Brain Peptides and Glial Growth. I. Glia-promoting Factors as Regulators of Gliogenesis in the Developing and Injured Central Nervous System

Dana Giulian, Robert L. Allen, Timothy J. Baker, and Yasuko Tomozawa

Program of Neuroscience and Department of Neurology, Baylor College of Medicine, Houston, Texas 77030

Abstract. Glia-promoting factors (GPFs) are peptides of the central nervous system which accelerate the growth of specific glial populations in vitro. Although these factors were first discovered in the goldfish visual system (Giulian, D., Y. Tomozawa, H. Hindman, and R. Allen, 1985, *Proc. Natl. Acad. Sci. USA.*, 83:4287–4290), we now report similar peptides are found in mammalian brain. The cerebral cortex of rat contains oligodendroglia-stimulating peptides, GPF_1 (15 kD) and GPF_3 (6 kD), as well as astroglia-stimulating peptides, GPF_2 (9 kD) and GPF_4 (3 kD).

The concentrations of specific GPFs increase in brain during periods of gliogenesis. For example, GPF_1 and GPF_3 are found in postnatal rat brain during a peak of oligondendroglial growth while GPF_2 and GPF_4 are first detected at a time of astroglial proliferation in the embryo. Stab wound injury to the cerebral cortices of rats stimulates astroglial proliferation and induces marked elevations in levels of GPF_2 and GPF_4. Our findings suggest that two distinct classes of GPFs, those acting upon oligodendroglia and those acting upon astroglia, help to regulate cell growth in the developing and injured central nervous system.

Glial cells and their precursors play an important role in organizing patterns of neuronal growth during development of or following injury to the central nervous system (1, 19, 25–28). Singer et al., for example, propose that primitive glia form channels which align and direct growing axons in the developing spinal cord of newt (28). In fetal mammalian brain, glia aid neuronal migration (25) and help to regulate growth of axons across the corpus callosum (27). During such glia–neuron interactions, it is likely that stimulatory signals are needed to activate glia which in turn create an environment suitable for growing axons. The biochemical basis for glial "activation" remains unknown.

Study of the regenerating goldfish visual system showed that peptides recovered from the optic tectum stimulated proliferation of fish macroglia (6, 8, 9). These fish peptides, referred to as glia-promoting factors (GPFs),[1] were also mitogens for mammalian cells in culture (12) with GPF_1 and GPF_3 acting upon oligodendroglia and GPF_2 and GPF_4 stimulating astroglia. Our observations suggested that GPF-like factors might also be found in neural tissues of mammals. We now report the presence of GPFs in the developing and injured rat brain. It appears that two distinct classes of GPFs, the astroglia-stimulating peptides and the oligodendroglia-stimulating peptides, stimulate gliogenesis in the central nervous system of mammals.

[1] *Abbreviations used in this paper:* Dulbecco's MEM, Dulbecco's minimal essential medium; EGF, epidermal growth factor; FCS, fetal calf serum; FGF, fibroblast growth factor; GC, galactocerebroside; GFAP, glial fibrillary acid protein; GMF, glial maturation factor; GPF, glia-promoting factor; GS, goat serum; IL-1 and -2, Interleukin-1 and -2.

Materials and Methods

Preparation of Cell Cultures

Mixed glial cultures were prepared from the brains of newborn rats and grown on coverslips coated with poly-L-lysine. Cerebral cortices from rat were stripped of meninges and isolated in ice-cold calcium- and magnesium-free phosphate-buffered saline (PBS) (pH 7.4) (10). Tissue was then placed in chemically defined medium (4) containing DNase (1 mg/ml; Sigma Chemical Co., St. Louis, MO), and minced with iridectomy scissors. After addition of trypsin (0.2% wt/vol; Sigma Chemical Co.), tissue fragments were dissociated by trituration. Cells in suspension were collected and washed twice with defined medium containing 10% fetal calf serum (FCS) (Gibco, Santa Clara, CA) which stopped the enzymatic dissociation. Cell viability was determined by erythrosin B exclusion using a hemocytometer; between 800,000 and 1,000,000 viable cells in defined medium with 10% FCS were added to 35-mm plastic culture dishes that contained 22-mm square coverslips (Fisher Scientific Co., Pittsburgh, PA) coated with poly-L-lysine (Sigma Chemical Co.). After 48 h, cells were washed three times with defined culture medium and used for bioassays as described below. Enriched populations of astroglia and oligodendroglia were obtained using the method of McCarthy and de Vellis (18). The glial cell line C6 (American Type Culture Collection, Rockville, MD; CCL #107) was grown in 100-mm plastic dishes containing defined medium.

Cell Identification

Identification of astroglia which contain glial fibrillary acidic protein (GFAP) was carried out using indirect immunofluorescence techniques (23, 24). Cells adhering to poly-L-lysine–coated coverslips were washed three times with Dulbecco's minimal essential medium containing 1% heat-inactivated FCS (Dulbecco's MEM–1% FCS; Gibco, Grand Island, NY). Cells were fixed at −20°C for 30 min with 90% acetone/10% acetic acid (vol/vol). Coverslips were

© The Rockefeller University Press, 0021-9525/86/03/0803/09 $1.00

dipped sequentially in 100% acetone, 70% ethanol, PBS, and Dulbecco's MEM-1% FCS. Rabbit anti-human GFAP-serum (whole serum; Accurate Chemical & Scientific Corp., Westbury, NY) was diluted with Dulbecco's MEM containing 1% goat serum (Dulbecco's MEM-1% GS) to a concentration of 1:200, and applied directly to the coverslips. Coverslips were incubated for 45 min at 37°C in 95% air/5% CO_2 with high humidity and then washed with Dulbecco's MEM-1% FCS. A goat anti-rabbit immunoglobulin conjugate of rhodamine isothiocynate (Accurate Chemical & Scientific Corp.) diluted to 1:100 with Dulbecco's MEM-1% GS was next applied for 45 min at 37°C. Coverslips were washed five times with Dulbecco's MEM-1% FCS and mounted in solution containing 1 mg/ml *p*-phenylenediamine, 10% PBS, and 90% glycerol at pH 8.0. Fluorescence-labeled cells were viewed at 200× magnification with a Nikon inverted microscope using epifluorescence. Oligodendroglia containing galactocerebroside (GC) were identified by indirect immunofluorescence using anti-GC serum. The technique was similar to that of astroglia except that cells were fixed after antibody binding. Rabbit anti-GC serum produced by our laboratory and by Dr. T. Inagami of Vanderbilt University (Nashville, TN) was diluted to 1:100 using Dulbecco's MEM-1% GS. In addition, monoclonal antibody against GC (ascites fluid was a gift from Dr. Barbara Ranscht) was diluted 1:200 and applied in a similar fashion with a goat anti-mouse immunoglobulin conjugate of Texas Red (1:50; Accurate Chemical & Scientific Corp.) serving as the fluorescent label.

Neuroglial progenitor cells containing the A2B5 surface antigen were also identified by immunofluorescence techniques (22). Mouse ascites fluid (A2B5 hybridoma from American Type Culture Collection #CRL 1520) diluted 1:50 was applied as described for GC with Texas Red (1:50) serving as the fluorescent conjugate. Rabbit anti-Thy-1 serum (1:100; Accurate Chemical & Scientific Corp.) was used to label fibroblasts (24) by the method described for GFAP. Microglia were identified as phagocytic cells, containing nonspecific esterase, acetylated low density lipoprotein receptors, and the Mac-1 and Mac-3 antigens (7, 24).

