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Peptides from regenerating central nervous system promote specific populations of macroglia

(astroglia/oligodendroglia/target tissue)

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ABSTRACT **The regenerating central nervous system of goldfish contains peptides referred to as glia-promoting factors (GPFs) that stimulate the proliferation of mammalian macroglia. We find that, *in vitro*, GPF_1 and GPF_3 promote the appearance of oligodendroglia and GPF_2 and GPF_4 stimulate proliferation of astroglia. The activities of GPF_1, GPF_3, and GPF_4 increase during regeneration of the goldfish visual system. These results suggest that brain peptides may play a role in the recovery of the injured central nervous system by regulating the growth and development of specific macroglial populations.**

Macroglia, the family of cells that includes astrocytes and oligodendrocytes, play an important role in both the developing and the injured central nervous system (CNS). Rakic argues that radial projections of astroglia guide the migration of neurons in the fetal mammalian brain (1). Singer *et al.* propose that nonneuronal cells form channels along which sprouting axons follow in the regenerating newt spinal cord (2). Silver and Sidman draw a similar conclusion for the development of the mouse visual system (3). Furthermore, manipulation of the glial environment alters patterns of axonal growth as shown by Aguayo *et al.* in mammals (4) and by Neuman *et al.* in fish (5). Their observations support a long-standing notion that interactions of the glia with neurons helps to determine the success or failure of axonal growth (6).

Gliogenesis occurs during the early phase of injury to the optic tract in the goldfish (7, 8). Recent work indicates that peptides are one class of glia-promoting substances that may be recovered from the regenerating goldfish visual system (9). These peptides, referred to as glia-promoting factors (GPFs), stimulate amino acid and thymidine incorporation in the glia of the fish optic tract (9). Our observation that GPFs act upon mammalian glia in culture forms the starting point for this study. We find that specific peptides stimulate the growth of select populations of macroglia.

MATERIALS AND METHODS

Tissue Culture. Cerebral cortices from the newborn rat were isolated in ice-cold calcium- and magnesium-free phosphate-buffered saline (pH 7.4).

The tissue was placed in defined medium (10) containing DNase (1 mg/ml; Sigma) and was minced with iridectomy scissors. After addition of trypsin (0.3 mg/ml; Sigma), tissue fragments were dissociated by mild agitation for 7 min. Enzymatic dissociation was stopped by the addition of defined medium containing 10% fetal calf serum (GIBCO, lot R781117). Cells in suspension were collected and washed twice with defined medium containing 10% fetal calf serum. Cell viability was determined by erythrosin B exclusion using a hemacytometer; between 80,000 and 100,000 viable cells in defined medium with 10% fetal calf serum were added to 35-mm plastic culture dishes (Falcon), which contained 22-mm^2 glass coverslips (Fisher) coated with poly(L-lysine) (Sigma). After 48 hr, cells were washed three times with defined culture medium. GPFs were added to dishes that contained 1.5 ml of defined medium.

Under the conditions described here, untreated 3-day-old cultures contained 1–2% galactocerebroside (galacto-1-β-glycosylceramide)-positive oligodendroglia and about 25–30% glial fibrillary acidic protein (GFAP)-positive astroglia. Examination of untreated 7-day-old cultures

