The Journal of Neuroscience, February 1991, 11(2): 327–336

A Growth Factor from Neuronal Cell Lines Stimulates Myelin Protein Synthesis in Mammalian Brain

Dana Giulian,[1] Beena Johnson,[1] Joseph F. Krebs,[1] Mischca J. Tapscott,[1] and Sandra Honda[2]

Departments of [1]Neurology and [2]Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Oligodendroglia growth factor (OGF) is a 16-kDa soluble protein produced by neuronal cell lines. This factor, when incubated with brain glia in culture, selectively stimulates growth of oligodendroglia, the myelin-producing cells of the CNS. OGF infused into the cerebral cortex of the adult rat accelerates the production of myelin proteins as shown by increased specific activity of the myelin enzyme 2′,3′-cyclic nucleotide 3′-phosphohydrolase (2′,3′-CNPase), by stimulated synthesis of myelin basic protein, and by elevations in levels of myelin proteolipid protein RNA. The ability of OGF to induce myelin protein production *in vivo* suggests that neuron-secreted growth factors help to regulate myelin formation within the CNS.

Secretion of growth-promoting molecules within the brain during development or after injury is now widely recognized as one mechanism to stimulate proliferation, accelerate differentiation, or alter functions of CNS glia (Giulian et al., 1988b). A number of proteins have been described as putative glial growth factors based upon observations of brain-cell behavior in culture. For example, fibroblast growth factor (FGF; Pruss et al., 1981), epidermal growth factor (EGF; Loret et al., 1989), platelet-derived growth factor (PDGF; Besnard et al., 1987), glia maturation factor (GMF; Lim et al., 1989), and interleukin-1 (IL-1; Giulian and Lachman, 1985) stimulate growth of cultured astroglia, while granulocyte/macrophage colony-stimulating factor (GM-CSF) and multipotential colony-stimulating factor (multi-CSF; Giulian and Ingeman, 1988) serve as mitogens for cultured microglia. FGF, PDGF, and ciliary neurotrophic factor (CNTF) are also found to alter patterns of proliferation or differentiation in glial progenitor cells grown *in vitro* (Hughes et al., 1988; Noble et al., 1988; Richardson et al., 1988). Some of these molecules may, in fact, be CNS regulatory factors because they are found within brain tissues during periods of elevated glial biosynthetic activity and because they demonstrate specificity of action upon certain classes of non-neural cells (Giulian et al., 1988a; Raff et al., 1988; Richardson et al., 1988).

At the present time, there is no well-characterized molecule that acts as a selective growth factor for myelin-producing oligodendroglia. Cell-culture experiments show that insulin and insulin-like growth factor 1 (IGF-1) promote proliferation and differentiation of oligodendroglia, as well as many other cell types (McMorris et al., 1986; Han et al., 1987). The cytokine interleukin-2 (IL-2) has been described by different laboratories as a growth promoter (Benveniste and Merrill, 1986), as a growth inhibitor (Saneto et al., 1986), or as having no effect upon oligodendroglia (Giulian and Lachman, 1985; Yong et al., 1988). All studies of factor effects upon oligodendroglia have been limited to cell-culture bioassays and thus await *in vivo* confirmation. When attempting to characterize biologic actions of a growth factor, there is a need to use highly stringent conditions with enriched cell populations and chemically defined culture medium. Under these conditions, it is difficult to know if the concentrations of a factor tested, the components of the culture medium, and the composition of the cell population adequately reflect physiologic states. Goldman et al. (1986) caution that differentiation pathways observed for glia *in vitro* may in fact not occur *in vivo*. One way to circumvent these problems is to infuse isolated growth factors directly into the brain (Giulian and Ingeman, 1988; Giulian et al., 1988a).

We have previously described isolation of several different glia-promoting factors (GPFs) found in brain tissue that selectively stimulate the growth of either cultured astroglia or oligodendroglia (Giulian et al., 1985, 1986). Soluble oligodendroglia-promoting proteins appear during periods of development or at times of axonal regeneration when peak levels of oligodendroglial growth and myelin membrane production occur (Giulian and Young, 1986). Some of these proteins are produced by neurons (Giulian and Young, 1986; Bottenstein et al., 1988), suggesting the existence of regulatory mechanisms to control myelination. We have isolated one of these proteins, oligodendroglia growth factor (OGF; previously referred to as GPF-1; see Giulian et al., 1985), from regenerating retinal ganglion cells of the goldfish (Giulian and Young, 1986). Goldfish-derived OGF has biochemical and biological properties identical to a growth factor found in the developing rat brain and to one secreted by a murine neuronal cell line (Giulian and Young, 1986). As described in this report, highly purified OGF infused into the cerebral cortex of the adult rat elevated production of 3 different myelin proteins. The ability of OGF to stimulate myelin protein synthesis *in vivo* suggests that neurons secrete molecules to trigger myelination of axons.

Materials and Methods

Glia cell culture. Mixed glial cell cultures were prepared as described earlier (Giulian et al., 1986) from dissociated cerebral cortex of newborn albino rats (Holtzman, Madison, WI). Cells (1.2×10^6 per dish) were placed upon poly-L-lysine-coated glass coverslips (22 mm square) within 35-mm plastic dishes containing chemically defined culture medium (Bottenstein and Sato, 1979) supplemented with 10% fetal bovine se-

Received July 17, 1989; revised June 4, 1990; accepted Aug. 17, 1990.

This work was supported by NINDS Grant NS 25637 and by NEI Grant EY-04915. We thank Jocelyn Chen, Neil Zeigler, Jeff E. Ingeman, and Yunnie Choe for their technical assistance. We also thank Dr. Robert Milner, The Scripps Institute, for the proteolipid protein probe PLP-1.

Correspondence should be addressed to Dana Giulian at the above address.

rum; after 48 hr, cells were transferred to chemically defined culture medium. All culture media contained 5 μg/ml insulin (Sigma, St. Louis) and 5 μg/ml transferrin (Sigma).

To test for biologic activity, cell cultures grown in 1.5 ml chemically defined medium were incubated with increasing concentrations of OGF for 72 hr. Appearance of oligodendroglia, ameboid microglia, and astroglia were monitored by scoring the number of specifically labeled cells in 9 randomly selected fields (0.314 mm^2) from each of 3 or more coverslips. The data were expressed as *n*-fold increase over control cultures incubated with matching aliquots of PBS ranging from 1 to 50 μl per ml of culture medium.

Glial cell identification. Indirect immunofluorescence techniques were used to identify astroglia containing glial fibrillary acidic protein (GFAP) and oligodendroglia containing galactocerebroside (GC; Raff et al., 1978; Giulian et al., 1986). Ameboid microglia were identified by fluorescence microscopy using acetylated low-density lipoprotein (ac-LDL) bound to the fluorescent probe 1,1′-dioctadecyl-1,-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Giulian and Baker, 1986) or fluorescently labeled polystyrene microspheres (Covaspheres, Duke Scientific, Palo Alto, CA). *In vitro* labeling was carried out in chemically defined culture medium with 200 ng/ml DiI–ac-LDL (Biomedical Technologies Inc., Cambridge, MA) for 12 hr at 37°C.

