true interannual differences rather than previous methodological bias. We need to re-evaluate both the assumption of steady state that underlies models of carbon or nutrient flux through the euphotic zone and our studies of community structure and dynamics.

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4. We are uncertain about the existence of seasonality in chlorophyll a concentration. We can detect no consistent relation between the 12 summers and 4 winters for which we have chlorophyll a data [T. L. Hayward, E. L. Venrick, J. A. McGowan, *J. Mar. Res.* **41**, 711 (1983)]. However, chlorophyll a shows a negative correlation with the number of days since 1 May ($P < 0.10$), indicating a compensating increase during the winter months. The high value in 1974 is our only datum from May, but seasonality does not completely explain the value. Removing seasonality does not reduce the long-term change or alter our conclusions.
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Trophic Stimulation of Cultured Neurons from Neonatal Rat Brain by Epidermal Growth Factor

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Epidermal growth factor (EGF) is a potent polypeptide mitogen originally isolated from the adult male mouse submaxillary gland. It also acts as a gastrointestinal hormone. EGF-immunoreactive material has recently been identified within neuronal fibers and terminals in rodent brain. In the present study, EGF was found to enhance survival and process outgrowth of primary cultures of subneocortical telencephalic neurons of neonatal rat brain in a dose-dependent manner. This effect was observed with EGF concentrations as low as 100 picograms per milliliter (0.016 nanomolar) and was dependent on the continuous presence of EGF in the medium. Similar effects were observed with basic fibroblast growth factor, but several other growth-promoting substances, including other mitogens for glial elements, were without effect. Thus EGF, in addition to its mitogenic and hormonal activities, may act as a neurite elongation and maintenance factor for select neurons of the rodent central nervous system.

POLYPEPTIDE GROWTH FACTORS ARE hormonelike agents that contribute substantially to the regulation of both hypertrophic and hyperplastic responses of eukaryotic cells, particularly in vertebrates (*1*). Neuronotrophic factors, a subset that acts on neurons of the peripheral and central nervous systems, primarily enhance neurite outgrowth and maintain cell viability (*2*). Nerve growth factor (NGF), a neuronotrophic agent with well-defined activity in the peripheral nervous system (*3*), is synthesized in the target region and acts via specific membrane-bound receptors located on the neuron (*4*). More recently, NGF has been identified within the central nervous system (CNS) and has been proposed to act as a trophic factor for a particular subset of neurons there (*5*). Other agents that act as trophic factors for the diverse types of CNS neurons have been identified, but have not been extensively characterized (*2*).

Epidermal growth factor (EGF) is a polypeptide mitogen for a number of cell types (*6*). It inhibits gastric acid secretion, a finding that has lead to the alternative designation, urogastrone (*7*). Recently, EGF immunoreactivity (*8*) and precursor messenger RNA (mRNA) (*9*) have been identified within the mammalian brain; the presence of EGF within the CNS is, however, the subject of controversy (*10*). EGF binding sites occur in brain tissue (*11*), and EGF stimulates the proliferation and differentiation of glial cells (*12*). In the present study we show that EGF also promotes the survival, and stimulates process outgrowth, of neonatal rat CNS neurons in vitro.

Dissociated cell cultures were derived from the subneocortical telencephalon of neonatal rats (*13*); this region contains areas that exhibit EGF immunoreactivity in the adult (*8*). The addition of mouse EGF (10 ng/ml) to these primary cultures resulted in a 15-fold increase in the number of surviving cells with a neuronal morphology (*14*) (Fig. 1A and Table 1). EGF also increased the number of processes and the degree of

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process branching (Fig. 1A). In contrast, control cultures maintained in the absence of EGF contained only a few scattered cells. Some of these cells exhibited moderate process outgrowth, although most cells cultured under these conditions extended only one or two truncated processes (Fig. 1B). In cultures treated with EGF, 90 to 95% of the surviving cells exhibited neuronal morphology and were labeled by antibodies to the neuronal marker (*15*) neuron-specific enolase (NSE) (Fig. 1C). These NSE-positive cells were not labeled with antibodies against glial fibrillary acidic protein (GFAP), a marker for glial cells. The remaining 5 to 10% of the cells stained positively for GFAP.