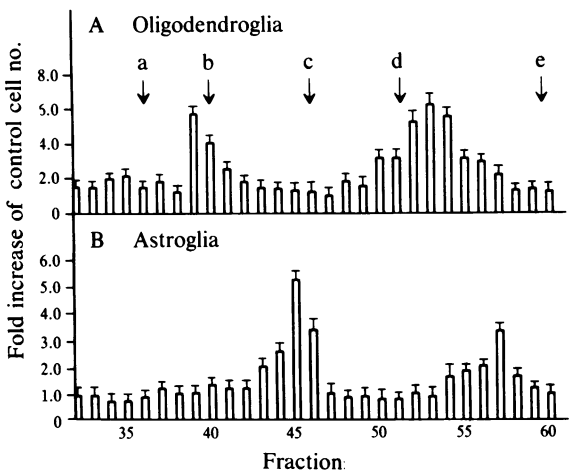

FIG. 1. Effects of GPFs on mammalian glia. Soluble material containing between 4 and 6 mg of total protein from sonicates of optic tecta of goldfish 10 days after ganglion cell axotomy was applied to a Bio-Gel P-10 column (100 × 0.9 cm) and eluted with sterile phosphate-buffered saline (pH 7.4). Fractions of 700 μl were collected. Aliquots (100 μl) from each fraction were tested for their ability to increase the number of rat brain oligodendroglia or astroglia in culture. GPFs were added to 35-mm culture dishes containing 1.5 ml of defined medium with cells grown on 22-mm^2 poly(L-lysine)-coated glass coverslips. Mean numbers of cells were determined by counting specifically stained glia seen in 10 randomly selected fields (0.314 mm^2) from two cultures incubated with each fraction. Arrows: protein molecular mass standards of 17 kDa (a), 14 kDa (b), 8 kDa (c), 6.4 kDa (d), and 2.5 kDa (e). Four peaks of biologic activity, designated GPF_1 through GPF_4, were seen, having apparent molecular masses of 125 kDa (GPF_1), 9 kDa (GPF_2), 6 kDa (GPF_3), and 3 kDa (GPF_4). (*A*) Oligodendroglia were identified as branched cells containing galactocerebroside. Data were expressed as the degree of increase in cell number compared with buffer-treated controls after a 5-day incubation period. As shown here, GPF_1 and GPF_3 increased the number of oligodendroglia *in vitro*. (*B*) Astrocytes were identified as those cells that contained GFAP. As shown here, GPF_2 and GPF_4 increased the number of astroglia *in vitro*. The protein concentrations for fractions containing GPFs ranged between 5 and 40 μg/ml.

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Abbreviations: GPF, glia-promoting factor; CNS, central nervous system; GFAP, glial fibrillary acidic protein.