Growth of neuroblastoma cell lines. The mouse neuronal cell line C1300 (CCL 147) was obtained from American Type Tissue Culture Company and grown in medium (Bottenstein and Sato, 1979) consisting of 50% Ham's F-12 nutrient mixture (Cellgro) with *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES, 1.19 gm/liter) and $NaHCO_3$ (7.84 ml/liter 7.5% $NaHCO_3$), and 50% Dulbecco's Modified Eagle Medium (Cellgro) with glucose (2.85 gm/liter), gentamycin (48 mg/liter), an antibiotic/antimycotic solution (Sigma, 10 ml/liter), L-glutamine (0.584 gm/liter), putrescine (16.11 mg/liter), sodium selenate (5.2 μg/liter), and progesterone (6.28 μg/liter), enriched with 2.5% fetal bovine serum and 15% horse serum. The rat neuronal cell line B103 (Schubert et al., 1974), a gift from Dr. Yasuko Tomozawa, Baylor College of Medicine, was grown in serum-free conditions using the same culture medium supplemented with transferrin (100 mg/liter) and insulin (5 mg/liter). Approximately 10^7 cells from either cell line were transferred to 700 cm^2 (Wheaton borosilicate roller bottles (Fisher, Pittsburgh, PA), each containing 150 ml medium. The bottles were incubated at 37°C on a Wheaton modular cell production roller apparatus (Fisher). After 7 d, approximately 2×10^8 cells were harvested from each bottle. Cells were collected by centrifuging 250-ml bottles at 1500 × *g* for 20 min in GSA rotor (Sorvall, Newton, CT) at 4°C.

Coculture experiments were used to monitor the effects of neuronal-cell-line secretion products upon oligodendroglial growth. Mixed glial cultures were plated on poly-L-lysine-coated coverslips (1.2×10^6 cells) in 35-mm plastic dishes containing chemically defined culture medium with 10% fetal bovine serum. After 48 hr, the medium was replaced with fresh serum-free medium, and a 12-mm culture well (Millicell-CM, Millipore, Danvers, MA) containing 2×10^4 B103 cells was placed atop each coverslip for 24 hr. Control experiments included incubation with empty culture wells or wells containing formaldehyde-fixed B103 cells. At the end of 72 hr, the number of oligodendroglia found on the coverslips was quantitated using indirect immunofluorescence labeling.

Isolation of an oligodendroglia-stimulating growth factor. Pooled cells (5×10^9 per ml) were dispersed by sonication in 80 ml deionized H_2O. This sonicate was further fractionated by ultracentrifugation at 108,000 × *g* for 60 min at 4°C. The supernatant was filtered with a 0.2-μm Acrodisc (Millipore, Danvers, MA) and concentrated by ultrafiltration with a YM2 filter (Amicon, Danvers, MA). The retained material was then separated by gel filtration chromatography (1.5 × 100-cm P30 column; BioRad, Richmond, CA) using 150 mM PBS (pH, 7.4) as the eluting buffer. Biologically active fractions were concentrated using YM2 ultrafiltration or Centricon 10 tubes (Amicon Corp., Danvers, MA). This material was next fractionated by reverse-phase high-performance liquid chromatography (RP-HPLC) using a 4.6 × 75-mm C8 column (Beckman, Palo Alto, CA) with elution by an acetonitrile gradient (from 0 to 55% over 52 min) with deionized H_2O containing 0.08% trifluoroacetic acid. Fractions containing biologic activity were then pooled, lyophilized, redissolved in PBS, and rechromatographed by RP-HPLC under identical gradient conditions. The growth factor under study here was first described by Giulian et al. (1985) as one of several glia-promoting factors (GPF-1) found in the regenerating visual system of goldfish. We now refer to this protein as oligodendroglia growth factor (OGF) in view of its ability to stimulate a specific class of CNS glia both *in vitro* and *in vivo*.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) used the method of Neville (1971) with a 20% polyacrylamide separating gel and a 4% stacking gel (10 × 15 × 0.15 cm). OGF samples were mixed with sample buffer (30% glycerol, 2.8 M 2-mercaptoethanol, 0.25% SDS, and 1% bromophenol blue, in Tris-HCl buffer, pH 8.4). This mixture was placed in boiling water for 5 min and centrifuged for 2 min at 15,000 × *g*. The samples were separated by electrophoresis at room temperature using 16 mA constant current for 3.5 hr. The gels were fixed and stained with silver as described by Poehling and Neuhoff (1981).

Preparative isoelectric focusing. Preparative isoelectric focusing (IEF) involved a horizonal column technique using the Rotofor preparative isoelectric focusing cell (Bio Rad, Richmond, CA). Fractions recovered from gel-filtration chromatography were pooled and dialyzed against sterile deionized H_2O overnight. Following dialysis, 2.5 ml Servalyt (pH, 3–10) isodalt-grade ampholyte solution (40% ampholyte w/v; Serva, NY) was added to the sample, and the volume was brought up to 50 ml with deionized H_2O to give a final 2% ampholyte concentration. The sample was separated by isoelectric focusing for 4–5 hr at 12 W constant power at 4°C into 20 fractions. After a pH measurement, each fraction was sterilized by filtration and tested for biologic activity. Control experiments showed no effect of recovered ampholytes upon oligodendroglia cultures.

Intracerebral infusion of growth factors. To study the effects of OGF *in vivo,* small volumes of material were injected into the cerebral cortex of albino rats. Adult rats (250–300 gm; Holtzman, Madison, WI) were anesthetized by intraperitoneal injection of a mixture of 8.5 mg/ml xylazine, 42 mg/ml of ketamine hydrochloride, and 1.4 mg/ml of acepromazine maleate using 0.7–0.9 cc per kg body weight. The animals were held in a stereotaxic device (David Kopf Instruments, 900) as burr holes were spaced 2 mm apart at 4 mm lateral to the sagittal suture. Injections were placed at a depth of 1.1 mm from the surface of the cerebral cortex at a rate of 1 μl per 2 min using a microliter syringe (Hamilton, 702-N). Injections included 2 μl vol PBS (pH, 7.4) containing 100 ng OGF, 100 ng cytochrome C, 100 ng native basic fibroblast growth factor (FGF; from Collaborative Research, Bedford, MA), 100 ng recombinant basic FGF (Amgen, Thousand Oaks, CA), 100 ng of recombinant insulin-like growth factor-1 (IGF-1; Amgen, Thousand Oaks, CA), 2 U recombinant interleukin-1 α (IL-1; Cistron, Pinebrook, NJ), 10 U recombinant interleukin-2 (IL-2; Cetus, Norwalk, CT), or 20 ng native epidermal growth factor (EGF; Collaborative Research). The amount of IL-1 injected matched the levels of IL-1 measured after penetrating injury in the cerebral cortex of adult rats (Giulian et al., 1988a). The injected quantitites of the other growth factors were based upon concentrations reported to stimulate maximal glial growth *in vitro* for epidermal growth factor (Leutz and Schachner, 1981), basic fibroblast growth factor (Pruss et al., 1981), insulin-like growth factor (McMorris et al., 1986), and interleukin-2. The *in vitro* action of interleukin-2 upon oligodendroglia is controversial, with reports of no growth effect (Giulian and Lachman, 1985), a mitogenic effect (Benveniste and Merrill, 1986), or an antimitotic effect (Saneto et al., 1986).

