot (about 10-20 plaques) of the corresponding area on the original plate was transferred into SM with a glass pipet, and put through a second round screening plating at a low density (about 100 plaques/80mm plate). The single positive clone of these eight individual clones at the second round screening was selected for the subsequent excision.
2.2.3.3 Excision of pBlueScript SK(−) plasmid from lambda ZAPII clones.

100μl (about 2x10⁶ phages) of these individual positive recombinant clones selected at the second round screening were mixed with approximate equal numbers of helper phages (R408, from Stratagene) and 200μl of infectious XL1-Blue. This mixture was incubated at 37°C for 20 min, then 5ml of 2xYT media (1.6% bacto-tryptone, 1% bacto-yeast extract and 0.5% NaCl) were added and incubated for 2.5 hours at the same temperature with continuous shaking. After heating at 70°C for 20 min, the mixture was centrifuged at 4,000g for 15min. 2μl of the supernatant, which contained the excised pBlueScript SK(−) plasmid, were used for the transformation. The positive clones were then analyzed by restriction digestion and sequencing.

2.3 Mammalian Cell Culture.

2.3.1 Cell culture.

The following cell lines were used in the experiments: Swiss 3T3 (a mouse fibroblast cell line), CG4 (a rat cerebellar O-2A progenitor cell line), B104 (a rat neuroblastoma cell line), cos (an African green monkey kidney cell, CV-1, transfected with SV40 large T antigen).

Confluent cells were washed with Hank’s solution (400mg/L KCl, 60mg/L KH₂PO₄, 8g/L NaCl, 47.88mg/L Na₂HPO₄, 1g/L D-glucose, 11mg/L Phenol Red.Na and 350mg/L Na₂CO₃) three times, then were treated with trypsin at a final concentration of 0.0125% until the cells came off the plate. The dissociated cells were collected into a 15ml tube and fetal calf serum (FCS) was added to a final
concentration of 10% to inhibit the trypsin. The cells were centrifuged at room temperature at 1,200rpm for 7min. The cells were resuspended in an appropriate volume of DMEM (Dulbecco’s Modification of Eagle’s Medium, with 0.45% dextrose, 4mM glutamine and 100IU/ml penicillin/streptomycin) with 10% FCS and cultured in a humid incubator with 5% CO2 at 37°C.

2.3.2 Dissociating embryonic rat spinal cord cells.

Pregnant rats were killed by asphyxiation with CO2. Embryos were taken out and washed with PBS A (0.8% NaCl, 0.025% KCl, 0.143% Na2HPO4 and 0.025% KH2PO4) briefly to remove blood. Under the dissection microscope, spinal cords were dissected out and chopped into small pieces in Hank’s solution. An equal volume of trypsin solution (0.1% trypsin and 0.5% BSA in Hank’s solution) was added and incubated at 37°C for 30min. EDTA was added to a final concentration of 0.2%; and the mixture was incubated at 37°C for a further 10min. The mixture was then pipetted up and down 10 times, and passed through a mesh before centrifugation at 1,200rpm. The cell pellet was resuspended in an appropriate volume of DMEM with 10% FCS and plated onto PDL-coated plates or glass cover slips and cultured at 37°C.

2.3.3 Culture of spinal cord explants.

Pregnant rats or mice were killed by asphyxiation with CO2 or cervical dislocation. Embryos were dissected out and washed briefly with PBSA. Under the dissecting microscope, the spinal cord was dissected out with a small razor blade and a tungsten needle in minimal essential medium (MEM). After the surrounding tissue was removed, the spinal cord was opened with a fine tungsten needle from the dorsal tip. The opened spinal cord was then transferred onto a culture plate with a drop of
medium. It was positioned with the ventricular surface facing up. With a fine tungsten needle, the spinal cord was then chopped transversely into small pieces. A drop of chicken plasma was pipetted around the tissues and a equal volume of thrombin (1mg/ml) was added to form a clot that attaches the explants onto the culture plate. Once the clots formed, the plates were left at room temperature for about one hour or until the clots slightly dried without addition of medium. Then DMEM medium with 10% FCS was added and the explants were cultured in a humidified 35.5°C incubator with 5% CO₂ on a four-direction rotating platform to make the explants half time of culture inside the medium and half time exposed in the air.

2.4 Cell Biology Methods.

2.4.1 In situ hybridization with RNA probes.

2.4.1.1 Preparation of glass slides and devices.

All the devices and solutions used for in situ hybridization should be RNase free unless indicated. All containers and slide racks were washed with 0.1% DEPC (diethylpyrocarbonate) and autoclaved. All solutions were treated with 0.05% DEPC for half an hour and autoclaved. Commercial glass slides (e.g. from BDH) were washed with detergent, rinsed thoroughly with water and baked at 250°C overnight. To coat the slides, they were immersed in the following solutions for 30sec each: 2% 3-aminopropyltriethoxysilane in IMS, 9% IMS, and DEPC-treated water. The slides were then dried at room temperature.

2.4.1.2 Radioactive RNA probe labelling with ³⁵S-UTP by in vitro transcription.

Plasmid DNA was linearized with appropriate restriction enzymes and purified
by GeneClean. The reaction was set up by mixing the following solutions, prewarmed to room temperature, in order: 2μl of 10x transcription buffer (400mM Tris.Cl pH7.9, 60mM MgCl2, 20mM spermidine and 100mM DTT), 0.5μl (20units) of RNasin (ribonuclease inhibitor), 4μl of dNTP (2.5mM each of ATP, GTP and CTP), 2.4μl of 100μM UTP, 5μl (1μg) of linearized template DNA, 5μl (50μCi) of α-35S-UTP and 1μl (10units) of SP6 or T7 or T3 RNA polymerase. The reaction was carried out at 37°C for 90 min with an addition of another 1μl of RNA polymerase half way through. To remove the DNA template, 20 units of RNasin, 25μg of tRNA and 1 unit of DNase (RNase free) were added and the mixture was incubated at 37°C for a further 15min. After 200μl of 1mM DTT were added, 0.5μl was taken out to check the length of the transcripts on a formaldehyde gel. Another 0.5μl of the reaction was taken and added to 0.4ml of 0.5M NaH2PO4 to determine the percentage of radioactive incorporation. The rest of the sample was extracted with phenol/chloroform once and chloroform once, then precipitated with ethanol. The pellet was redissolved in 50μl of 10mM DTT and 50μl of 100mM carbonate buffer (pH 10.2) was added. The solution was incubated at 60°C for an appropriate time to digest the radioactive transcripts to 100-150 bp in length. The appropriate time, ‘X min’, can be calculated by the following formula:

\[ X \text{ min} = \frac{(L_0-L_f)}{(K \cdot L_0 \cdot L_f)} \]

where

L₀ = Original transcript length in kb
Lₖ = Final transcript length in kb (0.1-0.15)
K = 0.11

After incubation, the solution was neutralised with 100μl of neutralising buffer (0.2M NaOAc, 1% HOAc and 10mM DTT). At this stage, 1μl of the reaction was removed for gel analysis and the rest was precipitated with ethanol. The final pellet was redissolved in 20-50μl of 10mM DTT containing 50% deionised formamide and

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stored at -20°C.

2.4.1.3 Preparation and sectioning of tissue.

Dissected tissue or whole embryos were fixed with 4% paraformaldehyde overnight at 4°C and then immersed into 0.5M sucrose overnight at 4°C. They were then embedded into OCT and frozen on dry ice. The frozen embedded tissues were stored at -80°C until used for sectioning.

Sections were cut with a frozen section-slicer--cryostat. 10μm thick sections were cut and mounted onto the coated glass slides, dried at room temperature for at least half an hour, then fixed with 4% paraformaldehyde for 15min at room temperature. The slides were briefly washed with PBSA for 3min, then either dehydrated through a series of alcohol concentrations (30%, 60%, 80%, 95% and 100%), for 1min each and stored at -80°C, or used directly for in situ hybridization.

2.4.1.4 Hybridization with radioactive probes and subsequent washes.

Frozen tissue sections were rehydrated through 100%, 95%, 80%, 60% and 30% ethanol for 1min each before use. PBSA washed sections from above or rehydrated sections were incubated in Proteinase K buffer (50mM Tris.Cl and 5mM EDTA pH 7.5) at room temperature for 5min. The sections were then treated with proteinase K (20μg/ml) for 5-7.5min (depending on the thickness of the sections) at room temperature. Treatment was stopped by incubating the sections with 0.2% glycine for 30sec. The sections were washed twice with PBSA, 30sec each, and fixed with 4% paraformaldehyde for 15min. The sections were then washed with PBSA for
3 min and 0.1 M triethanolamine (pH 8.0) for 5 min, and treated with freshly made 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0) twice, for 10 min each, to prevent non-specific binding of the probe by reducing hydrostatic charges on the sections. The sections were then washed in PBSA for 3 min and dehydrated through the series of alcohols and dried at room temperature before processing further.

Radiolabeled probes were diluted to a proper concentration (usually, one labelling reaction with 40-50% incorporation was diluted to 4 ml) with hybridization buffer (0.3 M NaCl, 10 mM Tris.Cl pH 6.8, 5 mM EDTA, 10% dextran sulphate, 100 µg/ml yeast tRNA, 1x Denhardts, 10 mM dithiothreitol (DTT) and 50% deionized formamide) and heated at 80°C for 5 min before applying onto the sections. 80 µl of the diluted probe were applied onto each slide and a 22 x 50 mm coverslip was placed on top. The slides were then placed in a humid container and left in a 55°C oven overnight. Negative controls were usually processed at the same time with a "sense" probe instead of an "antisense" probe.

It is not necessary to keep the slides RNase-free from this point. After hybridization overnight, the coverslips were removed by dipping the slide in 4x SSC (20x SSC: 3 M NaCl and 0.3 M sodium citrate, pH 7.5) until they slipped off. The sections were incubated in 4x SSC for a further one hour at room temperature, then washed in wash buffer (2x SSC, 2 mM DTT and 50% deionized formamide) at 65°C for 30 min. The sections were then washed in RNase A buffer (0.5 M NaCl, 10 mM Tris.Cl and 0.1 mM EDTA pH 7.5) twice for 5 min each at room temperature, and treated with RNase A (20 µg/ml) at 37°C for 30 min to remove the unhybridized single stranded RNA. The sections were washed in RNase A buffer for another 15 min at room temperature, then in wash buffer at 65°C, 2x SSC at 45°C and finally, in 0.1x SSC at 45°C for 30 min each. Slides were dehydrated through ascending alcohol
concentrations and dried before coating with emulsion.

2.4.1.5 Coating slides with autoradiographic emulsion.

Under safelight conditions, an appropriate amount (usually 5ml) of autoradiographic emulsion (Ilford K5) was melted in a 40°C water bath. Then an equal volume of water (containing 2% glycerol) was added. The solution was mixed gently by inverting the tube. The processed slides were dipped in the emulsion and excess emulsion was wiped off the back of the slides with a piece of tissue, and then dried on a platform in total darkness for 1.5 hours. The exposure proceeded in a dark dry box at 4°C.

2.4.1.6 Developing and photography of the autoradiographic slides.

Slides were developed with D-19 developer (Kodak) at 20°C for 2min. Developing was stopped by placing the slides into 1% acetic acid for 1min and fixed with 30% sodium thiosulphate for 5min. After washing with distilled water for at least 20min, the slides were stained with 20% hematoxylin for 5min. The slides were then briefly washed with distilled water, dehydrated through ascending alcohol concentrations and cleared in xylene. After mounting with a xylene based mountant (Xam), slides were viewed and photographed under bright field or dark field illumination.

2.4.2 Immunostaining of dissociated spinal cord cells with DAB after in situ hybridization.

In one experiment, it was necessary to view cells both by in situ hybridization
and by immunostaining with a GFAP antibody. Cells on Lab-Tex slides (from Nunc) were fixed with 4% paraformaldehyde in PBSA for 15 min and used in the in situ hybridization procedure (see above) except eliminating the proteinase K treatment. Before dehydrating and dipping in emulsion, the slides were immunostained by DAB as follows: After the final wash cells were washed with PBSA for 5 min, permeabilized with 0.4% Triton X-100 in PBSA for 20 min, and washed three times in PBSA. Cells were incubated with 10% sheep serum (diluted with PBSA) for 20 min to block non-specific antibody binding. The cells were briefly washed with PBSA, and the first antibody, a mouse monoclonal antibody against GFAP (from Sigma, using 1 to 400 dilution), was applied onto the slides and incubated at room temperature for one hour. Slides were then washed with PBSA three times for 5 min each. The second antibody, biotinylated goat-anti-mouse IgG, was applied, and incubated for a further hour at room temperature. A DAB staining kit (Vector Stain ABC kit) was used for signal development. Cells were incubated with a Streptavidin/peroxidase conjugate (5 ml of water containing one drop of reagent A and one drop of reagent B—made 30 min before use), at room temp for one hour, and the colour was developed using a mixture of 5 ml of water, 2 drops of buffer, 4 drops of DAB, and 2 drops of \( \text{H}_2\text{O}_2 \). The staining reaction was stopped by washing the slides with PBSA after the colour developed. Slides were dehydrated by dipped in ascending alcohol concentrations containing 0.3M ammonium acetate and dried at room temperature. The subsequent procedure for autoradiography was identical to the general protocol except that ammonium acetate was added to the emulsion to a final concentration of 0.3M.

2.4.3 Double immunofluorescence staining of cultured cells.

Cultured cells on coverslips were washed with PBSA once and fixed with 1% formalin in PBSA for 20 min at room temperature. The cells were then permeabilized
with 0.4% Triton X-100 in PBSA and preblocked with 10% sheep serum for 20min each. The following antibodies were applied onto the coverslips in order: rabbit-anti-FGFR-3 polyclonal antibody IgG, goat-anti-rabbit IgG conjugated with FITC, mouse-anti-GFAP monoclonal antibody, and goat-anti-mouse IgG conjugated with Texas Red. Incubations were carried out in a humid light-proof box for one hour each at room temperature. Coverslips were washed after each incubation by dipping them in PBSA three times. At the end of the last incubation, cells were washed with PBSA three times and mounted onto a microscopy slide with a drop of citifluor or equivalent mountant up side down. Then they were examined by fluorescence microscopy.

2.4.4 ACTH staining of frozen tissue sections.

10μm sections were cut and briefly dried at room temperature. The sections were soaked with PBSA for 10min, and then the staining medium was applied to the sections and incubated at room temperature until colour developed, (usually 2-6 hours). The staining medium was made up by dissolving 5mg of acetylcholine iodate into 6.5ml of 0.1M phosphate buffer pH6.0, and mixed with the following reagents in order: 0.5ml of 0.1M sodium citrate, 1ml of 30mM CuSO₄, 1ml of water and 1ml of 5mM potassium ferricycnide. The staining reaction was stopped by washing the slides with distilled water. Slides were then dehydrated through ascending alcohol concentrations, cleared with xylene, and mounted with Xam to make a permanent record.

2.4.5 The immunofluorescence staining of cultured explants of spinal cord.

After culturing, the spinal cord explants were washed with PBSA once, and fixed with 4% paraformaldehyde in PBSA at room temperature for 20min. The
explants were then washed with PBSA containing 0.1% Triton X-100 (PBSA-T) three times, 5min each, the explants were permeabilized with 0.5% Triton X-100 in PBSA for 20min at room temperature, then washed briefly with PBSA-T. The explants were incubated with 20% sheep serum in PBSA-T for another 20min at the room temperature, then incubated with the first antibody (against MBP) either for 2 hours at room temperature or overnight at 4°C, followed by three washes with PBSA-T, 5min each. The second antibody was then applied for one hour. The explants were then washed with PBSA-T three times, then with PBSA once and finally mounted with citifluor under cover slips and examined by fluorescence microscopy.