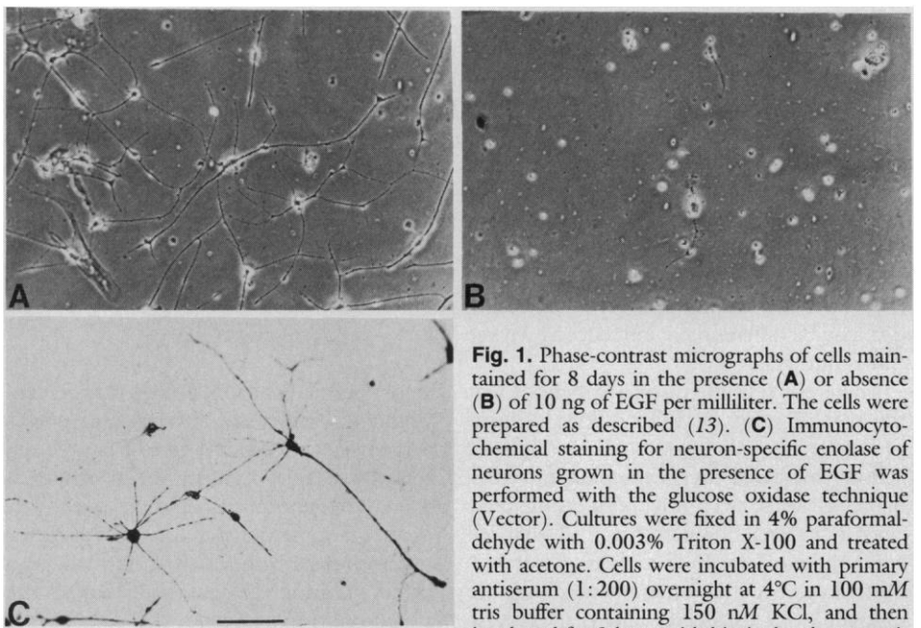

Fig. 1. Phase-contrast micrographs of cells maintained for 8 days in the presence (**A**) or absence (**B**) of 10 ng of EGF per milliliter. The cells were prepared as described (*13*). (**C**) Immunocytochemical staining for neuron-specific enolase of neurons grown in the presence of EGF was performed with the glucose oxidase technique (Vector). Cultures were fixed in 4% paraformaldehyde with 0.003% Triton X-100 and treated with acetone. Cells were incubated with primary antiserum (1:200) overnight at 4°C in 100 m*M* tris buffer containing 150 n*M* KCl, and then incubated for 1 hour with biotinylated goat antibody to rabbit immunoglobulin G (1:250). The cells were then incubated for 1 hour with a mixture of biotin-avidin and glucose oxidase and visualized, with glucose being used as the substrate and TNBT (tetranitro blue tetrazolium) as the chromagen. Cells did not stain when incubated in the absence of primary or secondary antiserum. Scale bar, 180 μm in (A) and (B), 100 μm in (C).

