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Development and regeneration in the central nervous system

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As part of our attempts to understand principles that underly organism development, we have been studying the development of the rat optic nerve. This simple tissue is composed of three glial cell types derived from two distinct cellular lineages. Type-1 astrocytes appear to be derived from a monopotential neuroepithelial precursor, whereas type-2 astrocytes and oligodendrocytes are derived from a common oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell. Type-1 astrocytes modulate division and differentiation of O-2A progenitor cells through secretion of platelet-derived growth factor, and can themselves be stimulated to divide by peptide mitogens and through stimulation of neurotransmitter receptors.

In vitro analysis indicates that many dividing O-2A progenitors derived from optic nerves of perinatal rats differentiate symmetrically and clonally to give rise to oligodendrocytes, or can be induced to differentiate into type-2 astrocytes. O-2A^{perinatal} progenitors can also differentiate to form a further O-2A lineage cell, the O-2A^{adult} progenitor, which has properties specialized for the physiological requirements of the adult nervous system. In particular, O-2A^{adult} progenitors have many of the features of stem cells, in that they divide slowly and asymmetrically and appear to have the capacity for extended self-renewal.

The apparent derivation of a slowly and asymmetrically dividing cell, with properties appropriate for homeostatic maintenance of existing populations in the mature animal, from a rapidly dividing cell with properties suitable for the rapid population and myelination of central nervous system (CNS) axon tracts during early development, offers novel and unexpected insights into the possible origin of self-renewing stem cells and also into the role that generation of stem cells may play in helping to terminate the explosive growth of embryogenesis. Moreover, the properties of O-2A^{adult} progenitor cells are consistent with, and may explain, the failure of successful myelin repair in conditions such as multiple sclerosis, and thus seem to provide a cellular biological basis for understanding one of the key features of an important human disease.

Introduction

Understanding the development of a complex multicellular organism from a single fertilized egg entails understanding the three processes of cell division, cellular diversification along alternative pathways of differentiation and morphogenesis. Although all of these processes have been of scientific interest for many years, there are few tissues for which significant insights into the control of morphogenesis have been achieved, and still fewer tissues where much is known about the specific cellular or molecular interactions that modulate either cell division or cell diversification.

Our own attempts to understand the principles which underly organism development have been focused on the optic nerve, the simplest part of the CNS. We have chosen the optic nerve

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for several reasons. This tissue is composed of a few cell types that can all be grown, and unambiguously distinguished from each other, in tissue culture conditions. Moreover, some of these cell types can be grown in highly enriched or pure form, so that cell–cell interactions and the effects of defined mitogens or differentiation-modulating agents on particular cells can be studied. In addition, the *in vivo* development of the optic nerve is well-described anatomically, and this tissue is accessible to experimental manipulation. Thus data gathered *in vivo* can be analysed in the context of normal development, and hypotheses generated through this analysis can eventually also be tested *in vivo*. Finally, although axons pass through the optic nerve, this tissue contains no intrinsic neurons. Thus, by studying the optic nerve it is possible to focus attention on glial cells, the non-neuronal residents of the CNS that outnumber neurones by as much as 20:1 throughout most of this tissue. Large portions of these studies have been extensively reviewed elsewhere (Anderson 1989; Raff 1989), and therefore, we provide only a brief summary of recently reviewed material, so as to concentrate on current investigations.

Definitions

For discussion of our studies, an acquaintance with the following terms is necessary: symmetric division and differentiation occurs when the cells arising from a division are identical in their properties. Asymmetric division and differentiation occurs when the cells arising from a division are different from each other. Progenitors are cells that are committed to differentiate within a limited period of time, but which are able to progress along two or more distinct pathways of differentiation. The specific timing of differentiation of progenitor cells can be controlled by biological clocks, which work through as yet unknown mechanisms to induce differentiation within a defined period of time, or by the presence of specific inducing factors in the cells environment. Stem cells (or pre-progenitor cells), unlike progenitors, are capable of both extended self-renewal and of generating differentiated progeny. The capacity for asymmetric division and differentiation is a general property of stem cells. Precursor cell is a term used in two ways, to describe cells committed to a single pathway of differentiation and as a generic term indicating all types of predecessor cells, including stem cells and progenitor cells; unfortunately, both usages are necessary in this paper, but the content in which either definition is used shows clearly which usage is intended.

The cells of the optic nerve

There are three different glial cell types that can be identified in the optic nerve, each of which is functionally distinct. Type-1 astrocytes, and their precursors, seem to contribute to the morphogenetic development of the optic nerve by offering a preferred substrate for growing axons (Noble *et al.* 1984). These cells also appear to interact with endothelial cells to induce formation of the blood–brain barrier (Janzer & Raff 1985), and (as discussed below), are a source of mitogen for other cells of the nerve. Oligodendrocytes enwrap large axons of the CNS with myelin sheaths, whereas type-2 astrocytes extend processes that encircle axons at the nodes of Ranvier (ffrench-Constant & Raff 1986*b*), the regions between consecutive myelin sheaths where ion fluxes occur during transmission of impulses along the myelinated axon. Thus oligodendrocytes and type-2 astrocytes appear to be specialized to create the anatomical specializations that characterize the myelinated tracts of the CNS.

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The three glial cell types of the optic nerve are generated at specific developmental periods from two distinct cellular lineages. The neuroepithelial cells that form the optic stalk and the embryonic anlage of the optic nerve, seem to give rise only to type-1 astrocytes (Small *et al.* 1987). The initial differentiation of type-1 astrocytes, at day 16 of embryogenesis (E16), is followed developmentally by the appearance within the nerve of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells, which can be induced *in vitro* to differentiate into either oligodendrocytes or type-2 astrocytes (Raff *et al.* 1983*b*). The O-2A progenitors arise in a germinal zone located in or near the optic chiasm, and first migrate into the optic nerve at E17 (Small *et al.* 1987). Several days later, at the time of birth of the rat (E21 or P0), oligodendrocytes are first seen in the nerve (Miller *et al.* 1985). Type-2 astrocytes are the third glial cell to appear, and do not begin to develop until around eight days after birth (P8; Miller *et al.* 1985).

We have recently found that the progenitors for oligodendrocytes and type-2 astrocytes are different in developing and adult rats. Initially identified in the optic nerves of adult (not younger than eight months) rats, the O-$2A^{adult}$ progenitors differ from their perinatal counterparts in antigen expression, morphology, cell-cycle length, motility, timecourse of differentiation, the manner in which these cells generate oligodendrocytes, and in other properties discussed in detail in later sections of this paper (Wolswijk & Noble 1989; Wren *et al.* 1990; see figure 1 herein, for summary). As discussed later, the O-$2A^{adult}$ progenitors appear to be derived from a subset of O-$2A^{perinatal}$ progenitors (Wolswijk & Noble 1989; Wren *et al.* 1990). Thus some O-$2A^{perinatal}$ progenitors appear to be tripotential, rather than bipotential, cells.