2.4.6 Cos cell transfection.

Cos cells were cultured to 75% confluence in DMEM containing 10% FCS. The cells were washed with Hepes-buffered saline (20mM Hepes pH 7.05, 137mM NaCl, 5mM KCl, 0.7mM Na₂HPO₄ and 6mM D-glucose) twice, harvested with trypsin, washed with 10ml (per 10cm dish) cold Hepes-buffered saline once and then resuspended in 0.25ml of cold Hepes-buffered saline. 5-10µg plasmid DNA in 0.25ml of cold Hepes buffered saline were mixed with the above prepared cells and transferred to a chilled electroporation cuvette (0.4cm). The electroporation was performed at 0.3kV/125µF. With these conditions the time constant was 5sec. After electroporation, the cuvette was returned to ice immediately and cells were transferred to a 10cm dish with DMEM containing 10% FCS. Dead cells were removed from the culture dish next day by changing the medium.

2.4.7 The detection of β-galactosidase.

Cells were washed with PBSA (plus 1mM CaCl₂ and 0.5mM MgCl₂) once and
fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were then rinsed with PBSA and then stained in the staining buffer for three hours at 37°C. The staining buffer was made by diluting the X-gal stock solution (20 mg/ml in formamide) in a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM MgCl₂ just before use.

2.5 Image processing.

Sections hybridized with radiolabeled probe and subsequently stained with haemotoxylin were photographed under dark- and bright-field illumination. The dark- and bright-field images were converted to digital format imported into a Macintosh computer using a video camera and image grabber. Corresponding dark- and bright-field images were then assigned false colours and superimposed using Adobe Photoshop software, and photographed either directly from the computer monitor or through a computer image maker.
Chapter Three

Embryonic Expression of Myelin Genes:
Evidence for a Focal Source of Oligodendrocyte Precursors
in the Ventricular Zone of the Neural Tube
Myelin is formed by oligodendrocytes in the CNS and by Schwann cells in the PNS. Oligodendrocytes and Schwann cells express overlapping, but not identical, sets of specialized proteins, known collectively as myelin proteins. Among these myelin proteins are: proteolipid protein (PLP), myelin basic protein (MBP) and 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNP). PLP, a transmembrane protein, is the most abundant protein in CNS myelin, accounting for about half of the total myelin protein content. PLP is also expressed in Schwann cells, but is restricted to the cell bodies and does not play a structural role in the myelin membranes (Kamholtz et al 1992, Puckett et al 1987). MBP is another major constituent of CNS myelin, which is also expressed by Schwann cells at a low level. CNP has an enzymatic activity which can hydrolyse 2',3'-cyclic nucleotides in vitro, and occurs in both CNS and PNS (Vogel and Thompson 1988). It is not thought to be a structural component of myelin per se, and its function in myelin formation is obscure.

It is now known that some of the myelin proteins are expressed, not only in myelinating oligodendrocytes and Schwann cells, but also in the premyelinating CNS and PNS and even outside of the nervous system. For example, an alternatively spliced isoform of PLP known as DM-20 (Nave et al 1987) is expressed at low levels in the embryonic and perinatal rodent CNS (LeVine et al 1990, Ikenaka et al 1993, Timsit et al 1992) and in the developing mouse heart (Campagnoni et al 1992). Alternatively spliced CNP mRNA (Bernier et al 1987, Kurihara et al 1990) has been detected in circulating lymphocytes, erythrocytes, and platelets (Bernier et al 1987, Sprinkle et al 1985), as well as in the premyelinating CNS (Scherer et al 1994). Some MBP-related mRNAs and proteins are also expressed in the developing CNS before the onset of myelination (Sorg et al 1987, Verity and Campagnoni 1988). The expression of myelin genes either outside the nervous tissue or in the premyelinating
CNS and PNS suggest that they might fulfill some other functions in addition to myelinating axons.

Oligodendrocytes differentiate from bipotential glial precursor cells, known as oligodendrocyte-type-2 astrocyte progenitors (O-2A progenitors), which can give rise to either oligodendrocytes or type-2 astrocytes in vitro, depending on the culture conditions (see Chapter one). O-2A progenitors were initially isolated from the developing rat optic nerve (Raff et al 1983) and subsequently were found throughout the developing CNS. In the spinal cord, oligodendrocyte precursors are believed to be generated from neuroepithelial precursor cells in the ventricular zone. Moreover, by culturing cells derived from the ventral or dorsal halves of the rat spinal cord, it has been shown that oligodendrocyte precursors are generated in the ventral, but not the dorsal half of the E14 cord (Warf et al 1991). Consistent with this finding, Pringle and Richardson (1993) showed that putative O-2A progenitors, visualized in situ with a probe to PDGF alpha-receptor (PDGFRα) (Pringle et al 1992), first appear in narrow columns at the ventricular surface in the ventral half of the E14 rat spinal cord (see Discussion).

To verify the idea that the oligodendrocyte lineage arises at a defined position in the ventricular zone of the spinal cord, it was important to examine the expression pattern of other putative genetic markers of this cell lineage. Since some myelin genes are known to be expressed in the embryonic CNS, we wondered whether one or more of these genes might be expressed specifically in oligodendrocyte precursors. We first tested this idea by probing Northern blots of mRNA isolated from pure (>95%) cultures of rat oligodendrocyte progenitors for transcripts encoding three myelin proteins: PLP, CNP and MBP. We found that proliferating oligodendrocyte progenitor cells express low levels of one alternative-splice isoform of CNP, but not
PLP or MBP, suggesting that CNP might be an useful marker of oligodendrocyte precursors in situ. I therefore examined the expression of CNP and PLP in the developing rat spinal cord by in situ hybridization. The results of these experiment are described in this Chapter.

Result


Two alternatively spliced isoforms of CNP transcripts with sizes of 2.4kb and 2.6kb respectively have been detected in both embryonic and postnatal brain tissues (Bernier et al 1987, Kurihara et al 1990, Scherer et al 1994). Previous in situ studies of CNP expression concentrated on postnatal tissues, where myelinating oligodendrocytes have developed. Since an alternatively spliced transcript encoding one of the isoforms of CNP could be detected on Northern blots prepared from cultured oligodendrocyte progenitor cells (Fig 3.1), it was interesting to see whether CNP transcripts could also be detected in situ in the embryonic CNS before the appearance of myelinating oligodendrocytes.

I examined embryonic rat spinal cords aged E12, E14, E16 and E18, using a \(^{35}\)S-radiolabeled CNP antisense RNA probe. This probe does not discriminate between the 2.4kb and 2.6kb CNP transcripts. Transverse sections were cut through the cervical, thoracic and lumbar regions of rat spinal cord. These sections represent successively earlier stages of development, because of the general temporal lag of development from the anterior to the posterior parts of the neural tube. CNP expression in the PNS, e.g. dorsal root ganglia (DRG) and peripheral nerves, was
detected at all ages examined, from E12 to E18 (E16 shown as Fig 3.2). There is no notable change of signal strength during this period in the PNS; this expression presumably arises in mature Schwann cells. CNP was also expressed in the CNS at all ages examined. At E12, there was a low level of CNP expression throughout the entire ventral half of the spinal cord, in both the ventricular zone (dividing cells) and the ventral horns (predominantly postmitotic motor neurons). The dorsal boundary of CNP expression corresponded approximately to the sulcus limitans (Fig 3.3 panel a). Two days later, at E14, the CNP expression domain had regressed and was restricted to a narrow column of cells running along the length of the spinal cord in the ventral ventricular zone. In transverse sections through the cervical region of the E14 cord, CNP expression was restricted to two small foci of two or three cells, one each side of the central canal (Fig 3.3 panel c). However, in the thoracic region of the same cord, where development is slightly delayed relative to more anterior regions, the column of CNP-positive cells had not yet regressed as fully as in the cervical region (Fig 3.3 panel b). Two more days later, at E16, the CNP-positive foci in the ventricular zone had disappeared, and instead there was a speckled appearance to the surrounding area, suggesting that individual cells in the grey matter of the spinal cord were expressing low levels of CNP mRNA (Fig 3.2 panel a). The signal at this stage was close to the limit of sensitivity of our in situ hybridization procedure. After another two days (at E18) in the cervical region of the cord, in addition to the same low-intensity speckled pattern in the grey matter of the spinal cord, there were a few very intensely labelled cells in the ventral-most white matter and the adjoining grey matter, which presumably represent the earliest differentiated oligodendrocytes in the cord (Fig 3.4 panel a). The contrast in signal intensity was very great between these latter intensely labelled cells and the weakly labelled cells; the intensely labelled cells were clearly visible, even in bright-field illumination (Fig 3.4 panels b, c), after an autoradiographic exposure of 2 or 3 days, whereas the weakly labelled cells required
from 4 to 6 weeks and were only visible under dark-field illumination. There was a
greater number of intensely labelled cells in the ventral half of the brainstem of the
same animals (Fig 3.5 panel a), consistent with the idea that these represent
differentiating oligodendrocytes, since development is even further advanced in the
brainstem, being more anterior than the spinal cord. In addition to the ventral aspect
of the brainstem, these intensely labelled cells also appeared in the dorsal aspect at
E18 (Fig 3.5 panel a). Negative controls using a radiolabeled probe which was
homologous to the mRNA (sense probe) rather than complementary (antisense probe)
gave no signal above background in this or any other of the experiments in this
Chapter.

The very restricted pattern of CNP expression in the ventricular zone of E14
spinal cord was similar to the pattern of PDGFRα expression. I therefore directly
compared the CNP expression pattern in the E14 cervical spinal cord with the
PDGFRα expression pattern in the equivalent region of an age-matched littermate.
The positions of the foci of CNP and PDGFRα expression were coincident (Fig 3.3
panels c, f). This position was approximately 0.15 of the way along the dorsoventral
axis, measuring along the lumen of the spinal cord in a ventral-to-dorsal direction.

2. Embryonic Expression Pattern of PLP/DM-20 Transcripts in the Spinal Cord

DM-20 is an alternative-splice isoform of PLP, which has 35 amino acids
missing from one of the extracellular loops of PLP (Nave et al 1987). It has been
reported to be expressed in the embryonic CNS and PNS (Ikenaka et 1993, Timsit et
al 1992) and even outside nervous system (Campagnoni et al 1992). Particularly, in
one report, DM-20 was shown to be expressed in the ventricular zone in the ventral
half of the E14.5 mouse spinal cord; this was interpreted as evidence of PLP
expression in early precursors of oligodendrocytes (Timsit et al 1995). This interpretation seemed at odds with our finding that PLP mRNA is not expressed in vitro in cultured rat oligodendrocyte precursors (Fig 3.1). I performed in situ hybridization on transverse sections of the developing rat spinal cord from E12 onwards with a probe that recognizes PLP/DM-20. At all ages examined, PLP/DM-20 was expressed in the PNS just as for CNP (Fig 3.2 panel b). In the spinal cord, however, the expression pattern of PLP/DM-20 was clearly distinguishable from that of CNP at the same age. Two bilateral foci of expression of PLP/DM-20 were observed just above the floor plate in the E12 spinal cord (Fig 3.3 panel d). From E14, there was no visible signal under my in situ hybridization conditions in the developing spinal cord, even when the autoradiographic slides were exposed for up to 6 weeks (Fig 3.3 panel e, Fig 3.2 panel b), except for the appearance of intensely-labelled cells in the ventral-most white matter of the cord at E18 (Fig 3.4 panel d). These intensely PLP/DM-20-labelled cells had a similar signal intensity to the corresponding intensely CNP-labelled cells in the same position and at the same age. These intensely-labelled cells doubtless correspond to the first differentiating oligodendrocytes. The only difference between them was that on the consecutive sections of the same spinal cord, the number of the intensely PLP/DM-20-labelled cells was slightly less than that of CNP-labelled cells suggesting that the expression of PLP/DM-20 in mature oligodendrocytes may slightly delayed relative to CNP (Fig 3.4 panels e, f). After E18, the expression patterns of PLP/DM-20 and CNP in the CNS were indistinguishable (Fig 3.6).

As mentioned above, PLP/DM-20 has been shown to be expressed in embryonic mouse spinal cord (Timsit et al 1995). Surprisingly, the PLP/DM-20 expression pattern of mouse was clearly different to what I found in the rat. To investigate this further, I directly compared PLP/DM-20 expression patterns of mouse
and rat by in situ hybridization. In this experiment, I used a mouse PLP/DM-20 probe on mouse sections and a rat PLP/DM-20 probe on rat sections. PLP/DM-20 expression was clearly detected in the DRG of E11 mice and E12 rats (developmentally equivalent ages), suggesting that the expression of PLP/DM-20 is similar in the mouse and rat PNS (Fig 3.7 panels a, c). However, in the CNS, unlike the E12 rat where there were two foci of PLP/DM-20 expression adjacent to the floor plate, no PLP/DM-20 signals were detected in the E11 mouse spinal cord (Fig 3.7 panel a). In situ hybridization of later ages showed that the PLP/DM-20 signal began to be expressed in the mouse spinal cord at E14 to E14.5, at which time individual PLP/DM-20 positive cells were visible in the ventral half of the ventricular zone. The density of PLP/DM-20 positive cells was so low in the E14.5 spinal cord that most individual transverse sections only contained 1 to 3 cells (Fig 3.7 panel b). These PLP/DM-20-expressing cells do not correspond to PDGFRα-expressing oligodendrocyte progenitors; the latter cells are much more numerous at E14.5 and are dispersed throughout the spinal cord at this age (Fig 3.7 panel d). Additionally, this pattern of PLP/DM-20 expression in the E14.5 mouse bore no similarity to the PDGFRα or CNP expression patterns of the E14 or E15.5 rat. These results demonstrated that PLP/DM-20 expression pattern was clearly different between mouse and rat, and suggested that the PLP/DM-20-positive cells in the E14.5 mouse are not related to the oligodendrocyte lineage.

Discussion


   I have presented evidence that oligodendrocyte progenitors arise in the ventral
half of the spinal cord and migrate to the dorsal half, eventually becoming distributed throughout the cross-section of the cord. Warf et al (1991) dissected embryonic rat spinal cord into dorsal and ventral halves, dissociated the cells and cultured them separately in vitro. They found that oligodendrocytes always developed on schedule in cultures of E14 ventral cells, but not in cultures of E14 dorsal cells. In contrast, oligodendrocytes developed in both ventral and dorsal cell cultures derived from E16 cord. These results suggested that oligodendrocyte progenitors originate in the ventral half of the E14 spinal cord, and migrate to the dorsal half the cord by E16. My results presented in this Chapter together with previous in situ hybridization data from this laboratory (Pringle and Richardson 1993) are clearly consistent with this interpretation, and extend the results of Warf et al by showing the precise source of the oligodendrocyte lineage in the ventricular zone of the cord.


Previous work in our laboratory has also suggested a ventral origin for oligodendrocyte precursors in the embryonic rat spinal cord (Pringle and Richardson 1993). PDGFRα is expressed widely outside the CNS. However, in the CNS, its expression pattern seems to be restricted to oligodendrocyte precursors except for a transient expression by developing dorsal neurons between E12 and E14 in the rat. By in situ hybridization, a narrow column of PDGFRα+ cells was discovered in the ventricular zone in the ventral half of the E14 rat spinal cord. In cross section, this column appeared as bilateral foci of cells sitting at the ventricular surface. Initially, there were only one or two cells in each of these two foci. Subsequently, the PDGFRα+ cells increased in number and spread throughout the cross section of the spinal cord, presumably by migrating away from their germinal site in the ventricular
zone. There are several lines of evidence suggesting that these PDGFRα* cells represent oligodendrocyte progenitors: 1) oligodendrocyte progenitor cells from various regions of the developing CNS are known to express PDGFRα in vitro (Hart et al 1989, McKinnon et al 1990) and in vivo (Hart et al 1989) and proliferate in vitro in response to PDGF (McKinnon et al 1990, Raff et al 1988, Pringle et al 1989, Richardson et al 1988, Noble et al 1988). 2) oligodendrocyte progenitors appear to be the only glial cells in cultures of perinatal rat optic nerve (Hart et al 1989) or embryonic rat spinal cord (Hall and Richardson, unpublished data) that express PDGFRα. 3) the way that PDGFRα* cells accumulate in the cerebral cortex, cerebellum, optic nerve, and spinal cord during development is consistent with what is known from other studies of oligodendrocyte development in these regions of the CNS (Le Vine and Goldman 1988, Hardy and Reynolds 1991, Pringle et al 1992, Mudhar et al 1993, Pringle and Richardson 1993). 4) the absolute number of PDGFRα* cells in the perinatal rat optic nerve (Pringle et al 1992) matches independent estimates of the number of oligodendrocyte progenitors in the nerve, identified either by electron microscopy (Vaughn 1969) or by quisqualate-induced cobalt uptake in whole nerves (Fulton et al 1992).

