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Type-2 Astrocyte Development in Rat Brain Cultures Is Initiated by a CNTF-like Protein Produced by Type-1 Astrocytes

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Summary

O-2A progenitor cells are bipotential glial precursors that give rise to both oligodendrocytes and type-2 astrocytes on a precise schedule in the rat CNS. Studies in culture suggest that oligodendrocyte differentiation occurs constitutively, while type-2 astrocyte differentiation requires an exogenous inducer such as fetal calf serum. Here we describe a rat brain cell culture system in which type-2 astrocytes develop on schedule in the absence of exogenous inducers. Coincident with type-2-astrocyte development, the cultures produce an ~20 kd type-2-astrocyte-inducing factor(s). Purified cultures of type-1 astrocytes can produce a similar factor(s). Under conditions where they produce type-2-astrocyte-inducing factor(s), both brain and type-1 astrocyte cultures produce a factor(s) with ciliary neurotrophic (CNTF)-like activity. Purified CNTF, like the inducers from brain and type-1 astrocyte cultures, prematurely induces type-2 astrocyte differentiation in brain cultures. These findings suggest that type-2 astrocyte development is initiated by a CNTF-like protein produced by type-1 astrocytes.

Introduction

We have been studying the role of cell–cell interactions in regulating the development of macroglial cells in the rat CNS. There are three major classes of CNS macroglial cells, and they differentiate on a precise schedule in vivo: type-1 astrocytes develop first, beginning at embryonic day 15–16 (E15–E16); oligodendrocytes develop next, beginning around birth; and finally type-2 astrocytes develop, beginning in the second postnatal week (Abney et al., 1981; Miller et al., 1985; Williams et al., 1985). Using antibodies to identify and manipulate glial precursor cells and their differentiated progeny, we have shown that oligodendrocytes and type-2 astrocytes develop in vitro from a common precursor, termed the O-2A progenitor cell, while type-1 astrocytes develop from a different precursor (Raff et al., 1983a, 1983b, 1984).

In vitro studies suggest that the timing and direction of O-2A progenitor cell development are greatly influenced by environmental factors. In cultures of perinatal rat optic nerve cells, for example, which contain all three types of macroglial cells and their precursors but no neurons, the timing of O-2A progenitor cell differentiation into oligodendrocytes has been shown to be regulated by a growth factor secreted by type-1 astrocytes (Noble and Murray, 1984; Raff et al., 1985). The growth factor, recently identified as platelet derived growth factor (PDGF) (Richardson et al., 1988), stimulates the proliferation of O-2A progenitor cells and prevents their premature differentiation into oligodendrocytes. Even in the continuous presence of PDGF, however, progenitor cells eventually stop dividing and differentiate into oligodendrocytes, a sequence precisely timed by a mechanism that appears to be intrinsic to the progenitor cell (Raff et al., 1988). Oligodendrocyte differentiation occurs in optic nerve cultures grown in defined, serum-free medium (Raff et al., 1983b), even in the absence of growth factors reported to promote oligodendrocyte differentiation, such as insulin and IGF-1 (McMorris et al., 1986; Dubois-Dalcq, 1987). Type-2 astrocytes, on the other hand, do not develop in optic nerve cultures grown in defined medium; their differentiation requires an exogenous inducer such as fetal calf serum (FCS) (Raff et al., 1985). These observations suggest that oligodendrocyte differentiation occurs constitutively, whereas type-2 astrocyte differentiation is induced.

To analyze the mechanism by which type-2 astrocyte differentiation is normally induced, we have attempted to reconstitute the normal induction process in vitro. We demonstrate the following: First; type-2 astrocytes can develop on schedule in cultures of perinatal rat brain cells grown without FCS. Second, starting at the time type-2 astrocytes first appear, the cultures produce an ~20 kd protein that induces O-2A progenitor cells to begin to develop into type-2 astrocytes. Third, O-2A progenitor cells can respond to this inducing factor at least 1 week earlier than it would normally appear in the culture medium, suggesting that the production and release of the inducing factor, not the onset of progenitor cell responsiveness to it, are responsible for timing type-2 astrocyte development. Fourth, purified type-1 astrocytes release a similar inducing protein within 24 hr of mechanical injury, suggesting that they may be the source of this factor in brain cell cultures. Fifth, under conditions where they produce factors with type-2-astrocyte-inducing activity, both brain and type-1 astrocyte cultures produce factors that mimic the effect of ciliary neurotrophic factor (CNTF) in supporting the survival of ciliary neurons in vitro. Finally, purified CNTF prematurely induces type-2 astrocyte differentiation in brain cultures. These findings suggest that the timing of type-2 astrocyte differentiation depends on a CNTF-like protein produced

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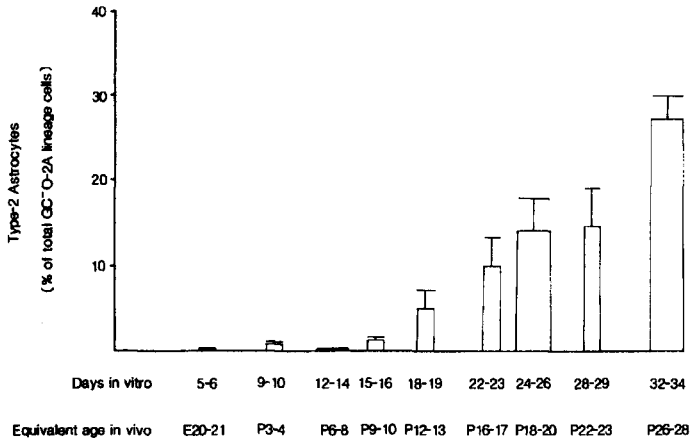

Figure 1. Time Course of Type-2 Astrocyte Development in Cultures of E15 Brain Cells

Type-2 astrocytes were identified as A2B5$^+$, GFAP$^+$, GC$^-$ process-bearing cells. A2B5$^+$, GC$^-$ O-2A lineage cells could readily be distinguished from neurons that were also A2B5$^+$, GC$^-$ process-bearing cells by their distribution and intensity of A2B5 labeling: O-2A lineage cells tended to grow in clusters (probably clones) near the periphery of the cultures, and they stained more intensely with A2B5 antibody than did neurons, which tended to form tight aggregates. These assignments were confirmed in some experiments by staining with the RT97 anti-neurofilament monoclonal antibody. The cultures were grown in defined medium, with or without 0.5% FCS; the addition of 0.5% FCS did not alter the results. The results are expressed as the mean ± SEM of cultures from two to four different platings.

by type-1 astrocytes. The CNTF-like inducing protein apparently only initiates type-2 astrocyte differentiation; however, an additional factor(s) seems to be needed to complete the process.