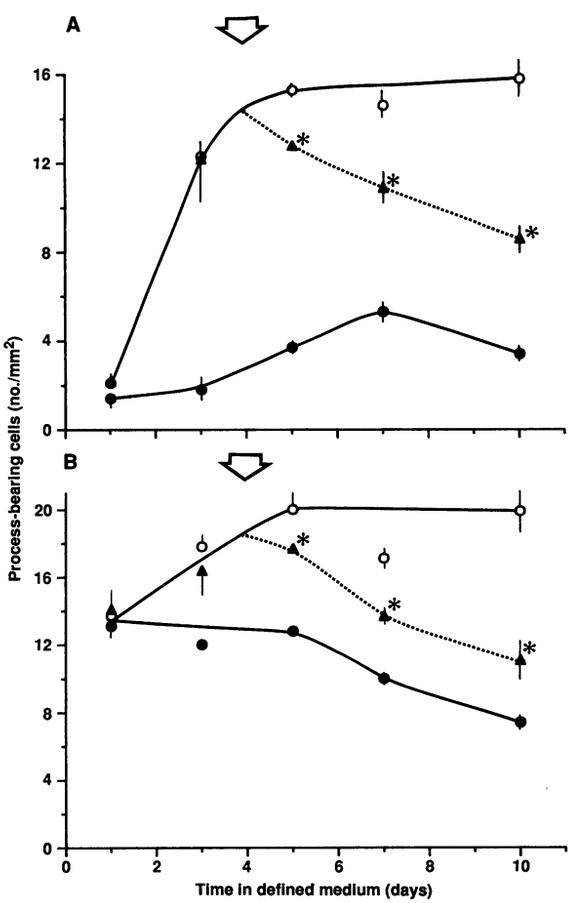

Fig. 2. Dependence of survival and process outgrowth on the continued presence of EGF. Neurons were plated and maintained as described (*13*). At day 0, cells were converted to chemically defined medium (CDM) plus (open circles) or minus (closed circles) EGF. Four days after conversion to CDM (arrow) one set of cultures that previously contained EGF was converted back to EGF-deficient medium (triangles). The number of cells bearing processes greater than or equal to 100 μm in length (**A**) or bearing processes of any length (**B**) were scored by counting ten fields across the diameter of the dish. Three dishes were counted for each condition; the same dishes were counted throughout the experiment. The data are expressed as the average number of cells per square millimeter ± SEM and represent one of two similar experiments. The asterisk indicates that values are less than EGF-treated cultures after conversion to EGF-deficient medium ($P < 0.05$) (Fisher exact probability test). All control values were significantly lower than all experimental values. Statistical comparisons were made with values obtained on the same day.

The number of cells bearing processes greater than 100 μm in length increased significantly within 24 hours of addition of EGF (Fig. 2A). This effect reached a peak after 3 to 5 days of continuous treatment. Neurons treated with EGF could be maintained in culture for at least 6 weeks without marked cell loss or process degeneration. However, when EGF was removed from the culture medium after 4 days (Fig. 2A), there was a cessation of process outgrowth and a decline in the number of process bearing cells. The effect of EGF removal was rapid: a significant decline in the number of process-bearing cells was observed within 24 hours. This rapid reversal suggests that EGF acts as a soluble factor and not through a mechanism of substrate attachment, as has been proposed previously for laminin (*16*).

When the total number of cells with neuronal morphology and with processes of any length was measured, incubation with EGF produced a similar effect (Fig. 2B). However, this effect had a greater latency to onset than that observed when only cells with long processes were counted (Fig. 2A), where significant effects were observed after only 1 day. This indicates that the EGF-mediated induction of neurite extension occurs more rapidly than does the effect on neuronal survival. After 4 days of continuous treatment with EGF, approximately

Table 1. Influence of growth-promoting substances on neuronal survival. Cells were plated and maintained as described in Fig. 1. At day 0, cultures were converted to CDM with no additions or with the additions shown. Eight days after conversion the number of cells bearing processes 200 μm or longer were scored in ten fields from three different wells, and the data were expressed as the mean ± SEM of the three wells. Data presented are from one of two similar experiments.

Factor	Process-bearing cells (no./mm²)
Control	1.67 ± 0.30
EGF (10 ng/ml)	25.67 ± 0.50*
FGF (1 ng/ml)	20.00 ± 0.72*
β-NGF (10 ng/ml)	2.00 ± 0.20
Thrombin (10 ng/ml)	1.00 ± 0.20
IL-2 (10 units/ml)	0.67 ± 0.23
PDGF (5 ng/ml)	0.33 ± 0.12

*Denotes significantly greater than controls ($P < 0.05$) from the Fisher exact probability test.