PDGF and bFGF together are sufficient to keep oligodendrocyte progenitor cells proliferating in vitro and prevent them from differentiating into oligodendrocytes. In the presence of these two growth factors, a 2.4kb CNP isoform but not the alternatively-spliced 2.6kb isoform can be detected by Northern hybridization in the oligodendrocyte progenitor cells. Following withdrawal of the growth factors, these progenitor cells differentiated into oligodendrocytes within two days, and concurrently, both 2.4kb and 2.6kb CNP isoforms were upregulated strongly (Yu et al 1994). This result suggested that oligodendrocyte progenitors might express the 2.4kb CNP isoform in vivo. In order to check this idea and trace the development of
oligodendrocyte lineage, I did in situ hybridization with a CNP probe that can pick up both CNP isoforms on the developing rat spinal cord. Indeed, in the E14 rat spinal cord, two foci of CNP-positive cells were found that co-localized with PDGFRα+ cells in sections of the same age and at the same position in the cord. This result has provided strong evidence on the precise origin of the oligodendrocyte lineage in the rat spinal cord.

3. PLP/DM-20 Is Expressed by Unidentified Precursor Cells in the Ventricular Zone Adjoining the Floor plate in Rat.

DM-20, an alternatively spliced isoform of PLP, has been shown to be expressed in the CNS (Nave et al. 1987). In order to determine whether PLP/DM-20 is, like CNP, expressed by oligodendrocyte progenitors, I performed in situ hybridization with a probe that can hybridize to both PLP and DM-20 transcripts in transverse sections of embryonic rat spinal cord. However, I did not detect PLP/DM-20 transcripts in the spinal cord between E14 and E18 except for the PLP/DM-20 intensely-labelled cells in the ventral white matter at E18, which undoubtedly represent differentiating oligodendrocytes. In the E12 spinal cord, there were two clusters of PLP/DM-20-expressing cells located just above the floor plate. These two clusters of cells were clearly distinct from the two foci of CNP-positive cells in the E14 spinal cord both temporally and spatially. By E14, these PLP/DM-20-expressing cells in the spinal cord were gone, and there was no detectable PLP/DM-20 signal above background until E18. This result was consistent with the observation that there is no detectable PLP/DM-20 signal on Northern blots of mRNA prepared from cultured oligodendrocyte progenitor cells (Fig 3.1). It is not known what these two clusters of PLP/DM-20-expressing cells are. Presumably, they are some kind of neural precursors which express PLP/DM-20 transiently before differentiating into
either neurons or glia. Motor neurons are still being generated in the ventral region of the spinal cord at E12 (Altman and Bayer 1984), so one possibility is that the PLP/DM-20 expressing cells might be motor neuron precursors. I think it is less likely that the PLP/DM-20 expressing cells are oligodendrocyte precursors because, as mentioned above, no significant quantities of PLP/DM-20 transcripts were detected in purified oligodendrocyte progenitors, although it remains a formal possibility that there may be two, or more, distinct cell lineages which can give rise to oligodendrocytes in vivo.

4. Differences in PLP/DM-20 expression in embryonic rats and mice.

Expression of PLP/DM-20 in the embryonic spinal cord appears to differ between mouse and rat. Before the first differentiating oligodendrocytes appear at E18, PLP/DM-20 is only transiently expressed in the E12 spinal cord of rats whereas, in mice, there is no PLP/DM-20 signal until E14.5, which is developmentally equivalent to E15 to E16 in the rat. The PLP/DM-20-expressing cells in the E14.5 mouse spinal cord were distinct from either the PLP/DM-20-expressing cells in the E12 rat spinal cord or the CNP/PDGFRα-expressing cells in the E14 rat spinal cord. It is not known whether the PLP/DM-20-expressing cells in the embryonic rat and mouse are the same cell lineage but generated at a different place and time in the two different species, or whether they represent two unrelated cell lineages. In my opinion the latter seems more likely.

Conclusions

1) The precise co-localization of the two foci of CNP-positive and PDGFRα+ cells in the E14 rat spinal cord provides strong evidence for a focal origin of
oligodendrocyte precursors in the ventral ventricular zone of the rat spinal cord.

2) PLP/DM-20 is expressed at a different time and location to CNP in the embryonic rat spinal cord, suggesting that PLP/DM-20 marks a different population of neural precursors, not related to oligodendrocytes.

3) PLP/DM-20 expression in the embryonic CNS is significantly different between mouse and rat. In neither animal does PLP/DM-20 expression correlate with PDGFRα expression, suggesting that PLP/DM-20 is expressed by different, unidentified populations of neural cells in the rat and mouse.

4) Both CNP and PLP/DM-20 are expressed intensely by differentiating oligodendrocytes. There is no significant difference in CNP and PLP/DM-20 expression in differentiating oligodendrocytes except that CNP appears slightly earlier than PLP/DM-20.
Fig. 3.1 Expression of CNP transcripts in oligodendrocyte precursor cells.

(A) Alternatively spliced transcripts encoding CNP and the probes used to detect them. The CNP gene structure is shown at the top (not to scale). Numbered boxes represent exons. Beneath this are diagrams of the two major CNP mRNAs, which are produced from primary transcripts with different 5' ends (Bernier et al 1987, Kurihara et al 1990) and encode proteins with different amino termini. The larger CNP mRNA encodes the smaller protein (CNPI), and the smaller mRNA encodes the larger protein (CNPII) (Kurihara et al 1992). Shaded boxes, coding sequence. Unshaded boxes, 5' and 3' noncoding sequences.

(B) Northern blot analysis of mRNA from cultures of immunoselected O-2A progenitors, grown in the presence of PDGF-AA and bFGF (+GF) or in medium lacking these growth factors (-GF) for the indicated periods of time. The blot was sequentially probed for CNP transcripts (probe 2; see Fig. 3.1A), then specifically for the larger CNP transcript (probe 1; see Fig. 3.1A), PLP/DM-20 transcripts, MBP transcripts and pyruvate kinase (PK) transcripts (a control for gel loadings). Proliferating O-2A progenitor cells (+GF) express the smaller, 2.4kb CNP transcript, but little or no 2.6kb CNP transcript, MBP transcript, or PLP/DM-20 transcripts. In each case, the autoradiographic exposure was between 3 and 4 days, except for MBP, in which case the exposure was 24 hours.

This figure is provided by Dr. Ellen Collarini in the Lab.
MYELIN GENE EXPRESSION IN CULTURED 0-2A LINEAGE CELLS

+ GF  - GF  6h  24h  72h

2.6 kb  2.4 kb

CNP

PLP

MBP

PK
Fig. 3.2 Visualization of CNP and PLP/DM-20 transcripts in an E16 rat embryo by in situ hybridization.

(Panel A) CNP probe, dark-field illumination. (Panel B) PLP/DM-20 probe, dark-field illumination. (Panel C) Bright-field micrograph. Positive hybridization signal is evident throughout the PNS in both (A) and (B): dorsal root ganglia (DRG), spinal roots (SR), many peripheral nerves (PN) sectioned transversely and longitudinally, and the skin (Sk) of the embryo. SC, spinal cord. The CNP and PLP/DM-20 expression patterns in the PNS are very similar in these consecutive sections. Autoradiographic exposure, 5 weeks. Scale bar, 500µm.
Fig. 3.3 CNP, PLP/DM-20, and PDGFRα transcripts in transverse sections through the spinal cords of E12 and E14 rat embryos, visualized by in situ hybridization.

Panels a-c were hybridized with a probe against CNP, panels d, e were hybridized with a probe against PLP/DM-20, and panel f was hybridized with a PDGFRα probe. Panels a, d are consecutive sections from the thoracic region of an E12 embryo, panels b, e are consecutive sections from the thoracic region of an E14 embryo, and panels c, f are from the equivalent cervical region of age-matched E14 littermates. Images are computer-generated superimpositions of corresponding bright-field (blue) and dark-field (yellow) micrographs. Autoradiographic exposures: 5 weeks (a-e); 2 weeks (f). Scale bar, 200μm.
Fig. 3.4 CNP and PLP/DM-20 transcripts in transverse sections through an E18 rat spinal cord, visualized by in situ hybridization.

Panels A-C, CNP probe. Many weakly CNP-positive cells can be seen throughout the gray and white matter of the E18 cord in dark-field illumination (panel A), whereas a few very intensely CNP-positive cells can be seen in the ventral-most white matter (arrows). These intensely CNP-positive cells can be seen after a few days' exposure even in bright-field illumination (panels B, C). Panels D-F, PLP/DM-20 probe. In the E18 spinal cord, a very small number of strongly PLP/DM-20-positive cells can be seen (arrows) in either dark-field (panel D) or bright-field (panels E, F) illumination. These strongly PLP/DM-20-positive and the strongly CNP-positive cells presumably represent the first differentiating oligodendrocytes in the cord. Sections present in panels A-C and panels D-F are consecutive. Panels C and F are higher magnification, higher contrast views of the ventral white matter illustrated in panels B and E, respectively. Autoradiographic exposure, 5 weeks (but note that the intensely labelled cells were visible after only a few days). Scale bar, 500μm.
Fig. 3.5 Strong expression of CNP and PLP/DM-20 by the first differentiating oligodendrocytes the rat brain stem.

Panels A-C are consecutive sections from an E18 rat brain stem hybridized with a CNP probe (panel A), a PLP/DM-20 probe (panel B) and a PDGFRα probe (panel C). In addition to the ventral strongly CNP and PLP/DM-20-positive cells, some CNP and PLP/DM-20-positive cells appeared at the dorsal white matter. These strongly CNP and PLP/DM-20-positive cells are presumably the first differentiating oligodendrocytes. There is no co-localization between the strongly CNP, PLP/DM-20-positive cells and PDGFRα° cells (panel C) which mark the oligodendrocyte precursors. Scale bar, 500μm.
Fig. 3.6 CNP and PLP/DM-20 transcripts in sagittal sections through a P10 brain and cerebellum, visualized by in situ hybridization.

(A) Bright-field illumination. (B) In situ autoradiograph with a CNP probe, dark-field illumination. Intensely CNP-positive cells can be seen throughout the brain, especially in the corpus callosum (CC), superior colliculus (SC), brain stem (Bs), and in the foliar white matter of the cerebellum (Cb). Hc, hippocampus. (C) PLP/DM-20 probe. Intensely PLP/DM-20-positive cells are found in the brain and cerebellar white matter in a similar distribution to the CNP-positive cells. Autoradiographic exposure, 1 week. Scale bar, 1mm.
Fig. 3.7 The expression pattern of CNP is different between mouse and rat.

Panels A and B, transverse sections of E11 and E14.5 mouse embryos at the throracic level were hybridized with a mouse PLP/DM-20 probe. PLP/DM-20 transcripts were expressed by the DRG at both ages. In the mouse spinal cord, no PLP/DM-20 transcripts were detected at E11 except the DRG (arrows). At E14.5 transcripts were expressed by some cells in the ventral ventricular zone. Panel B and D are consecutive sections of an E14.5 mouse embryo, showing that there is no co-localization between the PLP/DM-20 expression (panel B) and the expression of PDGFRα (panel D). Panel C, a transverse section of an E12 rat embryo was hybridized with a rat PLP/DM-20 probe showing some PLP/DM-20-expressing cells adjacent the floor plate (arrows).
Chapter Four

Specification of the Oligodendrocyte Lineage in the Ventricular Zone of the Neural Tube Requires Signals from the Floor Plate.
The identification of early markers for oligodendrocyte progenitor cells has enabled us to trace the development of the oligodendrocyte lineage right back to its origin in the ventricular zone of the embryonic spinal cord (Pringle and Richardson 1993, Yu et al 1994, Chapter 2). However, the mechanism by which these earliest recognizable oligodendrocyte precursors are set apart from their neighbours which do not give rise to oligodendrocytes, is still unknown. In this Chapter, I describe studies designed to shed light on this question.

During vertebrate development all the diverse cell types in the mature CNS are generated from the seemingly identical neuroepithelial cells in the embryonic neural tube. There are two notable features of the spatial distribution of differentiated cells in the developing neural tube: 1) cells are arranged with bilateral symmetry along the antero-posterior axis, and 2) different classes of neural cells are often located at different dorsoventral levels of the neural tube. For example, differentiated motor neurons are located ventrally, whereas commissural neurons and neural crest cells appear in dorsal positions. Although there is little or no direct evidence that the final destination of a class of neurons is directly related to their site of origin within the ventricular zone, it does seem likely that there should be some such relationship, if only because the migration routes of postmitotic neurons away from the ventricular surface are believed to be restricted to the radially oriented processes of radial glia. Thus, it is to be expected that ventrally-located motor neurons, for example, should be derived from precursor cells near the ventral extremity of the central canal. Support for this idea comes from the early experiments of Wenger (1950), who showed that removing the dorsal part of the chick neural tube, including the dorsal ventricular zone, did not affect the formation of motor neurons in the ventral part of the spinal cord. However, since there are no known markers for the ventricular
precursors of any specified neuronal cell type, including motor neurons, it has not been possible to locate the precursors of any defined neuronal population within the ventricular zone. So far, the only defined ventricular precursors whose future fate can be predicted with confidence are oligodendrocyte precursors, which reside in the ventricular zone in the ventral half of the E14 rat (E13 mouse) spinal cord. Despite the fact that oligodendrocytes in the mature animal are distributed more-or-less uniformly throughout the white matter of the cord, at all dorso-ventral levels, their earliest recognizable precursors are located at a precise point in the ventricular zone, 15% of the way along the central canal in a ventral-to-dorsal direction (Pringle and Richardson 1993, Yu et al 1994).

It is known that certain aspects of the dorso-ventral polarity of the spinal cord are controlled by two cellular structures at the ventral midline of the neural tube, the notochord and the floor plate (Yamada et al 1991). The floor plate is a defined group of cells located at the ventral midline of the neural tube. Differentiation of the floor plate appears to be induced by an underlying group of mesodermal cells known as the notochord (Watterson et al 1955, van Straaten et al 1988, Smith and Schoenwolf 1990, Placzek et al 1990). A strong candidate for the floor plate-inducing signal from notochord is the product of Sonic hedgehog (Shh, also known as vhh), a vertebrate homologue of the Drosophila patterning gene hedgehog (hh) (Roelink et al 1994, Echelard et al 1993, Krauss et al 1993). Following induction by the notochord, the floor plate becomes a secondary source of Shh and other signalling molecules (Pourquiè et al 1993, Kennedy et al 1994, Serafini et al 1994), which further control dorso-ventral patterning of the spinal cord.

It is known that signals derived from the floor plate are required for the differentiation of ventrally-located motor neurons. In the absence of a floor plate,
motor neurons do not differentiate (Yamada et al 1993, Yamada et al 1991), and
ingrafting an additional notochord or floor plate to ectopic positions close to the spinal
cord can induce the appearance of additional motor neuron columns (Yamada et al
1991). In these experiments, it was shown that the floor plate affects motor neuron
differentiation by secreting diffusible signal molecules, whereas the notochord
probably controls motor neuron differentiation indirectly by inducing an extra floor
plate. There is also evidence that, in the absence of a floor plate, commissural axons
are mis-routed, failing to converge on the ventral midline or to execute their normal
rostral turn into the ventral funiculus (Bovolenta and Dodd 1991, Bernhardt et al 1992,
commissural axons has recently been identified as a member of a family of laminin-
related molecules known as netrins (Serafini et al 1994, Kennedy et al 1994), whereas
the motor neuron-promoting signal has not yet been characterized.