Results

Type-2 Astrocytes Develop on Time in Brain Cell Cultures in the Absence of FCS

In a previous study, type-2 astrocytes were found to develop on schedule in cultures of early embryonic brain cells grown in 10% FCS but not in serum-free or low serum (0.5%) medium (Williams et al., 1985). In that study, type-2 astrocyte development could only be assessed in cell suspensions prepared from the brain cultures, as the cultures were too dense to permit accurate identification of these cells in the cultures themselves. We have circumvented the density problem by growing brain cells at low density; this was possible only when the low density cultures were sustained by coculture with cultures containing 20-fold more of the same cells.

When brain cells from E15 rats were grown in serum-free defined medium (or defined medium containing 0.5% FCS) using this coculture system, oligodendrocytes, identified by the binding of anti-galactocerebroside (anti-GC) antibody (Raff et al., 1978), first appeared after about a week in vitro, as previously reported (Abney et al., 1981; Williams et al., 1985). This is equivalent to the time of birth. Moreover, type-2 astrocytes also developed in these cultures and could first be seen in significant numbers after 15–18 days (Figure 1), which is the same time they begin to develop in substantial numbers in cultures grown with 10% FCS and is equivalent to the time they begin to develop in vivo (Williams et al., 1985). Type-2 astrocytes (Raff et al., 1983a) were identified as process-bearing cells that were stained on their surface with the A2B5 monoclonal antibody (Eisenbarth et al., 1979) (but not with the anti-GC antibody) and intracellularly with antibodies against glial fibrillary acidic protein (GFAP), an astrocyte-specific marker in the rat CNS (Bignami et al., 1972). The type-2 astrocytes developed in clusters, which could be seen most easily at the periphery of the cultures (Figure 2).

Brain Cultures Produce an ~20 kd Type-2-Astrocyte-Inducing Factor at the Time Type-2 Astrocytes Develop

The development of type-2 astrocytes in the absence of FCS in brain cultures suggested that these cultures might produce an endogenous type-2-astrocyte-inducing factor(s). As shown in Figure 3, beginning at the equivalent of P10–P12 (i.e., after 16–18 days in vitro), cultures of E15 brain released into the medium a diffusible factor(s) that could induce O-2A progenitor cells in neonatal optic nerve cultures to express GFAP. (Type-2-astrocyte-inducing activity was assayed in optic nerve cultures rather than in brain cultures because O-2A lineage cells are more prevalent and more easily identified in neuron-free optic nerve cultures.) The timing of the appearance of this factor(s) thus coincided with the development of type-2 astrocytes in the embryonic brain cultures. The timing was not simply a reflection of time in culture since the diffusible factor(s) appeared after only 6–9 days in cultures prepared from newborn rather than E15 brain (Figure 3). Nor was the timing a reflection of a critical cell density since type-2-astrocyte-inducing activity did not appear earlier in cultures plated at a cell density 3-fold higher than normal (data not shown). Cultures of brain cells continued to release type-2-astrocyte-inducing factor(s) for at least 8 weeks (data not shown).

The time of appearance of the type-2-astrocyte-inducing activity in the culture medium apparently reflected an increase in its production, since extracts of cultures of E15 brain made after 8–12 days in vitro had relatively little inducing activity whereas extracts of older cultures (25–34 days in vitro) had at least 50-fold more activity (Figure 4). The inducing activity in conditioned medium was usually present at limiting concentration: a dilution of even 1:1 resulted in a partial or complete loss of activ-

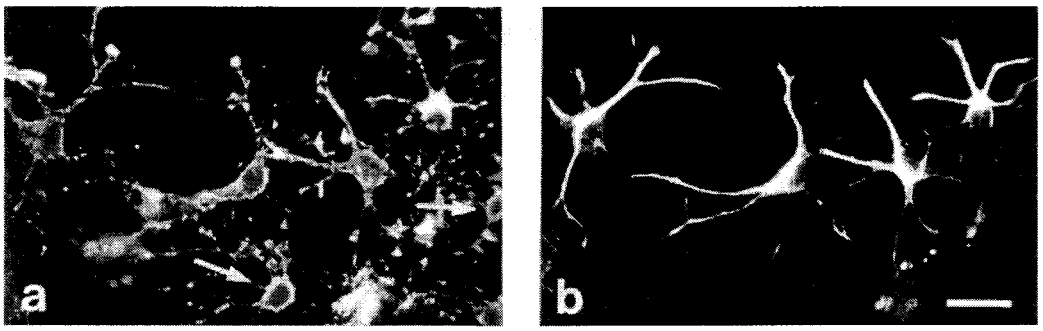

Figure 2. Type-2 Astrocytes in Cultures of E15 Brain Cells after 24 Days In Vitro in Serum-Free Defined Medium

The cultures were stained with A2B5 antibody (followed by G anti-MIgRd) and, after fixation in acid-alcohol, with anti-GFAP antiserum (followed by Sh anti-RIgFl). They were photographed with rhodamine (a) and fluorescein (b) optics. There are four type-2 astrocytes ($A2B5^+$, $GFAP^+$) and two O-2A progenitor cells ($A2B5^+$, $GFAP^-$) (arrows) in the field. Bar, 25 μm.

ity. Extracts of older cultures contained at least 50-fold more inducing activity per ml than conditioned medium (CM) from the same cultures. Even high concentrations of extracts, however, induced only 20%–35% of the O-2A progenitor cells in optic nerve cultures to express GFAP, which was the same proportion induced by CM.

