Brain Peptides and Glial Growth.
II. Identification of Cells That Secrete Glia-promoting Factors

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Abstract. Glia-promoting factors (GPFs) are brain peptides which stimulate growth of specific macroglial populations in vitro. To identify the cellular sources of GPFs, we examined enriched brain cell cultures and cell lines derived from the nervous system for the production of growth factors. Ameboid microglia secreted astrogial-stimulating peptides, while growing neurons were the best source of the oligodendroglia-stimulating factors. These secretion products co-purified by gel filtration, anion exchange chromatography, and reverse-phase high performance liquid chromatography with GPFs isolated from goldfish and rat brain. Our findings suggest that glial growth in the central nervous system is regulated in part by a signaled release of peptides from specific secretory cells.

As described in the preceding report, peptides recovered from the central nervous system stimulated the proliferation of oligodendroglia or astroglia in culture (10). Elevated concentrations of some of these glia-promoting factors (GPFs) were found during periods of glial proliferation in developing rat brain, in the regenerating goldfish visual system, or after injury to the adult rat brain (10). Since our findings suggested that secretion of growth-stimulating peptides might be associated with gliogenesis, we sought to identify the cells which were responsible for the production of GPF-like factors. In this report, we isolated GPFs from cells in culture using gel filtration, ion-exchange chromatography, and reverse-phase high-performance liquid chromatography (HPLC). Astrogial-stimulating GPFs were secreted by microglia, and oligodendroglia-stimulating GPFs were produced by neurons. Proliferation of glia in the brain may, therefore, be regulated by the release of peptides from specific secretory cells.

Materials and Methods
Tissue Preparation and Cell Cultures
Optic tecta obtained from common goldfish (Ozark Fisheries, Ozark, MO) or cerebral cortices from newborn albino rat (Holtzman Co., Madison, WI) were isolated as described previously (5, 10, 11). Pooled tissues were dispersed by mild sonication in phosphate-buffered saline (PBS) and centrifuged for 30 min at 15,000 g (Microfuge) at 4°C. The supernatant was filtered (0.45 µm; Millipore/Continental Water Systems, Bedford, MA) and applied to a P-10 column isolated as described previously (5, 10, 11). Pooled tissues were dispersed by mild sonication in phosphate-buffered saline (PBS) and centrifuged for 30 min at 15,000 g (Microfuge) at 4°C. The supernatant was filtered (0.45 µm; Millipore/Continental Water Systems, Bedford, MA) and applied to a P-10 column (100 x 0.9 cm, Bio-Rad Laboratories, Richmond, CA). The column was eluted with sterile PBS (pH 7.4) and 700-µl fractions of eluate were collected in sterile plastic collecting trays. The cell lines C-1300 (CCL #147), C6 (CCL #107), GH3 (CCL #821), N2A (CCL #131), and 3T3 (CCL #92) were obtained from American Type Culture Collection, Rockville, MD. The B65 neuronal cell line from rat brain tumor was a gift from Dr. Yasuko Tomozawa (Baylor College of Medicine, Houston, TX: reference 22). Astroglia were isolated by the method described by McCarthy and de Vellis (19) with 5 mM 1-leucine methyl ester used to destroy contaminating microglia (24). Ganglion cells were isolated from the goldfish retina by microdissection as described previously (4). The fluorescent compound, true blue (Sigma Chemical Co., St. Louis, MO), was used as a retrograde tracer (13) to confirm the identity of isolated ganglion cells (Fig. 1).

