An inducer protein may control the timing of fate switching in a bipotential glial progenitor cell in rat optic nerve

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Summary

In rat optic nerve, oligodendrocytes and type-2 astrocytes develop from a common (O-2A) progenitor cell. The first oligodendrocytes differentiate at birth, while the first type-2 astrocytes differentiate in the second postnatal week. We previously showed that the timing of oligodendrocyte differentiation depends on an intrinsic clock in the O-2A progenitor cell. Here we provide evidence that the timing of type-2 astrocyte differentiation, by contrast, may depend on an inducing protein that appears late in the developing nerve. We show that extracts of 3- to 4-week-old, but not 1-week-old, rat optic nerve contain a protein (apparent Mr ~ 25 000) that induces O-2A progenitor cells in culture to express glial fibrillary acidic protein (GFAP), an astrocyte-specific marker in the rat central nervous system.

Key words: rat, optic nerve, glial progenitor cell, fate switching, inducing, astrocyte, GFAP-induction, oligodendrocyte.

Introduction

The various cell types in a multicellular organism differentiate on a predictable schedule, but the mechanisms responsible for controlling the direction and timing of cell differentiation are largely unknown. We have been studying cell differentiation in the rat optic nerve, a relatively simple myelinated tract in the central nervous system (CNS). We have focused on a population of bipotential glial progenitor cells in the nerve that gives rise to both oligodendrocytes (which make myelin in the CNS) and a subclass of astrocytes (type-2 astrocytes) (Raff, Miller & Noble, 1983). One aim of our studies is to discover what determines whether an individual progenitor cell becomes an oligodendrocyte or a type-2 astrocyte and why the first oligodendrocytes appear on the day of birth while the first type-2 astrocytes do not appear until the second postnatal week (Miller et al. 1985).

An important clue to the mechanisms underlying the choice of developmental pathway and the timing of differentiation in this system is the difference in behaviour of the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells in vivo and in vitro. In vivo, proliferating O-2A progenitors give rise to post-mitotic oligodendrocytes (Skoff, Price & Stocks, 1976a,b) and type-2 astrocytes (Miller et al. 1985) over a period of several weeks, whereas in cultures of dissociated perinatal optic nerve the majority of O-2A progenitor cells stop dividing and differentiate within 2 or 3 days: if cultured in ≤0-5 % fetal calf serum (FCS), most of the progenitor cells become oligodendrocytes, while in ≥10 % FCS most become type-2 astrocytes (Raff et al. 1983; Raff, Williams & Miller, 1984b). The normal in vivo timing of oligodendrocyte production can be reconstituted in vitro by coculturing embryonic optic nerve cells (in ≤0-5 % FCS) on a monolayer of type-1 astrocytes: the O-2A progenitor cells continue to proliferate in such cocultures, and the first oligodendrocytes appear at the in vitro equivalent of the day of birth; moreover, new oligodendrocytes continue to develop for several weeks, just as in vivo (Raff, Abney & Fok-Seang, 1985). These and other findings led us to postulate that oligodendrocyte differentiation is the constitutive pathway of development, the timing of which is controlled by a clock in the O-2A progenitor cell (Raff et al. 1985; Temple & Raff, 1986). Although the clock is intrinsic to the progenitor cell, it is not cell autonomous. It is driven by factors secreted by type-1 astrocytes (Noble & Murray, 1984; Raff et al. 1985),
the first glial cell type to differentiate in the nerve (Miller et al. 1985).

Type-2 astrocyte differentiation seems to be controlled in a different way. When embryonic optic nerve cells are cocultured on type-1 astrocytes in ≤0.5% FCS for several weeks, type-2 astrocytes fail to develop, even though some type-2 astrocytes would have developed by this time in vivo (Raff et al. 1985). Thus the timing of type-2 astrocyte differentiation is not intrinsic to the O-2A progenitor cell: some component required for type-2 astrocyte development, which is presumably present in the optic nerve from at least the second postnatal week, seems to be missing from the cocultures. On the other hand, if 10% FCS is added at the start of the cocultures, O-2A progenitor cells develop into type-2 astrocytes within several days (Raff et al. 1985). Thus O-2A progenitor cells are capable of developing into type-2 astrocytes long before they normally do so in vivo. We hypothesized that FCS mimics a physiological inducer that appears in the developing optic nerve around the second postnatal week and is responsible for timing type-2 astrocyte differentiation in vivo (Raff et al. 1985).