All of the diverse cell types of the optic nerve can be readily distinguished from each other *in vitro* on the basis of antigen expression and morphology. The identifying characteristics of the cells of the O-2A lineage are summarized in figure 1. As also shown in figure 1, O-2A progenitors grown in the presence of appropriate inducing factors (for example, foetal calf serum or ciliary neurotrophic factor) differentiate into type-2 astrocytes, whereas growth in the absence of inducing agents is associated with differentiation of O-2A progenitors into oligodendrocytes (Raff *et al.* 1983*b*; Hughes *et al.* 1988; Lillien *et al.* 1988). We will not discuss differentiation of type-2 astrocytes in this paper, and the reader is referred to reviews by Anderson (1989) and Raff (1989) for further information about this differentiation pathway.

The role of type-1 astrocytes in modulating division and oligodendrocytic differentiation of O-$2A^{perinatal}$ progenitors

When we originally identified the O-$2A^{perinatal}$ progenitor cell we were puzzled by the observation that these cells were not dividing in tissue culture conditions (Raff *et al.* 1983*b*), even though they were being removed from the rat optic nerve at times previously identified as peak periods for division of glial precursors (Skoff *et al.* 1976*a*, *b*). The failure of O-$2A^{perinatal}$ progenitor cells to divide *in vitro* suggested that a necessary component of the *in vivo* environment was missing in the tissue culture dish. The two most likely cellular candidates for this missing component were type-1 astrocytes and axons. Subsequent research showed that purified type-1 astrocytes secrete a soluble mitogenic activity capable of causing extensive division of O-$2A^{perinatal}$ progenitor cells (Noble & Murray 1984). In contrast, removal of axons from the nerve by removal of the eye of newborn rats had no effect on the division, or

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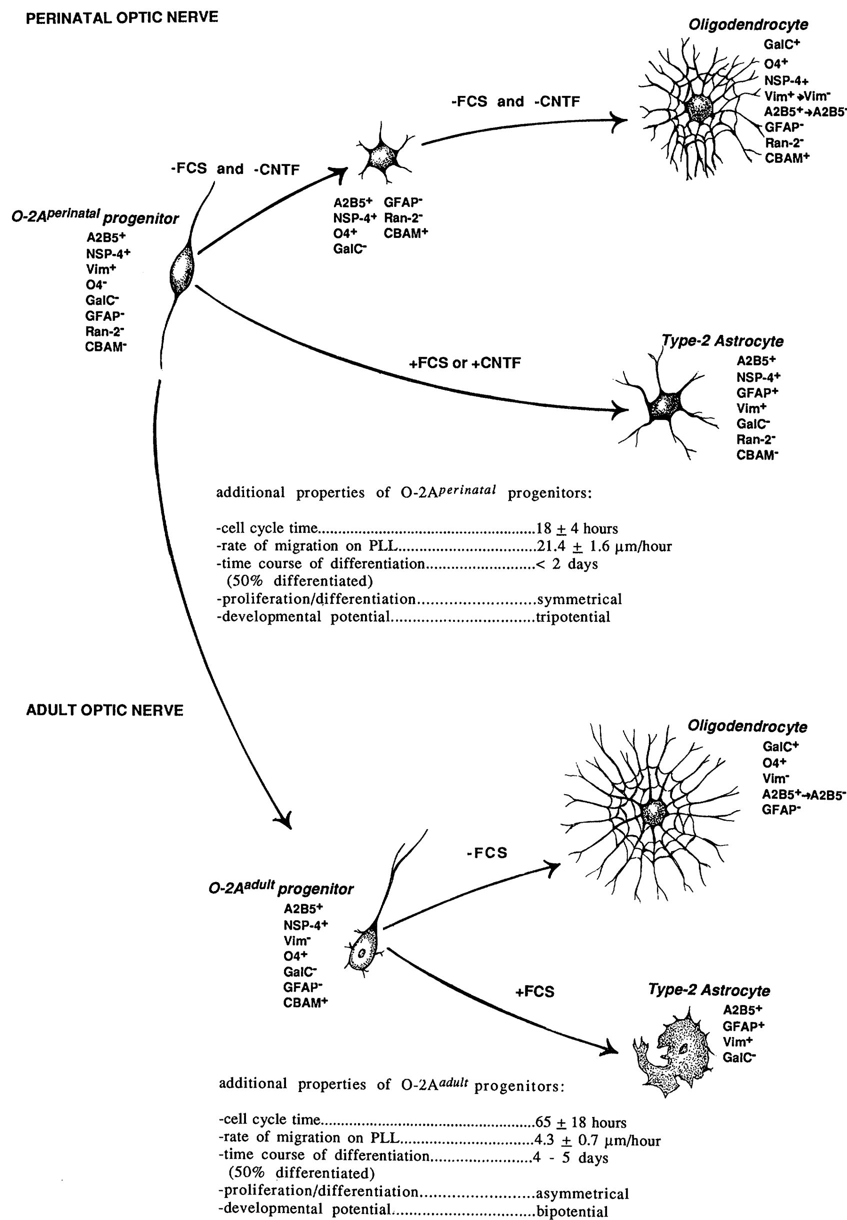

FIGURE 1. This figure summarizes the defining characteristics of O-2A$^{\text{perinatal}}$ and O-2A$^{\text{adult}}$ progenitor cells and their derivatives. O-2A$^{\text{perinatal}}$ progenitors are bipolar cells which divide with a cell cycle of about 18 h and migrate with an average speed of 21 μm h^{-1} when grown in the presence of type-1 astroyctes or platelet-derived growth factor (Small *et al.* 1987; Noble *et al.* 1988). These cells can be distinguished antigenically by labelling with monoclonal antibodies A2B5 and NSP-4, their expression of vimentin intermediate filaments (IFS) and their lack of labelling with the O4 monoclonal antibody when dividing *in vitro* (Raff *et al.* 1983*b*; Raff *et al.* 1984; ffrench-Constant & Raff 1986*b*; Sommer *et al.* 1990). O-2A$^{\text{perinatal}}$ progenitors divide predominantly in a symmetrical fashion, followed by differentiation of all cells within a single family into oligodendrocytes after 1–8 division. O-2A$^{\text{perinatal}}$ progenitors differentiating into oligodendrocytes first pass though an intermediate stage of differentiation that can be identified by labelling with the O4 monoclonal antibody (Sommer *et al.* 1990). O-2A$^{\text{adult}}$ progenitors have a characteristic unipolar morphology, divide with a cell cycle time of 65 h and migrate with an average speed of only 4 μm h^{-1} when grown in the presence of type-1 astrocytes

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differentiation, of progenitors still in the optic nerve (David *et al.* 1984), suggesting that type-1 astrocytes may be primarily responsible for promoting division of the cells of the O-2A lineage.