Treatment of CM or extracts of older brain cultures with trypsin completely destroyed the GFAP-inducing activity (data not shown), suggesting that the inducing factor(s) is a protein(s). To determine the approximate size of the protein(s), extracts of older cultures (E15 + 34 days in vitro) were fractionated by fast protein liquid chromatography (FPLC) on a Superose 12 gel filtration column. Type-2-astrocyte-inducing activity was found in a single peak centered at about 20 kd (Figure 5a). No inducing activity could be detected in any fractions prepared from extracts of younger cultures (E15 + 12 days in vitro), suggesting that the absence of inducing activity was not due to the presence of a smaller or larger inhibitory molecule.

Cultures of Type-1 Astrocytes Release an ~20 kd Type-2-Astrocyte-Inducing Protein When They Are Injured

To identify the cellular source of the type-2-astrocyte-inducing factor(s), we tested media conditioned by several types of cultures, including cultures of newborn retina (which contains neurons, Müller cells, and some type-1 astrocytes, but no O-2A lineage cells) (ffrench-Constant et al., 1988) and cultures enriched for oligoden-

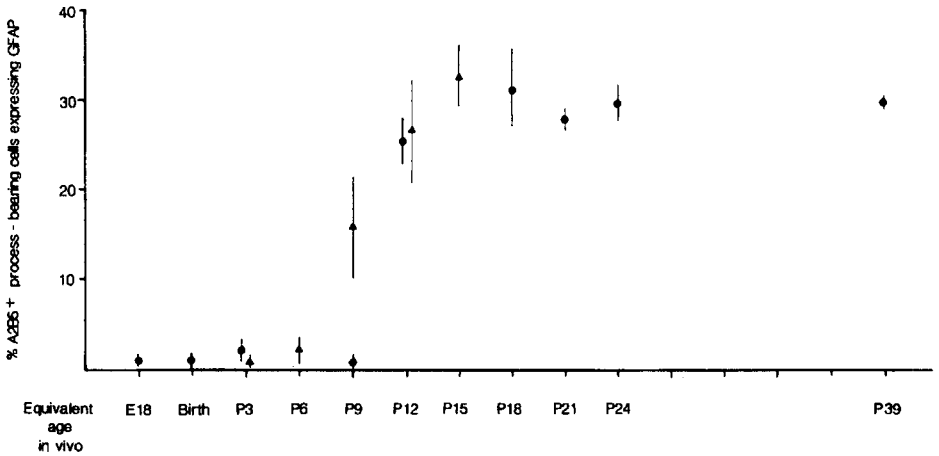

Figure 3. The Time Course of Appearance of Type-2-Astrocyte-Inducing Activity in Conditioned Medium (CM) from E15 and Newborn Brain Cell Cultures

CM was collected at 3 day intervals and tested on cultures of newborn optic nerve cells for type-2-astrocyte-inducing activity. The optic nerve cultures were grown in undiluted CM for 1 day and then fixed and stained with A2B5 and anti-GFAP antibodies as in Figure 2. The proportion of $A2B5^+$ process-bearing cells expressing GFAP was determined. The E15 brain cultures (●) were grown in defined medium, with or without 0.5% FCS, while the newborn brain cultures (▲) were grown in defined medium containing 0.5% FCS. The presence of 0.5% FCS did not alter the results. At least 100 $A2B5^+$ process-bearing cells were counted per coverslip, and the results are expressed as the mean ± SEM of three to four coverslips.

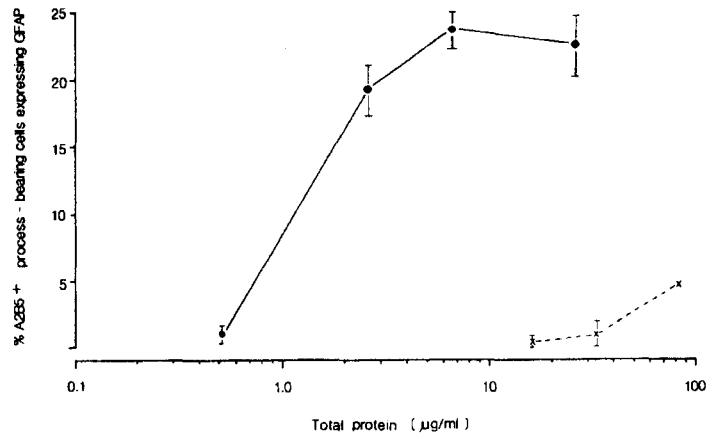

Figure 4. Type-2-Astrocyte-Inducing Activity in Extracts of Young and Old Brain Cell Cultures

Extracts of E15 brain cell cultures after 8 days (x) or 34 days (●) in vitro were assayed using cultures of newborn optic nerve cells as in Figure 3. Results are expressed as the mean ± SEM of two (E15 + 8) or three (E15 + 34) cultures. Similar results were obtained with extracts of several other young and old brain cultures.