In this paper we have tested an important prediction of the induction hypothesis. We report that extracts of 3- to 4-week-old rat optic nerve contain a protein that, in the absence of FCS, rapidly and directly induces O-2A progenitor cells to express glial fibrillary acidic protein (GFAP), a widely used marker of astrocyte differentiation in the CNS (Bignami, Eng, Dahl & Uyeda, 1972) and one that we have used to help define the type-2 astrocyte (Raff et al. 1983); the concentration of the GFAP-inducing protein is at least 50 times lower in extracts of 1-week-old optic nerve. These findings strongly support the hypothesis that type-2 astrocyte differentiation in vivo depends on an inducer that appears late in CNS development.

Materials and methods

Cell culture

Optic nerves were removed from E17–18, newborn (P0–1) or P7 Sprague–Dawley rats and dissociated into single cells using trypsin, collagenase and EDTA as previously described (Miller et al. 1985). Approximately 7500 cells were cultured on poly-D-lysine-coated coverslips in 0.4–0.5 ml Dulbecco's modified Eagle's medium (DMEM) with 0.5% FCS and additives modified from Bottenstein & Sato (1979) (B-S medium), as previously described (Miller et al. 1985). Conditioned medium experiments were performed by placing a coverslip containing P0 optic nerve cells on the upper surface on top of a second coverslip containing type-1 astrocytes prepared from neonatal brain as described previously (Noble & Murray, 1984); the two coverslips were separated by several small bits of broken glass slide.

Microcultures were carried out by limiting dilution in Terasaki microwell plates (Falcon) coated with poly-D-lysine. Approximately 15–25 cells were added to wells in 10 μl of B-S medium; after one day, cells were detected in less than half of the wells and in these only 1–10 cells were seen. For low-density cultures, all wells were assessed. For single-cell experiments, wells with only one cell after 20 h were studied.

Immunofluorescence staining

Cells on coverslips were fixed for 5 min at room temperature in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7). After washing in minimal Eagle's medium buffered with 0.02 M-Hepes (MEM–Hepes), the cells were incubated with A2B5 (Eisenbarth, Walsh & Nirenburg, 1979) or anti-GFAP (Ranscht et al. 1982) monoclonal antibodies (ascites fluid diluted 1:500 in MEM–Hepes with 10% FCS), followed by rhodamine-coupled F(ab')2 goat anti-mouse immunoglobulin (G anti-Mlg-Rd, Cappel, diluted 1:100). In some experiments, the anti-GFAP antibody was visualized with fluorescein-coupled, class-specific G anti-IgG3 (Nordic, 1:100). After being washed, the cells were fixed in 5% glacial acetic acid:95% ethanol (acid–alcohol) at −20°C for 5 min before staining with rabbit anti-GFAP serum (Pruss, 1979; diluted 1:1000) followed by fluorescein-coupled sheep anti-rabbit Ig (Sh anti-Rig-F1, Wellcome, diluted 1:100) as previously described (Raff et al. 1978). In some experiments, the G1 monoclonal anti-GFAP antibody (Gheuens, de Schutter, Noppe & Lowenthal, 1984) was used, followed by G anti-MlG-F1 (Cappel, diluted 1:100). Coverslips were mounted on glass slides in glycerol–PBS (9:1) containing 2.5% (w/v) triethylenediamine to prevent fading of the fluorescence and were examined in a Zeiss Universal fluorescence microscope using a ×63 objective and photographed using Tri-X film rated at 400 ASA.

Terasaki plates were stained using a similar protocol except that the cells were not fixed with paraformaldehyde and the first antibody, which was added for 60 min, was detected with biotin-coupled G anti-Mlg followed by Texas-red-coupled streptavidin (both from Amersham and diluted 1:100). The wells were examined using a ×40 objective as previously described (Temple & Raff, 1985).

Cells were scored as GFAP+ only if they contained fluorescent filaments.

