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Murine oligodendroglial cells express nerve growth factor

(brain-derived neurotrophic factor/neurotrophic factors/oligodendrocyte/immortalized cell line)

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ABSTRACT **The studies reported here present evidence for the expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) by an oligodendroglial cell line and of NGF by oligodendrocytes in mouse primary culture. An immortalized oligodendroglial cell line (N19) expressing markers for immature oligodendrocytes stimulated PC12 cells to elaborate processes. Polymerase chain reaction analysis with degenerate primers indicated that the N19 cells expressed the mRNAs for the neurotrophic factors NGF and BDNF. Northern blot analysis confirmed that the N19 cells expressed the 1.3-kb NGF mRNA and the 1.4- and 4-kb BDNF mRNAs. *In situ* hybridization histochemistry identified the presence of NGF mRNAs in 9-day primary oligodendroglial cultures. Combined immunocytochemistry and *in situ* hybridization histochemistry colocalized NGF mRNA within primary cultured cells that immunostained for the oligodendrocyte marker galactocerebroside (GC). Double-immunofluorescence analysis also colocalized NGF protein within GC^+ cells and within $A_2B_5^+$ cells, a marker for oligodendrocyte progenitors. These results show that oligodendroglia and their precursor cells can express the neurotrophic factor NGF. They suggest that cells in the oligodendrocyte lineage may play an active role in neurite extension through fiber tracts in addition to myelination.**

Neuronal survival and differentiation are regulated by many endogenous and target-derived neurotrophic factors. Nerve growth factor (NGF) is the prototype of this growing family (1), which includes brain-derived neurotrophic factor (BDNF) and neurotrophins 3, 4, and 5 (2–5). Other molecules, not part of the NGF family, that exhibit neurotrophic activity are ciliary neurotrophic factor (6), leukemia inhibitory factor (7), fibroblast growth factor (8), and glial-derived neurotrophic factor (9).

Numerous studies indicate that targets of neuronal innervation, usually referred to as the target tissue, synthesize and release trophic factors. These are taken up by the axon terminals and transported retrogradely to the cell somas, where they regulate multiple cellular functions including protein phosphorylation, gene induction, and differentiation (10, 11). For example, NGF is synthesized and released by the target tissue of NGF-dependent axons (12–14). In addition to their primary role in neuronal survival and differentiation, trophic factors may play an important part in nerve regeneration (15, 16) and in protection against neuronal necrosis following brain insults (17).

There is growing evidence that factors released by the target tissue may not be the only source of trophic support for developing neurons. They may derive trophic support from other cell types. For example, astrocytes have been shown to synthesize NGF mRNA and protein (18, 19). Schwann cells in the peripheral nervous system also appear to produce NGF (20). Neurons themselves have been found to synthesize trophic factors both in the central and in the peripheral nervous systems (21, 22).

Glial cell biology has been considerably enhanced through the analysis of a number of well developed *in vitro* model systems, most notably primary astrocyte and oligodendrocyte cell cultures. Adding to this rich repertoire of model systems, we have recently generated several immortalized mouse oligodendroglial cell lines by using the retroviral vector pZIPSVtsA58, which contains a gene encoding the temperature-sensitive simian virus 40 large tumor antigen (23, 24). One of these lines, N19, expresses the characteristics of immature oligodendroglial cells (24). As part of a larger study to examine the *in vitro* interactions of oligodendrocytes with neurons and other glia, we observed that this cell line greatly influenced the formation of neurites by PC12 cells. Traditionally, expression of neurotrophic factors has been low and there seemed to be little reason to seriously examine oligodendrocytes for the expression of neurotrophic factors. Because of this effect of the immortalized cell line on PC12 cells, we were prompted to examine further the expression of neurotrophic factors by oligodendrocytes. Our results indicate that the oligodendrocyte cell lines express NGF and BDNF and cultured oligodendroglial cells express NGF. In light of these findings, we were prompted to reexamine neuron–oligodendrocyte interactions.

MATERIALS AND METHODS

Cell Culture. PC12 cells, a gift of Erik S. Schweitzer (University of California at Los Angeles), were grown in an atmosphere of 9.5% CO_2/90.5% air in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum and 5% horse serum at 37°C. N19 and N20.1 oligodendroglial cells were grown as described (24).