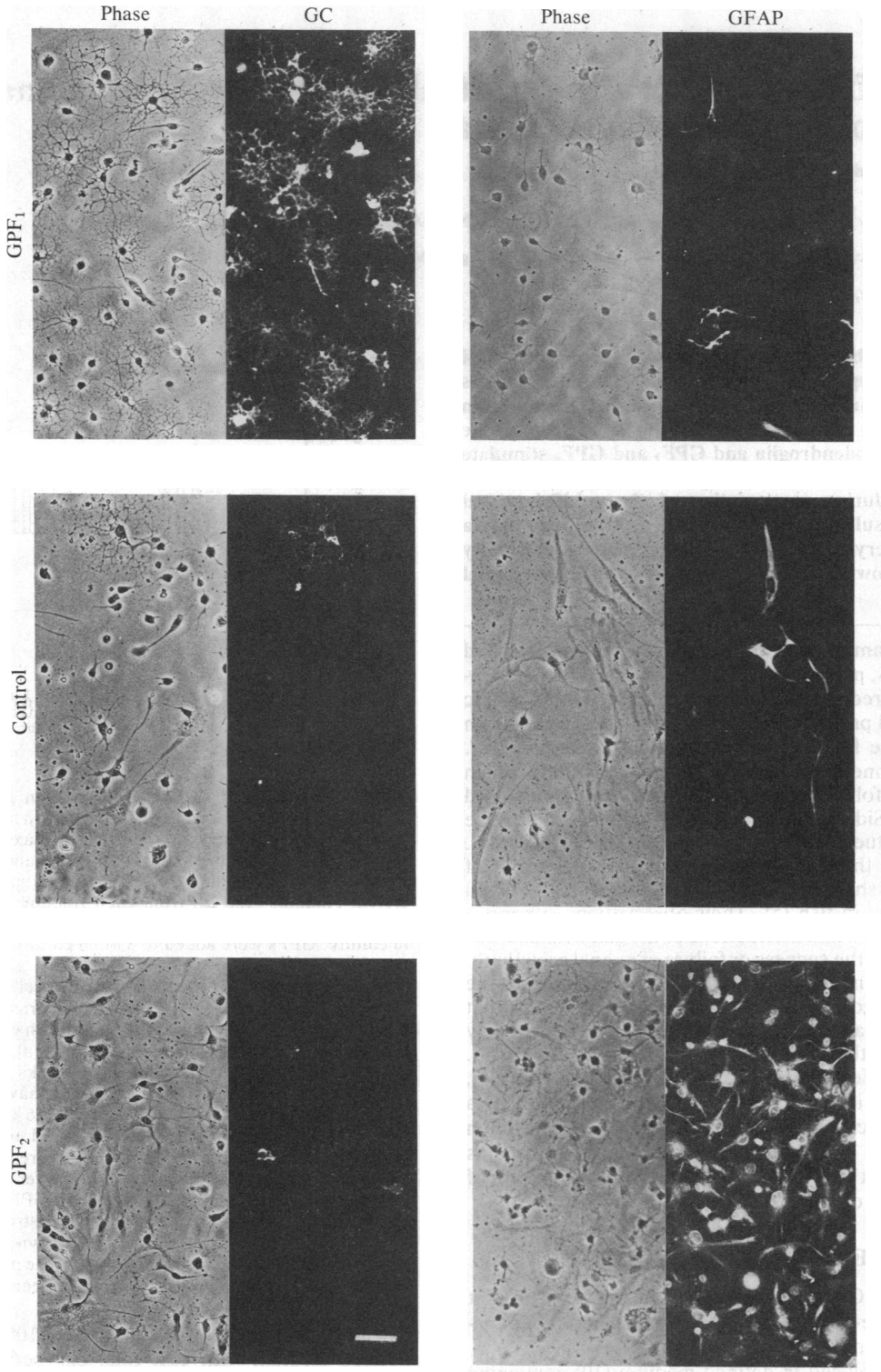

FIG. 2. Effects of GPFs on specific macroglial populations. Glial cells grown in culture were treated with partially purified GPF_1 or GPF_2. Photomicrographs show the cell populations seen by phase and after fluorescence staining for galactocerebroside (GC) or GFAP. Galactocerebroside serves as a specific marker for oligodendroglia and GFAP for astroglia. These representative photomicrographs were obtained from different cultures. (Bar = 25 μm.) (*Top*) Seven-day cultures after 5-day treatment with GPF_1 (1 μg of protein per ml of medium). Cells are predominantly galactocerebroside-positive oligodendroglia, with a few cells that stained for GFAP. (*Middle*) Cell populations in control 7-day cultures contain 3–5% galactocerebroside-positive oligodendroglia and 37–40% GFAP-positive astroglia. (*Bottom*) Seven-day cultures after 5-day treatment with GPF_2 (1 μg of protein per ml of medium). Most of the cells are GFAP-positive astroglia. Few cells contained galactocerebroside.

showed 3–5% of the total cell population to be oligodendroglia and 37–40% to be astroglia; the majority of the cells that lacked specific markers were presumed to be microglia and undifferentiated macroglia (11, 12). Enriched populations of astroglia (96% of all cells containing GFAP) and oligodendroglia (90% of all cells containing galactocerebroside) were obtained by modifications of the adhesion method of McCarthy and de Vellis (13).

Identification of Macroglia. GFAP served as a marker for astroglia and galactocerebroside as a marker for oligodendroglia (11, 13, 14). Cells adhering to poly(L-lysine)-coated glass coverslips were washed three times with Dulbecco's minimal essential medium (DMEM) containing 1% heat-inactivated fetal calf serum (GIBCO). Identification of cells that contained GFAP involved an indirect immunofluorescence technique. Cells were fixed at −20°C for 30 min with 90% acetone/10% acetic acid (vol/vol). Coverslips were dipped sequentially in 100% acetone, 70% ethanol, phosphate-buffered saline, and DMEM containing 1% fetal calf serum. Rabbit anti-human GFAP-antibody (whole serum; Accurate Chemicals, Westbury, NY) was diluted 1:200 with DMEM containing 1% goat serum and was applied directly to the coverslips. Coverslips were incubated for 45 min at 37°C in 95% air/5% CO_2 with high humidity and were washed with DMEM containing 1% fetal calf serum. A goat anti-rabbit immunoglobulin G conjugate of rhodamine isothiocyanate diluted 1:100 with DMEM containing 1% goat serum was applied next, for 45 min at 37°C. Coverslips were washed with DMEM containing 1% fetal calf serum five times and mounted in media containing 1 mg of *p*-phenylenediamine per ml, 10% phosphate-buffered saline, and 90% glycerol at pH 8.0. Fluorescence-labeled cells were viewed at a magnification of ×200 with a Nikon inverted-phase microscope using epifluorescence. Identification of cells that contained galactocerebroside was carried out in a similar fashion, with cell fixation after antibody binding. Rabbit anti-galactocerebroside serum, some of which was a gift from T. Inagami of Vanderbilt University and some of which was produced in our laboratory, was diluted 1:100 with DMEM containing 1% goat serum.

Isolation of GPFs. Freshly isolated fish tecta were sonicated at 4°C in sterile phosphate-buffered saline (pH 7.4) with 30 μl of buffer per tectum. Soluble material was recovered after 30 min of centrifugation (15,000 × *g*; Microfuge), filtered with a 0.45-μm membrane (Millipore), applied to a Bio-Gel P-10 column (100 × 0.9 cm; Bio-Rad), and eluted with phosphate-buffered saline (pH 7.4). Protein concentrations were estimated by the fluorescamine method with bovine serum albumin standards (15).