The cellular composition of untreated 3-d cultures of newborn rat cerebral cortex is shown in Table I. Approximately 35-45% of cells were fibroblastic astroglia containing glial fibrillary acidic protein ($GFAP^{+}$). These cells were $A2B5^{(-)}$ and corresponded to the Type 1 astrocyte described by Raff et al. (22). The Thy-$1^{(+)}$ fibroblasts made up <1% of all cells in culture and were not evaluated further in this study. Microglia represented ~5-7% of all cells in culture, while $GC^{(+)}$ oligodendroglia contributed between 3-6% of the cell populations. Approximately 20% of cells in rat brain cultures were $A2B5^{(+)}$; nearly all of these cells were $GC^{(-)}$, $GFAP^{(-)}$, round in shape, and some contained thin, short processes. We assumed that most of the $A2B5^{(+)}$ cells and the remaining unstained fibroblastic cells were progenitor cells of macroglia (22-24).

Selective Cell Destruction

Oligodendroglia were selectively destroyed by a complement lysis technique (22). Cells grown on poly-L-lysine-coated coverslips were washed three times with defined medium, covered with 100 µl of medium containing anti-GC serum (1:100 dilution), and placed in a humidified incubator with a 95% air/5% CO_2 atmosphere for 30 min at 37°C. After two washes with defined media, 100 µl of medium containing guinea pig complement (1:10 dilution, Sigma Chemical Co.) were added for an additional 60 min at 37°C. With these conditions, <0.1% of remaining cells were intact $GC^{(+)}$ oligodendroglia.

A similar method was used to lyse $A2B5^{(+)}$ cells with an incubation of A2B5 hybridoma ascites fluid (1:100) for 30 min followed by complement lysis (1:50) for 30 min. Under these conditions, >99% of all $A2B5^{(+)}$ cells were eliminated.

To destroy microglia, cell suspensions were incubated with 5 mM L-leucine methyl ester in defined medium for 3 h at 37°C (7, 30).

Table I. Cellular Composition of Dissociated Cultures from Newborn Rat Cerebral Cortex

	%
$GFAP^{(+)}$ astroglia	35-45
$A2B5^{(+)}$ cells	20-22
$GC^{(+)}$ oligodendroglia	3-6
Nonspecific $esterase^{(+)}$ microglia	5-7
Thy $1^{(+)}$ fibroblasts	<0.5

Glia populations found in cultures of cerebral cortex from brains of newborn rat. After 3 d in vitro, cells were identified using the specific markers described in Materials and Methods.

GPF Isolation and Bioassay

Transection of the goldfish optic tract was carried out 1.0-1.5 mm before its entry into the tectum. An intact contralateral visual system provided control tissue (6). Optic tecta were isolated 10 d after axotomy and frozen at −20°C. Tissues were dispersed by mild sonication in PBS (pH 7.4) and centrifuged at 15,000 *g* for 30 min (Microfuge). Supernatants were pooled, passed through a 0.45-µm filter (Millipore/Continental Water Systems, Bedford, MA), and separated by a gel filtration column (100 × 0.9 cm, P10; Bio-Rad Laboratories, Richmond, CA). Embryos and neonates from albino rats (Holtzman Co., Madison, WI) were the source of GPFs during development. Stab wound injury to the cerebral cortices of anesthetized adult rats was carried out as described earlier (10). GPFs were recovered from rat brain in a fashion identical to that of the fish factors.

Partially purified GPFs were identified by monitoring glia-stimulating activity in fractions recovered from gel filtration. Pooled fractions were added to culture dishes that contained 1.5 ml of chemically defined medium to give final GPF concentrations ranging from 0.1 to 10 µg protein/ml medium. Protein determinations were estimated by the fluorescamine method using bovine serum albumin standards (3).

Peptide Hormones

Partially purified fibroblasts growth factor (FGF) and epidermal growth factor (EGF) were obtained from Collaborative Research (Lexington, MA). Partially purified glial maturation factor (GMF) was a gift from Dr. R. Lim of the University of Iowa (Iowa City, IA). Human Interleukin-1 (IL-1) was a gift from Dr. L. B. Lachman of M.D. Anderson Hospital and Tumor Institute (Houston, TX) and cloned Interleukin-2 (IL-2) from Dr. A. Mazumder of Baylor College of Medicine (Houston, TX).

Glutamine Synthetase Assay

200,000 cells of the C6 glial line were grown on 100-mm plastic culture dishes in defined medium with 10% FCS. Dishes were washed three times with defined medium and incubated with 3.0 ml of defined medium containing 200 µl of partially purified GPFs or PBS. After 48 h, cultures were washed twice with PBS and lysed with 500 µl of 50 mM *N*-morpholino ethane sulfonic acid buffer (pH 7.0) containing 1% Nonidet P-40 (Sigma Chemical Co.). Cells were removed with a rubber policeman and dispersed by sonication into 1.5-ml conical tubes. Enzyme assays were carried out in triplicate with protein concentrations ranging from 0.27 to 0.39 mg/ml. To measure glutamine synthetase activity, 100 µl of the lysate was incubated with 900 µl of the substrate solution (50 mM imidazole-HCL buffer, [pH 7.2]; 20 mM $MgCl_2 \cdot 6\ H_2O$; 50 mM L-glutamic acid; 0.11 M hydroxylamine; 30 mM 2-mercaptoethanol; 10 mM phosphoenolpyruvate; 20 mM ATP; and 50 U of pyruvate kinase (Sigma Chemical Co.) (15). After 60 min at 37°C, the reaction was stopped by the addition of 1.5 ml of solution containing 370 mM $FeCl_3$ and 200 mM trichloroacetic acid, and the mixture was centrifuged at 15,000 *g* (Microfuge) for 15 min at 4°C. The reaction product was measured at 535 nm (Model 250, Gilford Instrument Laboratories, Inc., Oberlin, OH) against blanks containing 50 mM *N*-morpholino ethane sulfonic acid buffer only. Specific activity for glutamine synthetase was expressed as µmol of γ-glutamylhydroxamic acid formed per microgram of protein per hour.

Results

GPFs in Mammalian Brain

As reported earlier (12), peptides isolated from the goldfish visual system stimulated growth of specific populations of rat brain glia. The response of mammalian cells to fish GPFs suggested that growth factors with similar biological activities existed within mammalian brain. Accordingly, we screened for the presence of GPFs in the central nervous system of rat. Supernatants from newborn rat cerebral cortex contained glia-stimulating factors. Gel filtration separated two peaks of growth activity specific for oligodendroglia. These factors with molecular masses of 15 and 6 kD (Fig. 1) corresponded to GPF_1 and GPF_3 found in the goldfish optic tectum (Fig. 2; reference 12). Astroglia-stimulating factors recovered from newborn rat brain (Fig. 1) included 9- and 3-kD peaks corresponding to fish GPF_2 and GPF_4 (Fig. 2). The glia-stimulating