Preparation of Enriched Oligodendroglial Cells. Primary mixed glial cultures were prepared from neonatal BALB/c ByJ mice as described by Amur-Umarjee *et al.* (25). Enriched oligodendroglial cultures were prepared from these at 7 days *in vitro* (DIV) by the method of Suzumura *et al.* (26).

PCR, Cloning, and Sequencing. The primers used to amplify neurotrophic factors in the N19 cell line were as follows: 5′ primer, 5′-GAATTCAAACAATACTTCTTCGAAACG-CAATG-3′; 3′ primer, 3′-CCTATCTGTGAAGAACACA-CACATCTAGA-5′. The PCR was carried out as described by Kawasaki and Wang (27) as modified by Foster *et al.* (28). Analysis of the PCR product on a miniagarose gel revealed a band of the expected size of 197 bp. The PCR products were directly cloned into the PCR II cloning vector (Invitrogen). The clones identified were sequenced by the dideoxynucleotide method using the Sequenase version 2.0 system (United States Biochemical) according to the manufacturer's instructions.

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; DIV, days *in vitro*; GC, galactocerebroside.

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Probes Used in Our Analysis. The mouse NGF probe was a gift from Eric Shooter (Stanford University). The BDNF probe used for Northern blot analysis was the cloned 197-nt PCR-generated fragment isolated in this study.

***In Situ* Hybridization Histochemistry and Immunohistochemistry.** A nonradioactive *in situ* hybridization technique with digoxigenin was employed using the Genius kit from Boehringer Mannheim. The use of this procedure has been described (29). Immunohistochemical staining for NGF, galactocerebroside (GC), and A2B5 was carried out using the same general procedure described previously (24, 25). The combined procedure for *in situ* hybridization and immunohistochemical staining followed by confocal microscopy was carried out as described (29) except that digoxigenin antibody conjugated to alkaline phosphatase was used to detect the digoxigenin-labeled mRNAs.

Antibodies Used. The antibody against NGF was a rabbit antiserum (1:100 dilution) purchased from Collaborative Research. The antibody against GC (1:20 dilution) was a mouse monoclonal antibody purchased from Boehringer Mannheim. The antibody against A2B5 was a mouse monoclonal antibody culture supernatant derived from hybridoma cells (a gift from Wendy Macklin, University of California at Los Angeles). The secondary antibodies IgG anti-rabbit rhodamine (1:500 dilution) and IgG anti-mouse fluorescein isothiocyanate (1:100 dilution) were purchased from Boehringer Mannheim.

RESULTS

Neurite Extension by PC12 Cells in Response to the N19 Cell Line. The adrenal chromaffin cell line PC12 has been used as a model for neuronal differentiation since its characterization (30). We were interested in investigating the interactions between neurons and glia by coculturing these cells with the N19 oligodendroglial cell line, primarily to see if the PC12 cells had any effect on the N19 cell line. Surprisingly, in these cocultures the PC12 cells elaborated processes that could be seen at 18 hr after coculture. With increasing time of coculture the PC12 cell processes became longer and more elaborate.

During this period, the shape of the PC12 cells changed considerably. The cells flattened out and elaborated processes, which gradually increased in length and number. The processes were sometimes as much as 3–5 times the length of the cell soma. These morphological and neurite-producing effects could be observed when N19 conditioned medium was added to a culture of PC12 cells (data not shown), suggesting that a soluble factor was responsible for the effects. Fig. 1*A* illustrates the appearances of the long processes elaborated by PC12 cells stained with neuron-specific enolase after coculturing with the N19 cell line (48 hr) when compared with PC12 cells that were grown without N19 cells (Fig. 1*B*). This neurite-promoting effect on PC12 cells was destroyed when the N19 conditioned medium was treated with trypsin (data not shown). These observations suggested that the N19 cell line synthesized and secreted one or more trypsin-sensitive factors that stimulated the PC12 cells into elaborating cellular processes, an effect similar to that observed when PC12 cells were exposed to NGF (data not shown).