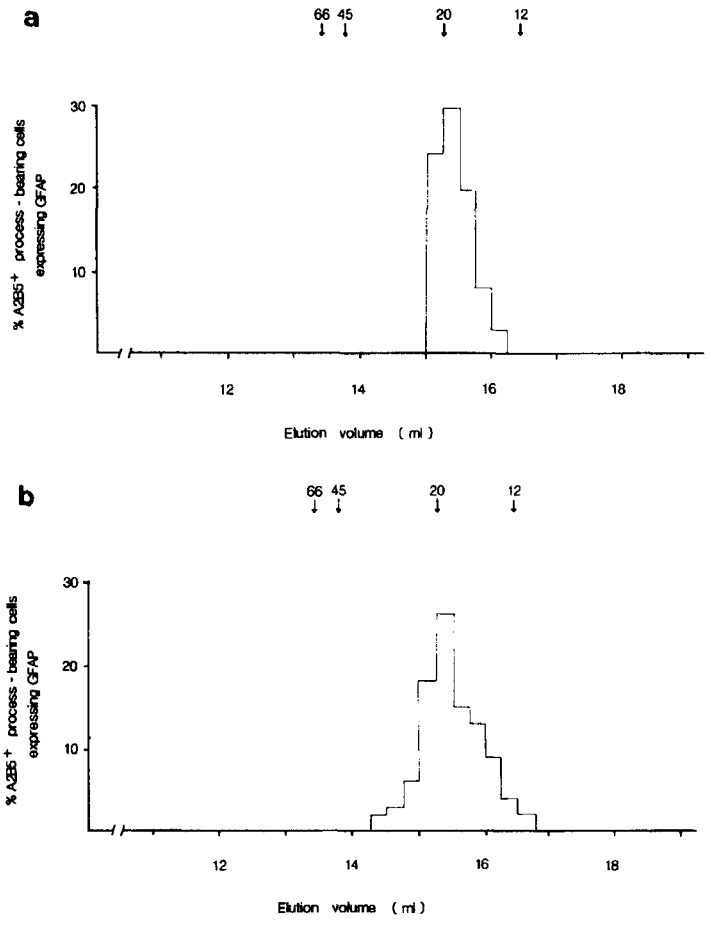

Figure 5. FPLC Gel Filtration Fractionation of Extracts of E15 Brain Cell Cultures after 34 Days In Vitro and CM from Injured Type-1 Astrocyte Cultures

(a) Brain culture extracts (100 µl; approximately 250 µg total protein) and (b) concentrated astrocyte CM (100 µl) were separated into 0.25 ml fractions, diluted 1:10, sterilized by filtration, and assayed for type-2-astrocyte-inducing activity using newborn optic nerve cultures as in Figure 3. Arrows indicate the positions of size markers: BSA (66 kd), ovalbumin (45 kd), soybean trypsin inhibitor (20 kd), and cytochrome C (12 kd).

drocytes, type-1 astrocytes, or meningeal cells. As shown in Table 1, none of these cultures released type-2-astrocyte-inducing factor(s). Following mechanical injury (scratching the monolayer or passaging the cells), however, type-1 astrocyte cultures, but none of the other types of cultures, released a factor(s) with type-2-astrocyte-inducing activity. Like the inducing activity produced by older brain cell cultures, medium conditioned by injured

Table 1. Cellular Source of Type-2-Astrocyte-Inducing Factor(s)

CM from Cultures of		% of A2B5$^+$ Process-Bearing Cells Expressing GFAP
Type-1 astrocytes	–uninjured	0.5 ± 0.1
	–injured	27.7 ± 2.7
Meningeal cells	–uninjured	0.1 ± 0.05
	–injured	0.07 ± 0.03
Retina	–uninjured	0.03 ± 0.03
	–injured	0
Oligodendrocytes	–uninjured	0.6 ± 0.2
	–injured	0.3 ± 0.3

Newborn optic nerve cells were either cultured for 1 day in CM from type-1 astrocytes or meningeal cells, or cocultured on separate coverslips with these cells, retinal cells, or optic nerve oligodendrocytes. The induction of GFAP was assayed as in Figure 3. The results are expressed as the mean ± SEM of at least three coverslips.

Table 2. O-2A Progenitor Cells in Brain Cultures Respond Prematurely to Type-2-Astrocyte-Inducing Factors

Additive	Total Number of A2B4$^+$, GFAP$^+$ Process-Bearing Cells per Culture
None	6.0 ± 2.2
Extract of E15 brain culture after 25 days in vitro (42 μg/ml)	189.2 ± 28.3
Medium condition by injured type-1 astrocyte culture (diluted 1:50)	211.5 ± 3.7
CNTF (3 ng/ml)	255.7 ± 17.9
CNTF (3 ng/ml) + Extract of E15 brain culture after 25 days in vitro (42 μg/ml) + Medium conditioned by injured type-1 astrocyte culture (diluted 1:50)	220.3 ± 23.9

Newborn brain cells (20,000) were cultured on poly-D-lysine-coated coverslips in the additives indicated for 1 day and then stained with A2B5 and anti-GFAP antibodies as described in Figure 2. The results are expressed as the mean ± SEM of four coverslips.

type-1 astrocytes, and extracts made from such injured cells, induced only 20%–35% of optic nerve O-2A progenitor cells to express GFAP. The inducing factors produced by brain and injured type-1 astrocyte cultures were not additive or synergistic when applied together at optimal concentrations (data not shown).

We concentrated medium conditioned by injured type-1 astrocyte cultures and fractionated it by FPLC on a Superose 12 gel filtration column. As shown in Figure 5b, the type-2-astrocyte-inducing activity was found in a single broad peak centered at approximately 20 kd. Extracts prepared from these cultures contained a similar single peak of inducing activity (data not shown).

O-2A Progenitor Cells Are Prematurely Responsive to Type-2-Astrocyte-Inducing Factors

In the experiments described above, CM and culture extracts were tested for type-2-astrocyte-inducing activity using O-2A progenitor cells in cultures of newborn optic nerve. The results indicated that these progenitor cells can respond to the type-2-astrocyte inducer(s) at least 1 week before type-2 astrocytes normally begin to develop. To determine whether O-2A progenitor cells in brain cultures, which contain neurons, are also prematurely responsive to type-2-astrocyte-inducing factors, we exposed cultures of newborn brain cells to inducing factor(s) from either old brain cultures or injured type-1 astrocyte cultures. In both cases many O-2A progenitor cells were prematurely induced to express GFAP within 24 hr (Table 2).

Brain and Astrocyte Cultures Produce a Factor(s) with CNTF-like Activity, and Pure CNTF Induces Type-2 Astrocyte Differentiation in Brain Cultures

It has been reported that type-1 astrocyte cultures release a number of growth-promoting factors following mechanical injury (Rudge et al., 1985). One of these factors is thought to be CNTF (Manthorpe et al., 1986a), which is similar in size (Barbin et al., 1984; Carnow et al., 1985; Manthorpe et al., 1986b) to the type-2-astrocyte-inducing factor(s) produced by cultures of brain cells and injured type-1 astrocytes. Ion-exchange chromatography of medium conditioned by injured type-1 astrocytes and extracts of brain cultures indicated that, like CNTF (Barbin et al., 1984; Manthorpe et al., 1986b), these inducing factors were acidic (unpublished observations). When pure CNTF was added to cultures of newborn brain cells, it mimicked (but did not increase) the effect of type-2-astrocyte-inducing factors from brain and type-1 astrocyte cultures, inducing the premature expression of GFAP in a substantial number of A2B5$^+$ process-bearing cells (Table 2; Figure 6).