To see if the appearance and subsequent differentiation of the oligodendrocyte
lineage in the developing spinal cord also requires signals from the floor plate, we
took advantage of a mouse mutant, the Danforth's short tail (Sd). The Sd mutation
causes early degeneration of the notochord during embryogenesis, resulting in the loss
of a variable portion of the floor plate in caudal regions of the spinal cord. This in
turn has been shown to result in failure of ventral cell types to develop, where the
floor plate is absent (Yamada et al 1993, Yamada et al 1991).

Results

1. Conservation of PDGFRα expression in the mouse and rat spinal cords.

During later development of the rat CNS (after E14) PDGFRα expression is
restricted to oligodendrocyte precursors although it might be expressed transiently by some immature neurons during earlier embryonic development (Pringle and Richardson 1993). In the E14 rat spinal cord, PDGFRα+ cells appear at the ventricular surface in the ventral half of the cord; this is believed to be the origin of the oligodendrocyte lineage (Pringle and Richardson 1993). Before making use of the Sd mutant mouse to investigate the role of the floor plate on the specification of oligodendrocyte precursors, it was necessary to examine PDGFRα expression in the developing mouse spinal cord to determine whether PDGFRα also marks oligodendrocyte precursors in mice.

Fig 4.1 shows that in the E13 mouse spinal cord, PDGFRα+ cells first occur at a corresponding position to where they appeared in E14 rat spinal cord (Fig 4.1 panel a). In the E15 mouse, as in the E16 rat, PDGFRα+ cells are dispersed throughout the spinal cord although they are predominantly towards the ventral side of the cord (Fig 4.1 panel b). The PDGFRα+ cells are distributed evenly in the E19 mouse spinal cord (Fig 4.1 panel c). Thus, there is no obvious difference in PDGFRα expression between rat and mouse at the ages examined except that, as expected, mouse development is advanced over the rat by about a day. This conservation between mouse and rat allows PDGFRα to be used as a probe to examine the development of oligodendrocyte precursors in Sd mice by in situ hybridization.

2. Oligodendrocyte precursors do not appear in regions of the Sd mouse spinal cord that lack a floor plate.

The Sd mutation causes early degeneration of the notochord and results in perinatal lethality of homozygotes (Sd/Sd). In heterozygotes (Sd/+) partial degeneration of the notochord leads to their having a discontinuous notochord during
early developmental life and consequently having a discontinuous floor plate in the
most caudal regions of the neural tube. The heterozygous animals were used for the
following investigation.

Since the first oligodendrocyte precursors appear in the wild-type mouse spinal
cord at E13, Sd/+ embryos of the same age were serially sectioned transversely from
the tip of the vestigial tail to the mid-thoracic region to examine whether lack of a
floor plate affects the development of oligodendrocyte precursors. The region lacking
a floor plate was easily identifiable by the morphology of the spinal cord. In
transverse sections of the floor plate-less regions, the dorsoventral extent of the central
canal was shortened, resulting in a wide margin between the ventral tip of the canal
and the most ventral side of the spinal cord. Oligodendrocyte precursors were
visualized by in situ hybridization with the PDGFR\(\alpha\) cRNA probe. In the E13 Sd/+ spinal
cord, bilateral foci of PDGFR\(\alpha^+\) cells were present in regions that possessed a
floor plate (Fig 4.2, panels a, c), but not where the floor plate was missing (Fig 4.2
panel b). This result suggests that the appearance of oligodendrocyte precursors at the
ventricular surface requires the presence of the floor plate.

To confirm that my morphological criteria (see above) accurately reflected the
presence or absence of the floor plate, consecutive sections were examined either by
in situ hybridization with the PDGFR\(\alpha\) probe or a histochemical assay for
acetylcholinesterase (Karnovsky and Roots 1964) that can be used to identify floor
plate cells and motor neuron pools. On the left of Fig 4.3 are sections from the
caudal region of a Sd/+ heterozygote hybridized with the PDGFR\(\alpha\) probe to show the
presence (Fig 4.3 panels a) or absence (Fig 4.3 panel c) of PDGFR\(\alpha^+\) oligodendrocyte
precursors in the ventricular zone. Consecutive sections stained for
acetylcholinesterase activity are shown on the right. The presence of PDGFR\(\alpha^+\)
oligodendrocyte precursors at the ventricular surface correlates with the presence of 
the floor plate (fp) and motor neuron pools (mn).

During these experiments, I found, unexpectedly, that there were PDGFRα+ cells 
distributed evenly throughout the spinal cords of E19 Sd/+ mice, even in caudal regions 
that would have lacked a floor plate at E13 (Fig 4.4). At E15, an intermediate age, 
there were still regions of spinal cord that lacked both a floor plate and PDGFRα+ cells 
(Fig 4.5, panel a). There were also regions that lacked a floor plate but nevertheless 
contained dispersed PDGFRα+ cells (Fig 4.5, panel b). These cells were never found 
in the ventricular zone, even when the section contained only a few PDGFRα+ cells, 
suggesting that they were not generated locally but had invaded those regions without 
a floor plate by migrating longitudinally between E13 and E15 from neighbouring 
regions (anterior and posterior) where spinal cord development was wholly normal 
(Fig 4.5 panel c). The experiments described in the next section were designed to test 
this idea.

3. Oligodendrocytes are generated in vitro from explants of the normal spinal 
cord but not from explants without an associated floor plate.

The question of whether mature oligodendrocytes can be generated locally in 
regions of the spinal cord that lack a floor plate was addressed by using explant 
cultures, in which segments of E13 mouse spinal cord that lacked a floor plate were 
dissected away from neighbouring normal regions and cultured in vitro to avoid the 
possibility of longitudinal migration of oligodendrocyte progenitors. In this 
experiment, the E13 Sd/+ spinal cord was dissected from the surrounding tissues, 
opened along the dorsal midline with a tungsten needle and flattened (like opening a 
book) onto a tissue culture plate. Regions that lacked floor plate were cut away from
neighbouring, normal regions of the Sd/+ spinal cord and cultured in DMEM medium with a supplement of 10% FCS (Fig 4.6). For a control, the equivalent regions of a wild type littermate spinal cord were cut and cultured in the same plate. After culturing for 19 days to the equivalent of postnatal day 12 (P12), by which time mature oligodendrocytes have been present for several days in vivo, the explants were stained with an antibody against MBP. Explants of Sd/+ spinal cord that lacked a floor plate did not have any MBP⁺ cells (Fig 4.7 panel c), whereas the equivalent regions of wild type littermates had a significant number of MBP⁺ cells (Fig 4.7 panel d). There was no obvious difference in the numbers of MBP⁺ cells that developed in explants derived from those regions of Sd/+ spinal cord that did possess a floor plate, and the equivalent regions of wild-type cord (compare Fig 4.7 panel a and panel b).

Discussion

1. The emergence of the oligodendrocyte lineage requires signals from the floor plate.

The floor plate is a defined group of cells located at the most ventral tip of the central canal of the spinal cord. Its initial induction requires signals derived from the notochord (Placzek et al 1993). A strong candidate for this notochord-derived signalling molecule is Sonic hedgehog (shh), a vertebrate homologue of the Drosophila segment polarity gene hedgehog (hh) (Echelard 1993). In Danforth’s short tail mice, the floor plate is absent from a variable region of the caudal spinal cord (Theiler 1959). The absence of the notochord along most of the near axis in Sd mice may be the primary defect in this mutant (Gruneberg 1958, Theiler 1959) and may account for the discontinuous induction of the floor plate (Placzek et al 1990). In our
In the Sd colony, the position and extent of floor plate-less regions in the Sd/+ spinal cord was variable among different litters. In most litters (e.g., those used for the explant cultures and the in situ hybridization experiments at E15 and E19), the floor plate-less regions started right at the root-caudal region of the spinal cord, and extended about 0.5-1mm anteriorly. Embryos in some Sd/+ litters possess an additional normal region at the posterior end of the floor plate-lacking region (e.g., the embryos used for the in situ hybridization experiment at E13). It is possible that this variability among embryos results from slight variations in the time of notochord degeneration among different litters during early development; however, the precise reason for the variability is unknown.

There is accumulating evidence that PDGFRα is a specific marker for oligodendrocyte progenitor cells (Chapter 3). In this experiment, the absence of PDGFRα+ cells in the portion of the E13 spinal cord where the floor plate is missing indicated that the appearance of oligodendrocyte precursors requires the presence of the floor plate. There are two possibilities for this lack of oligodendrocyte precursors: 1) The occurrence of oligodendrocyte precursors might have been delayed where the floor plate was missing. 2) The specification of oligodendrocyte precursors might have been completely abolished in the absence of the floor plate. To distinguish between these two possibilities, some older embryos were examined by in situ hybridization with the PDGFRα probe. In the E19 Sd/+ spinal cord, PDGFRα+ cells appeared uniformly throughout the cord, even in the most caudal regions where the floor plate would have been absent at E13. At an intermediate age, E15, there was still a floor plate-less region where no PDGFRα+ cells were present but towards the rostral and caudal limits of the region lacking a floor plate, there were a few PDGFRα+ cells dispersed throughout the cross-section of the cord. However, these PDGFRα+ cells were never found at the ventricular surface of the spinal cord even there were only
very few PDGFRα⁺ cells present. This distribution of PDGFRα⁺ cells in the older $Sdl^+$ embryos suggested that they are not generated in the ventricular zone locally, but invaded from the neighbouring, normal region of the cord.

To test this idea, floor plate-less regions of $Sdl^+$ spinal cord were separated from their neighbouring, normal regions at E13 to avoid the possibility of long-range longitudinal migration of oligodendrocyte precursors, and cultured in vitro far beyond the time when myelinating oligodendrocytes normally occur. The explants were stained with an anti-MBP antibody to identify differentiated oligodendrocytes. In this experiment, the MBP⁺ oligodendrocytes were only present in explants of either wild-type or $Sdl^+$ spinal cord that had an associated floor plate, but not in the explants of $Sdl^+$ spinal cord that lacked a floor plate at E13. This result strongly supported the idea that the appearance of oligodendrocyte precursors requires signals from the floor plate.

2. Glial cells and neurons are specified by fundamentally similar mechanisms.

It has been shown that the floor plate and notochord play an important role in the dorsoventral patterning of the vertebrate spinal cord (Yamada et al 1991, Bovolenta and Dodd 1991, Yamada et al 1993). The previous work mainly concentrated on two aspects, 1) the pattern of differentiated neurons, such as motor neurons (Yamada et al 1993, Yamada et al 1991, Ericson et al 1992) and other ventral neurons (van Straaten and Hekking 1991), and 2) axon guidance, for example the extension of the axons of commissural neurons from the dorsal gray matter to the floor plate (Tessier-Lavigne et al 1988, Bovolenta and Dodd 1991). The elimination of the notochord and floor plate prevents differentiation of motor neurons (Yamada et al 1991, Ericson et al 1992), and an ectopic graft of notochord or floor plate tissue

The experiments described in this Chapter demonstrate that the influence of the notochord/floor plate complex extends to glial cells as well as neurons, suggesting that these two classes of neural cells are under similar developmental control. Moreover, the results also suggest that the notochord/floor plate complex can control the development of neural cells by influencing their specification from undifferentiated neuroepithelial precursors that are part of the neuroepithelial lining of the central canal. It has not previously been possible to draw this conclusion, because the antibody markers used to identify motor neurons, for example, recognize only the postmitotic cells after they have detached from the ependymal layer and started to migrate into the gray matter of the spinal cord.
Fig. 4.1 The expression of PDGFRα in the developing mouse spinal cord.

Transverse sections of the developing mouse spinal cord at the thoracic level were hybridized with a radiolabelled PDGFRα antisense cRNA probe and subjected to autoradiography and photographed under a dark field illumination. Panel a shows that at E13, PDGFRα expression is restricted to two discrete positions at the ventral ventricular surface of the spinal cord. Two days later at E15 (Panel b), the number of PDGFRα+ cells in the spinal cord is increased and the positive cells are dispersed throughout the cord. At E19 (Panel c), a more even distribution of PDGFRα+ cells is found throughout the spinal cord. The PDGFRα expression pattern of the developing mouse spinal cord is similar to that in the developing rat spinal cord except that it is one day earlier in the mouse than rat, which reflects the shorter period of gestation in the mouse. Scale bar, 200μm.
Fig. 4.2 Appearance of oligodendrocyte precursors at the ventricular surface of Sdl+ mouse mutant depends on the presence of a notochord/floor plate.

Transverse cryosections (10μm) were cut through the spinal cords of heterozygous E13 Sdl+ embryos, from the tip of the vestigial tail to the upper thoracic region. Four consecutive sections were collected at 100μm intervals. Two of each set were processed for in situ hybridization with a radiolabelled antisense cRNA probe against mouse PDGFRα. In three embryos examined, the most caudal sections possessed a floor plate (fp; panel a), but anterior to this was a region approximately 600μm in length that lacked a floor plate (panel b). More anterior still, the floor plate reappeared (panel c). The section in panel a came from the caudal spinal cord, 2mm from the tip of the tail; the sections in Panels b and c are approximately 1mm and 2mm anterior to Panel a, respectively. After hybridization to the PDGFRα probe, the sections were autoradiographed, photographed under dark-field illumination, stained with haematoxylin and rephotographed under bright-field illumination. Images were assigned false colours (bright-field, blue; dark-field, yellow) and superimposed using Adobe Photoshop software with a Macintosh computer Image grabber. PDGFRα is expressed in many tissues outside of the CNS (panels a-c). In regions of spinal cord that were associated with a floor plate (panels a, c) there were bilateral foci of PDGFRα+ cells in the ventral ventricular zone (arrows). Where there was no floor plate, there were no PDGFRα+ cells in that region of the spinal cord (panel b). Scale bar, 200μm.
Fig. 4.3 Acetylcholinestase assay for floor plate cells and motor neurons in E13 Sdl+ heterozygous.

The presence or absence of floor plate, judged by morphology of the ventral midline region, was confirmed in some sections by an immunohistochemical assay for acetylcholinesterase. Panels a and c depict sections from the caudal region of a Sdl+ heterozygote hybridized with the PDGFRα probe to show the presence (panel a) or absence (panel c) of PDGFRα' cells in the ventricular zone (within the boxed areas). Panels b and d show consecutive sections to those of panels a and c, respectively, stained for acetylcholinesterase activity. The presence of PDGFRα' oligodendrocyte precursor cells at the ventricular surface correlates with the presence of the floor plate (fp) and motor neuron pools (mn). The ventral-most tip of the central canal (cc) is indicated (arrowheads in b and d). Scale bars, 200μm.
Fig. 4.4 The PDGFRα expression in the caudal region of E19 Sd/+ embryonic spinal cord.

The presence of the floor plate cannot be detected at this stage of development. In order to include the regions which would have lacked a floor plate at the age of E13, sections were taken from the most posterior end of the caudal region of an E19 Sd/+ embryo and hybridized with the PDGFRα probe. PDGFRα+ cells appeared on all examined cross sections of the spinal cord. The figure shows sections from post-caudal region (panel a), middle caudal region (panel b) and lower lumber region (panel c). Scale bar, 200μm.
Fig. 4.5 Oligodendrocyte progenitor cells invade floor plate-less regions of Sd/+ spinal cord by longitudinal migration from neighbouring regions of normal spinal cord.