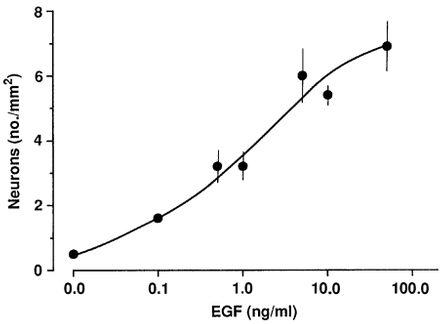

Fig. 3. Influence of EGF concentration on the survival of neuronlike cells of neonatal rat brain. Neuronal cultures were prepared as described (*13*). Upon conversion to CDM (day 0), EGF was added to cultures at various concentrations. Eight days after conversion to CDM the number of cells with processes 100 μm or longer were scored in ten fields across the diameter of the dish. Three dishes were used for each concentration of EGF tested. The results are expressed as the average number of cells per square millimeter ± SEM and represent one of three similar experiments. All values for EGF-treated wells were significantly greater than for the appropriate controls ($P < 0.05$, Fisher exact probability test).

80% of the surviving neurons exhibited processes greater than 100 μm.

Survival and process outgrowth of neuronlike cells were dependent on the concentration of EGF in the medium (Fig. 3). The effect appeared to plateau at concentrations between 5 and 50 ng/ml, with a midpoint at approximately 1 ng/ml (0.16 n*M*). However, concentrations as low as 100 pg/ml were effective in promoting enhanced survival and process outgrowth. These findings suggest a specific, receptor-mediated mechanism. This EGF effect was completely blocked by incubating the cells with antibodies to EGF at a dilution of 1:100 for 3 days. In EGF antibody–treated cultures the number of cells bearing long processes was 11% of that of cultures treated with EGF (10 ng/ml) and an equivalent amount of nonimmune sera. In contrast, antibodies to NGF did not significantly alter the EGF response.

To determine whether these trophic actions are specific to EGF or are shared by other growth-promoting substances, we tested the abilities of other polypeptide growth factors to support neuronal survival and process outgrowth. Of the other factors examined, only bovine basic fibroblast growth factor (bFGF), which supports the survival of cultured rat cerebral cortical, hippocampal, and striatal neurons (*17*), supported the development and survival of subneocortical telencephalic neurons (Table 1). Neither NGF, which influences fetal striatal neurons in organotypic culture (*18*), nor interleukin 2 (IL-2) significantly altered the parameters measured in the present study. Platelet-derived growth (PDGF) and thrombin, both of which are glial mitogens (*19*), had an adverse effect on the survival of process-bearing cells and increased the level of contaminating astrocytes. Although EGF is a mitogen for astrocytes (*20*), glial contamination of our EGF-treated cultures was only 5 to 10%. This is in sharp contrast to the results obtained after 2 weeks of growth in the presence of thrombin and PDGF, where astrocytes comprised greater than 90% of the cell population. The reason for increased glial proliferation in the presence of PDGF and thrombin, but not EGF, is unknown. One possibility is that the lethality of cytosine arabinoside on proliferating glia may be differentially influenced by these three mitogens.

There are several reasons to believe that the trophic effect of EGF reflects a direct action on neurons, although an indirect action, mediated by contaminating nonneuronal cells, has not been completely ruled out. First, the percentage of glial cell contamination in EGF-treated cultures is quite low. Second, other factors that are mitogenic for astrocytes and that increase the level of glial cell contamination within these cultures, have no neuronotrophic activity. Third, there is very little neuronotrophic effect of medium conditioned by astrocytes grown in the presence of EGF (Table 2). Neuronal cultures raised in this conditioned medium showed approximately fivefold fewer process-bearing cells than cultures treated directly with EGF, although there was a slight increase as compared to controls. The lack of neuronotrophic effect of medium conditioned by astrocytes raised in the absence of EGF is in contrast to previous findings (*21*) that astrocyte-conditioned medium is trophic for CNS neurons.

The present findings suggest that EGF, or an EGF-like peptide, may serve as a trophic factor for some neurons within the brain. While in the present experiments we have demonstrated that EGF exerts a neurotrophic effect on cells derived from brain areas that exhibit EGF immunoreactivity, we have also found that EGF produces similar effects on cells from other brain regions (*22*). It is possible that neurons that respond to EGF in vitro are not exposed to this factor in vivo. Alternatively, such cells may serve as targets for the action of an EGF-related peptide, such as transforming growth factor α, which can interact with the EGF receptor (*23*).

