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Brain neurons and glial cells express Neu differentiation factor/heregulin: A survival factor for astrocytes

(tyrosine kinase/epidermal growth factor family/central nervous system)

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ABSTRACT **Neu differentiation factor (NDF, also called heregulin) was isolated from mesenchymal cells on the basis of its ability to elevate phosphorylation of ErbB proteins. Earlier *in situ* hybridization analysis showed that NDF was transcribed predominantly in the central nervous system during embryonic development. To gain insights into the role of NDF in brain we analyzed its distribution by immunohistochemistry and *in situ* hybridization. Late-gestation (day 17) rat embryos displayed high NDF immunoreactivity in both motor (e.g., putamen) and limbic (e.g., septum) regions. Lower levels of the factor were exhibited by adult brain, except for the cerebellum, where NDF expression was increased postnatally. Both neurons and glial cells were identified by immunohistochemistry as NDF-producing cells (e.g., pyramidal neurons in the cerebral cortex and glial cells in the corpus callosum). By establishment of primary cultures of rat brain cells we confirmed that NDF was expressed in neurons as well as in astrocytes. In addition, by using such primary cultures we observed that NDF treatment exerted only a limited mitogenic effect, which was accompanied by significant acceleration of astrocyte maturation. Furthermore, long-term incubation with the factor specifically protected astrocytes from apoptosis, implying that NDF functions in brain as a survival and maturation factor for astrocytes.**

The group of subtype I receptor tyrosine kinases includes four transmembrane glycoproteins whose prototype is the epidermal growth factor receptor (1, 2). The search for a ligand that interacts with ErbB-2 led to the isolation of a 44-kDa glycoprotein from the medium of *ras*-transformed fibroblasts that elevated tyrosine phosphorylation of ErbB-2 in mammary cells (3, 4). Although this glycoprotein, called Neu differentiation factor (NDF), underwent coimmunoprecipitation with ErbB-2, its cellular binding displayed cell-type specificity, implying that the direct receptor for NDF is an ErbB-2-related tyrosine kinase that is expressed in mammary but not in ovary cells (5). This led to the identification of ErbB-4 as the direct receptor for NDF and to the realization that ErbB-2 and ErbB-4 are functionally coupled through heterodimer formation (6). A human homologue of NDF, termed heregulin, has been isolated and found to act as a mitogen for certain mammary tumor cells (7). Yet in other breast cancer cell lines the factor induced a differentiated phenotype that included synthesis of both the intercellular cell adhesion molecule 1 and milk components, and also induced cell growth arrest (8).

Only a single NDF gene was localized in the human genome (9). However, alternative splicing was found to generate at least 10 isoforms of NDF that fall into two groups, α and β, which differ in their epidermal growth factor (EGF)-like domains (10). In addition, all of the isoforms include an immunoglobulin-like domain, and most variants are produced as transmembrane proteins with a variable-length cytoplasmic domain (11). *In situ* hybridization with a nucleotide probe from a region that is common to all isoforms revealed that the predominant site of expression of NDF in mid-gestation mouse embryos is the nervous system (9). This raised the possibility that, in addition to its role in epithelia, NDF affects certain functions of the central and peripheral nervous systems. Two functions in the peripheral system have been more recently identified through the isolation of NDF isoforms from neuronal tissues: (*i*) certain brain- and pituitary-derived isoforms were found to accelerate proliferation of Schwann cells *in vitro* (12) and (*ii*) an activity that induced synthesis and clustering of acetylcholine receptors in chick neuromuscular junctions was found to correspond to a variant of NDF, called ARIA (13). We have used immunohistochemistry and *in situ* hybridization to characterize the spatial and temporal expression patterns of NDF in rat brain. A multisite distribution map was observed and the producing cells were identified as both neurons and glial cells. These cell types retained NDF synthesis when cultured *in vitro*. In addition, application of NDF to primary cultures of brain neurons and glia cells preferentially affected astrocytes, as was reflected by accelerated maturation, limited proliferation, and a remarkable lengthening of cell viability.

MATERIALS AND METHODS

Materials. NDF was purified from medium conditioned by *ras*-transformed Rat-1 fibroblasts (3). Recombinant isoforms of the factor were purified from bacterial cells to apparent electrophoretic purity by using a series of chromatographic steps (11). A rabbit polyclonal antiserum was raised against bacterial recombinant NDF-α2, and it recognized all isoforms of the factor (11). The antiserum was purified over a column of recombinant rat NDF. Mouse monoclonal antibodies to glial fibrillary acidic protein (GFAP) and ≈43-kDa growth-associated protein (GAP-43) were purchased from BioMakor (Rehovot, Israel), and antibodies to myelin basic protein were from Chemicon.