Biopsies from injection sites were used to monitor the *in vivo* effects of growth factors upon the production of myelin proteins. Freshly isolated brains were placed on glass slides over crushed ice, and injection sites were identified as erythematous patches on the surface of the brain. A sterile plastic pipet tip (250-μl size; West Coast Scientific, Emeryville, CA) trimmed to give an inner diameter of 2 mm was pressed over the site of injection through the cerebral cortex to the base of the brain. The core of tissue was pushed from the pipet tip, and the gray matter was tranferred using a pair of number 5 watchmakers forceps to ice-chilled sterile 1.5-ml conical centrifuge tubes.

Measurement of 2′,3′-cyclic nucleotide 3′-phosphohydrolase (2′,3′-CNPase). Biopsies of the injection sites (3 tissue samples, each from different animals) were placed in conical tubes containing 1.0 ml 50 mM 2-(*N*-morpholino)ethane sulfonic acid (MES; pH, 6.5) with 3% Triton X-100 (Kodak, Rochester, NY), dispersed by sonication, and centrifuged for 3 min at 15,000 × *g*. One hundred μl of the tissue sonicate was mixed with 100 μl of substrate solution [9 mM 2′,3′-cyclic adenosine monophosphate (Sigma, St. Louis, MO) in MES buffer] incubated for 4 min at 25°C (Giulian et al., 1983). The reaction was terminated by the addition of 40 μl glacial acetic acid and centrifugation for 3 min at 15,000 × *g*. Chromatographic separation of the nucleotides was performed by anion-exchange HPLC (4.6 × 250 mm; Zorbax SAX, DuPont, Wilmington, DE) with an eluting solution of 75 mM KH_2PO_4. The amount of 2′AMP product formed was determined from peak areas measured at 254 nm. Specific activities of the samples were expressed as micromoles of product formed per min per mg protein under the conditions described. Protein concentrations were estimated by the flu-

orescamine method (Bohlen et al., 1973) using bovine serum albumin as a standard.

Measurement of myelin basic protein synthesis. Bovine myelin basic protein (MBP) was obtained from Sigma. Rat myelin basic protein was isolated by the method of Norton (1981) and further separated into large (predominately the 18-kDa form) and small (predominately the 14-kDa form) components by elution from SDS-PAGE (Benjamins and Morell, 1978). Rabbit anti-bovine myelin basic protein serum (Bethyl Laboratories, Conroe, TX) was dialyzed (Spectra/Por 2, Fisher) against a buffer of 20 mM KH_2PO_4 (pH, 8.0) containing 0.02% NaN_3. Following dialysis, the serum was applied to a DEAE Affi-Gel Blue (BioRad, Richmond, CA) column prewashed with 2 bed vol buffer. IgG fractions were eluted with 2 bed vol buffer, and fractions were pooled and concentrated to 20% of the original volume. The anti-bovine myelin basic protein IgG bound both the large and small components of rat myelin basic protein. Electrophoretic transfers from the SDS-PAGE to an Immobilon PVDF (Millipore, Bedford, MA) membrane used the basic method of Towbin et al. (1979) with the Hoefer SE-600 apparatus at 35 mA for 4 hr. Immunostaining of membranes employed the 3-amino-9-ethylcarbazole technique described in the Immobilon Tech Protocol (TP007, Millipore Corp.).

Biopsies of brain tissue were incubated in 24-well tissue-culture plates (Falcon) at 37°C in 0.5 ml Dulbecco's Modified Eagle's Medium Deficient (lacking L-lysine, L-leucine, L-glutamate, and S-methionine; D4655, Sigma) with supplements of L-lysine HCl (0.146 gm/liter), L-leucine (0.105 gm/liter), L-glutamine (0.584 gm/liter), and 100 μCi L-^{35}S-methionine (Amersham, Arlington Heights, IL). After 24 hr, the biopsies were twice washed with PBS and dispersed by sonication (Kontes Micro-Ultrasonic cell disrupter) in 400 μl 50 mM HEPES (pH, 7.0) with 1% Triton X-100. Boiled sonicate mixed with radiolabel served as a background control. The samples were centrifuged for 10 min at 15,000 × *g*. About 95% of total radiolabeled material precipitated by trichloroacetic acid was released with this detergent sonication procedure.

To each 100 μg biopsy sonicate protein, 120 μg of the rabbit anti-bovine myelin basic protein IgG was added and incubated for 5 hr at 4°C with continuous shaking. The samples were next incubated with 50 μg of a secondary antibody, goat anti-rabbit IgG (Bethyl Laboratories, Conroe, TX), for 16 hr at 4°C with continuous shaking. A precipitate recovered by centrifugation for 10 min at 15,000 × *g* was washed twice with 1 ml PBS, solubilized with 70 μl 1% SDS in PBS, and placed in boiling water for 5 min. Cooled samples were centrifuged at 15,000 × *g* for 10 min. Liquid scintillant (Scintiverse E, Fisher) was added to triplicate samples of 5-μl aliquots of supernatant and radioactivity monitored by scintillation counting. Data [(incorporated cpm) − (background cpm)] were presented as mean values ± standard errors. Alternatively, myelin basic protein recovered by immunoprecipitation from 3 pooled biopsies was separated by 10% SDS-PAGE. Densitometric measurements of autoradiograms were obtained from these gels using the Joyce-Loebel Magiscan 2. In addition, the 18-kDa component of radiolabeled myelin basic protein was eluted from Coomassie-stained gel in 1 ml of tissue stabilizer NCS (NCS:H_2O 9:1; Amersham) by heating at 50°C for 2 hr. The samples, cooled to room temperature, were mixed with 15 ml OCS (Amersham) for scintillation counting.

Measurement of proteolipid protein RNA. RNA was prepared from the pooled cerebral cortex samples by the guanidinium isothiocyanate–cesium chloride method (Maniatis et al., 1982). Tissue samples frozen in liquid N_2 were dispersed by brief trituration through a glass pipet in 4 M guanidinium isothiocyanate (1.5 mg tissue: 1 ml guanidinium isothiocyanate), 5 mM sodium citrate (pH, 7.0), 0.5% sarcosyl (Fisher), and 0.1 M 2-mercaptoethanol. The homogenate was then vigorously mixed with CsCl (2.5 ml homogenate per gm CsCl) and gently layered onto a 1-cm bed of 5.7 M CsCl, 0.1 M EDTA, and 25 mM HEPES. This material was centrifuged at 100,000 × *g* for 24 hr at 20°C on a Beckman SW 27 rotor. After removal of the cellular debris, each pellet was carefully drained and mixed in 3 ml 10 mM Tris-HCl (pH, 7.5), 5 mM EDTA, and 1% SDS by vortexing for 15 min. The solution was then extracted twice with phenol (1 vol solution:2 vol phenol) and once with 0.3 ml chloroform/isoamyl alcohol (24:1). Total nucleic acid was precipitated overnight at −20°C by the addition of 0.5 vol 7.5 M ammonium acetate (pH, 7.5) and 2.5 vol absolute ethanol. Precipitates collected by centrifugation were resuspended in a minimal vol of 10 mM HEPES containing 1 mM EDTA and 0.3% iodoacetic acid. Yields ranged from 3 to 5 μg total RNA per biopsy sample.