Type-1 astrocytes, as well as promoting division of O-2A$^{\text{perinatal}}$ progenitors *in vitro*, also affected the morphology, motility and differentiation of these cells. O-2A$^{\text{perinatal}}$ progenitors grown in chemically defined medium (Bottenstein & Sato 1979) rapidly develop a multipolar morphology and express galactocerebroside (GalC) (Raff *et al.* 1983*b*), a glycolipid specifically expressed in the CNS by oligodendrocytes (Raff *et al.* 1978). In contrast, O-2A$^{\text{perinatal}}$ progenitors induced to divide by type-1 astrocytes expressed a characteristic bipolar morphology, an A2B5$^+$GalC$^-$GFAP$^-$ antigenic phenotype (Noble & Murray 1984; see figure 1 herein) and were extremely motile cells, capable of migrating at speeds of up to 100 μm h^{-1} (Small *et al.* 1987). In addition, dividing O-2A$^{\text{perinatal}}$ progenitors grown in the presence of type-1 astrocytes continued to produce increasing numbers of progenitors, and oligodendrocytes, for an extended period in tissue culture. Thus, just as *in vivo*, growth of O-2A$^{\text{perinatal}}$ progenitors in the presence of type-1 astrocytes was associated with maintenance of a dividing population of progenitors for extended periods, and also with the continued production of new oligodendrocytes (Noble & Murray 1984).

One effect of type-1 astrocytes on O-2A$^{\text{perinatal}}$ progenitor cells in which we have had a particular interest has been the promotion of a very particular pattern of oligodendrocytic differentiation, characterized by synchronous differentiation of clonally related cells after a limited number of divisions (Temple & Raff 1986). Raff *et al.* (1985) first observed that embryonic progenitors dividing *in vitro* consistently gave rise to oligodendrocytes after a period of time equivalent to the age of the embryo and the number of days that would have been required for the embryo to reach E21, the time when oligodendrocytes first appear *in vivo* (for example, E17+four days, E18+three days). These results suggested that the onset of oligodendrocytic differentiation was controlled by a biological clock. Temple & Raff (1986)

(Wolswijk & Noble 1989). Unlike O-2A$^{\text{perinatal}}$ progenitors, the O-2A$^{\text{adult}}$ progenitors divide as O4$^+$ cells *in vitro* (Wolswijk & Noble 1989). Moreover, these cells appear to divide and differentiate asymmetrically (Wren *et al.* (Submitted.)); such that a single clone of cells continues to generate more progenitors for at least several divisions after the first generation of oligodendrocytes within the clone. Both O-2A$^{\text{perinatal}}$ progenitors and -2A$^{\text{adult}}$ progenitors can differentiate into either oligodendrocytes or type-2 astrocytes. Astrocytic differentiation is induced by exposure to foetal calf serum (FCS) or ciliary neurotrophic factor (CNTF; Raff *et al.* 1983*b*; ffrench-Constant & Raff 1986*a*; Hughes *et al.* 1988; Wolswijk & Noble 1989; Lillien *et al.* 1989). As CNTF-like molecules are present in extracts prepared from optic nerves of three-week-old rats (Hughes *et al.* 1988), it appears that CNTF may play an important role in type-2 astrocyte differentiation *in vivo*. It is not yet known whether O-2A$^{\text{adult}}$ progenitors are induced to differentiate into type-2 astrocytes in the presence of CNTF. Oligodendrocytes can be identified *in vitro* by their multipolar morphology, their expression of galactocerobroside (GalC), by O4 labelling and by their lack of intermediate filaments (Raff *et al.* 1978; Sommer & Schachner 1981; Raff *et al.* 1983*b*; Kachar *et al.* 1986; Wolswijk & Noble 1989). Type-2 astrocytes generated from either O-2A$^{\text{perinatal}}$ progenitors or O-2A$^{\text{adult}}$ progenitors contain both vimentin and glial fibrillary acidic protein (GFAP) intermediate filaments, are A2B5$^+$ and NSP-4$^+$ (Raff *et al.* 1983*a*, *b*; ffrench-Constant & Raff 1986*a*, *b*; Wolswijk & Noble 1989). Whereas type-2 astrocytes generated from O-2A$^{\text{perinatal}}$ progenitors are process-bearing cells (Raff *et al.* 1983*a*, *b*), type-2 astrocytes generated by O-2A$^{\text{adult}}$ progenitors usually have a more flattened morphology (Wolswijk & Noble 1989). Oligodendrocytes and O-2A$^{\text{adult}}$ progenitors are capable of binding and activating complement in the absence of antibody, resulting in their lysis. O-2A$^{\text{perinatal}}$ progenitors are resistant to the effects of complement, and acquire the capacity to bind and activate complement while progressing through stages of differentiation intermediate between the progenitor cell and the GalC$^+$ oligodendrocyte (Wren & Noble 1989). Abbreviations: FCS, foetal calf serum; CNTF, ciliary neurotrophic factor; CBAM, complement binding and activating molecule(s); Vim, vimentin; GFAP, glial fibrillary acidic protein; GalC, galactocerebroside.

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then found that most O-2A^{perinatal} progenitors growing in the presence of type-1 astrocytes divide between one and eight times before all members of a clonally related family of cells synchronously differentiate into oligodendrocytes. The observations that sister cells grown in separate dishes divide an equal number of times before differentiating, and that different families within a culture dish can divide dissimilar numbers of times before differentiating, suggests that the timing of the clock is not regulated by alterations in the extracellular environment, but is internal to the progenitor cells themselves. However, function of the clock appears to be absolutely dependent upon maintenance of cell division, and embryonic progenitors grown in chemically defined medium generate oligodendrocytes within 48 h of plating, regardless of the starting age of the embryo (Raff *et al.* 1985).

Platelet-derived growth factor (PDGF) is the astrocyte-derived mitogen that modulates division and differentation of O-2A^{perinatal} progenitors *in vitro*

The dramatic effects of type-1 astrocytes on O-2A^{perinatal} progenitors led us to investigate the molecular basis for this cell–cell interaction.