Immunohistochemistry. Wistar rats were used for immunohistochemical analyses. After perfusion of the animals with 2.5% paraformaldehyde, brains were removed and stored for 5–10 days in 1% paraformaldehyde. Serial 50-μm-thick coronal or sagittal sections were subjected to immunohistochemical analysis with the avidin–biotin peroxidase technique (ABC Vectastain, Vector Laboratories) (14). The primary cultures of rat brain cells were characterized by

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Abbreviations: NDF, Neu differentiation factor; GFAP, glial fibrillary acidic protein; GAP-43, ≈43-kDa growth-associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride.

indirect immunofluorescent labeling with mouse monoclonal antibodies to GFAP, GAP-43, and myelin basic protein.

***In Situ* Hybridization.** NDF-specific 5′ (5′-TGAAGAGCCAGGAGTCAGCTGCAGG-3′) and 3′ (5′-GGCTCGAGACTCTGAGGACACATAGG-3′) oligodeoxynucleotide primers were used to amplify a 0.3-kb fragment of the rat NDF coding region from cDNA clone 44 (4). The amplification procedure was exactly as described (9), and the resulting DNA was cloned into the pBluescript plasmid (Stratagene). T3 and T7 RNA polymerases were used to generate [α-[^{35}S]thio]UTP-labeled sense and antisense transcripts that were used as probes. Embedding, sectioning, postfixation, and hybridization were as described (15).

Cell Culture. Primary cell cultures of embryonic day 17 rat brains were prepared by trypsinization (16). The dissociated cells were plated onto polylysine-coated 12-mm-diameter glass coverslips in 24- or 96-well plates, at a density of 10^5 cells per well for differentiation and thymidine incorporation assays or at 5×10^4 cells per well for cell survival assays. The cultures were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, penicillin (50 units/ml) and streptomycin sulfate (50 μg/ml). Astrocyte- and oligodendrocyte-enriched cultures were obtained from newborn rats (16). Neuron-enriched cultures were prepared from embryonic day 17 rat brains (17).

Thymidine Incorporation Assay. Incorporation of [*methyl*-^{3}H]thymidine into DNA in mixed neuron/glia cultures was measured as described (18). After 1 day in culture the medium was replaced with serum-free medium that was supplemented with 0.01% transferrin, 0.01% bovine serum albumin, 100 μM putrescine, 0.45 μM L-thyroxine, and 0.22 μM sodium selenite. After 24 hr of cell culture, growth factors were added and incubated with the cells for 48 hr. [^{3}H]Thymidine was added prior to the last 4 hr of incubation. The monolayers were then washed twice with cold phosphate-buffered saline and macromolecules were precipitated with cold 10% (wt/vol) trichloroacetic acid. Acid-soluble material was removed twice with ethanol, and the insoluble material was then solubilized in 0.3 ml of 0.5 M NaOH. Radioactivity was measured by β-scintillation counting.

Cell Survival Assays. Cell survival was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which determines mitochondrial activity in living cells (19), or by staining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), which labels nuclei. MTT was incubated with the cells for 2 hr at 37°C. Living cells can transform the tetrazolium ring into dark blue formazan crystals that can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic 2-propanol. Staining of nuclei with the fluorescent dye DAPI was used to estimate the number of living cells by first fixing them in 2% formaldehyde, washing three times with phosphate-buffered saline, and then staining with DAPI solution (10 μg/ml) for 10 min. After washing with phosphate-buffered saline and mounting on glass slides, the cells were either photographed or counted.

Reverse Transcription–PCR Analysis. Total RNA was purified from enriched primary cultures with an RNAzol kit (Cinna/Biotecx Laboratories, Friendswood, TX). cDNA synthesis and PCR amplification were performed with sense (5′-TGAAGAGCCAGGAGTCAGCTGCAG-3′; nt 461–484 of rat NDF) and antisense (5′-GGCTCGAGGAAGCTGTTATCACGA-3′; nt 1529–1548) primers. The resulting 1087-bp-long PCR product was tested by hybridization to an internal oligonucleotide probe (5′-GGCTCGAGACTCTGAGGACACATAGG-3′; nt 772–793). The primers for rat β-actin were as described (20), and the resulting 310-bp-long PCR product was checked for specificity by Southern blotting as described (20). The first strand of cDNA was synthesized at 37°C in a reaction mixture that contained reverse transcriptase (5 units, Promega), the sense primer (10 pmol), and RNA (2.5 μg). PCR was then performed with both primers (10 pmol of each) for 25 cycles of: 1 min (denaturation) at 94°C, 1 min (annealing) at 55°C, and 1.5 min (elongation) at 72°C. Products were resolved by electrophoresis through 1% agarose gel and capillary transferred with 20× standard saline citrate (SSC) to a GeneScreen membrane (New England Nuclear). The probe was end-labeled and hybridized with the blot at 42°C in the presence of 20% formamide. The blots were washed at 42°C in 0.2× SSC/0.2% SDS. Washed membranes were exposed for 7 days (NDF probe) or for 2 hr (actin probe) at −70°C to Agfa Curix RP2 x-ray films with intensifying screens.