Tissue extracts

Tissues were weighed and homogenized by hand using a small glass pestle and mortar in ice-cold sodium phosphate buffer (50 mM, pH 6.0) with 20 μM-phenylmethylsulphonyl fluoride (PMSF) added immediately beforehand. The homogenates were centrifuged for 10 min at 4°C in an MSE Micro Centaur, and the supernatants were recentrifuged at 80 000 g for 30 min at 4°C to remove particulate matter and then dialysed against DMEM for 24 h at 4°C in Spectropor dialysis tubing, grade 1. Protein concentrations were determined using a Bio-Rad protein assay kit with IgG (included in the kit) as standard. The extracts were stored at −20°C in 100 μl samples containing 0.2–2 mg total protein ml⁻¹.
Fast protein liquid chromatography (FPLC)
Optic nerve extracts were passed through a Millipore filter (0.22 μm pore size) and 100–300 μg total protein were loaded in 0.5 ml DMEM with 0.5 mg ml⁻¹ fatty acid-free bovine serum albumin (BSA, Sigma) and run at room temperature in the same medium at 0.3 ml min⁻¹ on a Pharmacia Superose-12 column linked to an LCC-500 FPLC system. BSA, ovalbumin, soybean trypsin inhibitor and cytochrome c (all from Sigma and all used at 2–8 mg ml⁻¹) were run as markers. Fractions of 0.25 ml were collected for assay.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
Extracts of 3- to 4-week-old optic nerve were incubated in sample buffer with 0.9 % SDS for 30 min at 37°C, loaded in ≤100 μl onto a 1 mm thick, 12.5 % polyacrylamide slab gel and electrophoresed under nonreducing conditions (Laemmli & Favre, 1974). The gel was cut into 5 mm slices, each of which was washed by vigorous shaking in 1 ml PBS for 30 min to remove the running buffer; the PBS was replaced and the wash continued for another 10 min. Protein was then eluted from the gel slices by rotating them overnight at 4°C in 0.5 ml DMEM with 0.5 mg ml⁻¹ BSA (Lemke & Brockes, 1984). The efficacy of elution was assessed by electrophoresing ¹²⁵I-labelled platelet-derived growth factor (PDGF) on the gel; 69 % of the loaded counts were found in two adjacent slices containing protein with apparent Mr between 30000 and 39000, and 27 % of these counts were eluted by the procedure described.

Results
The O-2A progenitor cells in cultures of perinatal rat optic nerve are readily identified by their characteristic process-bearing morphology and surface staining with the A2B5 monoclonal antibody (Eisenbarth et al. 1979; Temple & Raff, 1985). These cells do not express intracellular GFAP or cell-surface galactocerebroside (GC), a major myelin glycolipid and a definitive marker for oligodendrocytes in CNS cultures (Raff et al. 1978). When cultured in ≤0.5 % FCS, the majority of O-2A progenitor cells differentiate into GC⁺ oligodendrocytes, whereas when cultured in ≥10 % FCS, the majority differentiate into GFAP⁺ type-2 astrocytes. We have used the expression of GFAP, as detected by immunofluorescence, as a marker of astrocyte differentiation in studying the effects of optic nerve extracts on the development of O-2A progenitor cells in cultures containing ≤0.5 % FCS.

Extracts of ≥3-week-old optic nerve induce O-2A progenitor cells to express GFAP in the absence of FCS
When newborn optic nerve cells were cultured in 0-5 % FCS, very few, if any, O-2A progenitor cells were labelled by anti-GFAP serum after 1 day in vitro. However, when an extract of 3- to 4-week-old rat optic nerve was added to the culture, 15–45 % of the O-2A progenitor cells became GFAP⁺ after 1 day (Fig. 1). The extract had no appreciable effect on total cell numbers, the number of A2B5⁺ progenitor cells or GFAP expression in type-1 astrocytes. Similar results were obtained when cells were cultured with the extract in the absence of FCS or in the presence of medium conditioned by type-1 astrocytes in order to

Fig. 1. Induction of GFAP in an O-2A progenitor cell by optic nerve extract. Cells dissociated from P0 optic nerve were cultured on coverslips in B-S medium containing 0.5 % FCS and 14 μg ml⁻¹ 3- to 4-week-old optic nerve extract. After 24 h, the cells were stained with A2B5 antibody followed by G anti-MIg-Rd (B) and, after fixation in acid-alcohol, with R anti-GFAP serum followed by Sh anti-Rlg-F1 (C). Note that the A2B5⁺ progenitor cell (shown in phase contrast in A) has been induced to express GFAP by the extract; no GFAP⁺ progenitor cells were seen in sister cultures maintained without extract. While the A2B5⁻ type-1 astrocyte is also GFAP⁺, the extract had no detectable influence on GFAP expression by such cells. The A2B5⁻,GFAP⁺ cells are probably from the meninges, which ensheath the optic nerve. Bar, 50 μm.
stimulate O-2A progenitor cell proliferation (Noble & Murray, 1984; Raff et al. 1985) and when extract-treated cells were stained with a monoclonal anti-GFAP antibody (Gheuens et al. 1984). Assaying at various times between 6 and 30 h, we observed small but significant GFAP induction after only 6 h and maximal GFAP expression between 20 and 24 h (not shown).