Sections were taken from the caudal region of an E15 Sd/+ embryo and hybridized with the PDGFRα probe. The sections of panels a and b lack a morphologically recognizable floor plate, whereas the more anterior section of panel c possesses a floor plate (fp). The distance between sections a and b is approximately 150μm, the distance between b and c is 300μm. PDGFRα+ cells appear in section b despite the absence of a floor plate, presumably because of longitudinal migration of these PDGFRα+ cells from the more anterior, normal region of spinal cord. Since the region lacking a floor plate (and PDGFRα+ cells) in E13 Sd/+ mice is around 600μm (see Fig. 4.2), and PDGFRα+ cells are found at all levels of the neuraxis at E19 (see Fig. 4.4), this suggests that oligodendrocyte progenitors can migrate longitudinally at least 300μm during embryonic development. Scale bar, 200μm.
Spinal cords of E13 Sd/+ mice were dissected away from surrounding tissue, split along the dorsal midline and flattened out with the ventricular surface uppermost. The floor plate, where present, could be easily recognized at the ventral midline of such preparations. In the Sd/+ embryos used for these experiments, the region lacking a floor plate was about 1mm long at the caudal end; unlike the embryos described in Fig. 4.2, there was no adjacent normal spinal cord further posterior to this abnormal region. Transverse segments, approximately 0.5mm in the antero-posterior dimension were cut from the caudal region of the cord using a tungsten needle. First, the spinal cord was cut transversely into anterior (normal, with floor plate) and posterior (abnormal, no floor plate) sections at the visible boundary between normal and abnormal cord. The tail section was then removed and discarded. The remaining two sections were further divided into equal segments, the two most posterior segments without floor plate, and the more anterior segments with floor plate. Spinal cords from wild-type littermates were aligned with the Sd/+ cords, and cuts made in equivalent positions. Each spinal cord segment was attached to the surface of a tissue culture dish with 1μl of chicken plasma, which was clotted in situ by adding an equal volume of 1mg/ml thrombin (Sigma). The explants were cultured at 35.5°C in humidified chamber with 5% CO₂ on a rocking platform (12 cycles/min), so that the explant was exposed above the surface of the culture medium (4ml in a 6cm diameter dish) during about half of each cycle. Half of the culture medium (DMEM containing 10% FCS) was changed every other day.
Segments were cultured in DMEM + 10% FCS for 19 days separately.

**Fig. 4.6** Dissection of mouse spinal cord for explant culture.
Fig. 4.7 Oligodendrocytes do not develop in explant cultures of Sd/+ spinal cord in the absence of a floor plate.

After 19 days in culture (see Fig. 4.6), the explants were incubated with an anti-MBP polyclonal antibody followed by incubation with a fluorescein-conjugated goat-anti-rabbit IgG (Sigma). The explants were then examined under the fluorescence microscope. This procedure labelled only oligodendrocytes that lay at, or close to, the surface of the explants. Three Sd/+ and four wild-type embryos from two litters were analyzed. There were large numbers (>100) of MBP⁺ oligodendrocytes in anterior, floor plate-containing explants from both wild-type and Sd mice (panels a, b). In the two most posterior wild-type segments the number of oligodendrocyte decreased significantly, presumably reflecting the normal, systematic temporal lag of development towards the posterior end of the neuraxis (the most posterior segment of wild-type mice shown as panel d). However, in the equivalent Sd/+ segments (which lacked a floor plate) there was a precipitous drop in oligodendrocyte numbers to zero in each of the most posterior explants (panel c), and less than 10% of wild-type in the penultimate segments (data not shown).
Chapter Five

Expression of Fibroblast Growth Factor Receptor-3 in Putative Astrocyte Precursors in the Late Embryonic Rat Spinal Cord
The fibroblast growth factor family is one of the largest oncogene/peptide growth factor families known, with nine sequence-related members identified so far. An activity that can stimulate fibroblast division in vitro was detected in extracts of central nervous system (CNS) tissue (Trowell et al 1939, Hoffman 1940). Subsequently, two sequence-related molecules were identified that account for this activity. Because of their different isoelectric points (Bohlen et al 1984, Thomas et al 1984), they were named acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF). These have since been renamed FGF-1 and FGF-2 respectively. At the present time, the FGF family has expanded to nine members. Some of these were initially identified as oncogene products. For example, the FGF-3 gene is the preferred integration site of mouse mammary tumour virus (Smith et al 1988), FGF-4 is the hst oncogene (Taira et al 1987) and the Karposi sarcoma oncogene (Delli-Bovi et al 1987), and FGF-5 is a transforming gene when transfected into 3T3 fibroblasts (Zhan et al 1988). Others were identified as mitogens. For example, FGF-7 is a mitogen for keratinocytes (Finch et al 1989), FGF-8 is an androgen-induced mitogen for mammary carcinoma cells (Tanaka et al 1992), and FGF-9 is a mitogen for primary astrocytes (Miyamoto et al 1993). FGF-6 was first isolated from a low-stringency screening of a cDNA library with a FGF-4 probe (Marics et al 1989).

All these members of the FGF family share amino acid sequence homology, with 30-40% similarity in the core sequence. A most striking common property of FGFs is their ability to bind the proteoglycan heparin in the extracellular matrix. In addition, FGF-1, FGF-2 and FGF-9 lack an N-terminal hydrophobic signal peptide motif which is thought to be required for the efficient sorting of proteins into the secretory pathway. Because of the lack of this signal peptide, these three FGFs might
be present in the cytoplasm under normal physiological conditions. Interestingly, several different isoforms of FGF-2 can be translated from different initiation codons within the single mRNA, some of the resulting peptides possess a nuclear translocation signal which causes their accumulation within the cell nucleus (Renko et al 1990, Bugler et al 1991, Woodward et al 1992). The function of these nuclear-located FGFs is unknown.

By serving as signalling molecules, FGFs play important roles in cell proliferation, differentiation and survival of a wide array of cell types, such as epithelial cells, endothelial cells, mesodermal cells, neurons and glial cells in the CNS. FGFs bind to high-affinity transmembrane receptors of the tyrosine kinase (TK) class. Four high-affinity FGF receptors (FGFR-1 to FGFR-4) have been isolated so far (Lee et al 1989, Dionne et al 1990, Miki et al 1991, Keegan et al 1991, Partanen et al 1991). Analysis of the amino acid sequence of FGFRs reveals that the structure of all four receptors is very similar. They share about 60-70% similarity in amino acid sequence, have three immunoglobulin-like (Ig) domains in the extracellular part followed by a single hydrophobic transmembrane domain and two intracellular tyrosine kinase domains linked by a short insert sequence. There are indications that individual receptors have different ligand-binding properties. For example, FGFR-1 and FGFR-2 bind both FGF-1 and FGF-2 with high affinity (Dionne et al 1990), whereas FGFR-3 binds FGF-1 and FGF-7 twenty times tighter than FGF-2, and FGFR-4 binds FGF-1 with high affinity but does not bind FGF-2 (Mansukhani et al 1990). However, the biological significance of these ligand specificities is unclear. Further complexity is generated by alternative splicing, mostly affecting the extracellular portion of the FGFRs. In some FGFR variants, the first Ig-domain is lost. The signification of this modification is still unclear, but it is unlikely to affect ligand-binding (Johnson et al 1990, Shi et al 1993). In other FGFR variants, there are
alterations in the carboxyl half of the third Ig domain. There are three alternative exons for this portion of Ig-domain-3. One exon (exon IIIa) contains a termination codon and, therefore, products of transcripts containing this exon do not have the transmembrane domain or the tyrosine kinase domains; this should presumably result in a secreted form of the receptor. The other two alternative exons (IIIb and IIIc) do not contain termination codons and transcripts containing these exons encode full-length transmembrane receptors. Different ligand-binding affinities have been observed for these latter alternative splicing isoforms. For example, one of the FGFR-2 variants, FGFR-2(IIIb), binds FGF-1 and FGF-7 with equal high affinity but binds FGF-2 with much reduced affinity. Another variant of this receptor, FGFR-2(IIIc) binds FGF-1 and FGF-2 equally well but does not bind FGF-7.

In addition to the high-affinity receptors, FGFs can also bind with low affinity to heparan sulphate proteoglycans on the cell surface (Gallagher and Turnbull 1992, Guimond et al 1993, Maccarana et al 1993, Turnbull and Gallagher 1993). The fact that removal of heparan sulphate from the cell surface can abolish the ability of FGFs to successfully activate the transmembrane FGFRs suggests that heparan sulphate might play a role in the binding of FGFs to their high-affinity receptors (Rapraeger et al 1991, Yayon et al 1991). There is some evidence that heparan sulphate proteoglycan-binding can change the conformation of FGFs (Prestrelski et al 1992). There is also evidence that heparin itself can bind to FGFRs (Kan et al 1993). How this might influence FGF receptor binding is still unclear.

The biological functions of FGFs during vertebrate development are largely unknown. There is some evidence that FGFs play an important role in mesoderm induction during early amphibian development. The most direct evidence came from a dominant-negative mutant study of FGFR-1 in *Xenopus* (Amaya et al 1991). In this
study it was shown that explants from embryos expressing a truncated form of FGFR-1, which effectively abolishes the activity of endogenous FGFRs, fail to differentiate as mesoderm in response to FGF. In whole embryos, the mutant receptor causes specific defects in gastrulation and overexpression of wild type receptor can rescue these developmental defects. This strongly suggests that FGFs are crucial in early development of *Xenopus*, particularly in mesoderm induction.

In humans, several genetic disorders have been ascribed to mutations in genes of the FGFR family. Point mutations in the transmembrane domain of FGFR-3 causes achondroplasia, the most common genetic form of dwarfism in humans, (Shiang et al 1994, Rousseau et al 1994). In addition, mutations of FGFR-1 and FGFR-2 are associated with several autosomal dominant skeletal disorders. A point mutation in the extracellular domain of FGFR-1 causes Pfeiffer syndrome (Muenke et al 1994), and a variety of point mutations in the second half of the third extracellular Ig-domain of FGFR-2 cause several related syndromes, including Crouzon, Pfeiffer and Jackson-Weiss syndromes (Rutland et al 1994, Reardon et al 1994, Jabs et al 1994).

In addition to an extremely broad range of cells derived from ectoderm and mesoderm, several types of cells in the CNS have been shown to respond to FGFs. These include oligodendrocytes (Eccleston and Silberberg 1985, Bogler et al 1990, McKinnon et al 1990), astrocytes (Pettmann et al 1985), and some populations of neurons, like cerebrocortical, hippocampal, and motor neurons (Morrison et al 1986, Valicke et al 1986, Sendtner et al 1991). In vitro studies have shown that FGFs can function as mitogens, as neurotrophic factors and as differentiation-inducing factors. Apart from their putative roles in development, there is evidence that FGFs are partly responsible for the repair of injuries in vivo (Davidson et al 1985, Mellin et al 1988).
In order to try to understand the biological functions of FGFs in the developing CNS, I examined the expression patterns of FGFRs in the developing CNS by in situ hybridization and immunohistochemistry, in an attempt to identify the cell populations that express FGFRs and presumably respond to FGFs. These experiment are described in this Chapter. In particular, a restricted expression pattern of FGFR-3 was found in the embryonic rat CNS. Using a combined in situ hybridization and immunohistochemistry procedure, I found evidence for a population of FGFR-3\(^+\) astrocyte precursors in the rat spinal cord, and identified two possible sources of these astrocytes at the ventricular surface of the embryonic spinal cord.

Results

1. Cloning of rat FGFR-3 cDNA by RT-PCR.

Because of the lack of sequence information about rat FGFR-3, reverse-transcription PCR (RT-PCR) combined with restriction endonuclease digestion was applied to isolate a probe for rat FGFR-3, based on two structural properties of FGFRs. 1) The sequences in the tyrosine kinase domains are highly conserved among the different isoforms of FGFRs and across species boundaries, whereas the sequences in the "linker" region between the two tyrosine kinase domains is highly variable.

2) A restriction site for \textit{BstXI} appears within the linker region of both rat FGFR-1 and rat FGFR-2 but not FGFR-3. The former structural property of FGFRs facilitated the design of PCR primers able to amplify all FGFRs, while the latter property enabled me to discriminate FGFR-1 and FGFR-2 from FGFR-3 and subsequently allowed me to isolate a FGFR-3 cDNA from the mixture of FGFR cDNAs.
Two "universal" primers, $P^{up\text{FR-INTR}}$ and $P^{down\text{FR-INTR}}$ with sequences of 5' GAG AAG GAC CTG TCG GAT CTG 3' (upstream) and 5' GTC GCG CAT CAT CAT GTA CAG 3' (downstream), corresponding to amino acid sequences EKDLSDL and LYMMMRD respectively were synthesized to amplify fragments of the intracellular domain of all FGFRs including portions of each of the two tyrosine kinase domains and the entire "linker" region (Fig. 5.1). The first cDNA strand was reverse-transcribed from P5 rat brain mRNA and used as a template for the subsequent PCR reaction. Sequencing data showed that clones derived from this PCR product pool contained mostly FGFR-1 (flg) and FGFR-2 (bek) cDNAs. More than 40 randomly-selected clones were sequenced, none of which corresponded to FGFR-3 (data not shown). This could be due to a relatively low amount of FGFR-3 transcripts compared to FGFR-1 and FGFR-2 transcripts in the P5 brain. In order to remove FGFR-1 and FGFR-2 cDNAs from the pool of first-round PCR products, the amplified FGFR fragment mixture was digested with $BstXI$ which cuts FGFR-1 and FGFR-2 cDNAs in the "linker" region. The uncut PCR products (containing FGFR-3 fragments) were separated from the two cut segments of FGFR-1 and FGFR-2, recovered on an agarose gel and re-amplified with the original primers until there were no $BstXI$ digestible fragments remaining (usually, it took three rounds of amplification to achieve this) (Fig 5.1). The remaining undigested fragment was then cloned into a TA vector, and clones characterized by sequencing. These clones corresponded mainly to FGFR-3.

A downstream primer $P^{down\text{FR3LINKER}}$ with a sequence of 5' CAG GCA TCG AAG GAG TAA TCC 3' (the corresponding amino acid sequence is MDYSFDAC), from the highly variable "linker" region of rat FGFR-3 was then synthesized and used, together with a degenerate upstream primer $P^{up\text{FR-B}}$, to amplify a larger FGFR-3 fragment including most of the extracellular domain (see Chapter 2).
The products were cloned and sequenced. This larger fragment of FGFR-3 was subsequently used as a probe for in situ hybridization (see below).

2. The expression pattern of FGFR-3 in the developing rat CNS.

The expression pattern of FGFR-3 in developing rat embryos from E12 to birth was examined by in situ hybridization with a radiolabelled cRNA probe transcribed from the cloned PCR fragment described above. FGFR-3 was expressed at high level during rat embryogenesis especially in the CNS. Before E14, FGFR-3 expression was mainly restricted to the ventricular and subventricular zones throughout the CNS (Fig 5.2 panel a). In transverse sections of E14 spinal cord, it was evident that FGFR-3 was expressed predominantly in the ventricular zone in the ventral half of the cord (Fig 5.3 panel a). There were two "hotspots" of expression along the dorsoventral axis, one near the mid-point and the other beside the floor plate, perhaps including part of the floor plate itself. Two days later, at E16, two groups of FGFR-3^+ cells appeared to be streaming away from these regions of the ventricular zone into the developing grey and white matter (Fig 5.3 panel b). During the same period (E14-E16) expression levels in the rest of the ventricular zone decreased, and a clear gap between the two sources of presumptive migrating FGFR-3^+ cells developed. The two putative sources of migrating FGFR-3^+ cells extended into the brain and appeared to disappear at the hindbrain/midbrain border (Fig 5.2 panel c). At E18, FGFR-3^+ cells were dispersed throughout the spinal cord, although they remained predominantly in the ventral half (Fig 5.3 panel c). FGFR-3 expression had ceased in the ventricular zone, apart from the two putative sources of migratory cell. In the brain, scattered cells around the lateral ventricle expressed FGFR-3 at a very high level during the period of embryogenesis examined (Fig 5.2).
After birth, expression of FGFR-3 in the spinal cord and brain stem was gradually down-regulated, and became indistinguishable from background by postnatal day 5 (P5). In contrast, cells in the immature P5 cerebellum at this age began to express FGFR-3 (Fig 5.4 panels a, b). At P20, FGFR-3^ cells were mainly in the Purkinje cell layer (Fig 5.4 panels c, d). However, when examined by high-power microscopy, the FGFR-3^ cells were clearly not Purkinje neurons but neighbouring cells, possibly the Bergman glia (data not shown).