Ameboid microglia were obtained from mixed glial cell cultures grown for 1 wk in plastic flasks containing chemically defined medium described by Bottenstein and Sato (2) with 10% fetal calf serum. The flasks of 1-wk-old cultures were agitated for 15 h on a rotating shaker (180 rpm) at 37°C, and the suspended cells were transferred to new plastic flasks. Microglia selectively adhered to plastic within 3 h at 37°C, in defined medium containing 10% fetal calf serum (6). These adhering cells were re-suspended in a Ca2+, Mg2+-free PBS with 5 mM EDTA, and transferred to a plastic flask containing defined medium with 10% fetal calf serum. The population of cells adhering after a second 3-h period consisted of 95 ± 3% nonspecific esterase-positive microglia. These isolated ameboid microglia, proliferated in vitro, were capable of phagocytosing 5-µm latex beads and contained the acetylated low density lipoprotein receptor (Fig. 2) (6, 21) as well as the macrophage surface antigens, MAC-1 and MAC-3 (23). The fluorescent probe for acetylated low density lipoprotein, diododecyl-1,3,3',3'-tetramethyl-indo-carbocyanine (Di-ac-LDL) was a gift from Dr. David Via of the Department of Medicine, Baylor College of Medicine, Houston, TX.

Glia Cell Assays
Preparation of glial cultures and identification of specific cell populations by indirect immunofluorescence were carried out as described in the previous report (10, 11). Galactocerebroside (GC) served as a marker for oligodendroglia and glial fibrillary acidic protein (GFAP) was used to identify astroglia. Biosays to test for GPF activities used glia isolated from the cerebral cortex of newborn rat and plated on poly-L-lysine-coated glass coverslips in 35-mm plastic dishes containing 1.5 ml of defined medium with 10% fetal calf serum. After 48 h, these cultures were washed three times with defined medium (10). Between 10 and 100 µl of partially purified GPFs in PBS were added to glial cultures in 1.5 ml of defined medium. The final concentrations of the partially purified GPFs ranged from 0.05 to 10 µg/ml of culture medium (1). Matching control cultures were prepared with equivalent volumes (10-150 µl) of PBS. After an incubation period of 72 h, cultures were stained for GC+ or GFAP+ cells. The mean cell number was determined from 10 randomly selected fields (0.314 mm2) of each coverslip when viewed by a microscope with epifluorescence (Nikon). Data were expressed as a fold of increase in the mean cell number.
number over cultures incubated with matching aliquots of PBS controls. A unit of biological activity for a specific GPF was defined as the fold increase above the mean control cell number per microgram protein added to a milliliter of defined culture medium for 72 h.

**Column Chromatography**

GPFs separated by gel filtration were further purified using ion-exchange chromatography. Pooled fractions with GPF₁ or GPF₂ were applied to DEAE-5PW (Bio-Rad Laboratories, Richmond, CA) and eluted with a gradient of NaCl in 20 mM of sodium phosphate buffer (pH 7.4). In the case of GPF₁, the peak of oligodendroglia-stimulating activity was found routinely in fractions 17 through 25, while GPF₂ eluted in fractions 8 through 12 (Fig. 3). A combination of gel filtration and ion-exchange chromatography gave a final purification of ~2,000- to 3,000-fold for GPF₁ or GPF₂ (Table I). Between 80 and 90% of total biological activity for either GPF₁ or GPF₂ was recovered from the ion-exchange column.

GPFs partially purified by gel filtration were also separated on a C3 column.
GPFs have been identified as peptide growth factors present during gliogenesis in the central nervous system (10). Since the secretion of these peptides might represent an important mechanism for controlling glial growth, we sought to determine the cellular sources of GPFs in the brain. This type of investigation required us to distinguish GPFs from other factors that stimulated cell growth by several different biochemical criteria.

Identification of Cells That Promote Macrogial Growth

We screened cell lines derived from tumors of the nervous system or enriched cell preparations from newborn rat brain for the presence of glia-stimulating activities. Microglia, isolated by selective adhesion, were nearly homogenous with
Table I. Specific Activity and Degree of Purification of GPF₁ and GPF₂

| Techniques            | GPF₁            | GPF₂            |
|-----------------------|-----------------|-----------------|
|                       | Specific activity | Specific activity | Fold purification | Fold purification |
| Tectal supernatant    | 0.007 ± 0.003   | 0.008 ± 0.001   | 1                 | 1                 |
| P10                   | 2.40 ± 0.63     | 2.00 ± 0.29     | 350               | 250               |
| P10 + DEAE            | 19.00 ± 2.29    | 15.63 ± 2.03    | 2,700             | 1,950             |
| P10 + C3              | 61.00 ± 3.60    | 20.00 ± 2.00    | 8,700             | 2,500             |

A unit of biological activity represents a 100% increase in the mean numbers of oligodendroglia or astroglia per mm² when compared to control preparations treated with an equivalent volume of PBS. The increase in cell number is calculated from dose-response curves involving at least four cultures for each factor concentration assayed. Specific activity is expressed as U/μg protein in 1 ml of culture medium. Data are presented as mean values ± SEM.