The extract was active in inducing GFAP expression at concentrations of total protein as low as 300 ng ml\(^{-1}\). With higher concentrations, a plateau was reached where less than half of the progenitor cells were induced (Fig. 2); the plateau remained at this level with concentrations as high as 90 \(\mu\)g ml\(^{-1}\), the highest concentration of extract tested (not shown). Extracts of rat tissues that do not contain type-2 astrocytes, including 1-week-old optic nerve, 4-week-old retina and adult liver, were at least 50 to 100 times less active than extracts of 3- to 4-week-old optic nerve when normalized for either total extracted protein (Fig. 2) or wet weight of tissue (not shown), whereas extracts of adult optic nerve were about one third as active as extracts of 3- to 4-week-old nerve (Fig. 2).

The induction of GFAP in O-2A progenitor cells by 3- to 4-week-old optic nerve extract was completely inhibited if the cultures were treated with actinomycin D (0.5 \(\mu\)g ml\(^{-1}\) for 1 h) or cycloheximide (5 \(\mu\)g ml\(^{-1}\) for 24 h), suggesting that both RNA and protein synthesis are required for the induction (Table 1).

**The GFAP-inducing activity in optic nerve extracts resides in a protein(s) of apparent \(M_r \sim 25\,000\)**

The GFAP-inducing activity of 3- to 4-week-old optic nerve extract (which was used in subsequent experiments and henceforth will be referred to simply as optic nerve extract) was retained after dialysis for 24 h (using dialysis tubing that retains molecules larger than 6000–8000 \(M_r\)), but was lost after heating to 96°C for 10 min or treatment with trypsin (1-25 mg ml\(^{-1}\)) for 30 min at 37°C (Table 1), suggesting that the activity depends on one or more proteins in the extract.
Table 1. Effects of drugs, heating and trypsin treatment on GFAP-induction by optic nerve extracts

<table>
<thead>
<tr>
<th>Extract</th>
<th>Drug</th>
<th>% A2B5+ process-bearing cells expressing GFAP after 1 day in culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>Untreated</td>
<td>Actinomycin D</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Untreated</td>
<td>Cycloheximide</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Heated to 96°C, 10 min</td>
<td>0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>Treated with trypsin</td>
<td>0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

P0 optic nerve cells were cultured for 24 h in the presence or absence of 5-25 μg ml⁻¹ 3- to 4-week-old optic nerve extract. Actinomycin D was used at 0-5 μg ml⁻¹ for 1 h, while cycloheximide was used at 5 μg ml⁻¹ for the entire 24 h. For trypsin treatment, 50 μg of trypsin (Sigma, type XII) was added to 40 μl of extract containing 5 mg of total protein for 30 min at 37°C and was removed with 20 mg soybean trypsin inhibitor bound to DITC beads (Sigma). Results are expressed as means ± s.e. of at least three cultures.

To estimate the approximate size of the putative protein(s) responsible for the GFAP-inducing activity, we fractionated the extract by fast protein liquid chromatography (FPLC) on a Superose-12 gel filtration column. The activity eluted in a single peak in the range of 20000-30000 relative molecular mass (Mr) (Fig. 3). The activity yield from the column was ~90%, suggesting that the activity in the extract is associated with one or more protein(s) of apparent Mr ~ 25 000. As expected, when 1-week-old optic nerve extract was fractionated in this way, no activity was recovered from the column (not shown).

To test further whether the GFAP-inducing activity of the extract resided in a protein, we fractionated the extract by fast protein liquid chromatography (FPLC) on a Superose-12 gel filtration column. The gel was sliced and protein from each slice was eluted and tested for GFAP-inducing activity. Peak activity was found in a slice containing protein with apparent Mr between 22 000 and 25 000 (Fig. 4), although the yield was only about 2-5% of the activity applied to the gel.

Optic nerve extract can act directly on O-2A progenitor cells to induce GFAP expression

To test whether optic nerve extract acts directly on O-2A progenitor cells rather than indirectly via other cells in the optic nerve cultures, single-cell experiments were performed as previously described (Temple & Raff, 1985), except that cells were plated by limiting dilution instead of by micromanipulation. When individual cells from neonatal optic nerve were cultured for 24 h on their own in microwells in 0.5-1% FCS, none of the 43 O-2A progenitor cells (A2B5+ process-bearing cells) tested were GFAP⁺. On the other hand, when optic nerve extract (14 μg ml⁻¹) was added to the microcultures, 12 of 37 progenitor cells tested were GFAP⁺. Although the limiting dilution method does not definitively exclude the possibility that small numbers of undetected cells were present on the walls of the microwells, these experiments strongly suggest that the extract can act directly on O-2A progenitor cells to induce GFAP expression.