In screening a variety of known mitogens, we found that only PDGF was capable of mimicing the effects of type-1 astrocytes on O-2A^{perinatal} progenitor cells (Noble *et al.* 1988). PDGF promoted deoxyribonucleic acid (DNA) synthesis in O-2A^{perinatal} progenitors as effectively as type-1 astrocytes or astrocyte-conditioned medium (Astro-CM). With the best PDGF preparations, response plateaued at not more than 100 pM and PDGF induced DNA synthesis at not more than 5 pM. With a similar dose–response curve, PDGF also inhibited the premature differentiation of progenitors into oligodendrocytes otherwise seen when these cells are grown in the absence of type-1 astrocytes. After 72 h of growth in chemically defined medium virtually all 0-2A^{perinatal} lineage cells differentiate into GalC^{+} oligodendrocytes. In contrast, after 48 or 72 h of growth in the presence of PDGF or Astro-CM, over half of the O-2A^{perinatal} lineage cells still expressed the antigenic and morphological phenotype of O-2A^{perinatal} progenitors. Moreover, O-2A^{perinatal} progenitors grown in the presence of PDGF and analysed by time-lapse micro cinematography had similar rates of migration and cell cycle lengths to those grown in Astro-CM (24.6 ± 5.4 μm hr^{-1} against 21.4 μm hr^{-1}; 19.7 ± 6.4 h against 17.7 ± 4 h in PDGF and Astro-CM, respectively).

PDGF was also capable of driving the clock that modulates the timing of oligodendrocytic differentiation *in vitro* (Raff *et al.* 1988). O-2A^{perinatal} progenitors grown in the presence of PDGF generated families of cells in which all members of the family differentiated synchronously after a few divisions. In addition, if PDGF was added to cultures of embryonic rat optic-nerve cells grown in chemically defined medium, then the correct timing of oligodendrocyte differentiation was restored. In contrast, PDGF neither promoted differentiation of O-2A^{perinatal} progenitors into type-2 astrocytes nor inhibited such differentiation when foetal calf serum was present in the medium (Noble *et al.* 1988).

Several lines of evidence indicate that the effects of type-1 astrocytes on O-2A^{perinatal} progenitors are indeed mediated by PDGF: (i) antibodies to PDGF block the effects of type-1 astrocytes on O-2A progenitors derived from either embryonic or perinatal rats (Noble *et al.* 1988; Richardson *et al.* 1988; Raff *et al.* 1988); (ii) type-1 astrocytes make messenger ribonucleic acid (mRNA) for PDGF A-chain, and (iii) the mitogenic activity secreted by type-1

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astrocytes comigrates with PDGF on chromatography columns (Richardson *et al.* 1988). That this factor is physiologically relevant is supported by observations that: (i) PDGF is effective at picomolar concentrations (Noble *et al.* 1988; Richardson *et al.* 1988; Raff *et al.* 1988); (ii) mitogenic activity for O-2A^{perinatal} progenitors found in extracts of optic nerve is largely blocked by anti-PDGF antibodies (Raff *et al.* 1988), and (ii) *in situ* hybridization indicates that type-1 astrocytes express mRNA for PDGF A-chain *in vivo* (Pringle *et al.* 1989). Moreover, O-2A^{perinatal} progenitor cells express type-A PDGF receptors, in agreement with their capacity to respond to A-chain homodimers (Hart *et al.* 1989).

The programme of behaviour induced in O-2A progenitors by PDGF is mitogen specific

One of the next problems we have addressed is the extent to which individual aspects of the behavioural programme of O-2A^{perinatal} progenitor cells are separately regulable. The ability of PDGF to completely replace type-1 astrocytes in modulating the *in vitro* development of O-2A^{perinatal} progenitors suggested that these cells have a complex and constitutive behavioural phenotype, controlled by processes internal to the progenitors themselves, and that initiation of this programme might only require interaction of progenitors with a single polypeptide mitogen. But, does induction of cell division necessarily cause expression of the entire programme of behaviour seen in O-2A^{perinatal} progenitor cells grown in PDGF or the presence of type-1 astrocytes?

Our studies on the effects of basic fibroblast growth factor (bFGF) on O-2A^{perinatal} progenitor cells have shown that it is possible to separate the promotion of progenitor division from other possible behaviours of dividing O-2A^{perinatal} progenitor cells (Wren *et al.* (1990). Although bFGF is an effective mitogen for O-2A^{perinatal} lineage cells, we have found that O-2A^{perinatal} progenitors dividing in the presence of bFGF rapidly develop a multipolar morphology and show little or no migratory behaviour. In addition, most O-2A^{perinatal} progenitor cells grown in the presence of bFGF differentiate into oligodendrocytes within 72 h. Thus O-2A^{perinatal} progenitors can be induced to divide without necessarily expressing other properties associated with progenitors grown in the presence of type-1 astrocytes or PDGF.

If induction of division of O-2A^{perinatal} progenitors is not sufficient to induce expression of other potential behaviours of these cells, then what signals are missing in the bFGF stimulated cells? We are trying to identify these missing signals by activating second messenger pathways known to be involved in the control division of other cell types. In our initial experiments we have examined the behaviour of O-2A^{perinatal} progenitors grown in the presence of bFGF together with phorbol esters, which activate protein kinase C, or with dibutyryl cyclic adenosine monophosphate (cAMP). Our results so far show that if phorbol esters and bFGF are both applied to O-2A^{perinatal} progenitors, we now see a pattern of division and differentiation, which resembles that induced by PDGF, in that cells grown in the presence of bFGF and as little as 10 nM tumour promoting agent (TPA) continue to express a bipolar morphology and A2B5^{+}GalC^{-} antigenic phenotype after several days of *in vitro* growth (O. Bögler & M. Noble, unpublished data). Thus it may be that interaction with protein kinase C plays a necessary role in the induction of the complete behavioural programme induced by exposure of O-2A^{perinatal} progenitors to PDGF. In contrast, dibutyryl cAMP does

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not alter the pattern of differentiation seen when cells are grown in the presence of bFGF. Moreover, dibutyryl cAMP is itself not mitogenic for O-2A^{perinatal} progenitors, suggesting that FGF itself is not working solely, if at all, through the cAMP pathway.

Another difference between PDGF and bFGF is that bFGF can induce division of oligodendrocytes (Saneto & deVellis 1985; Eccleston & Silberberg 1985); thus, even though oligodendrocytes are not induced to divide by PDGF, they are none the less competent to do so. This finding suggests the functioning of the biological clock that modulates the timing of oligodendrocyte differentiation, although associated with a withdrawal from division before oligodendrocytic differentiation, is not associated with terminal loss of the ability to divide. In addition, the observation that oligodendrocytes can be induced to divide by bFGF focuses attention on the paradoxical observation that these cells express PDGF receptors (Hart *et al.* 1989), but are not induced to divide by PDGF (Noble *et al.* 1988). In future experiments, it will be of interest to determine which signal transduction pathways are being stimulated in oligodendrocytes by bFGF, but not by PDGF, and also to determine what role the PDGF receptors might serve on oligodendrocytes.