If the type-2-astrocyte-inducing factor(s) produced by injured type-1 astrocytes is CNTF, then extracts of, or medium conditioned by, such cells should also support the survival of ciliary neurons in vitro, as previously reported (Rudge et al., 1985). This was found to be the case, and the dose–response curves for type-2 astrocyte induction and ciliary neuron survival were very similar (data not shown). Moreover, extracts of old brain cultures (E15 + 25–34 days in vitro) supported the survival of ciliary neurons in culture, while extracts of young brain cultures (E15 + 8–12 days in vitro), which had little type-2-astrocyte-inducing activity, had little CNTF activity (Figure 7); in both cases, the dose–response curves for type-2 astrocyte induction and neuronal survival were similar (compare Figure 4 and Figure 7).

More than One Factor Seems to Be Required for Type-2 Astrocyte Development

When type-2-astrocyte-inducing factor(s) from cultures of brain cells or injured type-1 astrocytes was added to newborn optic nerve cell cultures, A2B5$^+$, GFAP$^+$ pro-

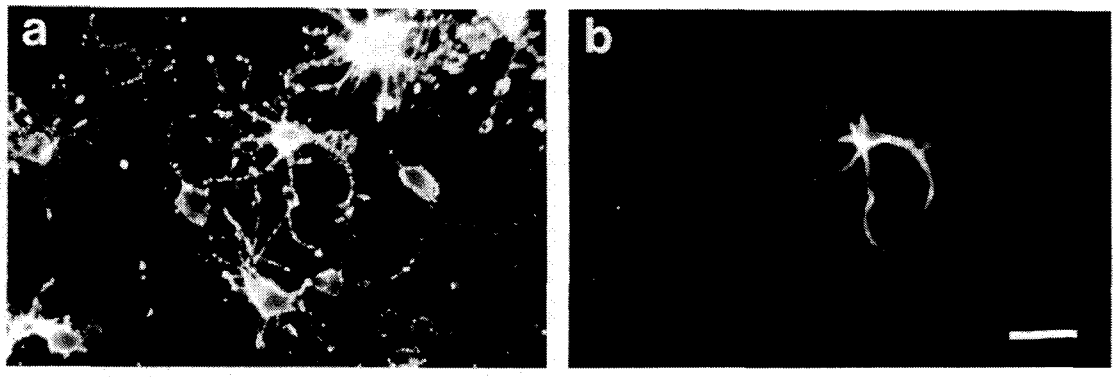

Figure 6. Premature Induction of a Type-2 Astrocyte by CNTF in a Culture of Newborn Brain Cells

The cells were cultured with CNTF for 1 day as described in Table 2, stained with A2B5 (a) and anti-GFAP (b) antibodies, and photographed with rhodamine and fluorescein optics. Bar, 25 μm.

cess-bearing cells, characteristic of type-2 astrocytes, were seen within 24 hr; after 3 days, however, few if any such cells remained (data not shown). The same results were obtained when the inducing factor(s) was added daily or when the optic nerve cells were cocultured with injured type-1 astrocyte cultures to ensure continuous exposure to the inducer. Preliminary experiments suggest that the cells in optic nerve cultures which are induced to express GFAP in response to these inducers subsequently develop into oligodendrocytes (L. E. L. and M. C. R., unpublished data). It seems that this inducing factor(s) can initiate type-2 astrocyte differentiation, but other signals, perhaps mediated by cell–cell contact, are apparently required to induce stable expression of the type-2 astrocyte phenotype.

Discussion

In this report we have provided evidence that the differentiation of type-2 astrocytes in brain cultures depends on the production of an endogenous inducing factor similar to CNTF. In this culture system the population of O-2A progenitor cells differentiates on the same schedule in vitro as in vivo, giving rise initially to oligodendrocytes and then to type-2 astrocytes. The advantage of this culture system over a similar one described previously (Williams et al., 1985) is that here type-2 astrocytes could be observed to develop in the absence of FCS, which itself induces type-2 astrocyte differentiation in cultures of optic nerve cells (Raff et al., 1983b). It was this feature that enabled us to demonstrate and characterize the type-2-astrocyte-inducing factor(s).

Type-2-Astrocyte-Inducing Factors Are CNTF-like Proteins

Hughes and Raff (1987) recently described a 20–25 kd protein in extracts of 3–4 week old rat optic nerve that induces O-2A progenitor cells in cultures of newborn optic nerve to express GFAP. Several lines of evidence suggest that the type-2-astrocyte-inducing factor(s) produced by brain cell cultures at the time type-2 astrocytes begin to develop is the same or very similar to the factor in optic nerve extracts. Both factors elute in the same position when fractionated by FPLC on a Superose 12 gel

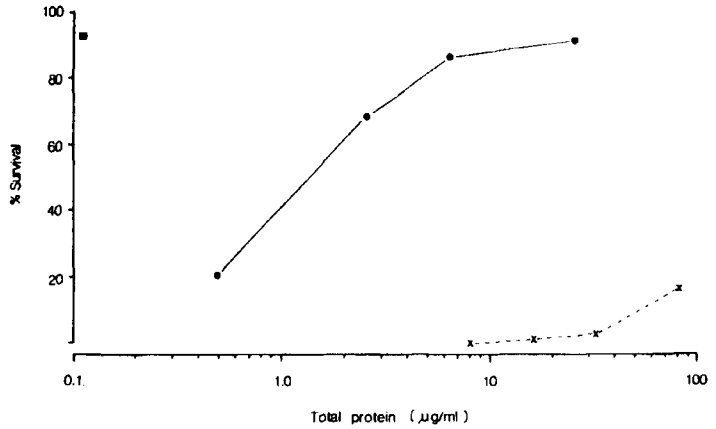

Figure 7. CNTF activity in Extracts of Old and Young Brain Cell Cultures

Old cultures (●) consisted of E15 + 34 days in vitro cells; young cultures (x) consisted of E15 + 8 days in vitro cells. The same extracts that were assayed for type-2-astrocyte-inducing activity in Figure 4 were tested for their ability to support the survival of E8 chick ciliary neurons for 1 day in culture. The results are expressed as the proportion of neurons present after 24 hr compared with the number counted 2 hr after plating. CNTF (5 ng/ml) was used as a positive control (■).

filtration column (this study; Hughes and Raff, 1987). Both factors induce only 20%–35% of the O-2A progenitor cells in newborn optic nerve cultures to express GFAP (this study; Hughes and Raff, 1987), and their effects are not additive or synergistic (unpublished data); in both cases the induction of GFAP is transient.