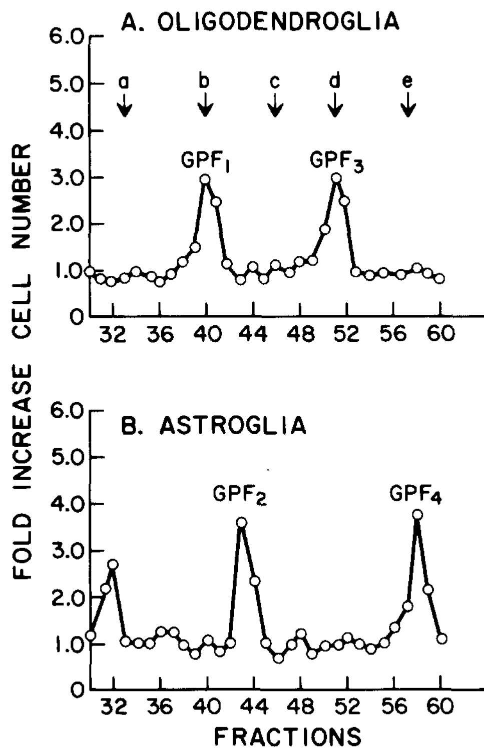

Figure 1. 20 mg of soluble protein from newborn rat cerebral cortex were applied to a gel filtration column (P-10, 100 × 0.9 cm) and eluted with sterile PBS (pH 7.4). 100-μl aliquots taken from the 700-μl fractions were incubated for 72 h with glial cultures grown in defined medium on poly-L-lysine–coated glass coverslips. Using immunofluorescence techniques, astroglia were identified as $GFAP^{(+)}$ cells and oligodendroglia as $GC^{(+)}$ ones. Mean cell counts were determined by scoring the number of glia found in 10 randomly selected fields. Data are expressed as fold increase in a specific glial population when compared with control cultures treated with equivalent volumes of PBS. Three coverslips were used for each data point (*A.*). Two peaks of oligodendroglia-promoting activity were recovered with apparent molecular masses of 15 kD (GPF_1) and 6 kD (GPF_3) (*B.*). Three peaks of astroglia-promoting activity were recovered with apparent molecular masses of 18 kD, 9 kD (GPF_2) and 3 kD (GPF_4). Molecular mass markers: (*a*) 17 kD; (*b*) 14 kD; (*c*) 6.4 kD; (*d*) 2.5 kD.

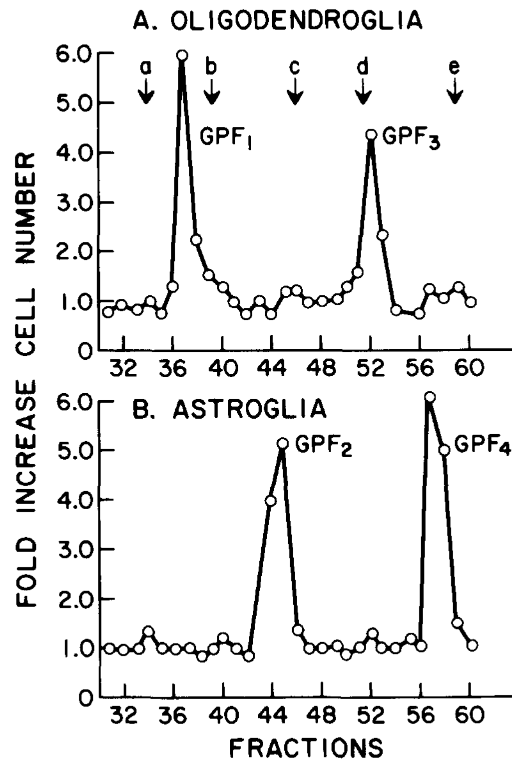

Figure 2. Recovery of GPFs from goldfish optic tecta 10 d after ganglion cell axotomy. 15 mg of soluble tectal protein were eluted from a P10 (100 × 0.9 cm) column with PBS (pH 7.2) and assayed as described in Fig. 1. GPF_1 and GPF_3 increased the number of oligodendroglia in culture while GPF_2 and GPF_4 increased the number of astroglia. Molecular mass markers: (*a*) 17.0 kD; (*b*) 14.5 kD; (*c*) 8.0 kD; (*d*) 6.4 kD; (*e*) 2.5 kD.

factors recovered from rat brain were trypsin-sensitive peptides (Fig. 3) and co-purified with fish GPFs isolated by anion exchange chromatography and by reverse-phase high performance liquid chromatography (11). Based upon similarities in biological activities, apparent molecular masses, and elution profiles, we concluded that GPFs from rat brain were structurally similar to GPFs isolated from goldfish optic tectum. In addition, the rat brain contained an 18-kD peptide (Fig. 1) that stimulated astroglia in culture; this factor was not detected in fish optic tecta and subsequently has been identified as IL-1 (10).

GPFs and Target Cell Specificity

GPFs might stimulate the appearance of differentiated glia (i.e., $GC^{(+)}$ or $GFAP^{(+)}$ cells) in culture by acting directly upon specific target cells or indirectly by eliciting growth factors from neighboring cells. To monitor the direct effects of GPFs, we used enriched cultures of astroglia or oligodendroglia. In these enriched cell preparations, we obtained populations of either 90% $GC^{(+)}$ oligodendroglia (with <1% astroglia) or 98% $GFAP^{(+)}$ astroglia (<0.1% oligodendroglia). 72-h incubations with GPF_1 increased the number of cells in oligodendroglial cultures by four- to fivefold (Figs. 4 and 5); in contrast, GPF_2 stimulated proliferation of astroglia by as much as 10-fold but did not affect oligodendroglial growth (Figs. 4 and 5). These findings support the idea that GPFs influenced cell growth by direct action upon specific target cell populations.

A variety of peptides isolated from mammalian tissues have been found to stimulate proliferation of astroglia or Schwann cells in culture (10, 13, 15–17). Since the relationship of such peptides to GPFs was unknown, we compared the biological actions of GPFs with GMF, EGF, and FGF. As shown in Fig. 6, partially purified GPF_1 stimulated the appearance of $GC^{(+)}$ oligodendroglia in a dose-dependent fashion, although it had little effect upon astroglial number. Other various peptides, including GPF_2, GMF, EGF, and FGF (Fig. 6), did not change the number of oligodendroglia found in culture. In addition neurotensin, IL-1, IL-2, and β-endorphin (data not shown) had no effect on the growth of oligodendroglia. The specificity of action for oligodendroglia-stimulating peptides suggested that GPF_1 and GPF_3 represented a unique class of glia-specific growth factor.