Isolated RNA was separated by gel electrophoresis in 1.2% agarose (Seakem, Rockland, ME). Samples were prepared by the addition of 7.2 μl RNA solution to 15 μl dimethyl sulfoxide, 7.5 μl deionized glyoxal, and 0.3 μl 1 M PBS (pH, 7.5). The mixture was incubated for 1 hr at 55°C and loaded onto the gel. Electrophoresis in 10 mM PBS (pH, 7.0) running buffer containing 0.26% iodoacetic acid was run at constant voltage (35 V) for 12 hr. For staining, the gel was treated for 20 min in 50 mM NaOH, washed with 500 mM ammonium acetate, then incubated for 30 min with 5 μg/ml ethidium bromide in 100 mM ammonium acetate. After a 10-min wash in deionized H_2O, gels were examined at 250 nm on a viewing box. Northern blots from these gels were prepared by contact transfer to a nylon membrane (Biotrans, ICN Biochemicals, Costa Mesa, CA) for 24 hr (Maniatis et al., 1982).

RNA from biopsy samples was dotted directly onto a nylon membrane in 1 or 2 μl aliquots containing 2 or 4 μg total RNA. Membranes were baked at 80°C for 1 hr, prehybridized in 50% formamide in a 5 × SSC solution (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 50 mM sodium phosphate buffer (pH, 6.8), 0.1% SDS, 0.2% bovine serum albumin, 0.2% ficoll, and 0.2% polyvinylpyrrolindone at 55°C for 1 hr. Hybridization to an RNA probe PLP-1 (Milner et al., 1985) for proteolipid protein was carried out in the same mixture as that for prehybridization, with the inclusion of 5% dextran sulfate. The probe was added to hybridization solution at a concentration of 5–10 × 10^6 cpm/ml and hybridized overnight at 55°C. Washes were performed at room temperature 3 times for 10 min each in 2 × SSC, followed by a wash at 65°C for 1 hr in 0.1 × SSC. Bound radioactivity was quantified for individual dots by scintillation counting using Scintiverse E.

Table 1. Appearance of GC(+) oligodendroglia in cultures containing secretion products from neuronal cell line B103

	Number of glia per field	
	GC(+)	GFAP(+)
Control	11.1 ± 1.2	137.0 ± 10.2
Coculture	35.5 ± 4.1*	107.7 ± 10.7

Coculture experiments examined the effect of secreted factors upon appearance of GC(+) oligodendroglia in culture. 2 × 10^4 cells of the B103 line were placed in miniwells in 35-mm dishes containing mixed brain-glia populations (1.5 × 10^6 cells) grown on poly-L-lysine-coated glass coverslips. After 24 hr, the miniwell containing B103 cells was removed, and GC(+) or GFAP(+) glia were identified at the end of 72 hr. Control cultures were incubated with an empty miniwell for 24 hr. Cell numbers were determined by scoring the numbers of a specific glia-type population in 9 fields from each of 6 coverslips. As shown, secretion products from the B103 cells led to a 3-fold increase in the number of GC(+) oligodendroglia, with little change in the number of GFAP(+) astroglia.

*, Student's t test = 5.96, df = 10, p = 0.0002.

Results

Neuronal cell lines secrete oligodendroglia-promoting activity

Our previous studies (Giulian and Young, 1986; Giulian et al., 1986) have suggested that neurons secrete proteins that help to accelerate the growth and proliferation of cultured oligodendroglia. Neuronal cell lines, including mouse C1300 and rat B103, produce these oligodendroglia-promoting factors (Giulian and Young, 1986). Coculture experiments in which cell lines are placed in filtered chambers among glia grown on glass coverslips demonstrate the secretion of such oligodendroglia-stimulating growth factors (Fig. 1). The presence of rat cells increases the number of [GC(+)] oligodendroglia in culture by 3–4-fold (Table 1) without proliferative effect upon astroglia. Neuronal cell-line-conditioned medium also promotes oligodendroglia to form elaborate processes (Fig. 1).

Isolation and characterization of oligodendroglia growth factor

We isolate an oligodendroglia-stimulating growth factor (Giulian and Young, 1986) from either the mouse neuronal cell line C1300 or the rat cell line B103. Soluble proteins from cell sonicates are concentrated 10-fold and fractionated by gel filtration. A 12–18-kDa fraction is further separated by ultrafiltration, then

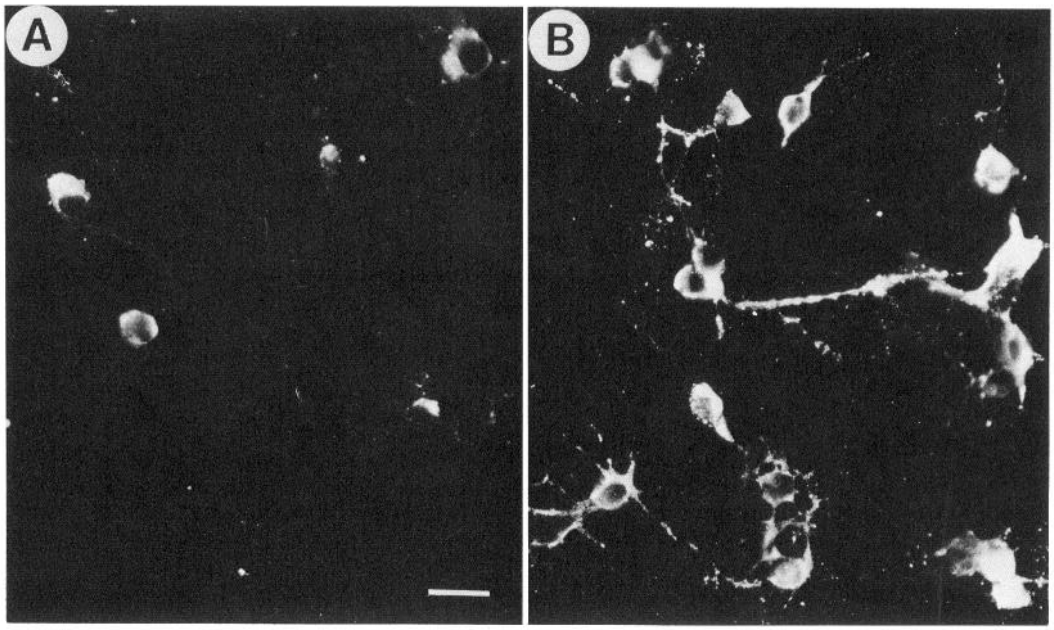


Figure 1. Fluorescent photomicrographs showing effects of neuronal cell-line-conditioned medium upon brain glial cultures. Oligodendroglia were identified as those cells containing the glycolipid GC. Panel *A* shows that there was a sparse population of GC(+) oligodendroglia found in control coculture preparations. When incubated with miniwells containing 2×10^4 B103 cells, the density of GC(+) oligodendroglia markedly increased (panel *B*); in addition, oligodendroglia often showed the formation of elaborate processes. No quantitative differences were found in the numbers of GFAP(+) astroglia in B103-containing cocultures (see Table 1) when compared to numbers of astroglia in control cocultures. Scale bar, 20 μm.