95% of cells containing acetylated lipoprotein receptors (Fig. 2). Astroglial cultures, prepared using McCarthy’s method (19), provided 98% GFAP⁺ cells. We also examined the neuronal cell lines B65, N-2A, and C-1300, as well as the C6 glial cell line. The fibroblast cell line 3T3 and the pituitary cell line GH3 provided nonneural control material.

Cell lines were harvested from 35-mm plastic dishes and dispersed by sonication. These sonicates were incubated with newborn rat brain cultures and after 48 h, glia populations were identified by immunofluorescence. Significant increases in the number of oligodendroglia were found in cultures incubated with sonicates from the neuronal lines N-2A and C-1300, but not with sonicates from microglia or the C6, GH3, or the 3T3 cell lines (Fig. 5). By comparison, the microglial sonicates stimulated the appearance of GFAP⁺ astroglia (Fig. 5). We next explored the possibility that the glia-stimulators found in cell sonicates were similar to GPFs isolated from brain.

Microglia Secrete Astroglia-stimulating GPFs

Putative astroglia-stimulating factors from cell preparations were fractionated by gel filtration. Once again, only microglial sonicates contained significant biological activity. Two of the microglial factors co-purified by gel filtration with fish GPF₂ and GPF₄ (Fig. 6).

As suggested earlier, a signaled secretion of growth factors might help to control glial proliferation in the brain. Our initial attempts to detect microglial secretion of GPF-like substances proved unsuccessful. We found, however, that a 24-h incubation of microglia with fixed *Staphylococcus aureus*, a known macrophage activator (3), significantly increased the amount of astroglia-stimulating activity released into culture medium. This growth activity, fractionated by gel filtration, contained factors similar to GPF₂ (9 kD) and GPF₄ (3 kD) (Fig. 7). We confirmed the identity of the 9-kD factor secreted by microglia as GPF₂ by co-purification with goldfish and rat brain factors using anion exchange chromatography (Fig. 8) and reverse-phase HPLC (Fig. 9). The similarities in apparent molecular masses, biological activities, and co-elution profiles suggested that microglia secreted GPF₂ in the central nervous system (20).

![Figure 4. Separation of GPFs by reverse-phase chromatography. GPF₁ and GPF₂, partially purified by gel filtration, were eluted from a reverse-phase C3 column with 0.01 M trifluoroacetic acid and a gradient of acetonitrile (20–70%). The total protein concentrations in each of the 700-μl fractions were determined by the fluorescamine method. Biological assay was carried out as described in Materials and Methods using three cultures per data point. (Upper panel) Two peaks of biologically active GPF₁ were recovered; (lower panel) the major peak of biologically active GPF₂ was eluted with 25–30% acetonitrile.](image)
Figure 5. Whole cell sonicates (25 μl) containing ~100 μg protein from glial cell line (C6), microglia (MIC), a fibroblast cell line (3T3), a pituitary tumor cell line (GH3), and two neuronal cell lines (N-2A and C-1300) were incubated with primary glial cell cultures grown in defined media for 48 h. The fold increase in GCsyn oligodendroglia or GFAPsyn astroglia were calculated from increases in mean cell numbers/mm² when compared to control cultures. These controls were incubated with 25 μl of PBS for 48 h. As shown, sonicates from the neuronal cell lines increased the number of oligodendroglia found in culture while microglia sonicates contained astroglia-stimulating activity.