The results presented here and elsewhere (S. M. H., L. E. L., M. C. R., H. R., and M. S., submitted) suggest that the type-2-astrocyte-inducing protein(s) found in cultures of brain cells and in extracts of optic nerve is related, if not identical, to CNTF. CNTF was originally discovered as an activity in heart cell–conditioned medium that promotes the survival of chick ciliary ganglion neurons in vitro (Helfand et al., 1976). Subsequently characterized as a 20–25 kd acidic protein (Barbin et al., 1984; Carnow et al., 1985; Manthorpe et al., 1986b) that promotes the in vitro survival of several types of peripheral neurons, it has been found in a variety of tissues (Ebendal, 1987), including brain (Carnow et al., 1985; Nieto-Sampedro et al., 1983), and has been purified from chick eye (Barbin et al., 1984) and rat sciatic nerve (Manthorpe et al., 1986b). At least three lines of evidence suggest that the type-2-astrocyte-inducing protein(s) is the same or similar to CNTF. First, it has similar chemical properties to CNTF. Second, CNTF purified from rat sciatic nerve is a potent inducer of type-2 astrocyte differentiation in cultures of optic nerve cells (S. M. H. et al., submitted) and brain cells (this study). Third, extracts of brain cultures (this study) and optic nerve (S. M. H. et al., submitted) support ciliary neuron survival in vitro at concentrations that correlate well with their ability to induce type-2 astrocyte differentiation.

The Timing of Type-2 Astrocyte Development Depends on the Production and/or Secretion of the CNTF-like Protein(s)

The timing of type-2 astrocyte development both in vitro and in vivo appears to depend only on the timed production and release of the CNTF-like protein(s) and not on the onset of O-2A progenitor cell responsiveness to it. In cultures of embryonic brain cells, both the large increase in production and the release of type-2-astrocyte-inducing factor(s) coincide with the development of type-2-astrocytes. Similarly, the concentration of inducing factor in extracts of optic nerve increases 20- to 50-fold between the first postnatal week, before type-2 astrocyte development has begun, and the third postnatal week, when their development is well under way (Miller et al., 1985; Hughes and Raff, 1987). The ability of extracts of brain cultures (this study) and optic nerve (S. M. H. et al., submitted) to support ciliary neuron survival shows a similar developmental time course. By contrast, O-2A progenitor cells in cultures of brain (this study) and optic nerve (Hughes and Raff, 1987; S. M. H. et al., submitted) are responsive to the type-2-astrocyte-inducing factor(s) at least 1 week before type-2-astrocyte development normally begins.

This timing mechanism differs from some other developing systems in which the regulation of timing has been studied. For example, Davies et al. (1987) have studied the timing of both NGF production and responsiveness to NGF during the development of sensory innervation to mouse whisker pads. In this case, the production of NGF and the responsiveness of sensory neurons to NGF are similarly timed, and responsiveness to NGF is not induced prematurely by exposure to NGF in vitro.

In cultures of brain cells, large scale production of type-2 astrocytes began at the equivalent of P10–P12. Not all of the O-2A progenitor cells in the cultures, however, differentiated into type-2 astrocytes at this time, despite the presence of a diffusible type-2-astrocyte-inducing factor(s) in the culture medium. Similarly, not all O-2A progenitor cells in cultures of newborn optic nerve respond to the type-2-astrocyte-inducing factor(s), and no obvious difference distinguishes the 20%–35% of the cells that respond from those that do not. Preliminary experiments (L. E. L. and M. C. R., unpublished data) suggest that the timing of O-2A progenitor cell responsiveness to the inducing factor(s) may depend on the same intrinsic timing mechanism that controls oligodendrocyte differentiation (Raff et al., 1985; Temple and Raff, 1986); whether a progenitor cell becomes a type-2 astrocyte or an oligodendrocyte when "time" is reached would then depend on whether or not a sufficient concentration of inducer protein is available.

Type-1 Astrocytes in Culture Can Produce a CNTF-like Protein

Which cells produce the type-2-astrocyte-inducing factor(s)? We found that cultures of purified type-1 astrocytes produced such a factor(s) when the cells were mechanically injured. The factor(s) was around 20 kd and was indistinguishable from the factor(s) derived from brain cultures or optic nerve in its ability to initiate type-2 astrocyte differentiation. Cultures of retinal cells, meningeal cells, or optic nerve oligodendrocytes, by contrast, did not produce such a factor(s) when injured. A CNTF-like molecule has been previously shown to be produced by cultures of purified (type-1) astrocytes following mechanical injury (Rudge et al., 1985). These findings suggest that type-1 astrocytes may be the source of the CNTF-like factor(s) that initiates type-2 astrocyte development.

In the absence of injury, the production and/or release of this factor(s) by type-1 astrocytes might require their interaction with another type of cell. In preliminary experiments we have studied the development of type-2 astrocytes in PDGF-containing, serum-free cultures of optic nerve cells, which were enriched for both O-2A progenitor cells and oligodendrocytes but contained few type-1 astrocytes. Very few type-2 astrocytes develop under these conditions unless the cultures are combined with established cultures enriched for type-1 astrocytes (L. E. L. and M. C. R., unpublished data). This finding suggests that, even in the absence of injury, type-1 astrocytes may be required for the development of type-2 astrocytes and that the production and/or release of the CNTF-like protein may require an interaction between type-1 astrocytes, O-2A progenitor cells, and/or oligodendrocytes.