We found that a number of peptides, including EGF, FGF, and GPF_2 (Fig. 6), served as mitogens for $GFAP^{(+)}$ astroglia. Although the astroglia-stimulating GPFs might be similar to other growth factors, GPF_2 did not co-purify with EGF, FGF, or IL-1 by gel filtration chromatography (data not shown). Moreover, GPF_2 was inactive in the thymocyte stimulation

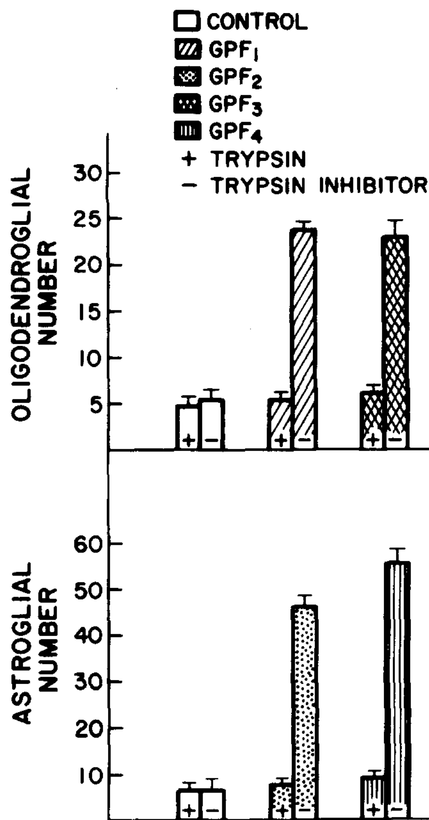

Figure 3. Effects of trypsin on GPFs. Partially purified GPFs isolated from newborn rat cerebral cortex were incubated with trypsin (100 μg GPF/1 μg trypsin) for 60 min at 37°C. Control preparations also contained soybean trypsin inhibitor (2 μg inhibitor/1 μg trypsin). Data are expressed as fold increase in the mean cell number when compared with cultures grown in equivalent volumes of PBS. Five cultures were used for each group. As shown, all rat GPFs were sensitive to trypsin.

assay for IL-1 (10) and did not compete for EGF-binding sites (data not shown). Further attempts to compare GPF_2 with other peptides will require biochemical analyses of purified factors.

We also examined the target selectivity of GPFs by measuring their ability to induce glutamine synthetase, an enzyme associated with brain astrocytes. Other investigators have established that the levels of glutamine synthetase correlate with the degree of astroglial differentiation (5, 14, 20). Our initial study showed that the basal activity of this enzyme increased by 1.5–2.0-fold in newborn rat brain cultures after incubations for 48 h with the astroglia-stimulating peptide GPF_2 (1 μg protein/ml culture medium). We followed this enzyme induction more closely by comparing the abilities of different GPFs to stimulate glutamine synthetase in the glial cell line C6, a population of poorly differentiated cells with astroglial properties (2). Incubations with GPF_2 increased the levels of enzyme activity by fivefold above those found in untreated C6 cultures and by threefold above those measured in cultures incubated with the oligodendroglia-stimulating peptide GPF_1 (Table II). The findings suggested that specific GPFs might induce astroglial differentiation in the developing brain.

Effects of GPFs Upon Survival, Differentiation, and Proliferation of Glia

Incubation of newborn rat brain cells with GPFs markedly increased the percent of differentiated astroglia or oligodendroglia when compared to control cultures (Table III). The greater number of glia found after GPF incubation might reflect an increase in the survival time of cells grown in chemically defined culture medium. Cell counts showed, however, that after 7 d, untreated cultures increased their total cell number by 150–250%, with no significant cell death under the culture conditions used. Thus, increased cell survival times alone did not account for the abilities of GPFs to increase glial cell number.

Alternatively, GPFs might act either by accelerating the expression of glial markers among undifferentiated cells or by stimulating proliferation of mature cells. To test the ability of GPF_1 to stimulate undifferentiated cells, we first destroyed differentiated oligodendroglia that contained the surface antigen GC by complement lysis. The remaining cells were then treated with GPF_1. By 3 d after GPF_1 incubation, more than 50% of the total cell population contained $GC^{(+)}$ oligodendroglia compared with 1.0% $GC^{(+)}$ cells found in the control cultures lacking GPF_1 (Fig. 7). These findings indicated that undifferentiated glia were sensitive to the effects of GPF_1.

The surface antigen A2B5 has been identified in a variety of neuro-epithelial cells during embryogenesis (22). Raff et al. (12) have found that $A2B5^{(+)}$ stem cells differentiate into $GC^{(+)}$ oligodendroglia and can be distinguished from $A2B5^{(-)}$ stem cells which develop into fibroblastic $GFAP^{(+)}$ astroglia. We monitored the $A2B5^{(+)}$ cell population in cultures of newborn rat brain treated with GPFs. There was a two-fold increase in the number of $A2B5^{(+)}$ progenitor cells after incubation with GPF_1 although not after incubation with GPF_2 (Table IV). These data supported the notion that GPF_1 acted upon a target cell population distinct from that of GPF_2.

The response of $A2B5^{(+)}$ cells to GPF_1 raised the possibility that oligodendroglia in the early stages of differentiation (i.e., $GC^{(-)}$ $A2B5^{(+)}$ and $GC^{(+)}$ $A2B5^{(+)}$) cells were targets for peptide factors. We could not rule out this possibility using routine cultures, for oligodendroglia (isolated by the method of McCarthy) contained fully differentiated $GC^{(+)}$ $A2B5^{(-)}$ cells as well as the less mature $A2B5^{(+)}$ ones. Destruction of all $A2B5^{(+)}$ cells by complement lysis allowed us to test the effects of GPF_1 upon fully differentiated cultures. Under these conditions we found that GPF_1 caused a proliferation of $GC^{(+)}$ $A2B5^{(-)}$ oligodendroglia (Fig. 8). GPF_1 also stimulated these cells to incorporate [^{3}H]thymidine (Table V).

GPFs and Brain Development

Since GPFs might help to regulate gliogenesis, we assayed for the presence of such factors in developing neural tissues known to have ongoing glial proliferation. The specific biological activities of rat astroglia-stimulating factors were greater in embryonic cerebral cortex than those recovered from newborn animals (Fig. 9). There was a decline in GPF_2 and GPF_4 concentrations during the first postnatal week such that only very low factor levels were found in normal adult brain. In contrast, GPF_1 and GPF_3 were not detected in significant quantities within the cerebral cortex until the postnatal period (Fig. 9). The peak levels of oligodendroglia-