Expression patterns of FGFR-1, FGFR-2 and FGFR-4 in the developing rat CNS were also examined by in situ hybridization. FGFR-1 and FGFR-2, like FGFR-3, were also strongly expressed in the ventricular zone of E12 to E16 rat spinal cord and there were no major differences among them except that, unlike FGFR-3 which appeared to be expressed predominately in the ventral half of the cord, FGFR-1 and FGFR-2 were expressed more uniformly all around the ventricular zone (Fig 5.5). After E16, expression of FGFR-1 and FGFR-2 in the ventricular zone gradually decreased. Neither FGFR-1^ nor FGFR-2^ cells were seen to leave the ventricular zone of the embryonic spinal cord. After birth, the expression patterns of FGFR-1 and FGFR-2 were clearly distinct in the brain and seemed to be restricted to different populations of neurons and, possibly, FGFR-2 was also expressed by some glial cells (data not shown). No FGFR-4 expression was seen anywhere in the developing rat CNS at the ages examined under the conditions of my in situ hybridization procedure.

3. FGFR-3-expressing cells in the embryonic spinal cord might be astrocyte precursors and/or newly-differentiated astrocytes.

The timing and pattern of FGFR-3 expression in the spinal cord after E16 suggested that the presumptive migrating FGFR-3^ cells could be some type of glial
cells since most neuronal precursors have left the ventricular zone by E14 (Altman and Bayer 1984). There are two major classes of glial cells in the CNS, oligodendrocytes and astrocytes. As described in Chapters two and three, oligodendrocytes originate at a different location in the ventricular zone than the FGFR-3^ cells. The FGFR-3^ cells might, therefore, be some type of astrocytes. To test this idea, I combined in situ hybridization and immunocytochemistry with the FGFR-3 cDNA probe and an antibody against glial fibrillary acidic protein (GFAP), respectively. In this experiment, cells from E17 rat spinal cord were dissociated and cultured in DMEM supplemented with 10% FCS for two days. They were then consecutively hybridized with a radioactive cRNA probe against FGFR-3, then stained with an anti-GFAP antibody before autoradiography. Fig 5.6 panel a shows that the FGFR-3 signal (silver grains) co-localize on cells that label for GFAP, suggesting that the FGFR-3^ cells belong to the astrocyte lineage. Additionally, there are some GFAP-staining cells that do not appear to express FGFR-3 (Fig 5.6 panel b). These GFAP-positive, FGFR-3-negative cells were frequently strongly labelled for GFAP and presumably represent astrocytes that are further advanced along their differentiation pathway. Staining of low-density cell cultures confirmed this result; the most dense silver grains (FGFR-3 signals) co-localized with cells that expressed rather low levels of GFAP, whereas some of the intense GFAP-stained cells expressed low levels of FGFR-3 (Fig 5.6 panels c, d). This result suggested that FGFR-3 might be expressed only by astrocyte progenitor cells or newly-differentiated astrocytes, but not by mature astrocytes.

4. FGFR-3 expression outside the CNS.

In addition to the CNS, FGFR-3 is also expressed in some other embryonic tissues. In particular, prebone cells in the developing vertebrae and other osteoblasts
express FGFR-3 at a high level (Fig 5.7 panel d). In the inner ear at E16, a subset of cells, possibly the developing cochlear duct, expressed FGFR-3 very strongly suggesting FGFR-3 might play a role on the development of this structure (Fig 5.7 panel c). FGFR-3 expression was also seen in developing skin cells, especially in the hair follicles (Fig 5.7 panel b).

Discussion

1. FGFR-3* cells in the later embryonic rat spinal cord and brain stem might be the astrocyte progenitors/newly-differentiated astrocytes.

FGFs have been shown to be able to stimulate the proliferation of glial precursors (Eccleston and Silberberg 1985, Saneto and DeVellis 1985, McKinnon et al 1990) and neuronal precursors (Gensburger et al 1987, Murphy et al 1990, Deloulme et al 1991, Drago et al 1991, Ray and Gage 1994). These dividing neuroblasts or glioblasts are predominantly localized in the ventricular zone of the CNS. In addition to FGFR-3, two other members of the FGF receptor family, FGFR-1 and FGFR-2, were also found to be highly expressed at the ventricular zone of the rat spinal cord before E14 (Wanaka et al 1991). However, the significance of the coexistence of different FGFRs in these regions is still unknown. Although there is some evidence suggesting that some precursors express all these three FGFRs simultaneously (see Appendix I of this Thesis), whether these three types of FGFRs are expressed by all the precursors in the ventricular zone, or whether different populations of precursors express different combinations of FGFRs, is unknown.

Shortly after E14, there are some FGFR-3* cells present outside the ventricular zone of the spinal cord. Considering the migratory properties of neural precursor
cells, it is likely that the FGFR-3\(^{+}\) cells outside the ventricular zone appear there by migration from the ventricular zone, rather than by differentiating in situ.

Neurons within the rat spinal cord are produced in a ventral-to-dorsal sequence between E11 and E16 (Nornes and Das 1972, 1974, Altman and Bayer 1984, Phelps et al 1988). For example, motor neurons in the ventral horns are born between E11 and E13 (Altman and Bayer 1984, Phelps et al 1988) and the cholinergic central canal cluster and dorsal horn neurons are born on E13-E14 (Phelps et al 1988). Therefore, because of their relatively late time of appearance around E16, it appears likely that the migratory FGFR-3\(^{+}\) cells in the ventricular zone are glial cells or their precursors. There are two major glial populations in the CNS, oligodendrocytes and astrocytes. As oligodendrocyte precursors have been shown to be generated at a defined locus at the ventricular surface of the E14 rat spinal cord, at a different position to the origin of the migratory FGFR-3\(^{+}\) cells, the FGFR-3\(^{+}\) cells might represent astrocytes or astrocyte precursors.

GFAP, a member of the family of intermediate filament proteins, is a well-documented astroglial-specific marker (Eng et al 1971, Bignami et al 1972). To determine whether the FGFR-3\(^{+}\) cells are co-localized with GFAP, immunohistochemistry with an antibody against GFAP was combined with in situ hybridization with the FGFR-3 probe. In practice, the in situ hybridization step was performed before antibody labelling, for two reasons: 1) the presence of RNase in the antibody solutions might degrade the mRNA target for in situ hybridization; and 2) the coloured product of the DAB-reaction can bind the radioactive probe used for in situ hybridization, resulting in a false positive reaction. In order not to destroy the epitopes for the GFAP antibody after the in situ hybridization procedure, the usual proteinase K treatment was omitted from the in situ procedure. This did,
unfortunately, result in a higher non-specific background hybridization. Nevertheless, a high degree of co-localization of FGFR-3 hybridization and GFAP immunoreactivity was observed in these experiments, indicating that FGFR-3\(^+\) cells do indeed belong to an astroglial lineage. It was noticeable that the most intense FGFR-3\(^+\) cells were labelled faintly by GFAP, whereas most intense GFAP\(^+\) cells were FGFR-3 negative, suggesting that FGFR-3 is expressed predominantly by astrocyte progenitor cells or newly-differentiated astrocytes since GFAP is mainly expressed by more mature differentiated astrocytes and not by early progenitors (Chiu et al 1981, Dahl 1981, Dahl et al 1981, Herpers et al 1986).

Recently, evidence has been presented that there are several morphologically distinct types of astrocytes present in cultures of neonatal rat spinal cord (Miller and Szigeti 1991). In E16 rat spinal cord, I have visualized two groups of FGFR-3\(^+\) cells migrating away from two different clearly defined regions of the ventricular zone, one around the middle of the spinal cord and the other abutting the floor plate (Fig 5.3 panel b). My experiments do not address the question of whether both these groups of FGFR-3\(^+\) cells, or only one of them, give rise to GFAP\(^+\) astrocytes, although the rather high proportion of FGFR-3, GFAP double-positive cells in spinal cord cultures might indicate that both groups of FGFR-3\(^+\) cells are of astrocyte lineage. Thus, it is possible that different classes of spinal cord astrocytes might be generated by glial precursors at different positions within the ventricular zone.

2. FGFR-3 expression outside the CNS.

In addition to the CNS, I found that FGFR-3 is expressed highly in several other structures, including bone cells, hair follicle cells and a group of unidentified cells in the inner ear. Similar results have been obtained from the examination of
FGFR-3 expression in developing mouse embryos by in situ hybridization (Peters et al 1993). The high level expression of the FGFR-3 in the developing bone cells suggests that it could be one of the mediators for FGF functions in bone development. The finding that a point mutation in FGFR-3 can directly cause the achondroplasia, a common genetic form of dwarfism in humans (Shiang et al 1994, Rousseau et al 1994) provides further evidence that FGFR-3 could be important for bone development. In addition to FGFR-3, FGFR-1 and FGFR-2 have also been found to be expressed in developing bone cells (Peters et al 1992), and to be mutated in some other hereditary bone disorders in humans (Muenke et al 1994, Rutland et al 1994, Reardon et al 1994, Jabs et al 1994).

FGF-3 (the ligand) has been shown to be expressed in hair cells of the developing inner ear (Wilkinson et al 1989). Furthermore, a targeted gene knockout experiment in mice has revealed that FGF-3 is required for normal development of the inner ear (Mansour et al 1993). However, whether FGF-3 can act via FGFR-3 is still not known. Because of their distinct expression patterns, it seems unlikely that FGFR-3 is the target of FGF-3 in the developing inner ear. It is also not clear whether FGF-3 can bind to FGFR-3 in vitro (Omitz and Leder 1992).

In another gene targeting experiment, it has been shown that FGF-5 is expressed by the outer root sheath of the hair follicle, and that disruption of FGF-5 expression causes the mouse to have abnormally long hair (Hebert et al 1994). This result suggests that FGF-5 functions as an inhibitor of hair elongation during the hair growth cycle. However, it is not known which receptors are activated by FGF-5. In spite of high expression of FGFR-3 by the developing hair follicle, it is unlikely that FGFR-3 is the target receptor of FGF-5 since it has been shown that FGF-5 does not bind to FGFR-3 in vitro (Omitz and Leder 1992). In addition to FGF-5, it has been
shown that some other members of the FGF family might play a role in hair follicle formation. For instance, ectopic expression of FGF-7 in the suprabasal epithelial layer inhibits the formation of primary hair follicles (Guo et al 1993), and repeated subcutaneous injection of newborn mice with large amounts of FGF-2 can inhibit both the formation of the primary hair follicle and the maturation of existing follicles (Du Cros, 1993). It is not yet clear whether these functions of FGFs are mediated by FGFR-3.

Conclusion

1) Before E14, FGFR-3 is expressed in the ventricular zone of the rat embryonic spinal cord, presumably by neural precursors.

2) Shortly after E14, two groups of FGFR-3⁺ cells apparently begin to migrate away from the ventricular zone and gradually become dispersed throughout the cord, being more-or-less uniformly distributed by E18.

3) Combined FGFR-3 in situ hybridization and GFAP immunocytochemistry on cultured spinal cord cells indicates that some of the migrating FGFR-3⁺ cells might be astrocyte precursors/newly-differentiated astrocytes.
Fig. 5.1 The PCR amplification of rat FGFR-3 intracellular domain combined with a restriction endonuclease digestion with BstXI.

(for description see text)
Fig. 5.2 Expression of FGFR-3 in the E14 and E16 rat embryo.

Panels a and c, sagittal sections of rat embryos hybridized with FGFR-3 antisense cRNA probe were subjected to autoradiography and photographed under dark field illumination. At E14 (panel a) in the CNS, FGFR-3 mRNAs are mainly distributed around the ventricular zones, including the lateral ventricle (LV), third (V3) and fourth (V4) ventricles and the central canal of the spinal cord (arrow). Outside the CNS, FGFR-3 mRNA was strongly expressed in developing vertebrae disks (arrowheads). At E16 (panel c) scattered cells around the lateral ventricle (LV) still expressed FGFR-3 strongly, whereas expression of FGFR-3 around the fourth ventricle (V4) had decreased. In the spinal cord, individual FGFR-3^ cells appeared outside the ventricular zone (arrowheads). These FGFR-3^ cells extended into the brain and appeared to disappear at the hindbrain/midbrain border (arrows). Panels b and d show same sections as panels a and c, respectively stained by haematoxylin and photographed under bright field illumination. Scale bars, 1mm.
Fig. 5.3 The FGFR-3$^*$ cells in developing rat spinal cord (transverse sections).

Each picture is a composite of a dark field image of an in situ autoradiograph hybridized with the FGFR-3 cRNA antisense probe (yellow) superimposed on a bright field image of the same section stained with hematoxylin (blue). At E14 (panel a), FGFR-3 is expressed around the ventricular zone of the spinal cord. Expression in the ventral half of the cord is much stronger than in that in the dorsal half. At E16 (panel b), two groups of FGFR-3$^*$ cells in the ventral half of the spinal cord appear to have migrated away from the ventricular zone. At E18 (panel c), the FGFR-3$^*$ cells have spread throughout the spinal cord, although they are predominantly in the ventral half. In the ventricular zone at this age, the FGFR-3$^*$ cells accumulated at two foci, possibly the sources of the migrating cells. All sections were cut at the thoracic level. Scale bar, 400\mu m.
Fig. 5.4 Expression of FGFR-3 in the developing cerebellum.

Panels a and c, sagittal sections of the developing cerebellum hybridized with FGFR-3 antisense cRNA probe were subjected to autoradiography and photographed under dark field illumination. Panel a shows that FGFR-3 mRNA appeared to be expressed at the pial surface of the P5 cerebellum. Panel c shows that FGFR-3 mRNA was mainly expressed by the cells in the Purkinje cell layer at P20. A high-power microscopic image of this section has shown that the FGFR-3 cells were not Purkinje neurons themselves but neighbouring cells, possibly Bergman glia (picture is not represented here). Panels b and d represent the same sections of Panels a and c, respectively, stained with haematoxylin and photographed under bright field illumination. Scale bar, 500μm.
Fig. 5.5 Expression of FGFR-1 and FGFR-2 in the developing rat spinal cord.

Tranverse sections of embryonic rat spinal cords were hybridized with radiolabelled FGFR-1 (panels a, c, e, g) or FGFR-2 (panels b, d, f, h) antisense cRNA probes. After autoradiography, pictures were taken under a dark field illumination. Panels a, b shows that FGFR-1 and FGFR-2 have begun to be expressed by cells around the E12 embryonic rat spinal cord. At E14, the expression of both the FGFRs increased and appeared to still be restricted to the ventricular zone (panels c, d). The expression did not change much at E16 (panels e, f). Shortly after E16, the expression of both FGFR-1 and FGFR-2 in the embryonic rat spinal cord decreased. By E18, only a few cells around the ventricular zone still express FGFRs (Panels g, h). Scale bar, 300μm.
Fig. 5.6 Co-localization of FGFR-3 signals with GFAP antibody staining on dissociated embryonic rat spinal cord cells.

E17 rat spinal cord was dissociated and cultured at either a high density (panels a, b) or a low density (panels c, d) in DMEM supplemented with 10% FCS for two days. Cells were consecutively hybridized with a radiolabelled cRNA probe against FGFR-3 by an ordinary in situ hybridization procedure (except that the proteinase K treatment was omitted), then stained with an anti-GFAP antibody before autoradiography. The antibody staining was visualized using DAB as substrate for HRP-conjugated second antibody. Pictures were taken under high power microscopy.