by 2-step RP-HPLC. This highly enriched preparation from either source appears as a single protein with an approximate 16-kDa molecular mass when separated by SDS-PAGE (Fig. 2). This material also shows a single peak of biological activity with a pI of about 6.3 ± 0.2 ($n = 5$) estimated from preparative isoelectric focusing (Fig. 3). This protein, now referred to as OGF, has biologic activity, an estimated molecular mass, and chromatographic properties using ion-exchange and RP-HPLC that are identical to the protein previously referred to as GPF-1 isolated from the goldfish visual system, developing rat brain, and C1300 cell line (Giulian et al., 1986). Past experiments employing complement lysis of progenitor cells and ^{3}H-thymidine incorporation suggest this factor stimulates both cell proliferation and differentiation of oligodendroglia when tested *in vitro* (Giulian and Young, 1986).

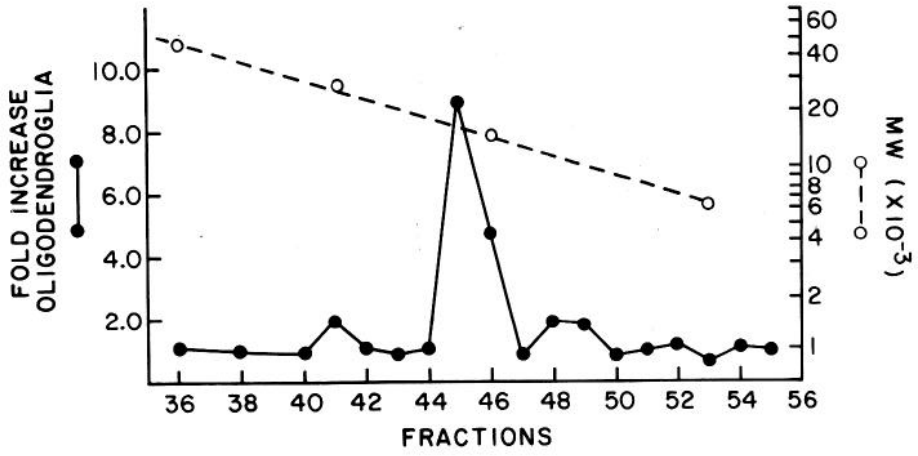
Myelin membranes contain approximately 70% lipid (Norton, 1981), including the glycolipid GC, which has been identified as a specific marker for myelin-producing cells (Raff et al., 1978; Mirsky et al., 1980). During development, oligodendroglia express GC prior to the synthesis of myelin proteins (Knapp et al., 1988). We find that OGF stimulates the appearance of GC(+) oligodendroglia in cultures of dissociated brain glia (Fig. 4). Importantly, this oligodendroglia-stimulating growth factor has specificity of action as demonstrated by the fact that OGF does not influence the growth of cultured astroglia or microglia (Fig. 4).

Oligodendroglia growth factor and 2′,3′-CNPase in vivo

The ability of OGF to activate oligodendroglia *in vitro* led us to test for similar effects *in vivo*. We monitored the effects of intracerebral infusions of OGF upon the myelin-associated enzyme 2′,3′-CNPase (Drummond et al., 1962, 1971; Sprinkle et al., 1978; Norton, 1981). Deeply anesthetized adult rats were injected with 100 ng OGF in 2 μl PBS, while control injections included 2 μl of either PBS alone, 100 ng cytochrome C in PBS, or one of several growth factors in PBS. Injections were placed using a stereotaxic device into the cerebral cortex at a depth of 1.1 mm from the surface of the brain, and 3 tissue specimens were pooled from different animals to monitor 2′,3′–CNPase-specific activity. A series of 5 consecutive experiments shows that a single injection of 100 ng OGF led to a 1.5–2-fold elevation in specific activity of 2′,3′-CNPase relative to controls (representative data shown in Fig. 5). Only the mean specific enzymic activities from sites of OGF injection were significantly elevated when compared with uninjected tissue controls (Student's $t = 6.04$, df = 54, $p < 0.005$) and with various other growth factors. This increase persists for at least 21 d (Fig. 5), suggesting a long-term change in the oligodendroglial cell population. Importantly, other growth factors reported to alter growth of glia in culture, including FGF, EGF, IGF-1, IL-1, or IL-2, had no effect upon the myelin-associated enzyme (Fig. 5); that is, OGF demonstrates an *in vivo* biologic activity unlike that observed for the other growth factors tested.

Oligodendroglia growth factor and biosynthesis of myelin basic protein

MBP comprises 30–35% of the protein in rat brain myelin and includes the predominate large forms with molecular masses of about 18–21 kDa and small forms of about 14 kDa (Barbarese et al., 1977; Benjamins and Morell, 1978; Norton, 1981). In order to monitor protein biosynthesis, we used an immunoprecipitation method that recovers both the 18- and the 14-kDa constituents of rat MBP (Fig. 6, left and right panels). Quantification of recovered radiolabeled protein shows that injection of OGF into the cerebral cortex leads to a 3–4-fold increase in the production of total MBP when compared to tissue from



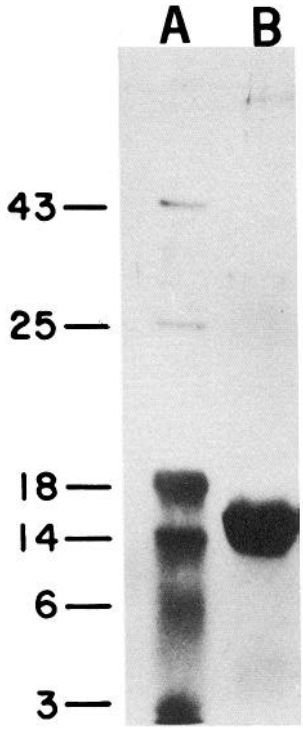
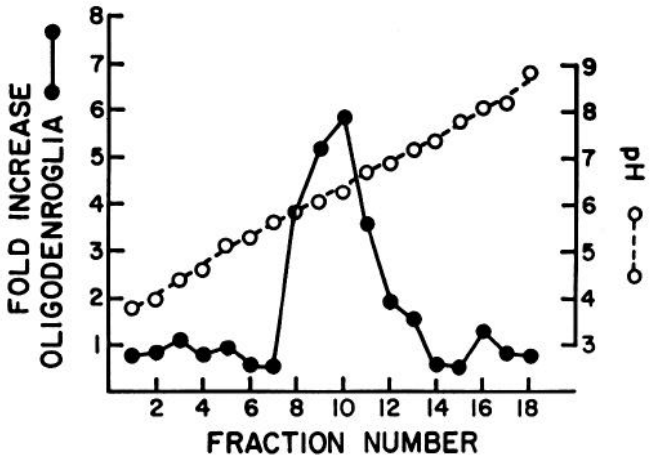