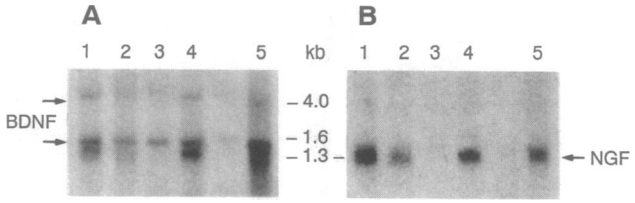

FIG. 2. Northern blot analysis of poly(A)$^+$ RNA isolated from mouse brain and several cell lines. Lanes 1–5 contain RNA from N19, N20.1, PC12, C6, and mouse brain (45 days), respectively. (*A*) Blot was probed with a BDNF cDNA probe. (*B*) Same blot was probed with a NGF cDNA probe.

Identification of the Neurotrophic Factors Expressed by the N19 Oligodendroglial Cell Line. Poly(A)$^+$ RNA from the N19 cells was reverse transcribed using degenerate PCR primers capable of amplifying mRNAs of the NGF neurotrophic family of factors. A strong band of the predicted size of 197 nt was observed when the PCR products were analyzed by agarose gel electrophoresis. The PCR products were cloned directly into the PCR II plasmid of Invitrogen. Fifteen clones were isolated and sequenced. Of the 15 clones, 10 (67%) were identified as mouse NGF cDNAs and 5 (33%) were identified as the mouse BDNF cDNAs as confirmed by comparison with the known sequences of these neurotrophic factor genes. These results indicated that the N19 cell line expressed both NGF and BDNF mRNAs.

Northern Blot Analysis of Oligodendroglial Cell Lines. Northern blot analyses were carried out on poly(A)$^+$ RNA isolated from the N19 cell line and another immortalized cell line, N20.1, which represents a more mature oligodendrocyte (Fig. 2). In Fig. 2, lanes 1–5 contain RNA from N19, N20.1, PC12, rat C6 astrocytoma cells, and mouse brain (45 days), respectively. The blot was hybridized with a cDNA probe for NGF (Fig. 2*B*) and, after stripping, it was reprobed with a BDNF cDNA probe (Fig. 2*A*). Later, the blot was stripped and probed with a cyclophilin-specific probe to confirm equivalence of loading (data not shown). The N19 and N20.1 oligodendrocyte cell lines clearly expressed NGF mRNA of

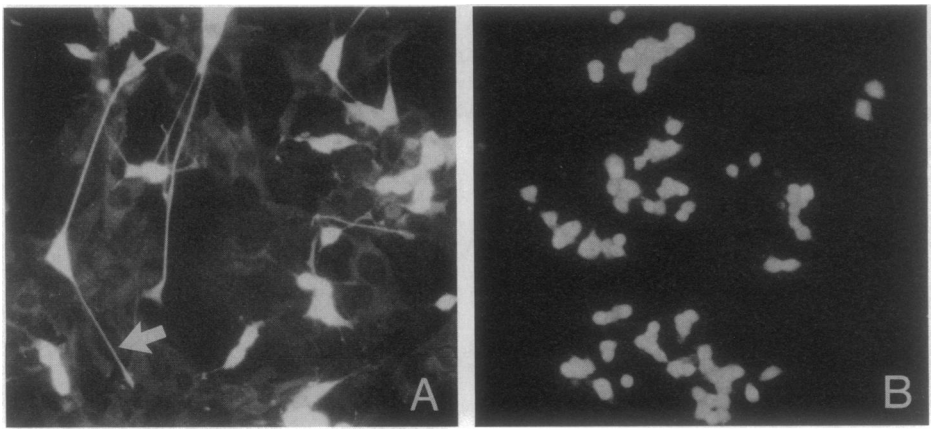

FIG. 1. Response of PC12 cells to coculturing with the N19 cells. (*A*) PC12 cells cocultured with N19 cells for 48 hr elaborated extensive processes, one of which is indicated by an arrow. (*B*) Control PC12 cells were grown in the absence of N19 cells.