Panel a shows the co-localization of the FGFR-3 signal (silver grains) with GFAP-positive cells (brown). However, some of the GFAP-staining cells do not appear to express FGFR-3 (panel b, arrow). The low density cultures show that the strongest FGFR-3 signals were co-localized with cells that expressed low levels of GFAP (panels c, d, arrowheads), whereas some of the intense GFAP-staining cells do not express or express at a rather low level FGFR-3 (Panel d, arrow).
Fig. 5.7 Expression of FGFR-3 outside the CNS.

Pictures were taken from a sagittal section of an E18 rat embryo hybridized with a radiolabelled cRNA probe against FGFR-3 under high power microscopy. Panel a shows FGFR-3\(^+\) cells in the spinal cord (arrows). Panel b shows a hair follicle that expressed FGFR-3. Panel c shows that a group of cells in the inner ear which expressed FGFR-3 at a high level. Panel d is a high magnification image of the developing vertebral disk that expressed FGFR-3 at high levels.
Chapter Six

Discussion
1. The ventricular zone of the developing spinal cord is a mosaic of different precursor cells.

   The vertebrate CNS consists of a huge variety of neurons and glial cells. Neurons can be subdivided according to their biological functions and their location, the neurotransmitters they express and respond to. Glial cells also can be subdivided into two major classes, oligodendrocytes and astrocytes. Both neurons and glial cells are initially derived from undifferentiated neuroepithelial cells in the ventricular zone of the CNS. An unsolved question is: are there separate precursors for neurons and glia and, if so, how are they arranged in the ventricular zone of the CNS? The developing spinal cord is a suitable part of the CNS to study this question because of the simplicity of its structure and its topological equivalence to the brain. Thus, in this Thesis I have concentrated my attention on the developing rat spinal cord.

   By surgically removing dorsal parts of the developing chick spinal cord (including the ventricular zone) and observing the subsequent normal development of ventral cell types, Wenger (1950) provided evidence that different parts of the ventricular zone of the cord give rise to specific cell populations. For example, the ventral-most basal plate can give rise to the cells between the medial motor columns and the lateral basal plate can give rise to the lateral motor column, whereas the internuncial cells and the sensory horn cells are generated from the dorsal basal plate and alar plate, respectively. Similar conclusions have been drawn from $^3$H-thymidine labelling studies in mouse (Nornes and Carry 1978). Together, these results suggest that the neural epithelium might consist of a mosaic of specialized precursors that can give rise to different cell populations during subsequent development.
Further evidence is accumulating in support of this idea: 1) $^3$H-thymidine labelling of proliferating precursors in the ventricular zone around the third ventricle (topologically equivalent to the central canal of the spinal cord) has shown that the major divisions of the hypothalamus and thalamus are derived during embryogenesis from different parts of the ventricular neuroepithelium; for example, neurons of the lateral habenular nucleus are derived from a portion of the superior neuroepithelial lobule and the neurons of the reticular nucleus are derived from the middle neuroepithelial lobe (Altman and Bayer 1979). 2) More recently, several classes of genes, including the Hox genes (for review see McGinnis and Krumlauf 1992), the Emx genes (Simeone et al 1992a), the Otx genes (Simeone et al 1992b), Wnt-3 (Robinson et al 1991), BF-1 (Tao and Lai 1992) and Pax-6 (Walther and Gruss 1991) have shown restricted overlapping expression patterns in the spinal cord or more anterior regions of the neuraxis. However, since it is not possible to precisely manipulate individual precursors in the ventricular zone either by surgical operation or by injection of $^3$H-thymidine, and because none of the genetic markers mentioned above specifically labels a class of precursors whose subsequent developmental fate is known, it has not been possible to say where the precursors for particular classes of spinal cord cells are located in the ventricular zone.

In this Thesis I have presented evidence, in strong support of previous studies from this laboratory (Pringle and Richardson 1993), that oligodendrocyte precursors arise from a highly restricted position in the ventral half of the ventricular zone of the E14 rat spinal cord (Chapter 3). In addition, I showed that there is another microdomain of the ventricular zone, just above the floor plate of the E12 spinal cord, that contains a mysterious group of cells, presumably neural precursors, that express mRNA encoding PLP/DM-20. I have also identified two localized sources of apparently migratory FGFR-3$^+$ cells, probably the precursors of some subclasses of
astrocytes, in the ventricular zone of the rat spinal cord. One of these groups of FGFR-3^+ cells is located close to the floor plate and the other near the sulcal limitans (Chapter 5). Together with previous results (Pringle and Richardson 1993), my results provide strong evidence that the ventricular zone of the developing rat spinal cord between E12-E15 is a mosaic of different precursors that express different molecular markers and are presumably pre-specified to generate distinct populations of differentiated cells. Strikingly, each microdomain that I have identified in the ventricular zone is limited in extent to just one or two cells along the dorsoventral axis.

2. Putative signalling molecules involved in neural tube patterning.

The floor plate is induced by the notochord during early stages of the neural development. An extra notochord grafted into an ectopic position relative to the neural tube induces the morphological, antigenic and functional properties of the floor plate in adjacent neural cells (van Straaten et al 1988, Smith and Schoenwolf 1989, Yamada et al 1991, Placzek et al 1990). After floor plate induction, the notochord and the floor plate together play an important role in subsequent spinal cord patterning. Two major aspects of neural development have been shown to be affected by the notochord/floor plate complex: 1) differentiation of spinal motor neurons, and 2) guidance of spinal commissural neuron axons. By examining the Danforth’s short tail mutant mouse, I have shown in this Thesis that the specification of oligodendrocyte precursors from undifferentiated neuroepithelial cells in the ventricular zone also requires the presence of the notochord/floor plate complex. This result shows that the influence of the notochord/floor plate extends to glial cells as well as neurons. Furthermore, my results show that for the oligodendrocyte lineage, and by implication other neural lineages like the motor neuron lineage, signals from the notochord/floor
plate act primarily on neuroepithelial precursor cells at the ventricular surface rather than the migrating progenitor cells near their final sites of differentiation. This raises the question—what is the identity of the crucial floor plate-derived signalling molecule(s)?

A strong candidate for the floor plate-inducing signal from notochord has been recently identified as *Sonic hedgehog* (Shh) (Roelink et al 1994, Echelard et al 1993, Krauss et al 1993). Subsequently, the floor plate itself becomes secondary source of Shh and, together with the notochord, induces motor neuron differentiation (Roelink et al 1994, Tanabe et al 1995). The Shh gene encodes a secreted polypeptide that contains an N-terminal signal sequence for secretion (Lee et al 1994). Following removal of the signal sequence, the Shh molecules are self-cleaved into two fragments (Lee et al 1994). Accumulating evidence has indicated that the N-terminal portion of Shh is sufficient for signalling (Fietz et al 1995, Porter et al 1995, Fan et al 1995, Roelink et al 1995). The C-terminal fragment contains the proteolytic activity necessary for self-processing of the Shh precursor protein (Lee et al 1994) and, additionally, it can restrict diffusion of Shh and hence increase its local concentration (Roelink et al 1995).

Shh is important, not only for floor plate and motor neuron induction, but more generally for setting up the dorso-ventral pattern of the developing neural tube. For example, in the developing forebrain where there is neither floor plate nor motor neurons, Shh is necessary for differentiation of some ventral forebrain neurons (Ericson et al 1995). There is also evidence that Shh plays a role in the determination of cell fate during somite differentiation (Johnson et al 1994, Fan and Tessier-Lavigne 1994). These results strongly suggest that Shh might also be responsible for specifying oligodendrocyte precursors in the developing spinal cord.
It has been shown that floor plate induction by the notochord requires contact between the notochord and responding tissue, while motor neuron induction by the notochord or floor plate does not (Roelink et al 1994). Furthermore, it has been shown that, although bacterially produced soluble Shh N-terminus is effective in inducing both floor plate and motor neurons, the concentration required to induce floor plate is five times higher than that required to induce motor neurons (Roelink et al 1995). Therefore, the apparent requirement for cell-cell contact for floor plate induction might simply reflect a requirement for a higher local concentration of Shh. These results suggest that the local concentration of Shh may be a crucial factor that affects cell fate determination. The site of origin of oligodendrocyte precursors in the embryonic spinal cord is not directly in contact with the source of Shh (i.e. the floor plate and notochord), so it seems likely that a lower concentration of Shh is required for oligodendrocyte specification than for floor plate induction. Given that the width of the oligodendrocyte precursor microdomain is so narrow (one or two cells), it is hard to believe that the position of this microdomain is specified directly by threshold concentrations of Shh in a long-range gradient of Shh. Instead, it seems more likely that a primary gradient of Shh from the floor plate initiates a cascade of short-range cell-cell interactions along the dorsoventral axis, much as the primary long-range gradient of Bicoid in the Drosophila blastoderm activates a hierarchy of secondary interactions that refine positional information in the anterior-posterior axis down to the single-cell level. Alternatively, it is conceivable that Shh might act, not as a long-range gradient, but as the initial trigger for a series of sequential cell-cell interactions in which each cell influences the fate of its next neighbour in the dorsal direction, rather like a "domino" effect. However, there is evidence from notochord-shift experiments in the chick (Pringle, Richardson and Guthrie unpublished data) that strongly favours a long-range gradient model. Certainly, this is an area that should be the subject of further investigation.
In addition to Shh, two other families of secreted molecules—the netrin family and the semaphorin family—have been identified as important floor plate-derived neural patterning molecules. Netrin-1 is a chemoattractant for spinal commissural axons (Serafini et al 1994, Kennedy et al 1994), whereas semaphorin-I and III are chemorepellants for axons of certain subtypes of sensory neurons, and semaphorin-II inhibits synapse formation of a subset of motor axons (Colamarino and Tessier-Lavigne 1995, Matthes et al 1995, Puschel et al 1995, Messersmith et al 1995, Shirasaki et al 1995). It is not known whether these molecules are also involved in cell fate determination in the developing neural tube, but the striking spatial and temporal correspondence of the expression patterns of semaphorin-III and PDGFRα in the ventricular zone of the spinal cord (Messersmith et al 1995) suggests that semaphorin-III might play a role in specifying oligodendrocyte precursors. Again further investigation is needed to address this question.

I also examined the expression of FGFR-3 in the developing Sd mutant mouse spinal cord (Chapter 4). These experiments showed that the lack of the floor plate did not completely abolish FGFR-3 expression in the ventricular zone. It seems that the lack of the floor plate only affects that focus of FGFR-3+ cells that lie close to the floor plate, but not the FGFR-3+ cells halfway along the dorso-ventral axis (data not shown). This result indicates that signals from the notochord/floor plate complex might affect only the ventral half of the spinal cord. The dorsal counterpart of the floor plate—the roof plate—might be more important for cell fate determination in the dorsal parts of the spinal cord.
Appendix I. Which subtype(s) of fibroblast growth factor receptors are expressed by O-2A progenitor cells?

O-2A progenitor cells were first identified in perinatal rat optic nerve cultures (Raff et al 1983) and subsequently in several other parts of the CNS including cerebellum, cerebral cortex, and forebrain (Levi et al 1987, Levine and Stallcup 1987, Behar et al 1988, Gard and Pfeiffer 1989, Hardy and Reynolds 1991). Proliferating O-2A progenitor cells first appear in the rat optic nerve around E16 (Small et al 1987), and start to differentiate into oligodendrocytes on the day of birth (Miller et al 1985). In vitro, dissociated O-2A progenitor cells from embryonic rat optic nerve stop dividing and differentiate into oligodendrocytes prematurely when cultured in chemically defined medium (Raff et al 1983), suggesting that this defined medium might lack a mitogen that is present in vivo and that keeps O-2A progenitor cells proliferating and inhibits their differentiation. Pure PDGF is able to fulfil this role in vitro (Noble et al 1988, Raff et al 1988, Richardson et al 1988), and PDGF-A has been shown to be important for oligodendrocyte development in vivo (unpublished results from this laboratory). Members of the fibroblast growth factor (FGF) family are also mitogenic for O-2A progenitors (Eccleston and Silberberg 1985, Saneto and DeVellis 1985, McKinnon et al 1990) in vitro. Strikingly, the combination of bFGF and PDGF can stimulate O-2A progenitor cells to proliferate indefinitely and prevents them from differentiating into oligodendrocytes in vitro (Bögler et al 1990).

Presumably, O-2A progenitor cells respond to PDGF and FGF because they express transmembrane receptors for these growth factors. There are two known isoforms of PDGF receptors, the α- and β-receptors (PDGFRα) and (PDGFRβ). It has
been shown that O-2A progenitor cells possess PDGFRα but not PDGFRβ (Hart et al 1989, McKinnon et al 1990); indeed, PDGFRα has been used as a specific marker for O-2A progenitors in situ and in vitro (Pringle et al 1992; Pringle and Richardson 1993; Hall and Richardson, unpublished data; Stallcup, personal communication). However, it is still not known which FGF receptors are expressed by O-2A progenitors. Four members of the FGF receptor family (FGFR-1 to FGFR-4) have been isolated so far. In order to determine which FGFR(s) is expressed by O-2A progenitor cells, probes for each of these were isolated from rat brain by PCR amplification of cDNA, degenerate primers (see Chapter 2).

Because of its simple cell composition and the fact that it is well-characterized, developing optic nerve was chosen to look for evidence of FGFR expression by O-2A progenitor cells in vivo, by in situ hybridization. FGFR-1 and FGFR-2 expression was diffuse and widespread within the developing optic nerve, and this did not change between P0 to P20 except that the expression of FGFR-1 seemed to be decreased from P5 (Fig AI-1). The expression of FGFR-2 in postnatal optic nerves seems consistently higher than that of FGFR-1, but neither of them shows the typical distribution of O-2A progenitors as determined by PDGFRα expression (Mudhar et al 1993). This result raises three possibilities: 1) FGFR-1 and FGFR-2 might be expressed in the optic nerve by cells other than O-2A progenitors. 2) The subcellular distribution of FGFR-1 and FGFR-2 mRNAs, unlike that of PDGFRα, might not be restricted to cell bodies but might also be present in cell processes so that the in situ hybridization signal is more diffuse. 3) FGFR-1 and FGFR-2 might be expressed at low levels by O-2A progenitors and at higher levels in other cell types whose expression masks that in O-2A progenitors. No significant FGFR-4 signal was detected in optic nerve sections by in situ hybridization. FGFR-3 has not yet been examined by in situ hybridization in the developing optic nerve. However, its expression pattern in the
developing spinal cord suggests that it is not expressed strongly by O-2A progenitors, although it is expressed strongly by other glia, probably astrocytes (Chapter 5).

As an alternative approach to determining which FGFRs might be expressed by O-2A progenitors, cDNA generated from CG4 cells, a permanent cell line that appears to be derived from and quite faithfully mimic O-2A progenitors (Louis et al 1992). The reason for using CG4 cells rather than primary O-2A progenitors was to avoid false positives due to possible FGFR expression in contaminating cells in primary cultures. In this experiment, 1 μg CG4 total RNA was reverse-transcribed to cDNA and subsequently amplified with sets of FGFR primers. The results indicated that both FGFR-1 and FGFR-2 are expressed by CG4 cells (Fig AI-2). FGFR-4 was not detected in the PCR reaction whereas, surprisingly, FGFR-3 was detected. In Chapter 5 I showed that, in the developing spinal cord, the major populations of FGFR-3-expressing cells probably correspond to astrocyte precursors. These apparently contradictory results could be explained in several ways. For example, O-2A progenitor cells might express low levels of FGFR-3 which are beyond the detection sensitivity of my in situ hybridization procedure or FGFR-3 expression might have changed during the creation of the CG4 cell line.