RESULTS AND DISCUSSION

Soluble proteins from goldfish optic tecta 10 days after retinal ganglion cell axotomy were separated by gel filtration. We observed changes in the numbers of astroglia and oligodendroglia within 48 hr after application of column fractions to glial cultures. Four peaks of biologic activity were observed with apparent molecular masses ranging from 3 kDa to 15 kDa (Fig. 1). These fractions are the GPFs; the largest factor was designated GPF_1, and the smallest was designated GPF_4. Partially purified GPF_1 stimulated the appearance of galactocerebroside-positive oligodendroglia in culture; under conditions of maximal stimulation, 60–70% of all cells were oligodendroglia, 20–25% were astroglia, and 5% lacked either the galactocerebroside or the GFAP antigen (Fig. 2). In contrast, partially purified GPF_2 stimulated the appearance of astroglia: 95% of the cells were GFAP-positive astroglia, 1% were galactocerebroside-positive oligodendroglia, and 4% lacked one or the other glial marker (Fig. 2). The effects of partially purified GPFs upon glia were dose-dependent (Fig. 3) and were decreased by prior treatment with trypsin (Table 1). For example, the number of oligodendroglia increased by >15-fold after GPF_1 treatment when compared to untreated or buffer-treated control cultures. Addition of bovine serum albumin (1 mg/ml) or 1% heat-inactivated fetal calf serum did not alter the action of GPFs.

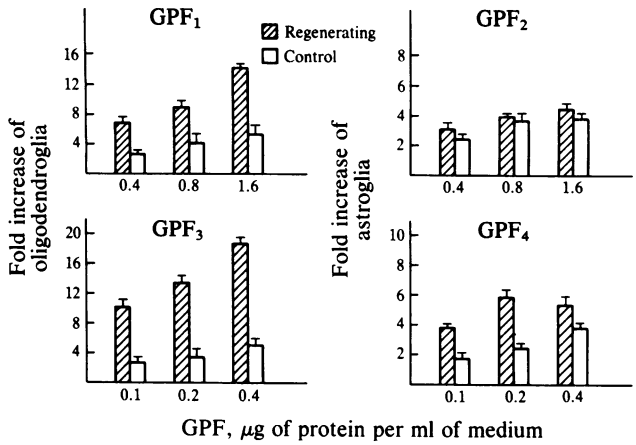

FIG. 3. Changes in activities of GPFs during regeneration of the goldfish visual system. As previously described, GPFs were isolated from optic tecta 10 days after ganglion cell axotomy (regenerating tecta) or from intact control tecta and were tested for abilities to increase glial cell number *in vitro*. Data were expressed as the degree of increase in cell number when compared with control cultures treated with volumes of phosphate-buffered saline that match the volumes of the GPFs added. Five cultures were used to arrive at a mean score ± SEM. Greater specific activities for GPF_1 and GPF_3 and a modest increase in the activity of GPF_4 were observed in tissues recovered from the regenerating visual system. The activity of GPF_2, which stimulates astroglia, was not altered by ganglion cell axotomy.

Partially purified GPF_1 and GPF_3 selectively increased the number of oligodendroglia, and partially purified GPF_2 and GPF_4 acted selectively on astroglia (Fig. 1). GPFs may increase specific macroglial populations in culture by increasing cell-survival times, by accelerating progenitor cell differentiation, or by stimulating proliferation of mature glia.

Table 1. Trypsin inactivation of GPFs

	Degree of increase in oligodendroglia			Degree of increase in astroglia	
Peptide	Without trypsin	With trypsin	Peptide	Without trypsin	With trypsin
GPF_1	4.7 ± 0.4	0.8 ± 0.1	GPF_2	3.9 ± 0.3	0.9 ± 0.1
GPF_3	5.4 ± 0.7	0.6 ± 0.1	GPF_4	4.7 ± 0.3	1.0 ± 0.2

Glia obtained from newborn rat brain were grown in 1.5 ml of Bottenstein and Sato's defined medium (10) in the presence of partially purified GPFs (0.5–1.0 μg of protein per ml of medium) for 3 days. Immunofluorescence staining was used to identify specific cell antigens with GFAP serving as a marker for astroglia and galactocerebroside for oligodendroglia. Trypsin treatment of each GPF (10 μg of enzyme for 1000 μg of protein) was carried out at 37°C for 60 min, and the reaction was stopped by the addition of soybean trypsin inhibitor (2:1 inhibitor/enzyme, by weight). The trypsin controls contained equivalent amounts of GPFs and were also incubated at 37°C for 60 min in the presence of soybean inhibitor. Data were the mean values ± SEM obtained from four cultures and were expressed as the degree of increase in cell number compared with control cultures containing equivalent volumes of medium with trypsin and with trypsin inhibitor.