Figure 6. Cell production of astroglia-stimulating GPFs. Soluble protein from goldfish optic tectum or cell sonicates were separated by gel filtration and assayed for the presence of astroglia-stimulating factors. Microglia contained biological activity which co-eluted with GPF2 (9 kD) and GPF4 (3 kD) found in goldfish brain. Such biological activity was not detected in astroglia, the glial cell line, or the neuronal cell lines, N-2A and C-1300.

Figure 7. Secretion of astroglia-stimulating factors. Media conditioned for 24 h by microglia, astroglia, glial cell line C6, and the neuronal cell line C-1300, were assayed for the presence of GPFs. All cell cultures were activated by the addition of a suspension of fixed Staphylococcus aureus (20 μl per 1.5 ml culture medium). The conditioned medium was collected after 24 h, and fractionated by gel filtration. Only microglia secreted detectable levels of GPF2- and GPF4-like factors. Microglia also released an 18-kD astroglia-stimulating peptide (not found in collected fractions) which has been identified as interleukin-1 (Guilian D., T. J. Baker, and L. B. Lachman, unpublished data).

Purification of GPF1
As a final step to confirm the identities of the oligodendroglia-stimulating factors, we purified GPF1 by combining the techniques of gel filtration, anion exchange chromatography, reverse-phase HPLC, and sieving HPLC. 200 mg of fish brain (400 optic tecta) yielded <10 ng of peptide with an estimated 100,000-fold purification (Fig. 14). This highly purified GPF1...
Figure 8. Co-purification of GPF₁ from fish, rat, and microglia using anion exchange chromatography confirms the similarity of these factors from different sources. Conditions for chromatography are described in Fig. 3.

Figure 9. Co-purification of GPF₁ from rat brain and from microglia using reverse-phase HPLC. Conditions for chromatography are described in Fig. 4.

appeared as a single peak of biological activity when separated by sieving HPLC with an apparent molecular mass of 15 kD. Using the same chromatographic techniques, we also isolated GPF₁, which was secreted by the C-1300 cell line. As shown in Fig. 15, highly purified GPF₁ from the neuronal cell line co-eluted by sieving HPLC with fish material. The co-purification of these GPFs suggest a high degree of structural homology between the oligodendroglia-stimulating peptides.
Figure 12. Co-purification of GPF₁ from fish optic tectum, rat brain, and the C-1300 cell line using anion exchange chromatography as described in Fig. 3.

Figure 13. Co-purification of GPF₁ from rat cerebral cortex and from neuronal cell line C-1300 by reverse-phase HPLC. Conditions for isolation as described in Fig. 4. Two peaks of biological activity are recovered from either source at 36–39% and 45–47% acetonitrile.

Figure 14. Dose-response curves showing purification of GPF₁. Supernatants of goldfish optic tecta were separated by gel filtration (P-10), anion exchange chromatography (DEAE), reverse-phase HPLC (C3), and sieving HPLC (TSK) as described in Materials and Methods. Specific biological activity suggests a 100,000-fold purification of GPF₁, when compared to tectal supernatants. The yield for 400 optic tecta was <10 ng of GPF₁.

Figure 15. Highly purified GPF₁ isolated from goldfish brain or secreted by the neural cell line C-1300 were separated using sieving HPLC. These peptides co-eluted with an apparent molecular mass of 15 kD. Goldfish GPF₁ was isolated as described in Fig. 14. 6 liters of conditioned media served as starting material for the C-1300 factor. This medium was concentrated by ultrafiltration (YM-2, Amicon Corp.) and then processed by the same techniques used for goldfish material. Molecular weight markers: (a) 68 kD; (b) 18 kD; (c) 12 kD; (d) 6 kD.

found in fish optic tectum and in a mammalian neuronal cell line.

**Regenerating Neurons Produce GPF₁ and GPF₃**

As noted before (10), brain levels of GPF₁ and GPF₃ were elevated during regeneration or development of the central nervous system. Since GPF₁ and GPF₃ were secreted by neuronal cell lines, we speculated that neurons might be a major source of oligodendroglia-stimulating peptides in vivo.
Figure 16. GPF production in neurons. Sonicates of 30 ganglion cell layer dissections (~1.5 mg protein) were separated by gel filtration. Oligodendroglia-stimulating factors GPF1 and GPF3 were found in retinal ganglion cells isolated from the regenerating goldfish visual system 12 d after axotomy (upper panel). No astroglia-stimulating activity was detected in the same preparation (lower panel).