Taken together, it seems that O-2A progenitor cells might express more than one isoform of FGFR, including FGFR-1, FGFR-2 and FGFR-3, but not FGFR-4. If so, the action of FGFs on O-2A progenitor cells might be complicated. In any event, the fact that oligodendrocyte differentiation does occur in vivo would suggest that 1) either FGFs are not available to O-2A progenitors during normal postnatal development or 2) expression of FGFRs on O-2A progenitors is induced by the process of dissociating the cells and placing them in culture.
**Fig. AI-1 Expressions of FGFR-1 and FGFR-2 in the rat developing optic nerve.**

Optic nerve sections at different ages, E18 (Panels A, B), P0 (Panels C, D), P2 (Panels E, F), P5 (Panels G, H) and P20 (Panels I, J) were hybridized with either FGFR-1 probe (FLG) (Panels A, C, E, G, I) or FGFR-2 probe (BEK) (Panels B, D, F, H, J) and photographed under dark-field illumination. Optic nerves are indicated by arrows. *Scale bar, 200μm*
Fig. AI-2  PCR amplification of FGFRs in CG4 cells.

cDNAs reverse-transcribed from 1µg of CG4 cell total RNA were amplified by PCR with different sets of primers. The products were checked on an agarose gel. The following pairs of primers were used for the amplification, and the expected size of the PCR products are indicated in the figure.

**FGFR-1:**
- \( \text{P} ^ \text{up}_1 \text{FR-B} \) (5'-attgagctctcagCA(A/G)CCNCA(T/C)AT(T/C/A)CA(A/G)TGG 3')
- \( \text{P} ^ \text{down}_1 \text{FR-C} \) (5'-tgtgcggccgctctagaCAT(T/C)TTCATCAT(T/C)TCCAT(T/C)TC 3')

**FGFR-2:**
- \( \text{P} ^ \text{up}_2 \text{BEK} \) (5'-tctctgcagGGN(A/C)GNGA(A/G)AA(A/G)GA(A/G)AT(T/C/A)ACNGC 3')
- \( \text{P} ^ \text{down}_2 \text{BEK} \) (5'-taattctgcagCACGGAACCCGCTCCTTGG 3')

**FGFR-3:**
- \( \text{P} ^ \text{up}_3 \text{FR-B} \) (5'-attgagctctcagCA(A/G)CCNCA(T/C)AT(T/C/A)CA(A/G)TGG 3')
- \( \text{P} ^ \text{down}_3 \text{FR-LINKER} \) (5'-CAGGCATCGAAGGAGTAATCC 3')

**FGFR-4:**
- \( \text{P} ^ \text{up}_4 \text{FR-B} \) (5'-AGCATTCGCTACAGCTATCT 3')
- \( \text{P} ^ \text{down}_4 \text{FR-C} \) (5'-GCTGGTTTTGTCCTGGGCGAGG 3')
Appendix II  Partial cDNA cloning of zebrafish PDGFRα.

Recently, the zebrafish \textit{(Brachydanio rerio)} has become an organism of choice for developmental analysis (Kemmel 1989). The reasons for this include their relatively simple body plan, fundamental similarity with other vertebrates and the availability of genetic mutants. In particular, two mutants have been isolated that are particularly interesting for the study of CNS patterning; "cyclops" (\textit{cyc}-1) (Hatta et al 1991) and "no tail" (\textit{ntl}) (Halpern et al 1993).

In \textit{cyc}-1 mutant embryos, the floor plate fails to develop in the caudal hindbrain and along the remaining neuraxis. Moreover, it has been shown that an ectopic graft of wild-type ectoderm into the mutant embryos can differentiate into floor plate, whereas an ectopic graft of wild-type notochord can not induce floor plate in mutant embryos, suggesting that the failure of floor plate development is caused by a cell-autonomous mutation in the ectoderm rather than a mutation of a signalling molecule in the mesoderm. Presumably, \textit{cyc}-1 carries a mutation in a receptor for a notochord-derived signalling molecule.

The \textit{ntl} mutation results in the loss of the notochord during development. However, \textit{ntl} mutant embryos have a floor plate in the hindbrain and along almost the entire length of the spinal cord except in the most posterior region. It appears that the mutant CNS develops normally where the floor plate is present. Therefore, it appears that the failure of notochord development in mutant embryos might be caused by early degeneration of notochord precursors (Halpern et al 1993). \textit{ntl} has been shown to be isogenic with the Brachyury mouse mutant (Green 1994).
As described in Chapter 4, the notochord and the floor plate have important roles in the developmental patterning of the CNS. I examined the role of the notochord/floor plate in oligodendrocyte development by taking advantage of the Sd mouse mutant. A disadvantage of this mouse mutant, however, is that the floor plate is missing in only a very restricted region of the spinal cord at a particular period of development (Chapter 4). In order to overcome this disadvantage of the mouse Sd mutant and to check if there is continuity between different species in the role of the notochord/floor plate in specifying oligodendrocyte precursors, an attempt was made to take advantage of the zebrafish mutants. As a prelude to this, I isolated a zebrafish PDGFRα cDNA as a probe for in situ hybridization experiments.

A 0.6kb fragment of zebrafish PDGFRα was first amplified with two primers, P\textsuperscript{up}AR-ZFA and P\textsubscript{down}AR-ZFC, that flank a portion of the tyrosine kinase domain. This fragment was then used as a probe to screen a post somitogenesis stage (20-28 hours) zebrafish cDNA library (constructed by R. Kiggleman and K. Helde at University of Utah). Eight positive clones were obtained, five of which were independent. The longest one, about 3 kb in length, was selected for subsequent sequence analysis. Sequencing data showed that this clone was a partial cDNA of zebrafish PDGFRα and included almost the entire intracellular tyrosine kinase domain and the whole 3’ untranslated region (for details see Attachment-1). This sequence has been submitted to EMBL-GenBank and the access number is Z32814.

While comparing this partial amino acid sequence of zebrafish PDGFRα with its counterparts from other species, I found that the sequence similarity reflected their phylogenetic relationship (Attachment-2). I also isolated a 0.4 kb fragment of chicken PDGFRα extracellular domain by PCR amplification with primers P\textsuperscript{up}AR-CHI and P\textsubscript{down}AR-CHI. The comparison of amino acid sequences with the corresponding
regions of PDGFRα in other species has also shown correspondance with their phylogenetic relationships (Attachment-3).

A Northern hybridization with my partial zebrafish PDGFRα cDNA fragment showed that the full-length cDNA is about 6kb (data not shown). My probe could be used in future to examine PDGFRα expression in developing wild type and mutant (cyc-l and ntl) zebrafish to provide more information on the origin of the signals that control development of the oligodendrocyte lineage in the CNS.
Attachment-1. The partial cDNA sequence of zebrafish PDGFRα.

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**DEFINITION** B.rerio mRNA for platelet-derived growth factor receptor alpha.  
**ACCESSION** Z32814  
**KEYWORDS** platelet-derived growth factor receptor alpha; tyrosine kinase.  
**SOURCE** zebrafish.  
**ORGANISM** Brachydanio rerio  
Eukaryota; Animalia; Metazoa; Chordata;  
Vertebrata; Osteichthyes; Actinopterygii;  
Cypriniformes; Cyprinoidei; Cyprinidae.  
**REFERENCE** 1 (bases 1 to 3075)  
**AUTHORS** Yu,W. and Richardson,W.D.  
**TITLE** Zebrafish Platelet-derived Growth Factor Receptor (Alpha subunit), partial cDNA sequence  
**JOURNAL** Unpublished  
**STANDARD** full automatic  
**REFERENCE** 2 (bases 1 to 3075)  
**AUTHORS** Yu,W.  
**JOURNAL** Submitted (29-APR-1994) to the EMBL/GenBank/DDBJ databases.  
Wei-Ping Yu, MRC Lab. for Molecular Cell Biology, University College London, Gower Street, London, WC1E 6BT, United Kingdom  
**STANDARD** full automatic  
**COMMENT** NCBI gi: 479105
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Attachment-2. Comparison of partial amino acid sequences of PDGFRα among different species--human, mouse, rat, Xenopus and zebrafish.

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153
A phylogenetic tree generated from the comparison of PDGF alpha receptor sequences of mouse, rat, human, Xenopus and zebrafish.
Attachment-3. The comparison of partial amino acid sequences of PDGFRα among different species—chicken, human, mouse, rat and Xenopus.

A phylogenetic tree generated from the comparison of PDGF alpha receptor sequences of mouse, rat, human, chicken and Xenopus.
Appendix III. Does FGFR-3 play a role in focal adhesion of cells to the substratum?

As argued in Chapter 5, FGFR-3 might be expressed by astrocyte precursors /newly-differentiated astrocytes in the embryonic rat spinal cord. In an attempt to visualize FGFR-3^ cells in dissociated embryonic rat spinal cord cultures by immunohistochemistry with a polyclonal antibody raised against FGFR-3, I found that this antibody did recognize some astrocytes that were distinguished by GFAP expression. Surprisingly, the FGFR-3 signal, when examined under high magnification, seemed to be restricted to focal adhesion sites (Fig AIII-1). The antibody used for this experiment was purchased from Santa Cruz Biotechnology Inc. (cat# sc-123) and was raised against a carboxy-terminal peptide of human FGFR-3 (aa. 792-806). The amino acid sequence of this peptide is DLLPPAPPSSGGSRT.

The question is, whether the positive signal is due to FGFR-3 itself or a cross-reaction with some other molecule. According to the manufacturer’s specifications, this antibody specifically recognizes FGFR-3 by both immunoprecipitation and Western blotting, and has no detectable cross-reactivity with FGFR-1, FGFR-2 and FGFR-4. Therefore, it seems unlikely that the positive signal is due to a cross-reaction with the other members of FGFR family. In order to verify the specificity of this antibody, I did the following experiments.

1) I tested whether the antibody recognizes the peptide epitope against which it was raised. In this experiment, the diluted antibody was preincubated with a 100-fold molar excess of peptide (from Santa Cruz Biotechnology Inc, cat# sc-123P)
at 4°C overnight before being used for staining. The preincubation completely blocked the focal adhesion staining (data not shown), suggesting that the antibody recognises the peptide epitope against which it was generated and the epitope that the antibody recognizes in focal adhesion sites has the same or a similar structure as the immunogen.

2) In order to determine whether the antibody can recognise FGFR-3 itself, I expressed a human FGFR-3 cDNA in Cos cells with a modified Cos cell expression vector, pHYK (Pollock and Richardson 1992). The FGFR-3-expressing Cos cells were strongly stained with the anti-FGFR-3 antibody, demonstrating that this antibody does recognise FGFR-3 immunohistochemically (Fig AIII-2).

3) Because the peptide epitope is located in the cytoplasmic domain of FGFR-3, the normal staining procedure involved a permeabilization step with 0.5% Triton X-100. When this step was omitted, cells could not be stained, showing that the positive signal is localized inside cells rather than on the cell membrane (data not shown). This result is consistent with the possibility that the positive signal originates from FGFR-3 itself rather than cross-reactive molecules on the cell surface. However, it does not eliminate the possibility that the positive signal was generated from cross-reactive molecules inside the cells.

4) Several other types of cells were examined by anti-FGFR-3 antibody staining including Swiss 3T3 cells, meningeal cells, untransfected Cos cells, B104 cells, and astrocytes from rat cerebral cortex. All of these were stained by this antibody (Fig AIII-3). Interestingly, in addition to the focal adhesion-like distribution, a filament-like distribution of immunoreactivity was observed in some cell types, such as untransfected Cos cells (Fig AIII-3 c). In order to determine whether these
immunocytochemically-stained cells express FGFR-3 mRNA, the rat-derived cells were examined by Northern hybridization with a rat FGFR-3 cDNA probe. Results showed that most of the immunocytochemically-stained cells also had positive signals at the RNA level except for B104 cells, which were immunocytochemically positive but negative by Northern hybridization (Fig AIII-4). The apparently contradictory result from B104 cells needs to be verified since it was a very preliminary experiment. A high-molecular-weight band did appear on the Northern blot of B104 cell RNA. The identity of this band is unknown, although it might correspond to an alternative-spliced isoform of FGFR-3.

Recently, a few lines of evidence have suggested that FGFRs could be involved in signalling via cell adhesion molecules. First, FGFRs possess a three amino acid motif, HAV (His-Ala-Val), that has been implicated in mediating homophilic interactions between some cadherins (Byers et al 1992). Further examination revealed that a 20 amino acid sequence around the HAV motif of FGFRs has significant homology with three different adhesion molecules--neural cell adhesion molecule (NCAM), N-cadherin and neural adhesion molecule L1 (Williams et al 1994, Mason 1994). It has been shown that the stimulation of neurite outgrowth by these three cell adhesion molecules might involve FGFR activation (Williams et al 1994). However, FGFR-3 is distinct from the other FGFRs in that it is the only FGFR not to possess an HAV motif.

In conclusion, it is still unclear whether the focal-adhesion-like immunolabelling is due to FGFR-3 itself. More experiments including, for example, Western blot analysis of cell extracts is required to take this line of investigation further.
Fig. AIII-1 Anti-FGFR-3 antibody staining of dissociated rat spinal cord astrocytes.

Cells dissociated from E17 rat spinal cord were cultured in DMEM containing 10% FCS for 3-4 days and double-stained with anti-FGFR-3 (polyclonal) and anti-GFAP (monoclonal from Sigma) antibodies. The binding of anti-FGFR-3 antibody on cells was visualized with an FITC-conjugated second antibody against rabbit IgG (green, upper panel), and the binding of anti-GFAP antibody on cells was visualized with a Texas-Red-conjugated second antibody against mouse IgG (red, lower panel). Focal adhesion-like signals of the FGFR-3 are indicated by arrows (top panel). The nuclei of all cells (also see Fig AIII-2 and 3) were stained strongly, presumably because of non-specific binding.
The full length clone of human FGFR-3 cDNA was modified by removing a 0.5kb fragment at the 3'-end. The resulting fragment, containing the entire 5'-end untranslated region, the entire coding region, and a fragment of 3'-end untranslated region, was then subcloned into a modified Cos cell expression vector, pHYK. Cells transfected with this plasmid were strongly stained by anti-FGFR-3 antibody (upper panel, transfected cells indicated by arrows). However, when examined under high power microscopy, the human FGFR-3 molecules expressed by the plasmid did not show a focal adhesion distribution pattern (lower panel, the transfected cell indicated by arrow), whereas the untransfected cell did (arrowhead). It is possible that the high level of expression of human FGFR-3 has hidden the signals at the focal adhesion sites.
Fig. AIII-3 Anti-FGFR-3 antibody stains different cell types.

Swiss 3T3 cells (panel a), meningeal cells (panel b), untransfected Cos cells (panel c), B104 cells (panel d) and cerebral cortex astrocytes (panel e) were stained with anti-FGFR-3 antibody. All cell types show a focal adhesion-like staining pattern. Additionally, in the untransfected Cos cells, this antibody stained some cell filament structure (panel c, arrow). Panel f is the same field with panel e but stained with an anti-GFAP antibody.
Fig. AIII-4 FGFR-3 expression of different cell types examined by the Northern hybridization.

Total RNA extracted from different populations of cells were hybridized with a rat FGFR-3 probe. 10μg of total RNA (except 2μg for the transfected Cos cells) were loaded on each lane. Transfer of RNA was checked by methylene blue staining and confirmed that the lanes contained equal amounts of RNA. The final washing condition was 0.5xSSC containing 0.5% SDS at 65°C for 1 hour. The RNA expressed by the transfected Cos cells is smaller in size because a 0.5kb fragment of the 3'-end untranslated region of FGFR-3 has been removed (see Fig. AIII-2). The relatively weaker signals of untransfected Cos cells and Swiss 3T3 cells might be due to the use of a heterologous (rat) probe. In addition to the normal FGFR-3 bands (4.3kb), a band with a higher molecular weight (about 6.2kb) was also seen.
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