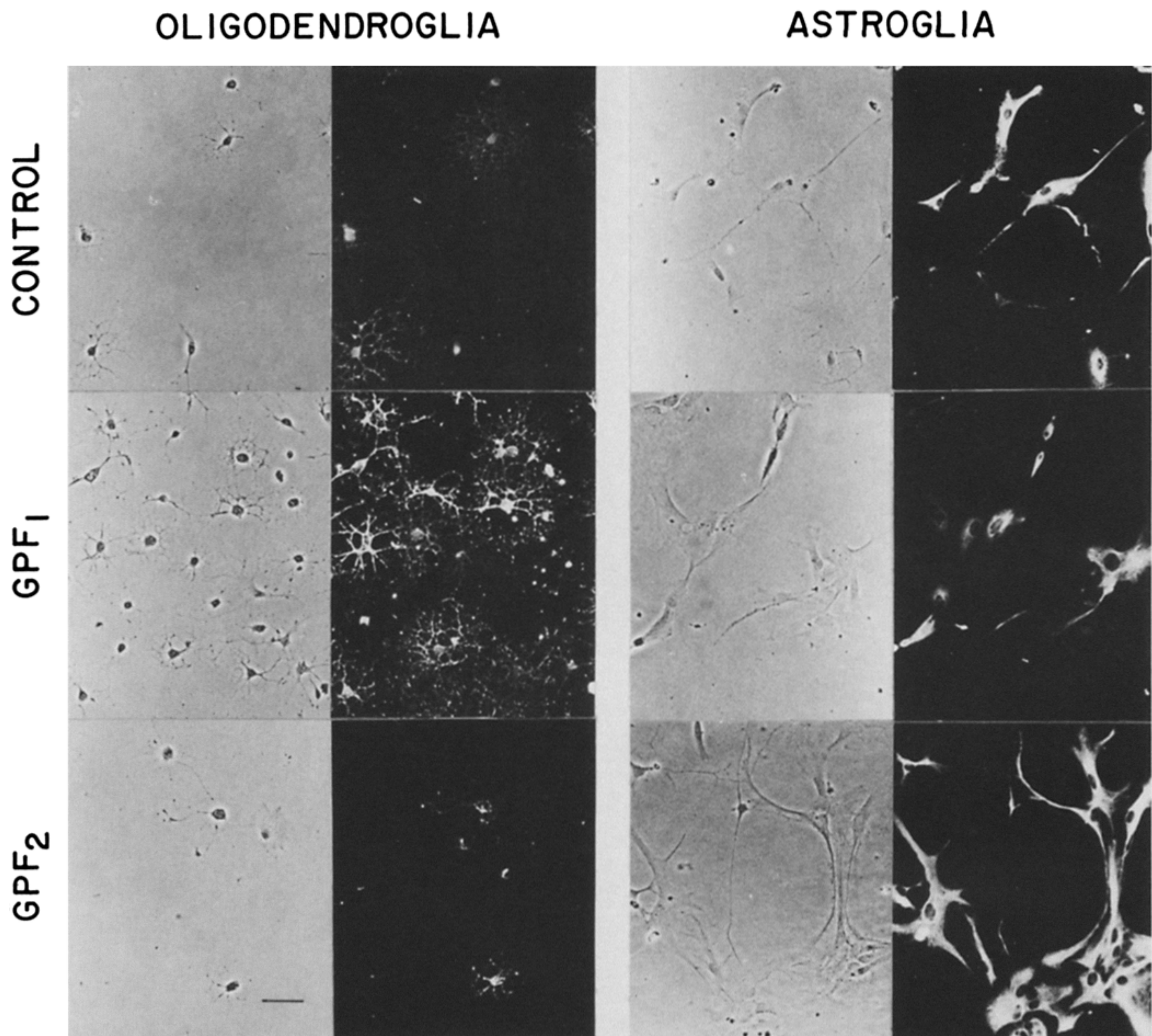

Figure 4. Photomicrographs of enriched oligodendroglial or astroglial cultures incubated with partially purified rat GPF_1 (1 μg protein/ml culture medium) or GPF_2 (0.8 μg protein/ml culture medium) for 72 h. The enriched glia cultures isolated by the method of McCarthy and de Vellis (18) consisted of differentiated cells with $GC^{(+)}$ oligodendroglia or $GFAP^{(+)}$ astroglia. As shown, GPF_1 stimulated the proliferation of oligodendroglia while GPF_2 acted as a mitogen for astroglia. Bar, 25 μm.

stimulating factors occurred during the first 10 d after birth declining to the low levels found in adult animals (Fig. 9). Astroglia-stimulating GPFs appeared, therefore, during embryogenesis or at that time when astroglia first appeared in vivo; the oligodendroglia-stimulating GPFs were found in the newborn animal and were associated with a postnatal period of oligodendroglial growth and differentiation (21, 29).

To assess age-dependent sensitivities of glia to GPFs, we assayed the action of GPF_1 and GPF_2 upon glia obtained from cerebral cortices of embryonic and newborn rats. As shown in Fig. 10, GPF_2 stimulated the appearance of $GFAP^{(+)}$ astroglia as early as Stage 15 of the embryo. By contrast, GPF_1 did not promote growth of $GC^{(+)}$ oligodendroglia until the time of birth. Based upon the appearance of certain GPFs in embryos (Fig. 9) and the response of embryonic glia to GPF_2 (Fig. 10), we concluded that the network of secretory and target cells associated with astroglia-stimulating peptides appeared earlier in development than those cells associated with oligodendroglia-stimulating peptides.

Tissue Levels of GPFs after Brain Injury

We also monitored levels of GPFs after inducing glial growth by inflicting damage to neural tissues. Stab wound injury was used to stimulate proliferation of astroglia in the adult rat brain (10) and axotomy of retinal ganglion cells to stimulate proliferation of oligodendroglia in the regenerating goldfish visual system (8, 9). Concentrations of astroglia-stimulating factors were selectively elevated in injured rat cerebral cortex

(Fig. 9), while oligodendroglia-stimulating ones were increased in the denervated goldfish optic tectum (Fig. 11). Since undamaged tissues in either the rat or goldfish showed far less GPF activity, we concluded that brain injury elicited production of specific GPFs that were associated with the growth of specific glial populations.

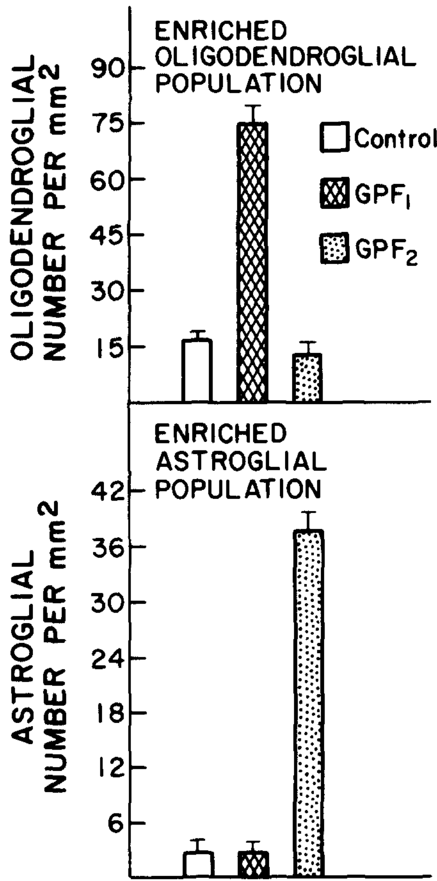

Figure 5. Effects of GPFs on differentiated populations of glia. Partially purified GPF_1 or GPF_2 (each 1 μg protein/ml culture medium) were incubated for 72 h with enriched populations of differentiated astroglia (98% $GFAP^{(+)}$ cells) or oligodendroglia. The oligodendroglia populations consisted of 90% $GC^{(+)}$ process-bearing cells, some of which contained the A2B5 surface antigen. Data are expressed as mean number of cells per mm^2 ± standard error observed in five cultures for each group. As shown, GPFs stimulated proliferation of specific cell populations.

Discussion

GPFs found in the mammalian brain served as mitogens for either astroglia or oligodendroglia in culture. Based upon estimated molecular masses and specificities of action, it appeared that the GPFs isolated from the rat brain were similar to those factors recovered from fish central nervous system. This conclusion was further supported by biochemical studies described in the following report (11).

In an attempt to distinguish classes of brain-derived growth factors, we compared the glia-stimulating activities of GPFs with those of known peptide hormones. Since no identified peptide selectively stimulated proliferation of oligodendroglia, we believe that GPF_1 and GPF_3 represent a new family of brain-derived growth factor. The identity of the astroglia-stimulating GPFs is less certain, however. Although several

Table II. Effects of Glia-promoting Factors on Glutamine Synthetase in Glial Cell Line C6

Treatment of cells	Glutamine synthetase
	U/mg protein
Control	5.7 ± 0.3
GPF_1	9.0 ± 1.4
GPF_2	30.1 ± 1.8

The C6 cell line was grown in chemically defined medium in 100-mm plastic culture dishes. Cultures were incubated for 4 d with GPF_1 or GPF_2 at concentrations of 1 μg protein per milliliter culture medium. Specific enzymatic activities (in U/mg protein), expressed as mean values ± standard error, were obtained from at least five cultures.