RESULTS

NDF Expression in Embryonic Rat Brain. Due to the cellular and regional heterogeneity of the brain, detailed expression patterns of NDF may provide clues as to the physiological role of this factor in the central nervous system. To this end we utilized immunohistochemical methods and a highly specific rabbit antibody to rat NDF that recognizes all isoforms of the factor (11). Use of this antibody in immunohistochemical staining of thin sections from brains of 17-day rat embryos resulted in intense signals in many brain regions. For a control, we preincubated the antibody with NDF (500 ng/ml) and observed no specific staining of brain slices (data not shown). A characteristic coronal section is shown in Fig. 1*A*. Intense immunostaining was observed in the cortical plate, piriform cortex, septum, putamen, and basal telencephalon. Moderate immunoreactivity was displayed by the neuroepithelium that lines the lateral ventricles and by the thalamus, the hippocampal-formation neuroepithelium, the anterior hypothalamic nucleus, and the cerebellum. Higher magnification suggested that the immunoreactivity in the cortex was confined to specific cellular layers in the cortical plate (Fig. 1 *A* and *B*).

NDF Expression in Adult Rat Brain. In serial sections of the brain of a 3-month-old rat, as in embryonic brain, NDF was found in multiple regions of adult brain (Fig. 2). These included various sites in the cerebral cortex (e.g., frontal, forelimb, hindlimb, parietal, piriform, olfactory tubercle, entorhinal, and occipital cortex) and other regions (e.g., medial septal nucleus, preoptic nucleus, mamillary body, medial habenular nucleus of the thalamus, amygdala, and subiculum). In addition, we observed staining in motor cortex, ventral pallidum, nucleus of the diagonal band, brainstem (vestibulocochlear nerve, red nucleus, reticulotegmental nucleus of the pons, and pons), and cerebellum. The extent of immunostaining in adult brain was in general lower than that observed in embryonic brain, except for the cerebellum, which displayed intense labeling in the granular layer, as well as in some Purkinje cells (Fig. 2*G*). In addition to these neuronal cells, we identified pyramidal cells of the hindlimb cortex (Fig. 2*D*) and neurons of the subiculum (Fig. 2*F*) as NDF-producing cells. However, NDF immunoreactivity was not limited to neurons, as morphologically identifiable glial cells displayed strong immunoreactivity. For example, cell bodies and their extensions were stained in the corpus callosum (Fig. 2*E*). Since this region contains no neuronal cell bodies but contains axons that interconnect cerebral cortices of the two hemispheres, the stained cells must be glial. The glial nature of the NDF-stained cells in the corpus callosum was confirmed by staining serial sections with antibodies to GFAP (data not shown).

The spatial distribution of NDF immunoreactivity in adult brain was compared with the distribution of the corresponding mRNA as shown by *in situ* hybridization. As a probe we used an antisense strand derived from a region of the NDF transcript that is common to all isoforms. The observed