Figure 2. Characterization of OGF after isolation by gel filtration and 2-step RP-HPLC. *Upper panel,* Sieving-filtration HPLC indicated that the oligodendroglia-stimulating activity has a molecular mass of about 14–18-kDa. *Lower panel,* SDS-PAGE demonstrated a single band, visualized with silver stain (*lane B*), with an estimated molecular mass of 16 kDa. *Lane A,* Molecular-mass markers (approximately 200 ng each), which include equine myoglobin (18 kDa), lysozyme (14 kDa), bovine trypsin inhibitor (6 kDa), and insulin (3 kDa). These markers were also used for SDS-PAGE shown in Figures 3 and 6.

controls (Student's $t = 3.28$, df = 61, $p < 0.01$) or from sites infused with cytochrome C (Fig. 6, lower panel). In a second set of experiments using pooled biopsies from 6 animals, densitometric measurements of autoradiograms show similar differences with a 3.7-fold increase of radiolabeled MBP in OGF-stimulated versus control injection sites.

Oligodendroglia growth factor and expression of proteolipid protein

Proteolipid protein (PLP), a hydrophobic transmembrane component of myelin, represents nearly 50% of the protein found in myelin (Folch and Lees, 1951; Laursen et al., 1984; Macklin et al., 1986). Milner et al. (1985) have cloned the gene for PLP and isolated a probe suitable for measuring expression of PLP mRNA. As shown in Figure 7, upper panel, 2 RNAs of about 3.2 and 1.6 kilobases (kb) are identified in Northern transfers of RNA isolated from brain biopsies using the PLP-1 probe; tissue specificity is shown by the lack of binding to RNA isolated from rat liver (Milner et al., 1985). Using this PLP-1 probe, we examined expression of PLP after OGF infusion into the brain. Quantification of specific binding by dot-blot analysis indicates a 2-fold increase in PLP RNA levels (Fig. 7) at those sites receiving 100 ng OGF (237 ± 16 cpm for 9 pooled tissue samples) when compared to those receiving 100 ng cytochrome C (93 ± 8 cpm for 8 tissue samples; Student's $t = 7.20$, df = 15, $p < 0.001$). No significant increases in myelin PLP RNA are found within tissues infused with basic FGF, IGF-1, IL-1, or IL-2 (data not shown).



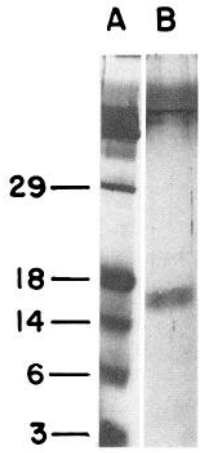


Figure 3. Highly purified OGF examined by preparative IEF. *Upper panel,* A plot of biologic activity and pH gradient for fractions separated by an IEF cell. OGF demonstrated a biologic activity with a pI of 6.3 ± 0.2 ($n = 5$). *Lower panel,* When stained by silver method, recovered protein from fraction 10 appeared as a single band (*lane B*) with a mass of about 16 kDa. *Lane A,* Molecular-mass markers (approximately 200 ng each), which include equine myoglobin (18 kDa), lysozyme (14 kDa), bovine trypsin inhibitor (6 kDa), and insulin (3 kDa).

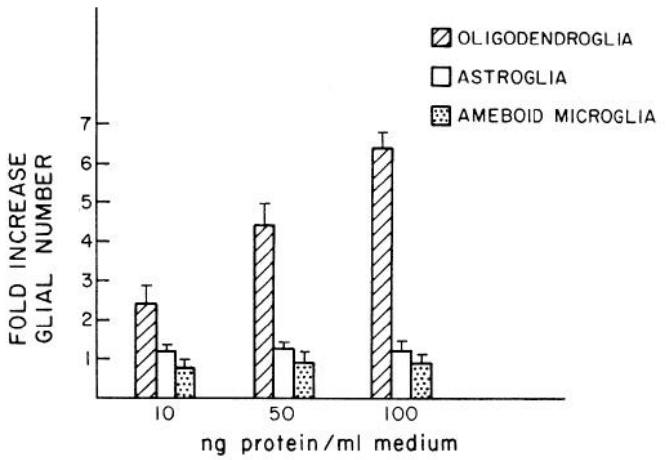


Figure 4. Effects of highly purified OGF upon CNS glia *in vitro.* OGF isolated by gel filtration, RP-HPLC, and IEF was incubated with cultures of glia grown in chemically defined medium for 72 hr. Quantification of the 3 glial-cell types found in dissociated brain cultures demonstrates that OGF is a growth factor specific for oligodendroglia. Data, mean values ± SE, are expressed as *n*-fold increase in cell number when compared to control cultures incubated with matching aliquots of PBS. Oligodendroglia are identified using indirect immunofluorescence techniques as cells containing GC, astroglia as those cells containing GFAP, and ameboid microglia as those containing ac-LDL receptors.

Discussion

Although myelin is essential for development and function of the nervous system (Bunge, 1968; Norton, 1981), the signals that initiate myelin protein synthesis and myelin membrane formation remain uncertain. In the PNS, it appears that both axonal membranes (Wood and Bunge, 1975; Salzer et al., 1980) as well as soluble factors (Lemke and Brockes, 1984; Le Beau et al., 1988) stimulate proliferation and protein synthesis in myelin-producing Schwann cells. Such observations argue that specific regulatory signals help to control glial populations and, ultimately, myelination of peripheral nerves (Lemke, 1988). There is indirect evidence that similar regulatory processes control myelination in the CNS. Histological investigations and coculture experiments, for example, show a close association between growing neurons and myelin-producing oligodendroglia (Weinberg and Spencer, 1975; Aguayo et al., 1976; Bologa et al., 1982; Wood and Williams, 1984; Macklin et al., 1986). It is also clear that the proliferation and differentiation of oligodendroglia may be facilitated *in vitro* by soluble factors obtained from neurons or neuronal cell lines (Bologa et al., 1986; Giulian et al., 1986; Bottenstein et al., 1988).