Finally, it is interesting to note that the effective dose (ED_{50}) of response to bFGF is not more than 2×10^{-10} M for O-2A^{perinatal} progenitors (Wren *et al.* 1990), a concentration suggestive of interaction with a physiologically relevant receptor. Moreover, bFGF is found at high concentrations in the brains of most mammals. It will be of importance to determine the localization and timing of bFGF expression in the rat optic nerve to develop some understanding of the possible roles of this mitogen in glial development.

The O-2A^{adult} progenitor cell

In the following sections we will discuss our research on a very different type of O-2A progenitor cell, the O-2A progenitor of the adult optic nerve. These cells differ from their perinatal counterparts in many important respects (as summarized in figure 1). For example, O-2A^{adult} progenitors have a cell cycle time of 65 h, over three times as long as that of O-2A^{perinatal} progenitors. Dividing O-2A^{adult} progenitors also migrate more slowly than their perinatal counterparts, express a different morphology and have a different antigenic phenotype. In addition, when grown in chemically defined medium, or in the presence of FCS, O-2A^{adult} progenitors differentiate several times more slowly than O-2A^{perinatal} progenitors.

The generation of O-2A^{adult} progenitor cells: basic question

The presence of precursor populations with different characteristics in developing and mature tissues raises the following questions.

1. When do adult-specific precursors first arise?
2. Do the distinguishing properties of adult precursors represent intrinsic properties of these cells , or would these cells express the properties of perinatal precursors if grown in the perinatal environment?
3. What are the cellular mechanisms that allow maintenance of a precursor population in adult tissue?
4. If adult precursors are derived from a self-renewing stem cell population, then what is the identity of the stem cell?

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5. What are the lineage relations between foetal and adult precursors?
6. What is the functional significance (and what are the functional consequences) of having distinct perinatal (or foetal) and adult precursor populations?

Our studies on the O-2A^{adult} progenitor cell have provided sufficient data to suggest answers to all of the above questions.

Developmental history and intrinsic properties of O-2A^{adult} progenitor cells

The first cells with the antigenic and morphological characteristics of O-2A^{adult} progenitors are seen in the optic nerves of P7 rats (Wolswijk & Noble 1990). By P21 about half of the O-2A progenitors in the optic nerve express the morphological and antigenic phenotypes of O-2A^{adult} progenitors, and by one month after birth most O-2A progenitors in the nerve appear to have the phenotype of adult progenitor cells.

Cells in the optic nerves of P21 and one-month-old rats with the characteristic morphology and antigenic phenotype of O-2A^{adult} progenitors also express other properties of these cells. For example, cells with the characteristic morphology of O-2A^{adult} progenitors isolated from optic nerves of P21 rats have lengthened cell cycle times and slow rates of migration *in vitro*. In addition, as presented in the next section, O-2A^{adult} progenitors isolated from optic nerves of eight-month-old rats divide and differentiate asymmetrically rather than in the symmetrical manner of O-2A^{perinatal} progenitors. Even in cultures derived from P21 rats, cells with other distinguishing characteristics of O-2A^{adult} progenitors also express the characteristic asymmetric behaviour shown by their counterparts in fully mature rats. In contrast, O-2A^{perinatal} progenitors in their cultures express the characteristic properties of their counterparts in younger rats.

The coexistence of O-2A^{perinatal} and O-2A^{adult} progenitors in the optic nerves of P7–1 month-old rats shows that O-2A^{adult} progenitors grown in an environment capable of supporting O-2A^{perinatal} progenitors retain their characteristic properties. This observation suggests that the properties of O-2A^{adult} progenitors are intrinsic to these cells.

O-2A^{adult} progenitors: asymmetry of division and differentiation

There are two possible explanations for the continued presence of O-2A progenitor cells in adult rats. Either optic nerves contain pre-progenitor cells that generate cells of the O-2A lineage throughout life (as suggested by ffrench-Constant & Raff (1986*a*)), or at least some O-2A^{adult} progenitor cells are themselves stem cells, and capable of self-renewal by means of asymmetric division and differentiation. To investigate this second possibility we examined the composition of colonies of clonally related cells derived from dividing O-2A^{adult} progenitors. If O-2A^{adult} progenitor cells behaved asymmetrically, then colonies would be expected to contain both oligodendrocytes and dividing O-2A progenitor cells. If progenitor division and differentiation was symmetric, as seems to be the case for most O-2A^{perinatal} progenitors (Temple & Raff 1986), then colonies containing oligodendrocytes would be expected to lack O-2A progenitor cells.

The *in vitro* behaviour of dividing O-2A^{adult} progenitor cells indicated that these cells were intrinsically asymmetric in their division and differentiation (Wren *et al.* 1990). More

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than 75% of colonies derived from individual O-2A^{adult} progenitors grown at clonal densities (not more than 1 cell/30 mm^2) contained both oligodendrocytes (which do not divide in these tissue culture conditions) and radiolabelled progenitors after either 15 or 25 days of growth on monolayers of type-1 astrocytes. Only 10% of the colonies visualized on day 25 (a length of time equal to not more than 10 divisions) consisted entirely of oligodendrocytes, and the remaining 14% contained oligodendrocytes and progenitors that were unlabelled by [^{3}H]thymidine. Although colony size increased, the proportion of colonies that contained both oligodendrocytes and radiolabelled O-2A progenitor cells remained as high on day 25 as on day 15, suggesting that asymmetric generation of oligodendrocytes, and of further O-2A^{adult} progenitors, represented the dominant pattern of division and differentiation in these colonies.

The size of individual colonies derived from O-2A^{adult} progenitors was also consistent with the view that the adult cells differentiated in a constitutively asymmetric manner. Symmetrically differentiating O-2A^{perinatal} progenitor cells isolated from perinatal rats generate colonies that cluster at sizes of 2, 4, 8, 16, 32 and so on in these culture conditions (Temple & Raff 1986; see also below). In contrast, the sizes of colonies derived from O-2A^{adult} progenitors showed no clustering at factors of 2 on either day 15 or day 25 of *in vitro* growth.

A further demonstration of the asymmetric behaviour of O-2A^{adult} progenitors was obtained by using time-lapse microcinematography to follow the behaviour of dividing cells. With such films we have directly observed that cells within a single clonal family of O-2A^{adult} progenitors continue to generate more progenitor cells for at least four divisions after the first generation of an oligodendrocyte (G. Wolswijk, unpublished data). Such behaviour is very different from that seen in most families of O-2A^{perinatal} progenitors, where clonally related cells generally all differentiate within one cell division of each other (Temple & Raff 1986: Raff *et al.* 1988).