As shown in Fig. 1, ganglion cells isolated from the goldfish retina by microdissection (4) consisted of cell bodies and unmyelinated axons. Sonicates of this glial-free preparation (4) contained significant levels of GPF1 and GPF3 (Fig. 16) with no evidence of astroglia-stimulating activity. Moreover, the concentrations of GPF1 and GPF3 were markedly elevated in neurons undergoing axonal regeneration (Fig. 17). We concluded that growing neurons found in the developing rat brain and the regenerating goldfish visual system were the likely sources of oligodendroglia-stimulating factors.

Discussion

We report here on the isolation of GPF1, an oligodendroglia-promoting factor, by several different chromatographic methods. Picomolar concentrations of fish GPF1, after a 100,000-fold purification, stimulated oligodendroglial proliferation in vitro. Based upon the specificity of its biological activity, GPF1 appears to represent a new class of brain growth factor. The stability of this factor after lyophilization, exposure to acetonitrile, and SDS PAGE (Giulian, D., unpublished data) increases the likelihood of obtaining bulk quantities of homogenous peptide. The neuronal cell lines as sources of GPF1 may provide sufficient material needed for such large-scale purifications.

Microglia are the principal phagocytic cells of the brain and consist of two forms (12, 18, 20): the ameboid cell, morphologically similar to the macrophage, is found in developing and injured brain, while the ramified cell is associated with the normal adult central nervous system (18). Investigators using histochemistry and electron microscopy (12, 18), have suggested that ameboid microglia serve as active scavenger cells and eventually differentiate into quiescent, ramified cells. Our study of enriched brain cell cultures indicate that "activated" ameboid microglia are the best secretors of the astro-

| Table II. GPFs in Brain |
|-------------------------|
|                         | Oligodendroglia-stimulating Factors | Astroglia-stimulating factors |
| Peptide factors         | GPF1 (15 kD)                      | GPF2 (9 kD)                     |
|                        | GPF2 (6 kD)                       | GPF4 (3 kD)                     |
| Developing mammalian brain | +++ (Postnatal)             | +++ (Prenatal)                  |
| Injured adult mammalian brain | ---                           | +++                              |
| Regenerating goldfish visual system | +++                         | ---                              |
| Brain cellular source   | "Growing" neurons                | Ameboid microglia               |

A summary of biological specificities and cellular sources for GPFs recovered from the central nervous system. The oligodendroglia-stimulating peptides are produced by neurons found in developing or regenerating tissues. Astroglia-stimulating factors are secreted by ameboid microglia found in embryonic brain or at sites of central nervous system injury.

We detect significant levels of GPF2 and GPF4 in such microglial-rich tissues as the cerebral cortex of embryonic rat and wound sites of brain-injured adults (12, 18). Such findings suggest that ameboid microglia serve not only as scavenger...
cells in the brain but also as regulators of astroglial growth by the release of peptide factors.

Neurons are the probable source of oligodendroglia-stimulating GPFs in developing rat brain and in the regenerating goldfish visual system (Table II). As reported here, neuronal cell lines secrete GPF, and GPFs, in vitro, implying such secretion also occurs in vivo. Although the conditions for eliciting production of GPFs, and GPFs are unknown, regenerating neurons showed greater factor concentrations than did quiescent cells. We suggest that peptides released by neurons stimulate oligodendrogial proliferation in the neighborhood of growing axons (7, 8).

Our findings point to the existence of a regulatory network whereby peptides released from specific secretory cells control the growth and proliferation of specific glial populations (7–9, 14–17). Perhaps application of these peptides will allow manipulation of cell growth in the developing or injured brain. Moreover, the identification of events which elicit the production and secretion of GPFs may help to elucidate mechanisms that control cellular organization of the nervous system.

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