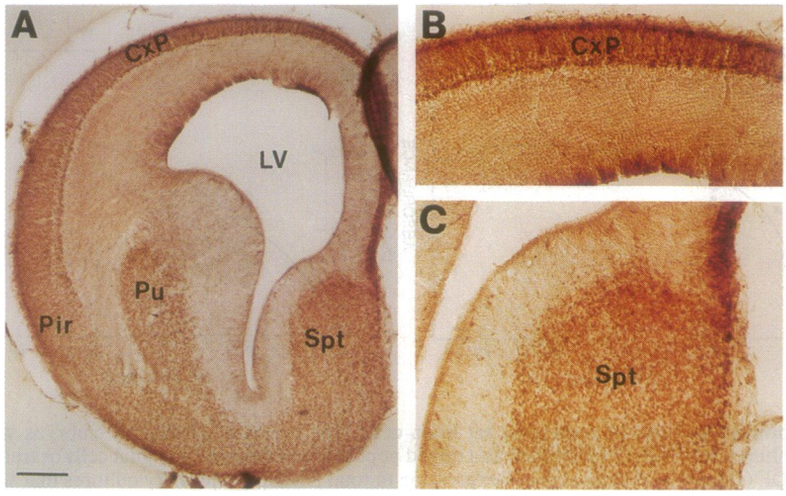

FIG. 1. Immunohistochemical localization of NDF in the brain of a day-17 rat embryo. An affinity-purified rabbit antibody to recombinant rat NDF was used to stain a paraformaldehyde-fixed thin section of an embryonic day 17 brain. (*A*) Whole coronal section. (Bar = 240 μm.) (*B* and *C*) Higher magnification of the cortical plate (*B*) and the septum (*C*). (Bar = 100 μm.) CxP, cortical plate; LV, lateral ventricle; Pu, putamen; Pir, piriform cortex; Spt, septum.

distribution of NDF transcripts was very similar to the distribution pattern that was determined immunohistochemically. An example of this is shown in Fig. 2 *H* and *I*, which depict RNA and protein localization, respectively, in the olfactory bulb.

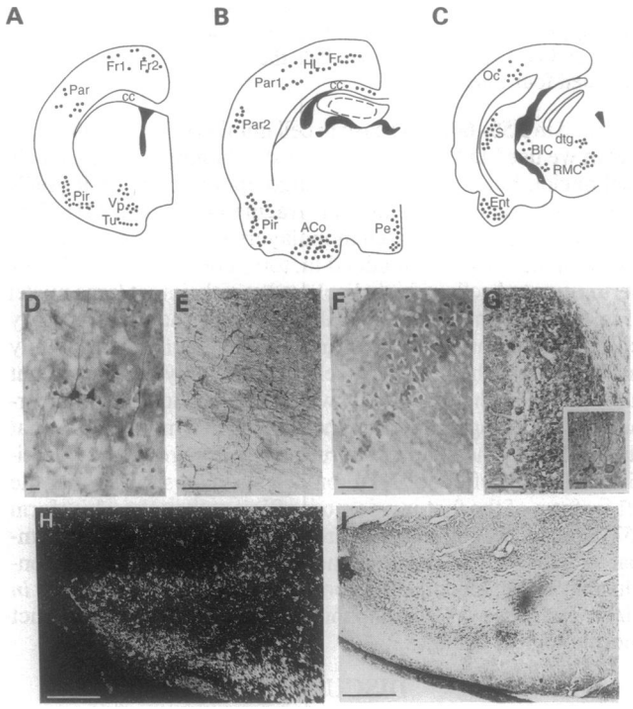

FIG. 2. Immunohistochemical and *in situ* hybridization analysis of NDF expression in adult rat brain. (*A–C*) Schematic representation of sites that display immunoreactivity (shown by dots) in three coronal sections of the brain of a 3-month-old rat. ACo, anterior cortical amygdaloid nucleus; BIC, brachium inferior collicular nucleus; cc, corpus callosum; dtg, dorsal tegmental bundle; Ent, entorhinal cortex; Fr1 and Fr2, areas 1 and 2 of the frontal cortex; HL, hindlimb cortex; Oc, occipital cortex; Par, parietal cortex; Pe, periventricular hypothalamic nucleus; Pir, piriform cortex; RMC, red magnocellular nucleus; S, subiculum; Tu, olfactory tubercle; Vp, ventral pallidum. (*D–G*) Higher-magnification immunohistochemical figures of the following NDF-positive cells: pyramidal neurons of the hindlimb cortex (*D*), glial cells of the corpus callosum (*E*), neurons of the subiculum (*F*), and granular and Purkinje neurons of the cerebellum (*G*). (*H* and *I*) NDF distribution in sagittal sections of the olfactory bulb. Shown are a darkfield figure of hybridization with an antisense probe of NDF (*H*) and a brightfield figure of immunoreactivity of a consecutive serial section of the same region (*I*). Control hybridization of the sense probe was performed and showed no specific labeling (data not shown). (Bars = 200 μm.)