Optic nerve extract induces a higher proportion of O-2A progenitor cells in young optic nerves than in older nerves to express GFAP

We have previously provided evidence that the population of O-2A progenitor cells in developing optic nerves of a single age consists of cells at different stages of maturation, suggesting that new O-2A progenitor cells are continually produced during development (Temple & Raff, 1986). Thus one possible explanation for the finding that less than half of the
Table 2. Effect of optic nerve extract on O-2A progenitor cells from rats of different ages

<table>
<thead>
<tr>
<th>Age of rats</th>
<th>Without extract</th>
<th>With extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>E17-18</td>
<td>0</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>P0-1</td>
<td>2 ± 1</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>P7</td>
<td>1 ± 0-5</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

Optic nerve cells from E17-18, P0-1 or P7 rats were cultured for 24 h with or without 3- to 4-week-old optic nerve extract (14-36 μg ml⁻¹). Results are expressed as means ± s.e. of 8, 28 and 7 cultures of E17-18, P0-1 and P7 optic nerves, respectively.

Progenitor cells are more sensitive than mature ones to the GFAP-inducing activity of the extract.

Significantly, in E17–18 cultures, GFAP⁺ type-1 astrocyte precursors (Raff, Abney & Miller, 1984a) were not induced to express GFAP by exposure to optic nerve extract for 24 h (not shown), even though most O-2A progenitor cells in the same cultures were so induced (Table 2).

O-2A progenitor cells in newborn optic nerve are also more sensitive than those in P7 optic nerve to the GFAP-inducing activity in FCS

To determine whether immature progenitor cells are more sensitive than mature ones to the GFAP-inducing activity in FCS, we compared the effects of various concentrations of FCS on progenitor cells in P0 and P7 optic nerve cultures. GFAP induction was assayed after 3 days in culture rather than after 1 day because most batches of FCS were found to act more slowly than optic nerve extract: whereas the extract had an effect at 6 h and reached a peak at 1 day, FCS often had relatively little effect at 1 day and reached a peak at 3 days (Raff et al. 1983). The relatively slow effect of FCS compared to optic nerve extract in inducing GFAP expression seemed not to be due to early-acting inhibitors in FCS since GFAP expression induced at 24 h by extract was not inhibited by the presence of 10% FCS (not shown). As can be seen in Fig. 5, progenitor cells in P0 cultures were more sensitive than those in P7 cultures to the GFAP-inducing activity in FCS, although, as previously described (Raff et al. 1983), in both P0 and P7 cultures, 10% FCS induced the majority of O-2A progenitor cells to express GFAP by 3 days.

Depending on age and culture conditions, GFAP-induction by optic nerve extract can be transient or long-lasting

The GFAP-inducing effect of optic nerve extract differed from that of FCS in another way. While 10%
Fate switching in a glial progenitor cell

Fig. 5. Comparative sensitivity to FCS of O-2A progenitor cells in P0 and P7 optic nerve. Optic nerve cells from P0 (●) or P7 (○) rats were cultured on coverslips in B-S medium containing various concentrations of FCS. After 3 days, the cells were stained and analysed as in Fig. 1. Approximately 40% of the A2B5+ process-bearing cells in P7 cultures seemed to be committed to oligodendrocyte differentiation, since they were GC+ after 3 days in 10% FCS; less than 10% of the A2B5+ process-bearing cells in P0 cultures under these conditions were GC+. Therefore, to make the results comparable at the two ages, they are expressed as a percentage of the A2B5+ process-bearing cells that were GFAP+ after 3 days in 10% FCS, a concentration of FCS that induces a maximum proportion of O-2A progenitor cells to become type-2 astrocytes (Raff et al. 1983). For the P7 cells, 100% represented 58% of total O-2A lineage cells, whereas for the P0 cells 100% represented 92% of total O-2A lineage cells.

FCS caused the majority of O-2A progenitor cells in P0 optic nerve cultures to become bona fide GFAP+, GC− type-2 astrocytes by 3 days in vitro, the progenitors induced by optic nerve extract in such cultures expressed GFAP only transiently, reaching a peak at around 24 h; after this time most of the induced cells apparently either stopped expressing GFAP, possibly in the process of becoming GC+ oligodendrocytes, or died (Fig. 6). This transient effect of optic nerve extract seemed unlikely to be due to inhibitors present in the extract as the active FPLC fraction of the extract gave similar results (not shown). Nor was it due to the lability or degradation of the active molecules contained in the extract because (1) the same result was obtained when fresh extract was added every 6 h and (2) GFAP-inducing activity was still detectable 3 days after a single addition of extract when the culture medium was retested on new P0 optic nerve cultures in a 1-day assay (not shown).