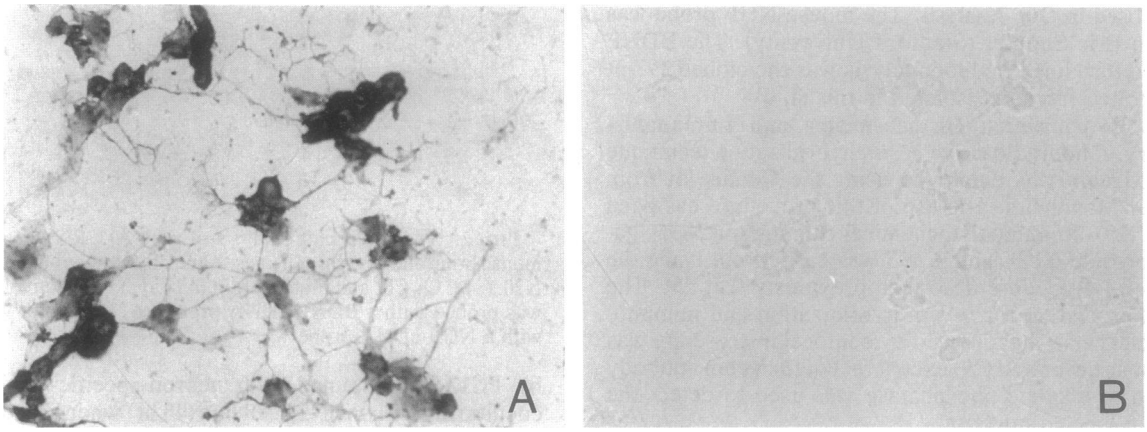

FIG. 3. *In situ* hybridization of oligodendroglial cells shaken off from mixed glial cultures at 7 DIV. (*A*) Digoxigenin-labeled NGF-specific cDNA probe. (*B*) No probe (control).

the appropriate size of 1.3 kb (31). The PC12 cells did not express the NGF mRNA. As expected the C6 glioma cell line known to express NGF (32) did express the NGF mRNA and served as a positive control in this experiment. The expression of the 1.6- and 4-kb BDNF mRNAs (31) was detected in all the samples examined including the PC12 cells themselves. Cross-hybridization of the BDNF probe was also observed with the 1.3-kb NGF mRNA, although there was very little cross-hybridization of the NGF probe with the 1.4- and 4-kb BDNF mRNAs.

Primary Oligodendroglial Cells Express NGF mRNAs. To determine whether oligodendrocytes expressed neurotrophic factors, mouse primary oligodendroglial cells and their precursors were prepared by standard procedures by shaking off mixed glial cultures at 7 DIV. The expression of NGF mRNAs in these cells was examined by nonradioactive *in situ* hybridization. Anti-digoxigenin antibody bound to alkaline phosphatase recognized the probe that was later identified by the colored alkaline phosphatase reaction product. In these experiments, the oligodendroglial cell bodies and some of their processes were darkly stained, showing the presence of NGF mRNA (Fig. 3*A*). In control cells no probe was used (Fig. 3*B*).

NGF Is Expressed in Oligodendroglial Cells at Different Stages of Differentiation. Since the primary oligodendrocyte preparations contained cells at different stages of differentiation, a series of experiments was carried out to examine the colocalization of the NGF mRNA and the protein with markers of specific stages of oligodendrocyte differentiation. Combined *in situ* hybridization/histochemistry was performed with a NGF cDNA probe and simultaneously the cells were stained with an antibody against GC, which is a marker for immature/mature oligodendroglial cells (33). As shown in Fig. 4, cells that stained for GC (Fig. 4 *Upper*) also expressed the NGF mRNA (Fig. 4 *Lower*). The blue reaction product deposited by the alkaline phosphatase activity quenches the fluorescence due to GC staining. Thus, all cellular processes normally visible by GC staining are not clearly evident in Fig. 4 *Upper*. Nonetheless, a comparison of Fig. 4 *Upper* and *Lower* indicates that NGF mRNA and GC are expressed in the same oligodendroglial cells in the two panels. These data confirm that NGF mRNA expression occurs within oligodendroglial cells.