In contrast to the asymmetric behaviour of O-2A^{adult} progenitors, the composition of colonies derived from O-2A^{perinatal} progenitors was generally consistent with symmetric division and differentiation of the perinatal cells (Wren *et al.* 1990). However, it is noteworthy that a small proportion (14%) of colonies derived from cells contained in the optic nerves of newborn rats were apparently asymmetric and contained both oligodendrocytes and radiolabelled O-2A progenitor cells after a length of time equivalent to not less than 10 cell divisions. This figure is consistent with other observations, showing that a minority population of O-2A^{perinatal} progenitor cells divide and differentiate asymmetrically *in vitro* (Temple & Raff 1986; Dubois-Dalcq, 1987; Wren *et al.* 1990). Thus it appears that not all O-2A^{perinatal} progenitors divide and differentiate in a symmetrical manner, a point we will return to later.

The origin of O-2A^{adult} progenitors: derivation from a subpopulation of O-2A^{perinatal} progenitors

Whereas the asymmetric division and differentiation of O-2A^{adult} progenitors might explain the maintenance of these cells in the optic nerve of adult rats, it leaves unanswered the question of the developmental origin of these cells. It could be that: (i) O-2A^{adult} and O-2A^{perinatal} progenitors represent two distinct progenitor lineages, (ii) are derived from a common pre-progenitor (or stem) cell that gives first to perinatal progenitors, and later in development to adult progenitors, or that (iii) O-2A^{adult} progenitors are directly derived from O-2A^{perinatal} progenitor cells.

Several observations now suggest that the most likely initial cell of origin for the O-2A^{adult} progenitor is an O-2A^{perinatal} progenitor-like cell. First, time-lapse microcinematographic

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analysis of cells derived from optic nerves of three-week-old rats (the time when the relative proportion of perinatal to adult progenitors changes most rapidly (Wolswijk & Noble 1990), has offered us clear examples of families that meet the following three stringent criteria.

1. The founder cell expressed the morphology, cell-cycle length and motility characteristic of an O-2A^{perinatal} progenitor.

2. The first division observed gave rise to more O-2A^{perinatal} progenitor-like cells.

3. Cells derived from subsequent divisions expressed the unipolar morphology, slow division time and slow migration rate typical of O-2A^{adult} progenitors (Wren *et al.* 1990). We have also provided with other methods a further example of a family of O-2A^{perinatal} progenitors that appear to give rise to O-2A^{adult} progenitors (Wren *et al.* 1990). Previous studies by Dubois-Dalcq (1987) have also suggested that some families of O-2A^{perinatal} progenitors can give rise to cells with at least some of the characteristics of O-2A^{adult} progenitors. As the only other cell of origin we have ever seen, by time-lapse microcinematography, for O-2A^{adult} progenitors is other O-2A^{adult} progenitors, and we have never seen the birth of O-2A^{perinatal} progenitors from anything other than O-2A^{perinatal} progenitors; there is at present no evidence for the existence of a pre-progenitor cell in this lineage. Indeed, our experiments show that the invocation of such a cell is not necessary to explain the development or maintenance in the adult of O-2A^{adult} progenitor cells.

The generation of O-2A^{adult} progenitors and the capacity for self-renewal in the O-2A lineage

Our studies suggest the following hypothesis about the origin and function of O-2A^{adult} progenitors: O-2A^{perinatal} progenitor cells can be divided into symmetric and asymmetric populations. In families of symmetric cells, all progenitors are lost by differentiation into oligodendrocytes (or, in the presence of appropriate inducing factors, into type-2 astrocytes). O-2A^{perinatal} progenitors also disappear from asymmetric families, but this occurs at least in part as a result of transition to the O-2A^{adult} progenitor phenotype. This perinatal–adult transition will occur if O-2A^{perinatal} progenitors are simply maintained in division, and does not require interaction of O-2A^{perinatal} progenitors with such components of the *in vivo* environment as axons, for a period of at least two divisions before the generation of O-2A^{adult} progenitors. The asymmetric behaviour of some O-2A^{perinatal} progenitors and of O-2A^{adult} progenitors will allow self-renewal within the O-2A lineage for extended periods, perhaps even throughout life.

By serially passaging O-2A^{perinatal} progenitors *in vitro* we have confirmed three predictions of the hypothesis stated above. First, using O-2A^{perinatal} progenitor cells as a starting population, we have been able to serially passage dividing O-2A lineage cells *in vitro* for an extended period. Secondly, this passaging was associated with replacement of the O-2A^{perinatal} progenitor cells with O-2A^{adult} progenitors. Thirdly, the generation of O-2A^{adult} progenitors occurred in cultures lacking retinal, or other, neurons.

Over the course of three months, we serially passaged perinatal optic nerve cells through six passages on monolayers of purified and irradiated type-1 astrocytes (to promote progenitor division (Noble & Murray 1984; Wolswijk & Noble 1989)). In two experiments we passaged optic-nerve cells from newborn rats, which contain no detectable adult-like cells (Wolswijk & Noble 1990). To allow for the possibility that O-2A^{adult} progenitor cells are derived from a subset of O-2A^{perinatal} progenitors not yet present in the optic nerves of newborn rats, we

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also separately passaged cells from optic nerves of seven-day-old rats, in which not more than 2% of the O-2A progenitors appear to be adult-like (as defined by antigen expression and morphology; Wolswijk & Noble 1990); in the latter case, the suspensions of optic nerve cells were first treated with the O4 monoclonal antibody (Sommer & Schachner 1981) and complement to lyse adult-like progenitors (which are $O4^+$, see figure 1) and also to lyse cells progressing through intermediate stages of differentiation that lie between the earliest $O\text{-}2A^{perinatal}$ progenitors and terminally committed oligodendrocytes (Sommer *et al.* 1990; see figure 1 herein). Thus, these passaging experiments were focused on the most immature members of the O-2A lineage, growing in the absence of neurons and in conditions which, in these experiments, did not generally promote differentiation of progenitors into type-2 astrocytes (D. Wren, unpublished data). All preparations yielded similar results.

Serial passaging of $O\text{-}2A^{perinatal}$ progenitors was associated with the generation of $O\text{-}2A^{adult}$ progenitors and the loss of $O\text{-}2A^{perinatal}$ progenitors, as judged by antigenic criteria, morphological criteria, changes in the population doubling times, and the generation of apparently asymmetric colonies containing both oligodendrocytes and dividing progenitors (Wren *et al.* 1989). However, just as we have seen during *in vivo* development (Wolswijk & Noble 1990), the transition to an adult phenotype *in vitro* did not occur simultaneously in the entire O-2A progenitor population, and cells with the phenotypes of $O\text{-}2A^{perinatal}$ and $O\text{-}2A^{adult}$ progenitors coexisted within the serially passaged populations for an extended period. This heterogeneity is reminiscent of that seen during oligodendrocyte development *in vitro* and *in vivo*, where some families of $O\text{-}2A^{perinatal}$ differentiate into oligodendrocytes whereas other families continue to divide and generate more progenitors.