Very little is known of the factors that may serve as trophic agents in the CNS. The heterogeneity of brain tissue has made it difficult to purify the very small quantities of material that are apparently present. In the present study, we demonstrate that EGF, a highly purified and extensively studied compound (*6*), enhances survival and process outgrowth in neurons derived from the subneocortical telencephalon of neonatal rat brain. This unexpected finding extends the view that some polypeptide growth factors and hormones with activities normally associated with nonneuronal tissue may also act as neuronotrophic agents.

Table 2. Effect of EGF and astrocyte–conditioned medium (CM) on neuronal survival and process outgrowth. Neurons were grown in the presence or absence of EGF or medium conditioned by astrocytes raised in the absence or presence of EGF. Results are the mean number of cells bearing processes greater than 200 μm in length per square millimeter ± SEM of three dishes. Data presented are from one of two similar experiments.

Culture conditions	Cells (no.)
EGF	8.7 ± 0.53*
Control	1.0 ± 0.02
CM: EGF-treated astrocytes	2.0 ± 0.18*
CM: EGF-untreated astrocytes	0.7 ± 0.30

*Denotes significantly different from controls ($P < 0.05$) from the Fisher exact probability test.

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13. Cultures were prepared by modifications of the method of K. D. McCarthy and J. deVellis [*J. Cyclic Nucleotide Res.* **4**, 15 (1978)]. One-day-old rats were killed by decapitation, and their brains were removed under sterile conditions. The neocortex, hippocampus, olfactory bulb, brainstem, and diencephalon were discarded, leaving a block of tissue containing the striatum, globus pallidus, olfactory tubercle, and septum. This tissue was trypsinized (0.1% trypsin) at 37°C and passed through a 130-μm nylon mesh. The cells were centrifuged and resuspended in plating medium [Ham's F-12 and Dulbecco's modified Eagle's medium (DMEM), 1:1, supplemented with 10% fetal calf serum, penicillin and streptomycin, and 5 μ*M* cytosine arabinoside]. The cells were passed through a 33-μm mesh and plated at a density of 1×10^6 cells per 2 ml of medium. At 18 to 24 hours later, cultures were converted to a chemically defined medium containing the same nutrient mixture (without serum) plus hydrocortisone (50 μ*M*), putrescine (100 μ*M*), prostaglandin $F_{2\alpha}$ (500 ng/ml), insulin (50 μg/ml),

cytosine arabinoside (5 μ*M*) and EGF (or other growth factor, where appropriate). Cytosine arabinoside was omitted from subsequent medium changes, which were done every 2 to 3 days. The number of cells bearing neuronal morphology in EGF-treated cultures was approximately 1 to 2% of the total number of cells plated. This is a low estimate of plating efficiency because nonneuronal cell types were included in the cells counted for plating, and both viable and nonviable cells were counted. In addition, of the total number of cells plated, less than 10% were found to be adherent to the plate after 24 hours in culture.
14. Cells were considered to be neurons if they possessed a rounded, phase-bright soma from which extended one or more slender processes. In most experiments, an additional criterion of process length was added. A cell was counted if the longest process extending from the cell body was at least 100 μm (200 μm in some experiments) in length.
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The IL-2 Receptor β Chain (p70): Role in Mediating Signals for LAK, NK, and Proliferative Activities

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Interleukin-2 (IL-2) induces cytolytic activity and proliferation of human blood lymphocytes. Yet, prior to activation, these cells do not express IL-2 receptors recognized by monoclonal antibodies to the Tac antigen. A novel glycoprotein (IL-2Rβ), identified on several lymphocytoid cell lines, has the ability to bind IL-2 alone and to associate with Tac antigen (IL-2Rα) to form high-affinity IL-2 receptors. It is now reported that IL-2Rβ is expressed on both circulating T lymphocytes and large granular lymphocytes in quantities approximately proportional to their responsiveness to IL-2. Studies of the responses of these cells to IL-2 suggest that IL-2Rβ mediates the initial phase of induction of lymphokine activated killer (LAK), natural killer (NK), and proliferative activities. Subsequently, IL-2Rα is induced and functional high-affinity IL-2 receptors are expressed.