In a companion set of experiments aimed at determining whether the NGF protein was expressed in primary oligodendrocytes, we performed double-immunofluorescence studies on the cultures with two-stage specific markers. Fig.

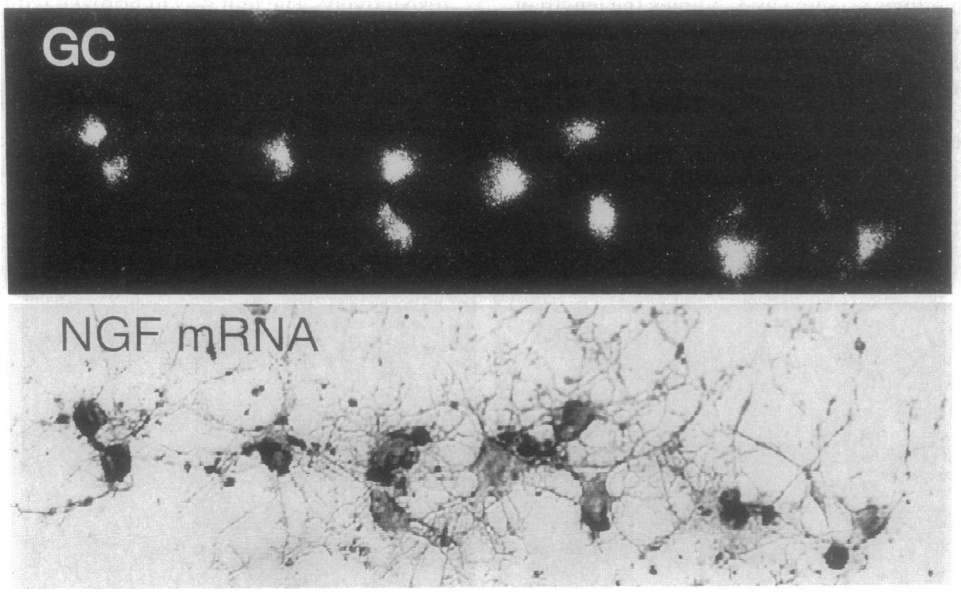

FIG. 4. Combined *in situ* hybridization/immunohistochemistry on oligodendroglial cells shaken off from mixed glial cultures at 7 DIV and analyzed by confocal microscopy. (*Upper*) GC immunoreactivity. (*Lower*) *In situ* hybridization with a NGF-specific cDNA probe. All the cells in the field showed colocalization of NGF mRNA with GC^+ immunoreactivity.