Of progenitors and stem cells

Is the $O\text{-}2A^{adult}$ progenitor cell really a stem cell? Our experiments have shown that the adult cells express both a slow cell-cycle time and the capacity for asymmetric division and differentiation, two of the defining characteristics of stem cells. Moreover, our serial repassaging experiments show that cells of the O-2A lineage can not only undergo extended self-renewal, but that extended self-renewal *in vitro* is associated with increasing prevalence of the $O\text{-}2A^{adult}$ progenitor phenotype among those cells engaged in proliferation. Thus it appears to be the $O\text{-}2A^{adult}$ progenitors, and not their perinatal counterparts, which are primarily involved in self-renewal. If we are correct in suggesting that a pre-progenitor cell is not involved in self-renewal in the O-2A lineage, then the capacity for self-renewal of $O\text{-}2A^{adult}$ progenitors must indeed be extensive, for the $O\text{-}2A^{adult}$ progenitors that develop *in vivo* during the first month after birth would then seem likely to be the direct ancestors of the $O\text{-}2A^{adult}$ progenitors in adult rats.

What is the biological significance of having a perinatal–adult transition in O-2A progenitor populations?

To achieve rapid myelination of CNS axon tracts during early development, it is necessary to rapidly generate large numbers of progenitors, and oligodendrocytes, within a relatively short period of time. Thus, a rapid cell cycle time, and the capacity for exponential expansion in cell number, appear to be requisite properties for an O-2A progenitor cell in the optic nerve of

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perinatal rats. Moreover, it appears that O-2A^{perinatal} progenitors originate from a germinal zone outside the optic nerve and populate the optic nerve in a wave of migration during perinatal development (Small *et al.* 1987). As oligodendrocytes are non-motile cells (Small *et al.* 1987; Noble *et al.* 1988), asymmetric division and oligodendrocytic differentiation of O-2A^{perinatal} progenitors would reduce the production of the large population of motile O-2A lineage cells necessary for the successful colonization of optic nerves during early development. A further property of O-2A^{perinatal} progenitors that is likely to be well-suited to the physiological requirements of the developing nervous system is the biological clock expressed by these cells, which induces symmetric differentiation of clonally related progenitor cells within a few cell divisions (Temple & Raff 1986; Raff *et al.* 1988). Regulation of differentiation with such a clock offers a simple means of generating large numbers of progenitors, and then generating large numbers of oligodendrocytes, at precisely the times when these cells are needed during early development.

Although the adult CNS does not have sufficient space to accommodate the large numbers of new cells produced by rapid exponential growth and symmetrical differentiation, O-2A progenitor cells are still likely to be required to replace oligodendrocytes and type-2 astrocytes lost through normal turnover, disease or injury. Asymmetric division and differentiation of O-2A^{adult} progenitors, coupled with greatly lengthened cell cycles, would allow maintenance of functional progenitor populations in adult tissue without the generation of large numbers of unrequired cells. In addition, the generation of cells with the stem cell-like characteristics of O-2A^{adult} progenitors from rapidly dividing O-2A^{perinatal} progenitors offers a previously unrecognized means of halting the rapid cell division of embryogenesis, by generating a population of cells that respond to particular cell–cell interactions in a manner different from their ancestors and more in keeping with the physiological requirements of adult tissues.

Implications for understanding the repair of CNS myelin and the disease of multiple sclerosis

Our results show that the O-2A^{adult} progenitor cells likely to be responsible for the regenerative events of remyelination, at least in the rat optic nerve, are not only significantly different in their biology from the cells relevant to developmental processes, but are likely to be much less effective than their perinatal counterparts in remyelinating large areas of damaged tissue. The potential relevance of these differences between O-2A^{perinatal} and O-2A^{adult} progenitors for human disease is readily apparent. For example, magnetic resonance imaging by McDonald and colleagues (W. I. McDonald, personal communication) indicates that the demyelinated plaques seen in patients with optic neuritis generally involve up to 25% of the fibres in the optic nerve (that is, 250000 fibres) and extend a distance of 1 cm (about 50 internodes). Oligodendrocytes are thought to myelinate an average of 25 axons, allowing an estimate of the number of oligodendrocytes needed to repopulate a demyelinated lesion. By using the optimal figures for oligodendrocyte production indicated by current data, 2000 O-2A^{perinatal} progenitors would produce the required numbers (5×10^5) of oligodendrocytes in eight days, whereas a similar number of O-2A^{adult} progenitors would require two months to produce the same number of oligodendrocytes. During these two months glial scars, seemingly formed by type-1 astrocytes (Miller *et al.* 1986), will also be forming within the demyelinated plaque, and these may prevent migration of O-2A progenitors into the lesion (Raff *et al.* 1987).

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Thus the differing properties of O-2A^{perinatal} and O-2A^{adult} progenitor cells may explain the reduced capacity for remyelination associated with ageing in patients with multiple sclerosis (Kriss *et al.* 1988) and also observed in organ culture experiments comparing the remyelinative capacities of tissue from perinatal mice with that in adult mice (Wolf *et al.* 1986).

An additional property of O-2A^{adult} progenitors that also bears upon our understanding of the disease of multiple sclerosis is that O-2A^{adult} progenitors, and oligodendrocytes, directly bind and activate complement in the absence of antibody, leading to their destruction (Wren & Noble 1989). This property is not shared by any other CNS glial cells, including O-2A^{perinatal} progenitors. Thus the cell that makes myelin and the cell most likely to be of central importance in the repair of demyelinated lesions can be destroyed *in vitro* simply by exposure to complement.

Several observations are at least consistent with the view that exposure of oligodendrocytes and O-2A^{adult} progenitor cells to complement *in vivo* could contribute to production of the demyelinating lesions of multiple sclerosis (MS). The blood–brain barrier is damaged in patients with perivascular leakage of cells and serum proteins into the CNS (Fog 1965). Similar vascular lesions are seen in the non-myelinated retina and in the myelinated white matter (Lightman *et al.* 1987), showing that the vasculature itself may be a primary site of damage in MS. In support of this view, blood–brain barrier breakdown is a consistent early event in new MS lesions (Miller *et al.* 1988), as shown by leakage of Gadalinium (which is visualized by magnetic resonance imaging), and may be seen before other changes (D. Miller & W. I. McDonald, personal communication). In addition, consumption of C9, which shows formation of membrane attack complexes, has been observed in patients with MS (Morgan *et al.* 1984; Sanders *et al.* 1986) and injection of normal serum into the CNS produces demyelination in experimental animals (Sergott *et al.* 1984).