As reported here, OGF, a protein growth factor secreted by neuronal cell lines (Giulian and Young, 1986), has the ability to stimulate 3 different specific markers for myelin: activity of 2′,3′CNPase, synthesis of MBP, and RNA levels of PLP. OGF has a molecular mass of 16 kDa and an isoelectric point of about 6.3, which distinguishes it from IGF-1, insulin, EGF, PDGF, and basic and acidic FGFs. The biological actions of OGF upon oligodendroglia in culture are interesting and include an ability

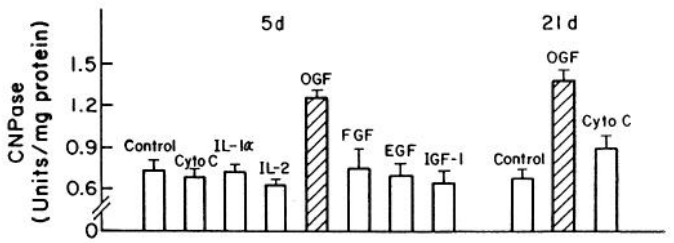


Figure 5. In vivo effects of OGF and other growth factors on specific activity of 2′,3′-CNPase. Highly purified OGF was injected into the cerebral cortex of adult rats. Biopsied tissues from the injection sites were examined for the 2′,3′-CNPase activity. Data, expressed as mean units of enzymic activity per mg protein ± SE, were measured from samples consisting of 3 pooled tissues. At least 4 samples were analyzed for each type of growth factor tested. Only OGF increased the specific enzymic activity when compared to uninjected cerebral cortex (*Control*) at 5 d (Student's $t = 6.04$, df = 54, $p < 0.005$) and at 21 d (Student's $t = 6.58$, $df = 11$, $p < 0.001$) after injection, with the adjusted confidence levels using the Bonferroni correction for these multiple comparisons as $p < 0.007$ and $p < 0.025$, respectively. Samples for injection included 2 μl vol containing 100 ng *OGF,* 100 ng cytochrome C (*Cyto C*), 100 ng *FGF,* 100 ng *IGF-1,* 2 U IL-1 (*IL-1α*), 100 U *IL-2,* or 20 ng *EGF.*

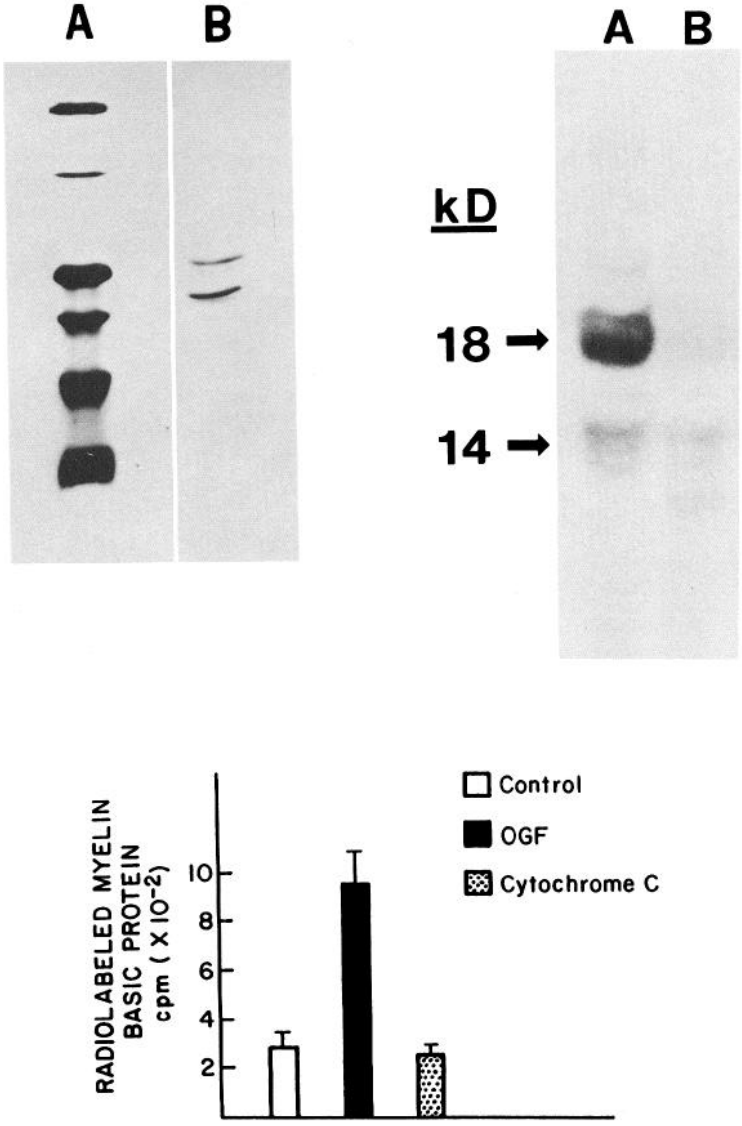


Figure 6. Effects of OGF upon synthesis of rat MBP. *Left panel,* Photograph of a protein transfer showing binding of rabbit anti-bovine MBP IgG to the large (about 18 kDa) and to the small forms (about 14 kDa) of MBP found in crude myelin membranes of the rat. A similar staining pattern was noted for isolated rat MBP (data not shown). Molecular-mass markers shown in *lane A* are described in Figure 2. *Right panel,* Autoradiogram of radiolabeled MBP recovered by immunoprecipitation from OGF injection site (*lane A*) or from a cytochrome C injection site (*lane B*) 5 d after injection of 100 ng of either protein. SDS-PAGE shows that a major portion of the radioactivity was associated with the 18-kDa component of rat MBP. Densitometry of autoradiograms indicates a 3.7 ± 0.3-fold increase in total radiolabeled MBP in the OGF-treated as compared to control tissues. *Lower panel, In vivo* effects of OGF on the synthesis of MBP. Five d after intracerebral infusions of 100 ng OGF or 100 ng cytochrome C, injection sites were isolated and labeled with ^{35}S-methionine. This histogram presents the amount of radiolabeled MBP recovered by immunoprecipitation. Data, mean values ± SE, are expressed as counts per min with the background subtracted (see Materials and Methods). The OGF injection sites produced significantly more MBP (Student's $t = 3.28$, $df = 61$, $p < 0.01$) when compared with control tissue.

to increase absolute cell numbers, stimulate ^{3}H-thymidine incorporation, and accelerate differentiation. Although OGF appears to have unique biologic properties with specific actions upon oligodendroglia, caution must be exercised when claiming the discovery of a novel growth factor. Determining the structural relationship of OGF to other classes of growth-regulating proteins awaits sequence analysis.