Table III. Effects of Glia-promoting Factors upon Cell Number in Cultures of Dissociated Brain of Newborn Rat

Treatment of cells	Total cell number per mm^2	% $GC^{(+)}$ oligodendroglia	% $GFAP^{(+)}$ astroglia
Control	158 ± 9 (n = 17)*	7.9 ± 0.9	34.8 ± 1.3
GPF_1	366 ± 20 (n = 13)	62.5 ± 5.1	26.0 ± 4.0
GPF_2	445 ± 25 (n = 18)	4.5 ± 1.3	89.9 ± 3.2

Mixed glial cell populations obtained from newborn rat brain were incubated with 50 μl of partially purified fish (1 μg protein/ml medium) or 50 μl of PBS in defined medium for 72 h. Oligodendroglia were identified by indirect immunofluorescence as those cells staining for GC and astroglia as those cells containing GFAP.

* n, number of coverslips scored.

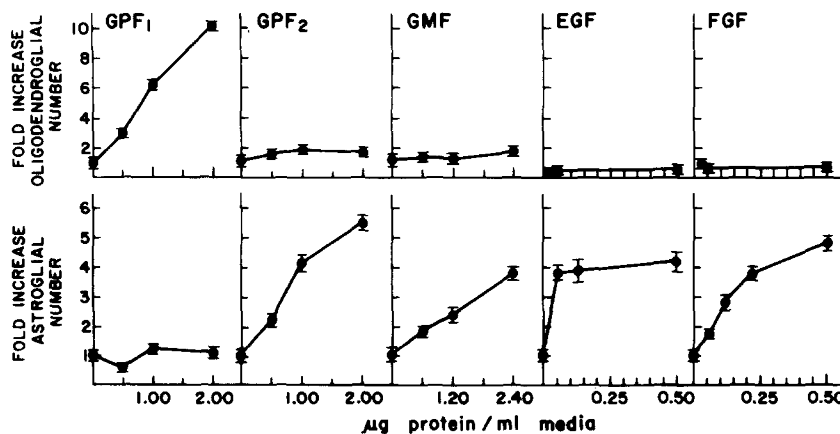

Figure 6. Dose-dependent stimulation of glia by peptide factors. Glial cultures grown on poly-L-lysine–coated coverslips were incubated with GPF_1, GPF_2, GMF, EGF, or FGF for 72 h. Mean values ± standard error express the fold increase in cell numbers when compared with control cultures treated with matching aliquots of PBS added to 1.5 ml of defined medium. Each value is calculated from at least four cultures. Only GPF_1 increased the number of oligodendroglia observed in culture.

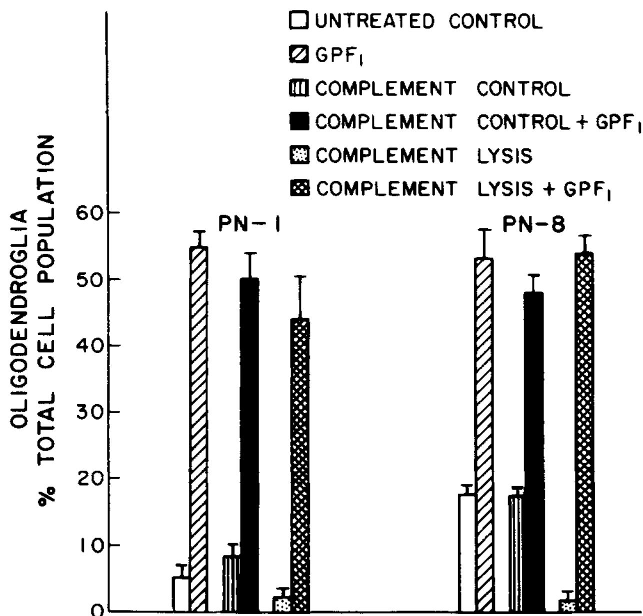

Figure 7. Effects of GPF_1 on undifferentiated glial cells. Glia obtained from rat brain on postnatal day 1 (*PN-1*) or postnatal day 8 (*PN-8*), were grown in defined medium on poly-L-lysine–coated coverslips in the presence or absence of GPF_1 (0.5 μg of protein/ml of medium) for 72 h. Differentiated oligodendroglia were lysed with anti-GC serum in the presence of guinea pig complement. A complement control was treated with only guinea pig complement. Mean values ± standard error express the percentage of number of oligodendroglia per total cell number. At least four cultures were used in each group. Complement lysis eliminated >99% of all $GC^{(+)}$ oligodendroglia. The greatest number of $GC^{(+)}$ cells appeared after incubation with GPF_1 despite prior complement lysis of differentiated oligodendroglia.

Table IV. Effects of GPFs upon $A2B5^{(+)}$ Cells

	Fold increase in cell number		
	$A2B5^{(+)}$	$GC^{(+)}$	$GFAP^{(+)}$
GPF_1	2.3 ± 0.2	4.7 ± 0.3	0.9 ± 0.1
GPF_2	1.4 ± 0.2	0.8 ± 0.3	3.1 ± 0.2

Dissociated cultures (900,000 cells/dish) obtained from newborn rat brain were incubated with partially purified GPF_1 (1.0 μg protein/ml medium) or GPF_2 (0.7 μg protein/ml medium) in defined medium for 72 h. Specific cell populations were identified by immunofluorescence for A2B5, GC, and GFAP antigens. The data are expressed as fold increase in specific cell number when compared to control culture. Control cultures contained 21% $A2B5^{(+)}$ cells, 5% $GC^{(+)}$ cells, and 37% $GFAP^{(+)}$ cells.

peptides (IL-1, EGF, FGF, GMF) were mitogens for astroglia, they could be distinguished from astroglia-stimulating GPFs by gel filtration chromatography. Moreover, GPF_2 and GPF_4 did not stimulate mouse thymocytes to incorporate thymidine as found for IL-1 and did not compete with EGF for receptor sites. Further biochemical study will, of course, be necessary to determine the structural relationships among GPFs and other classes of astroglia-stimulating peptides.

It is reasonable to suggest that GPFs regulate glial cell differentiation in developing brain. We found, for example, a correlation between the presence of GPF_2 and GPF_4 and the appearance of astroglia in embryos. Moreover, the levels of the oligodendroglia-promoting factors were greatest in the postnatal period, a time associated with oligodendroglial growth. Controlled release of GPFs by secretory cells might serve to regulate development of glial populations in specific regions of the central nervous system.

GPFs might also play a role in regulating glial growth after brain injury. The levels of astroglia-stimulating factors, GPF_2 and GPF_4, were elevated after stab wound injury to adult rat brain while, in contrast, the levels of oligodendroglia-stimulating factors, GPF_1 and GPF_3, increased during regeneration of the goldfish visual system. Moreover, such changes in biological activities were found only in the damaged tissues. Perhaps GPFs released by injured neural tissues (6, 8, 9, 12) act to stimulate growth of glial populations near the site of injury or to provide chemotaxic signals to affect glial cell migration (8, 9).

Our findings point to the existence of peptides within the central nervous system that regulate glial cellular responses.