Other Signals May Be Required to Generate Stable Type-2 Astrocytes

The CNTF-like proteins(s) produced by brain and astrocyte cultures and found in optic nerve extracts is apparently able to initiate type-2 astrocyte differentiation but is not able to drive the process to completion. Even when optic nerve cultures are exposed to it continuously, the factor(s) induces O-2A progenitor cells to express GFAP only transiently. The transient nature of the type-2 astrocytes induced by this factor in optic nerve cultures does not reflect the inability of optic nerve O-2A progenitor cells to develop into stable type-2 astrocytes in culture in the absence of FCS. We have recently found that stable type-2 astrocytes can develop in serum-free cultures of newborn optic nerve cells grown with PDGF (although PDGF itself neither induces nor stabilizes the development of type-2 astrocytes) (L. E. L. and M. C. R., unpublished data). Apparently other signals in addition to the CNTF-like protein(s) are required to generate a stable type-2 astrocyte.

Our results provide strong support for the hypothesis that type-2 astrocyte differentiation is initiated by a CNTF-like protein that is produced relatively late in development. They also raise a number of questions. If type-1 astrocytes are the normal source of the CNTF-like protein that initiates type-2 astrocyte development, what regulates the timing of the protein's production? What determines whether an individual O-2A progenitor cell responds to this inducer? What is the nature and the cellular source(s) of the additional signals apparently required to stabilize the type-2 astrocyte phenotype and how is the production of these signals controlled? The ability to reconstitute in vitro the control mechanisms responsible for timing type-2 astrocyte development is an important step toward answering these and other questions concerning the regulation of O-2A progenitor cell differentiation.

Experimental Procedures

Animals

Timed pregnant Sprague-Dawley rats were obtained from the breeding colony of the Imperial Cancer Research Fund. The age of embryos was confirmed by crown-rump length (Angulo Y Gonzales, 1932) and paw morphology (Long and Burlingame, 1938). Newborn rats used for brain and optic nerve cultures were less than 24 hr old.

Materials

FCS (Myoclone) was purchased from GIBCO. All other tissue culture reagents and molecular weight markers were purchased from Sigma. Plastic-ware was purchased from Falcon, while fluorescent conjugates were purchased from Wellcome (sheep anti-rabbit Ig-fluorescein is Sh anti-Rlg-Fl), Cappel (goat anti-mouse Ig-rhodamine is G anti-Mlg-Rd), and Nordic (goat anti-mouse IgG3-fluorescein is G anti-MIgG3-Fl).

Cell Cultures

Embryonic Brain

Brains from E15 embryos were dissected free of meninges and cut into small pieces. After two 15 min incubations in trypsin (1 mg/ml, type III), BSA (5 mg/ml, fraction V, fatty acid-free), and collagenase (0.2 mg/ml, type V), the tissue was dissociated by trituration in soybean trypsin inhibitor (50 μg/ml) and deoxyribonuclease (40 μg/ml, type IV) with a Pasteur pipette, followed by passage through a 23 guage needle on a 2 ml syringe. The cell suspension was filtered through nylon mesh, washed twice in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS (DMEM-FCS), and plated on glass coverslips in the wells of a 24-well culture dish or in 25 cm^2 culture flasks, all of which were coated with poly-D-lysine (10 μg/ml).

Low density cultures of brain cells (10^4 cells) died when cultured on their own. To maintain such cultures, the coverslips were grown in wells with 2×10^5 cells prepared at the same time. Between 2×10^5 and 2.5×10^5 cells in 100 μl of culture medium were plated in a ring around the edge of the plastic well, and 10^4 cells in 10 μl of medium were plated on a coverslip. After the cells had attached (0.5 hr), 0.5 ml of culture medium was added to the wells; glass chips made from microscope slides were placed in the center of each well, and the coverslip bearing the 10^4 cells were placed in the well on top of the chips.

E15 brain cells were initially cultured in DMEM-FCS. After 1 day, this was replaced with defined medium modified from Bottenstein and Sato (1979), which contained glucose (5.6 mg/ml), bovine insulin (5 μg/ml), human transferrin (100 μg/ml), BSA (100 μg/ml), progesterone (0.06 ng/ml), putrescine (16 μg/ml), selenium (40 ng/ml), thyroxine (40 ng/ml), and tri-iodothyronine (30 ng/ml). Cultures were maintained in defined medium for over 2 months and were fed every 3 days by replacing approximately half of the medium. In some cases 0.5% FCS was added to the defined medium, particularly for cultures grown in flasks where 3×10^6 to 1×10^7 cells were initially plated. The timing of type-2 astrocyte development and of the production of type-2-astrocyte-inducing factor(s) was identical with or without 0.5% FCS, but brain cells survived better in flasks containing 0.5% FCS.

Newborn Brain

Cultures were prepared as described above except that the incubation times in trypsin and collagenase were extended to 30 min, tissue was triturated with a Pasteur pipette only, and cultures were grown from the start in defined medium containing 0.5% FCS and maintained in this medium for over 1 month. Neurons (identified with the monoclonal antibody RT97, which recognizes neurofilament proteins [Wood and Anderton, 1981]) were present in the cultures throughout this period.

Optic Nerve

Cultures were prepared as previously described (Miller et al., 1985) except that pieces of optic nerve were incubated in trypsin plus collagenase for 30 min without EDTA and then for 15 min with EDTA, and 5000 cells in 15 μl of medium were cultured per coverslip.

Type-1 Astrocytes

Cultures were prepared as previously described (Noble and Murray, 1984) except that, after shaking the cultures to remove the top layer of cells, the cultures were treated twice with cytosine arabinoside (10^{-5} M)—once while in 10% FCS (to reduce the number of fibroblast-like cells) and again after switching the cultures to defined medium (to reduce the number of O-2A progenitor cells). Cultures were grown in serum-free defined medium for at least 2 weeks before use. More than 98% of the cells in the resulting cultures were $A2B5^-$, $GFAP^+$ astrocytes; the remaining cells were $A2B5^-$, $GFAP^-$ fibroblast-like cells. Cells were passaged by removing them from flasks with trypsin (0.25 mg/ml) and EDTA (0.2 mg/ml).

Meningeal Cells

Meninges from newborn rat brain were dissociated in trypsin (1 mg/ml) and grown in DMEM-FCS. To eliminate type-1 astrocytes, the cultures were passaged 2–3 times and maintained in DMEM-FCS until use.