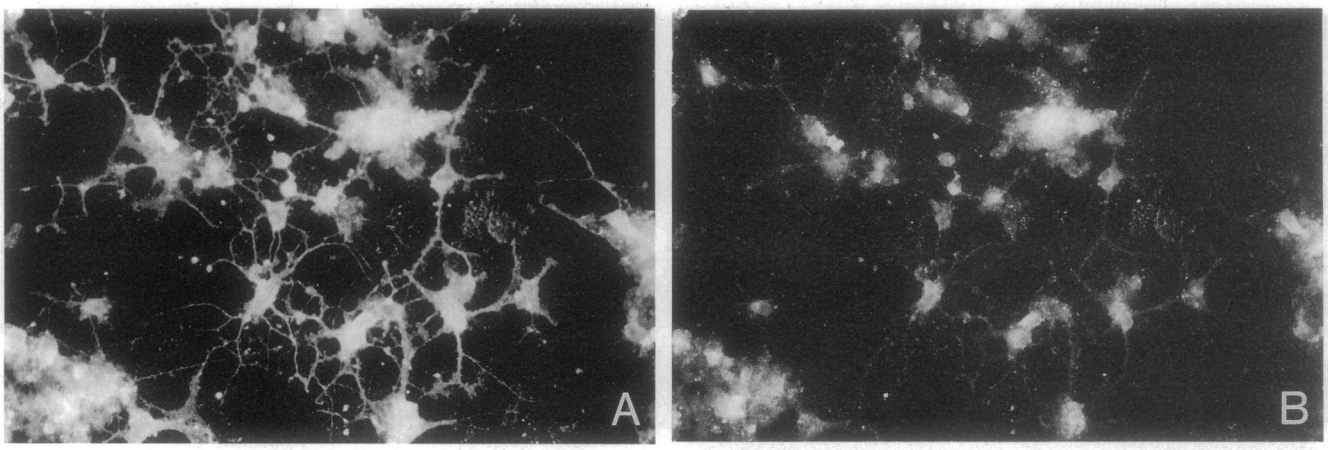

FIG. 5. Oligodendroglial cells shaken off at 7 DIV from mixed glial cultures were analyzed by double-immunofluorescence staining for NGF (*A*) and GC (*B*). Colocalization of NGF protein and the oligodendrocyte-specific marker GC was evident.

5 shows oligodendroglial cells shaken off at 7 DIV doubly stained with antibodies against NGF protein and GC. Fig. 5 shows identical fields stained for NGF with rhodamine (Fig. 5*A*) and for GC with fluorescein isothiocyanate (Fig. 5*B*). A comparison indicates that NGF and GC are coexpressed within the oligodendroglial cells. These two sets of experiments indicate that GC^+ oligodendroglial cells express both NGF mRNA and protein.

A2B5 is a marker for oligodendrocyte precursor cells (34). We were interested in determining whether oligodendroglial precursor cells also expressed the NGF protein. Fig. 6 shows the results of a double-immunofluorescence experiment with antibodies against both A2B5 and NGF. This figure illustrates the colocalization of NGF protein within precursor cells that are present in the population of oligodendroglial cells isolated at 7 DIV. Primary oligodendroglial cells shaken off at 7 DIV stained with antibodies against NGF (Fig. 6*A*) and the surface lipid A2B5 (Fig. 6*B*).

DISCUSSION

The observation of neurite formation by PC12 cells in response to coculturing with the N19 cell line led us to determine by several approaches that the N19 oligodendroglial cells expressed the neurotrophic factors NGF and BDNF. Because we could achieve greater specificity with our NGF probe, we focused subsequent efforts on examining NGF expression in oligodendrocytes. Primary mouse oligodendroglial cells, shaken off at 7 DIV, also expressed NGF mRNA. Colocalization of NGF mRNA with GC within the oligodendrocytes in primary culture was observed by immunohistochemistry and confocal microscopy. Furthermore, colocalization of NGF protein was observed with markers for mature oligodendrocytes and their precursors.

There have been a number of reports that astroglial cells synthesize and release several neuroactive compounds including steroids, growth factors, amino acid transmitters, neuropeptides, eicosanoids, and receptor agonists (35). Nonneural cells also have been observed to express neurotrophic factors. Cultured astrocytes have been shown to synthesize NGF mRNA and protein (18, 19). NGF-like immunoreactivity has been observed in Schwann cells (20), oligodendrocytes, and neurons (36). Immortalized rat hippocampal lines also have been found to release some neurotrophic factors (37).

NGF and BDNF function as target-derived neurotrophic factors that can be transported retrogradely through axons and exert their effects on the cell somas (12, 13, 14). Developmental studies suggest that the onset of NGF expression in the target tissue coincides with the arrival of axon terminals of NGF-dependent neurons (38, 39) and that this may not be sufficient for guiding axonal growth early in development. The cells of the oligodendroglial lineage have their origins in the subventricular zone of the brain and migrate subsequently into and along axonal tracts. During this and later periods when they myelinate mature axons, they are constantly in a neuronal environment. Synthesis and release of low levels of neurotrophic factors by oligodendrocytes would ensure that the developing neurons, in the process of extending their axonal processes, would be suffused with these trophic factors. This local stimulation of axonal outgrowth could be important in guiding the axonal processes through fiber tracts that will eventually be myelinated.