If the entry of complement into the CNS is capable of destroying oligodendrocytes and O-2A^{adult} progenitors, then why is it that the blood–brain barrier can be disrupted (for example, in head injury) without resulting in MS? Several factors may be important in considering this question. First, it appears that the development of MS, and the ability to induce MS-like conditions in experimental animals, are linked to expression of specific major histocompatibility complex (MHC) haplotypes. Thus not all individuals seem to be at risk when exposed to myelin antigens. Indeed, if different susceptible individuals were to find different myelin components to be immunodominant, one sees some reason for the consistent failure to identify universally present immunoreactivities in MS patients. In addition, it has recently been reported that the effects of repetitive exposure of oligodendrocytes to sublethal doses of complement appear to be cumulative, in that a dose that is initially sublethal eventually becomes lytic (Scolding *et al.* 1989). This last observation suggests an explanation for the continuous (and very confusing) success investigators have had at finding 'specific' viruses associated with MS, an association with which over 20 separate viruses have so far been implicated (Waksman 1989). It may be that all of the identified viruses are in fact associated with MS, but only through an ability to non-specifically cause transient disruption of the blood–brain barrier, leading eventually to oligodendrocyte destruction. Although it will be many years before we know the degree to which these suggestions accurately reflect the underlying causes of MS, it is striking that the fundamental biological properties of oligodendrocytes and O-2A^{adult} progenitors are consistent with observations on the genesis of MS lesions and on the failure of these lesions to be repaired.

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Beta-adrenergic stimulation of DNA synthesis in type-1 astrocytes of the cerebral cortex and optic nerve

Along with our interest in polypeptide mitogens, we have been interested in the possible regulation of glial cell division by neurotransmitter molecules. Previous studies have demonstrated that the beta (β)-adrenergic neurotransmitter norepinephrine stimulates cAMP production in astrocytes (Rougon *et al.* 1983; Evans *et al.* 1984) and that cAMP-modulated pathways can cause, or enhance, division in a wide range of cell-types (Rozengurt *et al.* 1981; Rozengurt 1982). These results suggested that β-adrenergic stimulation might promote astrocyte division.

When we applied isoproterenol, a β-adrenergic agonist, to purified cortical astrocytes (which have the antigenic and morphological characteristics of type-1 astrocytes), we saw a maximum threefold stimulation of DNA synthesis at a concentration of 10^{-6} M isoproterenol (Fok-Seang & Noble 1990). The degree of stimulation caused by epidermal growth factor (EGF), a known astrocyte mitogen (Leutz & Schachner 1981; Pruss *et al.* 1982), in the conditions of these experiments was similar to that caused by isoproterenol. Similar levels of stimulation were also achieved with cholera toxin or dibutyryl cAMP. Although this amount of stimulation is lower than usually published values; it is important to stress that all of our experiments were carried out in serum-free medium containing defined additives (Bottenstein & Sato 1979), so as to exclude effects of interaction with growth factors present in foetal serum.

Stimulation of DNA synthesis by isoproterenol, but not by EGF, was blocked by simultaneous application of propranolol (a β-adrenergic antagonist) showing that stimulation was mediated by β-adrenergic receptors. In contrast, activation of α-adrenergic receptors inhibited DNA synthesis. The α-adrenergic agonist phenylephrine, applied at 10^{-5} or 10^{-4} M, inhibited the stimulatory effects of 10^{-6} M isoproterenol. In addition, 10^{-5} M isoproterenol was less effective than 10^{-6} M isoproterenol at stimulating DNA synthesis, because of activation of inhibitory α-adrenergic receptors at the higher concentration. Thus 10^{-5} M isoproterenol stimulated DNA synthesis to an identical degree to 10^{-6} M isoproterenol if it was added in the presence of 10^{-5} M phentolamine, an α-adrenergic antagonist.

Whereas EGF or isoproterenol on their own only induced a maximal threefold increase in ^{125}I-uridine (IUdR) incorporation in the culture conditions used in our experiments, the addition of these compounds together caused a ninefold increase in DNA synthesis in the purified type-1 astrocytes. Thus these compounds in combination promoted astrocytical DNA synthesis at levels greater than the sum of the individual effects. The synergistic stimulation caused by simultaneous application of EGF and isoproterenol shows that these compounds stimulated overlapping populations of astrocytes, otherwise the combined effects of these compounds could only have been additive.

Determination of cell numbers confirmed that the stimulation of DNA synthesis measured in the above assays was associated with cell division. Exposure even to submaximal levels of isoproterenol or EGF was associated with increases in astrocytic cell number, and the increases associated with the simultaneous addition of isoproterenol and EGF were greater than the increases caused by either compound alone.

Type-1 astrocytes in cultures derived from optic nerves of P7 rats were also stimulated to synthesize DNA by exposure to isoproterenol. In contrast, isoproterenol had no effect on O-2A^{perinatal} progenitors or on oligodendrocytes, nor were these cells stimulated to synthesize

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DNA by direct application of dibutylyl cAMP or cholera toxin. Thus the expression of a cAMP-regulated pathway of cell division may be lineage specific, a possibility that can only be confirmed when we are able to identify conditions that induce differentiation of type-2 astrocytes from O-2A^{perinatal} progenitors, but do not induce progenitor division.

The ability to stimulate division of astrocytes by activation of β-adrenergic receptors suggests the possibility that neurotransmitter release could play a role in regulating cell number during CNS development. Such a developmental role for neurotransmitters might help to explain the surprising observations that norepineprine, as well as serotonin and acetylcholine, are detectable in the neural tube of one-day-old chicken embryos, well before synpases are formed (Ignarro *et al.* 1968*a*, *b*; Kirby *et al.* 1972).

Future questions

Of the many questions that remain to be answered in our studies on glial division and differentiation, some are of particularly immediate concern. We would like to understand the relation between the symmetrical and asymmetrical O-2A^{perinatal} progenitors, and also to understand why some families of O-2A^{perinatal} progenitors begin to generate O-2A^{adult} progenitors. Further information about the molecular mechanisms that underly function of biological clocks and multipotential differentiation is needed, as is information on the molecular control of asymmetric differentiation. Finally, it will be of interest to begin examining the effects of simultaneous application of polypeptide mitogens, or polypeptide mitogens and neurotransmitters, on division and differentiation, a situation likely to be relevant to unravelling the complexities of normal development.

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