Oligodendrocyte-Enriched Optic Nerve Cells

Cultures were prepared from P7–P8 rat optic nerve as described above: 2×10^4 to 4×10^4 cells were plated on poly-D-lysine-coated coverslips in serum-free defined medium, and after 2 days, they were treated with cytosine arabinoside in 0.5% FCS for 3 days. The cells were then maintained in serum-free defined medium until use, after a total of 10–14 days in vitro, when 90%–95% of the cells were GC^+ oligodendrocytes (Raff et al., 1978); the remaining cells were $A2B5^-$, $GFAP^+$ type-1 astrocytes and $A2B5^-$, $GFAP^-$ fibroblast-like cells.

Retina

Newborn rat retina was prepared as previously described, using

only the peripheral half of the retina in order to reduce the number of type-1 astrocytes (Watanabe and Raff, 1988): 2×10^5 cells were plated on poly-D-lysine-coated glass coverslips and grown in defined medium containing 0.5% FCS for up to 3 weeks.

Assay of Type-2-Astrocyte-Inducing Activity

CM, culture extracts, or purified CNTF was added to cultures of newborn optic nerve (5000 cells) or newborn brain (2×10^4 cells) 30–45 min after the cells were plated. Between 20 and 24 hr later, coverslips were fixed and stained with A2B5 and anti-GFAP antibodies as described below. The number or proportion of brightly stained $A2B5^+$ process-bearing cells that expressed GFAP was determined. Some type-1 astrocytes were weakly stained with A2B5 and were not counted.

Assay for CNTF Activity

Ciliary ganglion neurons were prepared from E8 chick embryos and cultured at a density of 1×10^3 to 2×10^3 cells per well in multiwell dishes (Costar, 16 mm) in F14 medium with 10% horse serum as previously described (Rohrer and Thoenen, 1978), except that the culture dishes were coated with laminin (BRL, 2 μg/500 μl per well) instead of heart-conditioned medium. The number of neurons initially plated per well was determined by counting the neurons 2 hr after plating. After 24 hr, the number of surviving neurons was counted.

Immunofluorescence

Brain and optic nerve cultures on coverslips were fixed in 4% paraformaldehyde at room temperature for 5 min and immunostained as previously described. To triple stain brain cultures, the cells were stained with monoclonal anti-GC antibody (Ranscht et al., 1982) (ascites, 1:500) followed by class-specific G anti-MIgG3-Fl (1:100) and then with A2B5 monoclonal antibody (Eisenbarth et al., 1979) (ascites, 1:500) followed by G anti-Mlg-Rd (1:100); after fixation with acid–alcohol at −20°C for 10 min, cultures were stained with rabbit anti-GFAP antiserum (Pruss, 1979) (1:100) followed by Sh anti-Rlg-Fl (1:100). All antibodies were diluted in HEPES-buffered MEM (MEM-HEPES) containing 10% FCS.

Coverslips were mounted in Citifluor and examined with a Zeiss Universal fluorescence microscope using a 63× objective. Cultures were photographed using either Ektachrome or Tri-X film rated 400 ASA.

Conditioned Medium

CM from brain cultures was collected at 3 day intervals, centrifuged to remove debris and dead cells, and stored at −20°C until used. In some assays CM was used fresh because freezing was found to reduce type-2-astrocyte-inducing activity. CM from type-1 astrocytes, oligodendrocytes, retina, and meningeal cells was either collected as described for brain culture CM or assayed by coculturing the optic nerve assay cultures on top of the CM-producing cells, separated by glass chips. Cultures were injured by passaging cells (using trypsin to remove cells from flasks) or scratched by drawing a plastic micropipette tip twice across a culture of cells on a coverslip.

Concentrated Conditioned Medium

Phenylmethylsulfonyl fluoride (1 mM) was added to 30–40 ml of CM from type-1 astrocyte cultures (3×10^6 to 6×10^6 cells) collected 1 day after passaging. After centrifugation, the CM was concentrated 40-fold by putting it in Spectropor dialysis tubing (6–8 kd cut off) and placing the tubing in polyethylene glycol (20 kd, BDH) for 3–4 hr at 4°C. Concentrated CM was dialyzed against DMEM overnight at 4°C, sterilized by filtering through a 0.2 μm Millipore filter, and stored at −70°C.

Culture Extracts

Extracts of brain and injured type-1 astrocyte cultures were prepared by homogenizing cells scraped from flasks in cold Ca^{2+}- and Mg^{2+}-free phosphate buffered saline containing 1 mM phenylmethylsulfonyl floride. Homogenates were contrifuged for 10 min at 4°C in an MSE Microcentaur and at 80,000 × g for 30 min at 4°C in an Airfuge (Beckman) to remove particulate material. Supernatants were dialyzed against DMEM overnight at 4°C. Protein concentrations were determined using a Bio-Rad kit with IgG as a standard. Extracts were stored at −70°C.

Gel Filtration

Concentrated CM and culture extracts (150–250 μg/ml total protein) were loaded in a volume of 0.1 ml and run at 0.3 ml/min at room temperature in DMEM containing 0.5 mg/ml BSA (fraction V, fatty acid–free) on a Superose 12 column linked to an LCC-500 FPLC system (Pharmacia); 0.25 ml fractions were collected for assay. BSA, ovalbumin, soybean trypsin inhibitor, and cytochrome c were used at 2–7 mg/ml as size markers.

Purification of CNTF

CNTF was purified from adult Wistar rat sciatic nerves by a modification of the method of Manthorpe et al. (1986b) using DEAE ion-exchange chromatography and preparative SDS-PAGE. The purified CNTF gave a single silver-stained band when analyzed by SDS-PAGE (S. M. H. et al., submitted) or isoelectric focusing (M. S., unpublished data).

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Note Added in Proof

The paper referred to as S. M. H. et al., submitted, is now in press (Hughes, S. M., Lillien, L. E., Raff, M. C., Rohrer, H., and Sendtner, M. [1988]. Ciliary neurotrophic factor (CNTF) induces type-2 astrocyte differentiation in culture. Nature).