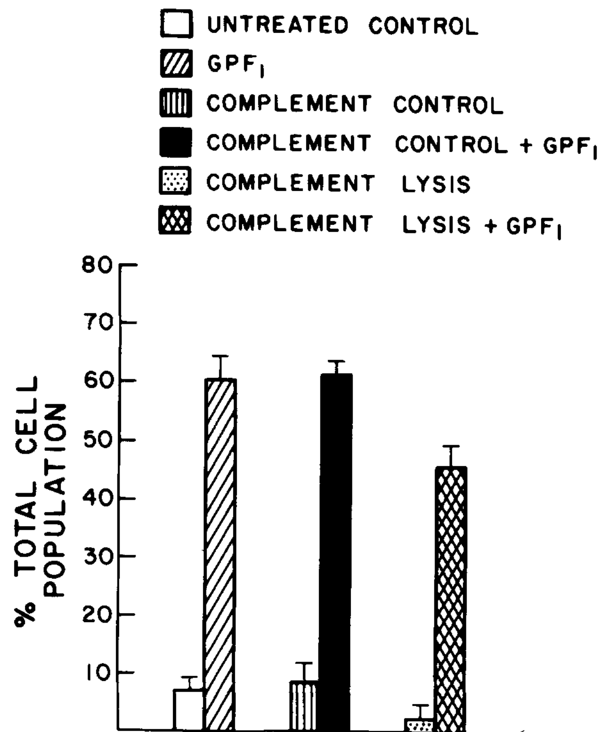

Figure 8. Effects of GPF_1 upon mature $GC^{(+)}$ oligodendroglia. Enriched populations of $GC^{(+)}$ oligodendroglia were freed of $A2B5^{(+)}$ cells by complement lysis. Cultures incubated with rat GPF_1 for 72 h (1 μg/ml) showed an increase in the number of differentiated oligodendroglia.

Table V. Incorporation of [^{3}H]Thymidine by Isolated $GC^{(+)}$ Oligodendroglia

	cpm/well	Fold increase
PBS Control	197 ± 70	—
GPF_1	1,291 ± 80	6.5 ± 0.4
GPF_2	399 ± 54	1.3 ± 0.1

100,000 isolated $GC^{(+)}$ oligodendroglia grown in 15-mm plastic wells containing 500 μl of defined medium were incubated for 72 h with 5 μl (1.1 μg/ml medium) of partially purified GPF_1, 5 μl (0.8 μg/ml medium) of GPF_2, or 5 μl PBS. Each culture received 5 μCi of [^{3}H]thymidine for the final 15 h of the incubation. The amount of incorporated radioactivity was determined using a glass filter assay (10). The data are mean values of cpm ± standard error obtained from at least four cultures and are expressed as a fold increase above the PBS control.

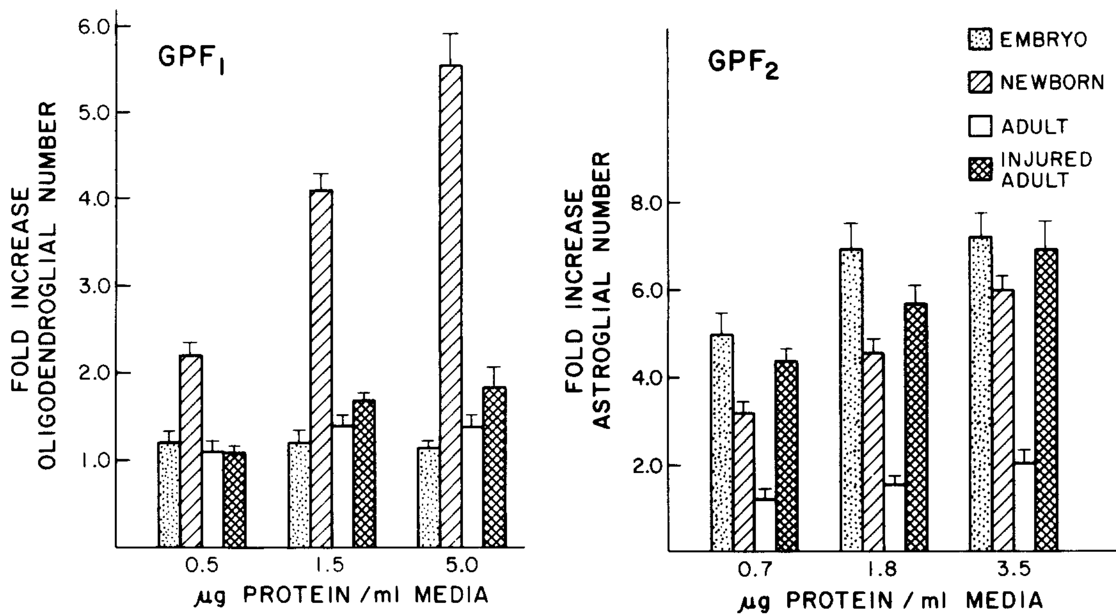

Figure 9. Recovery of GPFs from the cerebral cortices of embryonic (E-16), newborn, intact adult rats, and adult rats receiving stab wounds (injured). Glia-stimulating factors found in tissue sonicates were isolated by gel filtration. Specific GPFs from pooled fractions were incubated with glial cultures for 72 h. Data are expressed as fold of increase in cell number when compared with a control culture treated with aliquots of PBS. Five cultures were used for each data point. As shown, the astroglia-promoting factor, GPF_2, appeared during embryogenesis, or after stab wound injury to the adult brain. The concentration of the oligodendroglia-promoting factor, GPF_1, was greatest during the early postnatal period and could not be elicited by injury.

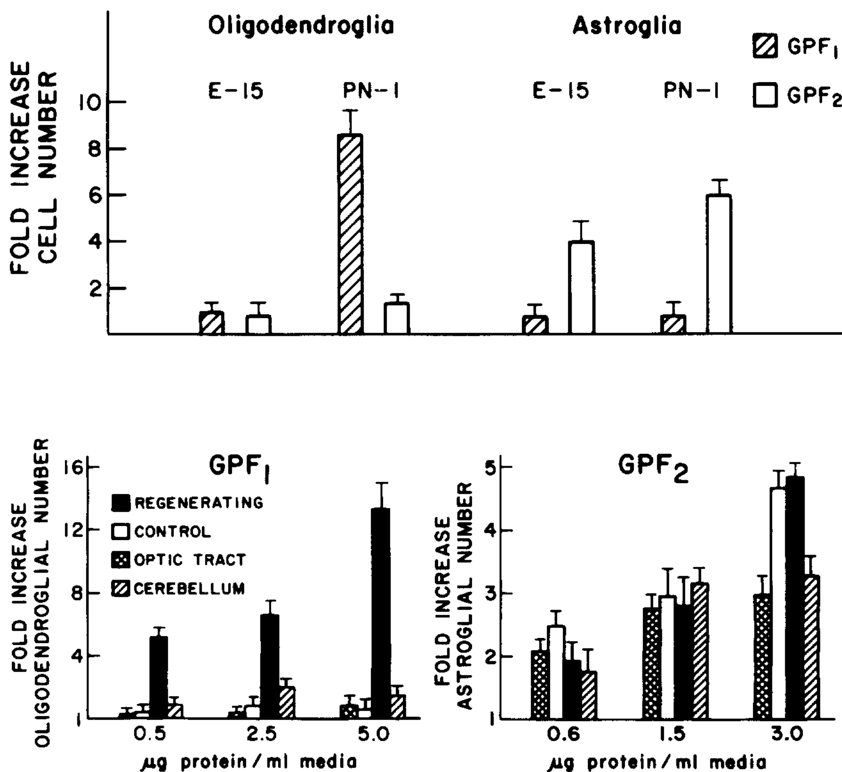

Figure 10. Sensitivity of glia to GPFs during development. Glial cultures obtained from embryonic (*E-15*) or newborn (*PN-1*) rat were incubated with partially purified rat GPF_1 or GPF_2 (1 µg protein/ml culture medium) for 72 h. After 5 d, the control embryonic cultures contained 10 ± 5% $GFAP^{(+)}$ astroglia and <0.1% $GC^{(+)}$ oligodendroglia. The control newborn cultures had 35% ± 5% $GFAP^{(+)}$ astroglia and 7% ± 2% $GC^{(+)}$ oligodendroglia. Data are expressed as mean fold increase in cell number when compared with control cultures. Five cultures were used for each data point. Embryonic cells did not respond to GPF_1 whereas glia isolated from the embryonic and postnatal brain were stimulated by GPF_2.