There were two conditions, however, where GFAP induction by optic nerve extract was more long-lasting. First, when E17–18 optic nerve cultures were treated with extract, approximately 25% of the O-2A lineage cells were still GC−GFAP+ after 3 days in culture (Table 3). Second, when P0 optic nerve cells were cultured in the presence of optic nerve extract in low-density microcultures (1–10 cells per well), about 25% of the O-2A lineage cells were GFAP+ after 3 days in vitro (Table 4), and most of these were GC− (not shown). Even in these experiments, however,

Table 3. Effect of optic nerve extract on E18 O-2A progenitor cells after 1 and 3 days in culture

<table>
<thead>
<tr>
<th>% O-2A lineage cells expressing GFAP after 1 day</th>
<th>3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without extract</td>
<td>3 ± 3</td>
</tr>
<tr>
<td>With extract</td>
<td>60 ± 4</td>
</tr>
</tbody>
</table>

E18 optic nerve cells were cultured for 1 or 3 days, with or without 3- to 4-week-old optic nerve extract (14 μg/ml), and then stained with A2B5 and anti-GC antibodies followed by anti-GFAP as in Fig. 6. The results are expressed as means ± s.e. of three separate experiments. When cultures were double labelled with anti-GC and anti-GFAP antibodies after 3 days in optic nerve extract, none of the process-bearing GFAP+ cells were GC+ (not shown).
Table 4. Effect of optic nerve extract on O-2A progenitor cells in low-density microwell cultures of neonatal optic nerve

<table>
<thead>
<tr>
<th>Days in culture</th>
<th>Without extract</th>
<th>With extract</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% (fraction) of A2B5&lt;sup&gt;+&lt;/sup&gt; process-bearing cells expressing GFAP</td>
<td>% (fraction) of A2B5&lt;sup&gt;+&lt;/sup&gt; process-bearing cells expressing GFAP</td>
</tr>
<tr>
<td>1</td>
<td>1 (1/115)</td>
<td>32 (30/94)</td>
</tr>
<tr>
<td>3</td>
<td>2 (2/98)</td>
<td>24 (19/79)</td>
</tr>
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</table>

P0 optic nerve cells were cultured at low density (by plating 15–25 cells per microwell, of which 0–10 adhered to the bottom) for 1 or 3 days, with or without 3- to 4-week-old optic nerve extract (14 μg/ml<sup>−1</sup>). Wells containing O-2A progenitor cells were double labelled with A2B5 and anti-GFAP antibodies as described in Materials and methods. The data from several different experiments are combined. In separate experiments, when wells containing O-2A progenitor cells were double labelled with anti-GC and anti-GFAP antibodies, almost all of the GFAP<sup>+</sup> cells were GC− (not shown).

The amount of GFAP detected in most cells induced by optic nerve extract was substantially less than that in most cells induced by 10% FCS in bulk cultures of P0 optic nerve.

Discussion

Previous in vitro experiments (Raff et al. 1985) suggested that oligodendrocyte differentiation and type-2 astrocyte differentiation in the rat optic nerve are controlled in different ways, even though both cell types are thought to develop from a common progenitor cell (Raff et al. 1983). Oligodendrocyte differentiation seems to be the constitutive pathway of O-2A progenitor cell development, the timing of which depends on a clock that is intrinsic to the progenitor cell (Raff et al. 1985). We proposed (Raff et al. 1985; Temple & Raff, 1986) that the clock may operate by counting cell divisions, which are stimulated by growth factors secreted by type-1 astrocytes (Noble & Murray, 1984; Raff et al. 1985). Type-2 astrocyte differentiation, on the other hand, seems to require inducing factors, which in culture can be supplied by FCS (Raff et al. 1983). To explain the late differentiation of type-2 astrocytes in vivo, we hypothesized that physiological inducing factors first appear in effective concentration in the developing optic nerve in the second postnatal week (Raff et al. 1985). The present results support this hypothesis.

A GFAP-inducing protein in extracts of optic nerve

We have found that crude extracts of 3- to 4-week-old rat optic nerve (a time when large numbers of type-2 astrocytes are thought to be developing in the nerve—Miller et al. 1985) induce O-2A progenitor cells in cultures of perinatal optic nerve to express GFAP within 24 h. Extracts of 1-week-old optic nerve (a time when type-2 astrocyte differentiation has not yet begun in the nerve—Miller et al. 1985) or of 4-week-old retina or adult liver (tissues in which type-2 astrocytes do not develop—C. French-Constant, R. Miller, J. Burne & M. C. Raff, unpublished data) contain at least 50-fold less GFAP-inducing activity than 3- to 4-week-old optic nerve extracts. The inducing activity in 3- to 4-week-old optic nerve extracts is nondialysable, heat-labile and trypsin-sensitive, indicating that it depends on one or more proteins in the extract. When such extracts are fractionated by FPLC on a gel filtration column or by SDS-PAGE, the GFAP-inducing activity is found in a single peak of apparent Mr ~ 25 000, suggesting that a single protein in the extract is probably responsible for the activity. Since the crude extract is active at a concentration of total protein of 300 ng ml<sup>−1</sup> and the polyacrylamide gel slice containing the activity is unlikely to contain more than 10% of the total protein (see Fig. 4), the GFAP-inducing protein probably acts at a concentration of ≈1·2×10<sup>−5</sup> M.