THE LYMPHOKINE INTERLEUKIN-2 (IL-2) has several effects on unstimulated human blood lymphocytes. Natural killer (NK) activity can be augmented (*1–4*); lymphokine-activated killer (LAK) activity (*5*) and proliferation (*6–8*) can be induced. Yet, unlike activated lymphocytes, which express both high-affinity (approximate dissociation constant, K_d, 10 p*M*) and low-affinity (approximate K_d, 10 n*M*) IL-2 receptors capable of binding monoclonal antibodies to the Tac antigen, such as anti-Tac (*9–12*), these circulating lymphocytes express little or no IL-2 receptor detectable by anti-Tac (*2, 3, 6, 7, 9*).

Sharon *et al.* detected (*13*) and directly identified (*14*) a 70-kD lymphocyte membrane glycoprotein (p70, IL-2Rβ) which, together with Tac antigen (p55, IL-2Rα), appears to form the high-affinity IL-2 receptor. Other reports have indicated that IL-2Rβ is capable of binding IL-2 and mediating its internalization in the absence of IL-2Rα (*15–17*). We now report evidence that IL-2Rβ mediates the activation of resting lymphocytes by IL-2.

Using Percoll density gradient centrifugation (*18*), we isolated a subpopulation of peripheral blood mononuclear cells (PBMC), large granular lymphocytes (LGLs), which are highly responsive to IL-2 (*3, 4, 7, 8*). As previously reported (*18*), LGLs, but not the denser small T cells, expressed NK activity and this activity is rapidly boosted by exposure to IL-2. After incubation in 1 n*M* IL-2, LGLs typically developed 3 to 5 times more LAK activity and 5 to 20 times more rapid proliferation (thymidine incorporation) than did small T cells.

After further enrichment for IL-2-responsive cells by sorting for the NK cell marker Leu-19 (NKH-1) (*19*), LGLs were examined for the expression of IL-2Rα and IL-2Rβ. Iodine-125–labeled IL-2 was bound and cross-linked to the cells; cell lysates were then analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). IL-2Rβ (p70), when cross-linked to IL-2 (15.5 kD), migrates at 85 to 92 kD, whereas cross-linked IL-2Rα (p55) migrates at 68 to 72 kD (*13*). In each of several experiments, Leu-19$^+$, CD5$^-$ LGLs were highly enriched in IL-2Rβ expression when compared to PBMC or various T cell populations (Fig. 1A). In contrast with our findings in activated T cells (*13*), unstimulated LGLs and T cells (including T cell populations highly purified by flow cytometric sorting) from each of several donors expressed IL-2Rβ in large excess of IL-2Rα (Fig. 1A and 1B). Nevertheless, some IL-2Rα expression was often detected on circulating lymphocytes. When the small subpopulations of LGLs and small T cells expressing IL-2Rα (<5% of total cells) were removed by panning out cells that bound the monoclonal antibody anti-Tac, the remaining cells expressed IL-2Rβ but no detectable IL-2Rα (Fig. 1B). In repeat experiments, these cells, which lack IL-2Rα, retained all of the IL-2–induced LAK and NK activity and >60% of the proliferative activity of the parent populations. These data suggested that IL-2Rβ might mediate the effects of IL-2 on these cells.

We therefore sought to correlate the concentrations of IL-2 required to bind to IL-2Rβ with those needed to activate LGLs and small T cells. In experiments testing the effects of IL-2 concentration—cross-linking to IL-2Rβ (Fig. 2A), induction of cellular aggregation (Fig. 2B), and induction of cytotoxic and proliferative activities (Fig. 2C, open symbols) in unstimulated LGLs—all effects were first observed at 30 to 100 p*M* IL-2, increased through 1 to 10 n*M*, and then reached a plateau. Half-maximal activation was generally observed in the 100 p*M* to 1 n*M* range (approximately 10 to 100 U/ml). Interleukin-2 binding and activation of small T cells was of lower magnitude but occurred at the same concentrations. In contrast, IL-2–induced proliferation of T lymphoblasts, a response mediated by the high-affinity IL-2 receptor, occurred at lower concentrations of IL-2 (Fig. 2C, closed circles).

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