The pattern of development for cultured oligodendroglia includes the early expression of A2B5 (Raff et al., 1988) and O4 surface antigens (Sommer and Schachner, 1981; Behar et al., 1988). Eventually, A2B5(+), O4(+) progenitor cells differentiate into highly branched GC(+) cells that produce such myelin proteins as 2′,3′CNPase, PLP and MBP (Knapp et al., 1987, 1988). A number of laboratories have examined conditions that alter the *in vitro* behavior of these developing cells. Raff et al. (1988) and others (Hughes et al., 1987; Nobles et al., 1988; Richardson et al., 1988) describe the ability of fetal calf serum, CNTF, FGF, and PDGF to promote proliferation or differentiation of glial progenitor cells. In the presence of these factors, glial cultures from the optic nerve (Raff et al., 1988; Richardson et al., 1988) or cerebral cortex (Behar et al., 1988) show an increase in the number of GFAP(+) astroglia with a concomitant decrease in the appearance of differentiated GC(+) oligodendroglia. Insulin and IGF-1, in contrast, accelerate proliferation and differentiation of many cell types, including cultured oligodendroglia (McMorris et al., 1986; McMorris and Dubois-Dalcq, 1988). [The effects of IGF-1 can be blocked by insulin, which binds to IGF-1 receptors. The chemically defined culture medium used here contains 5 μg/ml insulin, which precludes the action of IGF-1 upon glia (McMorris and Dubois-Dalcq, 1988).] In previous work (Giulian and Young, 1986; Giulian et al., 1986), we have shown that OGF applied to cultured brain glia increases the number of A2B5(+) progenitor cells, the number of differentiated GC(+) oligodendroglia, and the amount of ^{3}H-thymidine incorporated by enriched populations of GC(+) cells. Such experiments suggest that the biologic actions of OGF *in vitro* oppose those of growth factors, which promote glial progenitor cells but not GC(+) oligodendroglia. Thus, when examined by *in vitro* bioassays, OGF appears to be an oligodendroglia-specific mitogen distinct from such astroglial growth factors as EGF, FGF, and IL-1 (Giulian et al., 1986), from such progenitor cell factors as PDGF and CNTF, or from such mi-

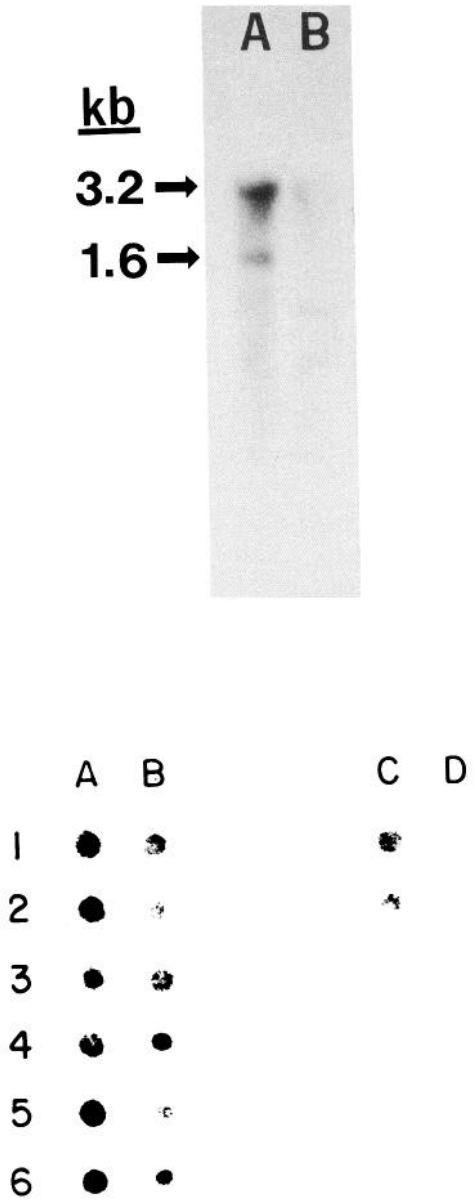


Figure 7. *In vivo* effects of OGF upon myelin PLP RNA levels. *Upper panel,* Northern blot of RNA isolated from rat brain biopsies (*lane A*) or from rat liver (*lane B*) showed that only the brain contained mRNA that bound specifically to the ^{32}P-PLP-1 probe. Two RNA species at 3.2 and 1.6 kb are found for PLP, as described by Milner et al. (1985). *Lower panel,* RNA, isolated from sites injected with 100 ng OGF (*lanes A, B*) or 100 ng cytochrome C (*lanes C, D*), was blotted onto nylon membranes and hybridized with a ^{32}P-labeled PLP-1 probe. This composite autoradiogram shows representative dot blots with 4-μg (*lanes A, C*) and 2-μg loads (*lanes B, D*) from pooled biopsies. Each *number* represents a tissue sample collected from injection sites of 3 different animals. Greater amounts of RNA for PLP were found in OGF infusion sites in 6 pooled tissue samples (*lanes A, B*) when compared to 4 pooled control samples (*lanes C, D*). Dot blots were exposed to X-ray film for 48 hr.

croglial mitogens as multi-CSF or GM-CSF (Giulian and Ingeman, 1988). It is necessary, of course, to be cautious in assessing the significance of such specificity of action *in vitro,* because stringent culture conditions may falsely indicate a growth factor's effect. However, as reported here, the monitoring of myelin proteins indicates that OGF, but not FGF, IL-2, or IGF-1, activates oligodendroglia *in vivo.* For these reasons, we believe that OGF is a true regulatory factor specific for a single class of CNS glia.

It is established that proliferating oligodendroglia appear in the adult mammalian CNS (Mori and LeBlond, 1970; Ludwin, 1984), as well as in nonmammalian neural tissues undergoing regeneration (Giulian and Iwanij, 1985). However, it is not understood whether proliferating oligodendroglia found *in vivo* originate from unrecognized progenitor cell populations or from activated, mature cells (Skoff, 1980). Although it is likely that OGF acts directly upon oligodendroglia *in vivo,* we do not know if the observed increases in myelin protein synthesis reflect action upon mature cells or a recruitment of undifferentiated glia. Further study of the adult mammalian brain will be needed to identify cell targets for OGF and to determine if, in fact, OGF accelerates myelination of axons. It may be that this growth factor will not trigger myelin membrane formation unless an appropriate axonal site is available. Future investigation will assess the putative role of OGF in myelination of growing, regenerating, and demyelinated axons.

Although sparse proliferation of oligodendroglia and an incomplete remyelination of axons may occur in adult mammals (Mori and LeBlond, 1970; Blakemore et al., 1977; Ludwin, 1984), the formation of new myelin membranes is quite limited in most neuropathic conditions (Prineas et al., 1969; McFarlin and McFarland, 1982). In general, a loss of myelin membrane impairs brain function, as noted in patients with multiple sclerosis, progressive multifocal leukoencephalopathy, or central pontine myelinolysis (Wright and Laureno, 1979; McFarlin and McFarland, 1982). We find the greatest concentrations of OGF in the cerebral cortex of the rat during the postnatal period (Giulian et al., 1986) or that time of peak oligodendroglial proliferation and myelin protein synthesis (Zeller et al., 1985; Kristensson et al., 1986; Verity and Campagnoni, 1988). Tissue levels of OGF are also high in the injured, regenerating goldfish visual system, but not in the injured, nonregenerating adult mammalian brain (Giulian et al., 1985; Giulian and Young, 1986). Studies using isolated neurons, various cell lines, and primary glial cultures suggest that growing neurons release OGF-like molecules to promote growth of neighboring oligodendroglia (Giulian and Young, 1986; Giulian et al., 1986). Perhaps the secretion of OGF-like proteins from neurons helps to control myelination during development and after brain injury.

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