Drug inhibition experiments indicate that extract-induced GFAP expression depends on both RNA and protein synthesis, while single cell experiments suggest that the inducing protein can act directly on the O-2A progenitor cells. The extract does not induce premature GFAP expression in type-1 astrocyte precursors in cultures of embryonic optic nerve, suggesting that the GFAP-inducing protein has some specificity for the O-2A cell lineage.

Optic nerve extracts are not equivalent to FCS in inducing GFAP expression

The GFAP-inducing effect of optic nerve extract differs from that of FCS in a number of ways, suggesting that the active components may be different in the two cases. First, FCS usually acts relatively slowly, reaching a peak at 3 days, while nerve extract acts within 6 h and reaches a peak at 1 day. Second, FCS induces GFAP expression in the great majority of progenitor cells, while nerve extract induces GFAP expression in a much smaller proportion of such cells. Third, when 10% FCS is present in cultures of perinatal optic nerve for 3 days, almost all of the progenitor cells become GFAP<sup>+</sup> type-2 astrocytes (Raff et al. 1984b) while, in the continuous presence of active nerve extract, responsive progenitor cells in neonatal optic nerve cultures express GFAP only transiently and then either lose GFAP (possibly in the course of developing into oligodendrocytes) or die. One possible explanation for the transient effect of the extract may be that the GFAP-inducing protein only initiates type-2 astrocyte differentiation and that other signals, which are not present in optic nerve
extracts, are required for progression to a more mature astrocyte phenotype; insoluble plasma membrane-bound signals, for example, may be required for such progression.

It has been shown previously that type-2 astrocyte differentiation in cultures of optic nerve containing 10% FCS involves at least two steps: an initial reversible induction of GFAP is followed by a stabilization process occurring on the third day in vitro, after which removal of FCS no longer reverses the induction (Raff et al. 1984b). It is uncertain whether a single factor in FCS induces both of these steps or whether separate factors are responsible. Even if separate factors are involved, it seems unlikely that the initiating factor in FCS is the same as that in optic nerve extracts, as required for progression to a more mature astrocyte phenotype; insoluble plasma membrane-bound signals, for example, may be required for such progression.

Although optic nerve extract induces only a transient expression of GFAP in O-2A progenitor cells in bulk cultures of P0 and P7 optic nerve, it has a more long-lasting effect in bulk cultures of E17–18 optic nerve and in low-density microcultures of neonatal optic nerve. Whether the GFAP+ O-2A lineage cells seen after 3 days in such cultures are bona fide type-2 astrocytes comparable to those found in vivo is uncertain. The finding that, for neonatal optic nerve cells, extract-induced GFAP expression is transient in bulk culture and long-lasting in low-density microculture suggests that cell–cell interactions in bulk cultures inhibit type-2 astrocyte differentiation initiated by optic nerve extract, although we cannot exclude the unlikely possibility that a rare population of cells is somehow selected in microculture. These putative inhibitory cell–cell interactions apparently operate less efficiently in bulk cultures of embryonic optic nerve and their role in normal type-2 astrocyte development in vivo remains to be determined.

Do O-2A progenitor cells become less responsive to the GFAP-inducing protein as they mature?

Our finding that only a proportion of O-2A progenitor cells are induced to express GFAP by plateau concentrations of optic nerve extract or by the active FPLC fraction of the extract confirms that the population of progenitor cells in a developing optic nerve is heterogeneous. We have previously shown that O-2A progenitor cells in P1 or P7 nerves are heterogeneous in their response to growth factors secreted by type-1 astrocytes: when individual progenitor cells were cultured in microwells on a monolayer of type-1 astrocytes (in 0-5% FCS), some divided eight times before differentiating into oligodendrocytes, while others divided fewer times, or not at all, before differentiating (Temple & Raff, 1986). We hypothesized that this heterogeneity in proliferative response reflected differences in progenitor cell maturation arising from the continual production of new O-2A progenitor cells and that the least mature cells divided most before differentiating.