Figure 11. Recovery of GPFs from brain regions during regeneration of the goldfish visual system. Specific activities of GPFs found in optic teetum of the regenerating visual system were compared with those of GPFs from intact optic tectum, optic tract, and cerebellum 10 d after retinal ganglion cell axotomy. Mean values ± standard error express the fold of increase in cell number over control cultures. At least five cultures were used in each group. The specific activity of GPF_1, but not GPF_2, increased within the optic tectum during regeneration of the visual system.

Although such peptides are unlikely to be the sole mediators of glial growth, shifts in GPF specific activities after brain injury and the changes observed in glial responsiveness to GPFs during development imply that these factors have important biological roles. In the following paper, we identify the probable cellular sources of GPFs in the brain (11).

Received for publication 2 January 1985, and in revised form 8 November 1985.

References

1. Aguayo, A. J., S. David, and G. M. Bray. 1981. Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. *J. Exp. Biol.* 95:231–240.

2. Benda, P., J. Lightbody, G. Sato, L. Levine, and W. Sweet. 1968. Differentiated rat glial cell strain in tissue culture. *Science (Wash. DC).* 161:370–371.

3. Bohlen, P., S. Stein, W. Dairman, and S. Udenfriend. 1973. Fluorometric assay of proteins in the nanogram range. *Arch. Biochem. Biophys.* 155:213–220.

4. Bottenstein, J. E., and G. H. Sato. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA.* 76:514–517.

5. Dennis, S. C., J. C. K. Lai, and J. B. Clark. 1980. The distribution of glutamine synthetase in subcellular fractions of the rat brain. *Brain Res.* 197:469–475.

6. Giulian, D. 1984. Peptides from the regenerating central nervous system of goldfish stimulate glia. *Proc. Natl. Acad. Sci. USA.* 81:3567–3571.

7. Giulian, D., and T. Baker. 1985. Characterization of ameboid microglia isolated from mammalian brain. *J. Neurosci.* In press.

8. Giulian, D., V. Iwanij, and H. Stuckenbrok. 1985. The response of optic tract glia during regeneration of the goldfish visual system. I. Biosynthetic activity within different glial populations after transection of retinal ganglion cell axons. *Brain Res.* 339:87–96.

9. Giulian, D., and V. Iwanij. 1985. The response of optic tract glia during regeneration of the goldfish visual system. II. Tectal factors stimulate optic tract glia. *Brain Res.* 339:97–104.

10. Giulian, D., and L. B. Lachman. 1985. Interleukin-1 stimulation of astroglial proliferation after brain injury. *Science (Wash. DC).* 228:497–499.

11. Giulian, D., and D. G. Young. 1986. Brain peptides and glial growth. II. Identification of cells that secrete glia-promoting factors. *J. Cell Biol.* 102:812–820.

12. Giulian, D., Y. Tomozawa, H. Hindman, and R. Allen. 1985. Peptides from regenerating central nervous system promote specific populations of macroglia. *Proc. Natl. Acad. Sci. USA.* 82:4287–4290.

13. Gospodarowicz, D., and J. S. Moran. 1976. Growth factors in mammalian cell culture. *Annu. Rev. Biochem.* 45:531–558.

14. Hallermayer, K., C. Harmening, and B. Hamprecht. 1981. Cellular localization and regulation of glutamine synthetase in primary cultures of brain cells from newborn mice. *J. Neurochem.* 37:43–52.

15. Lemke, G. E., and J. P. Brockes. 1984. Identification and purification of glial growth factor. *J. Neurosci.* 4:75–83.

16. Lemmon, S. K., M. C. Riley, K. A. Thomas, G. A. Hoover, T. Maciag, and R. A. Bradshaw. 1982. Bovine fibroblast growth factor: comparison of brain and pituitary preparations. *J. Cell Biol.* 95:162–180.

17. Lim, R. 1980. Glial maturation factor. *Curr. Top. Dev. Biol.* 16:305–322.

18. McCarthy, K., and J. de Vellis. 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* 85:890–902.

19. Neuman, D., A. Yerushalmi, and M. Schwartz. 1983. Inhibition of non-neuronal cell proliferation in the goldfish visual pathway affects the regenerative capacity of the retina. *Brain Res.* 272:237–245.

20. Norenberg, M. D., and A. Martinez-Hernandez. 1979. Fine structure localization of glutamine synthetase in astrocytes of rat brain. *Brain Res.* 161:303–310.

21. Privat, A., and J. Fulcrand. 1977. Neuroglia from the subventricular precursor to the mature cell. *In* Cell, Tissue, and Organ Culture in Neurobiology. S. Fedoroff and L. Hertz, editors. Academic Press, Inc., New York. 11–37.

22. Raff, M. C., R. H. Miller, and M. Noble. 1983. A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature (Lond.).* 303:390–396.

23. Raff, M. C., R. Mirsky, K. L. Fields, R. P. Lisak, S. H. Dorfman, D. H. Silberberg, N. A. Gregson, S. Leibowitz, and M. C. Kennedy. 1978. Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocyte in culture. *Nature (Lond.).* 274:813–816.

24. Raff, M. C., K. L. Fields, S. Hakomori, R. Mirsky, R. M. Pruss, and J. Winter. 1979. Cell-type-specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. *Brain Res.* 174:283–308.

25. Rakic, P. 1972. Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* 145:61–84.

26. Silver, J., and R. L. Sidman. 1980. A mechanism for the guidance and togographic patterning of retinal ganglion cell axons. *J. Comp. Neurol.* 189:101–111.

27. Silver, J., S. E. Lorenz, D. Wahlsten, and J. Coughlin. 1982. Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies in vivo on the role of performed glial pathways. *J. Comp. Neurol.* 210:10–29.

28. Singer, M., R. H. Nordlander, and M. Egar. 1979. Axonal guidance during embryogenesis and regeneration in the spinal cord of newt: the blueprint hypothesis of neuronal pathway patterning. *J. Comp. Neurol.* 185:1–22.

29. Sturrock, R. R. 1974. Histogenesis of the anterior limb of the anterior commissure of the mouse brain. III. An electron microscopic study of gliogenesis. *J. Anat.* 117:37–53.

30. Thiele, D. L., M. Kurosaka, and P. E. Lipsky. 1983. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotrophic agent, L-leucine methyl ester. *J. Immunol.* 131:2282–2290.