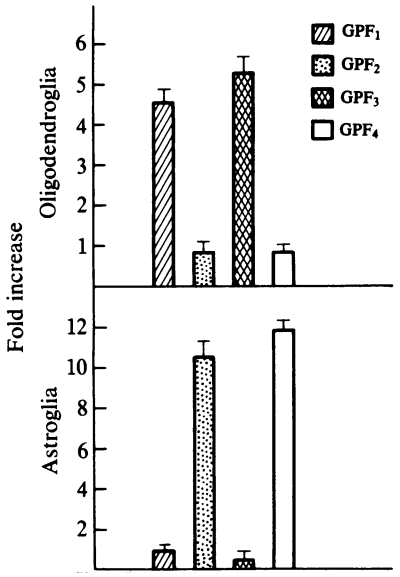

FIG. 4. Action of GPFs on enriched populations of mature galactocerebroside-positive oligodendroglia (*Upper*) or mature GFAP-positive astroglia (*Lower*). Cells were isolated by the adhesion method of McCarthy and de Vellis (13) and treated for 3 days with partially purified GPFs in the presence of defined medium. Control cultures were treated with equivalent volumes of phosphate-buffered saline. Data (mean cell numbers ± SEM) were calculated from five cultures per group and expressed as the degree of increase in cell number when compared with control cultures. Protein concentrations (μg of protein per ml of medium) were 0.5 μg/ml for GPF_1, 2.0 μg/ml for GPF_2, 0.5 μg/ml for GPF_3, and 2.0 μg/ml for GPF_4.

Accordingly, we tested the effects of GPFs on enriched populations of mature astroglia or oligodendroglia. Partially purified GPF_1 and GPF_3 caused proliferation of differentiated oligodendroglia but did not affect astroglial cultures; in contrast, the stimulatory actions of partially purified GPF_2 and GPF_4 were specific for mature astroglia (Fig. 4). It was apparent, therefore, that GPFs could stimulate proliferation of differentiated cells. Since untreated control cultures showed 150–250% increases in total cell numbers during the period of GPF treatment, it is unlikely that GPFs had a significant effect upon cell survival. Subsequent work has shown that GPF_1 also accelerated differentiation of oligodendroglia (unpublished data).

As reported previously (8, 9), GPFs might take part in the regulation of glial cell growth after injury to the CNS. We considered this possibility by determining the biologic activities of GPFs recovered from denervated and intact optic tecta of the goldfish. Greater specific activities (increase in glial cell number per μg of protein added) for partially purified GPF_1, GPF_3, and GPF_4 were noted in the injured visual system of fish, whereas GPF_2 activity was not altered during regeneration (Fig. 3). Perhaps the GPF activities that increased during regrowth of the CNS help to regulate cellular responses to injury by controlling proliferation of glia as well as recruitment of undifferentiated cells found near the site of injury (9, 12).

Other investigators have described peptide factors recovered from the mammalian pituitary or brain that stimulated glia (16–21). Lim and co-workers first reported a protein (glial maturation factor, having an apparent molecular mass of >40 kDa) that accelerated maturation of astroglia in culture (16–18). Lemke and Brockes have isolated a mitogenic factor for Schwann cells and astrocytes (glial growth factor, 31 kDa) (19, 20). In addition, epidermal growth factor (6.1 kDa) and fibroblast growth factors (between 13 and 17 kDa) were noted to stimulate proliferation of astrocytes (21, 22). None of these factors described by other investigators has been isolated from regenerating CNS and none acts upon oligodendrocytes (refs. 17, 19, and 21; our unpublished data). The relationships between these factors and the GPFs of fish are unknown and await further biochemical study.

Studies by Raff and co-workers support a long-standing idea that a common progenitor cell gives rise to either an astrocyte or an oligodendrocyte (12, 23). Raff's observation that culture medium affected the type of cell that appeared *in vitro* implied the existence of regulatory signals that control glial growth *in vivo*. GPFs might serve as such signals to regulate specific populations of macroglia during brain development. Increases in GPF activities after retinal ganglion cell axotomy suggest that peptide factors released by the damaged CNS also promote tissue healing (8, 9). Perhaps isolation of such factors will lead to new therapies for treatment of injury of the human brain.

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