Caroni and Schwab (40, 41) have noted that immature oligodendrocytes were frequently in contact with neurons and their processes in contrast to highly branched, differentiated (mature) oligodendrocytes that were nonpermissive for neurite growth. The same authors have identified two neurite growth inhibitors on the surface of adult central nervous system (CNS) myelin. Immature oligodendrocytes and their immediate precursors may release factors that promote growth of neuronal processes and participate in laying down fiber tracts for the nervous system preparatory to myelination. As the CNS matures, inhibitory molecules expressed on the myelin surface may prevent random neuronal process stimulation and maintain the architecture that has been established. Recent studies have shown that following trauma in the CNS, a number of molecules that mediate glial–neural interactions and promote wound healing are expressed (42, 43). It is possible that neurotrophic expression in glia is in response to wounding and trophic factors released by the oligodendroglial cells are important in wound healing and regeneration of the CNS. Another possibility is that the oligodendroglial neurotrophic factors may facilitate and play a specific role in the maturation and differentiation of immature glial cells themselves. Although neurotrophic factors were initially identified as promoters of neuronal growth, they clearly have roles far beyond that envisioned at first (44–47).

In our laboratory, we have demonstrated that the expression and timing of appearance of the key oligodendroglial markers in mouse primary tissue culture system are similar to that observed (25) *in vivo*. This leads us to suspect that the observations reported in this study may not be restricted to the culture system. Neurotrophic factors synthesized by oligodendroglia may serve as crucial determinants of the architecture of the nervous system.

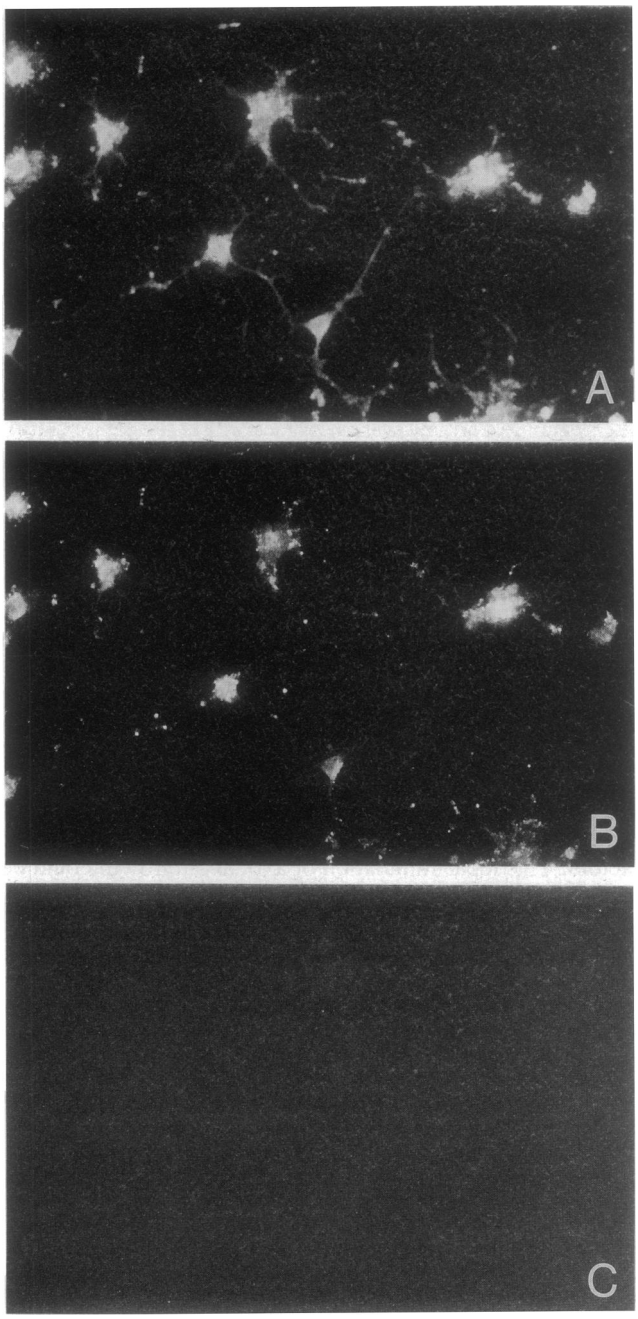

FIG. 6. Double-immunofluorescence staining for NGF (*A*) and A2B5 (*B*) in oligodendroglial cells shaken off at 7 DIV from primary glial cultures. In this field, NGF protein colocalized with cells expressing the oligodendrocyte precursor marker A2B5. Control cells were stained with only the secondary antibody (*C*).

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