It is possible that the heterogeneity of progenitor cells in their responsiveness to optic nerve extract demonstrated in the present study also reflects differences in maturation. The observation that the percentage of progenitor cells that can be induced to express GFAP by extract decreases with age from E17 to P7 is consistent with the possibility that only the least mature progenitor cells are induced by the extract. Moreover, O-2A progenitor cells in cultures of newborn optic nerve are more sensitive than those in cultures of P7 nerve to the GFAP-inducing activity in FCS. These findings are somewhat paradoxical because type-2 astrocyte differentiation in vivo only begins in the second postnatal week (Miller et al. 1985), when the proportion of O-2A progenitor cells that can be induced by optic nerve extract to express GFAP in vitro is reduced compared to that in the nerve at E17. However, if new O-2A progenitor cells are continually produced during CNS development, as we suspect (Temple & Raff, 1986), there would be a continual supply of immature progenitor cells that can be induced to become type-2 astrocytes. There is indirect evidence that new O-2A progenitor cells may be produced at a low rate throughout life (ffrench-Constant & Raff, 1986a), and, as extracts of adult optic nerve have substantial levels of GFAP-inducing activity, it is possible that new type-2 astrocytes may also develop in small numbers throughout life. Controlling both the time at which inducer is made and the time-window during which O-2A progenitor cells are responsive to it could provide for greater flexibility in regulating type-2 astrocyte production than if only one of these factors were controlled. Such a scheme could help explain why oligodendrocyte and type-2 astrocyte differentiation occur simultaneously during the second and third postnatal weeks in the rat optic nerve (Skoff et al. 1976b; Miller et al. 1985): when the level of inducer reaches effective concentration during the second postnatal week, only immature progenitor cells would respond and become type-2 astrocytes, while the more mature progenitor cells would continue to proliferate and develop into oligodendrocytes.

The GFAP-inducing protein may control the timing of type-2 astrocyte differentiation during development

While it seems likely that the induction of type-2 astrocyte differentiation involves multiple steps and factors, in principle the GFAP-inducing protein in optic nerve extracts could be solely responsible for timing the process. The cellular source of this protein remains to be discovered. Recent evidence suggests
that the O-2A cell lineage is specialized for myelination in the mammalian CNS: oligodendrocytes differentiate first and wrap their plasma membrane around axons to form myelin sheaths; then it seems type-2 astrocytes differentiate and extend processes that contact nodes of Ranvier (ffrench-Constant et al. 1986; ffrrench-Constant & Raff, 1986b), where the myelin sheath is interrupted. It would make teleological sense for an initiator of type-2 astrocyte differentiation to be produced only after a sufficient number of mature oligodendrocytes have developed in a region to ensure that all the axons can be myelinated. It would not be surprising, therefore, if mature oligodendrocytes were responsible for either producing the GFAP-inducing protein or triggering its production. The very low levels of GFAP-inducing activity in extracts of 4-week-old rat retina, a non-myelinated part of the rat CNS that does not contain O-2A lineage cells (C. ffrrench-Constant, R. Miller, J. Burne & M. Raff, unpublished data), is consistent with this possibility.

The control of O-2A progenitor cell differentiation apparently follows a general principle that may operate at many binary decision points as cells diversify during development— that one of two potential developmental pathways proceeds constitutively while the other is induced by interactions with other cells. Some of the most enlightening examples of this principle are found in invertebrates, where cell ablation and genetic techniques have greatly facilitated the analysis. In the nematode *C. elegans*, for instance, laser ablation of one of a pair of equivalent bipotential cells causes the remaining cell always to follow one fate (Kimble, 1981); in normal animals whichever cell follows this constitutive pathway induces the other cell to follow the second, alternative, fate. Several such cell pairs are affected by mutations in the lin-12 gene, which encodes an EGF-precursor-like protein (Greenwald, 1985) that is thought to mediate the cell–cell interactions involved in controlling the differentiation of these cells (Greenwald, Sternberg & Horwitz, 1983). Loss of function mutations in this gene are thought to alter cell fates by inactivating the induced pathway of differentiation so that both cells follow the constitutive pathway instead (Greenwald et al. 1983). By analogy, there should be mammalian mutants in which type-2 astrocyte induction fails. Such mutants would be invaluable for establishing the function of these enigmatic glial cells.

We thank Herb Geller for performing crucial pilot experiments, Laura Lillien for an important insight, Mike Mosley for advice, and Jim Cohen, Charles Jennings, Laura Lillien, Ivor Mason, Anne Mudge, Bill Richardson and Rochelle Small for helpful comments on the manuscript. S.M.H. was supported by a grant from the National Fund for Research into Crippling Diseases.

**References**


(Accepted 4 June 1987)