NDF Expression in Primary Cultures of Embryonic Brain Cells. Since the immunohistochemical analysis of NDF expression suggested that the factor was synthesized by both neuronal and glial cells at late gestation, we attempted to confirm this by *in vitro* studies. We used primary cultures of rat brain cells and enriched them for specific cell types–neurons, astrocytes, or oligodendrocytes—by established protocols (16, 17). RNA was extracted from neuron-enriched cultures at the day of their establishment, whereas the astrocyte- and oligodendrocyte-enriched cultures were extracted 2 weeks later, when they reached >95% purity. Because Northern blot analysis detected only faint signals, we used the more sensitive method of reverse transcription–PCR analysis. The cDNA of NDF was prepared, and a portion of it (nt 461–1548) was amplified by using specific primers. The resulting DNA products were separated by agarose gel electrophoresis and Southern blot transfer to a nylon filter. Probing of the filter with an NDF-specific oligonucleotide detected the expected 1.1-kb fragment in both neuron- and astrocyte-enriched primary cultures, but no signal was obtained with oligodendrocytes (Fig. 3*A*). This was not due to degradation of the corresponding RNA, as a positive signal was observed with the same RNA sample and an actin probe (Fig. 3*A*).

NDF Is Weakly Mitogenic for Primary Brain Cultures but Strongly Accelerates Astrocyte Maturation. NDF was reported to act *in vitro* either as a mitogen or as a differentiation factor for certain mammary tumor cells (5, 7), and a variant of the factor exerted a strong proliferative effect on Schwann cells (12). To determine the effect of NDF on cultured brain cells of embryonic origin, we employed a thymidine incorporation assay to monitor DNA synthesis and an MTT assay to monitor cell survival. After serum deprivation of the primary cultures for 24 hr, incubation with increasing concentrations of NDF for 48 hr resulted in a relatively small effect on DNA synthesis, only a 1.6-fold increase (Fig. 3*B*). By contrast, under the same conditions 10% fetal bovine serum induced a 13.2-fold increase in DNA synthesis, indicating that the small effect of NDF was not due to a limited proliferative potential of the cell culture. This conclusion was supported by the results of the MTT assays. No significant effect of NDF at ≤20 ng/ml was observed in this assay after a 3-day incubation of serum-starved cultures with the factor. However, longer incubations (9 days) with NDF resulted in a maximal effect, 3-fold (Fig. 3*C*), but stimulation with serum yielded a 12-fold increase in mitochondrial activity. Unlike staining for a neuron-specific marker (GAP-43) and an oli-

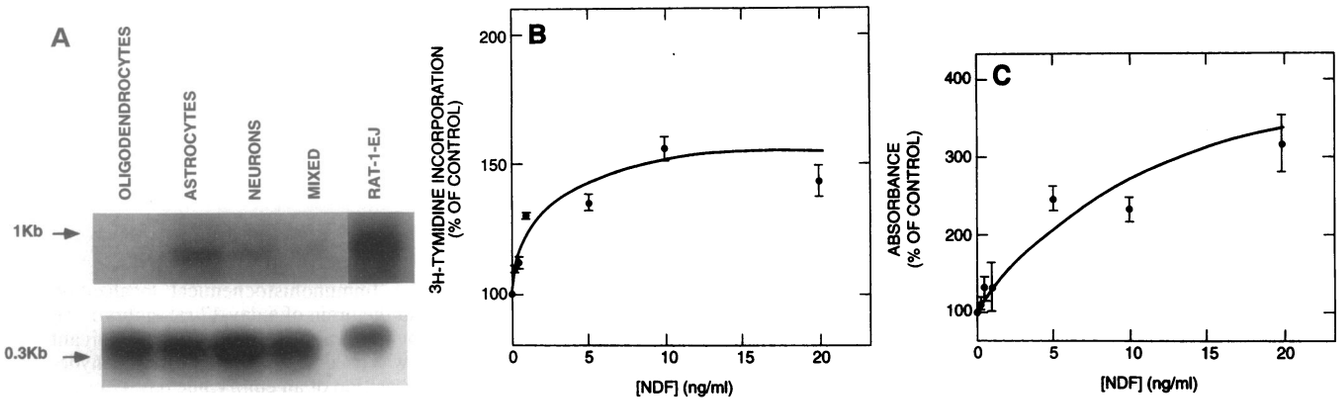

FIG. 3. NDF expression and biological effects in primary cultures of embryonic rat brain cells. The brains of 17-day rat embryos were dissociated into single cells and cultured *in vitro*. The cultures were either maintained as a mixed population of neurons and glial cells or treated to enrich for a specific cell type. (*A*) PCR analysis of NDF (*Upper*) or β-actin (*Lower*) expression in total RNA from primary cultures that were enriched for the indicated cell types. For a positive control we used RNA from *ras*-transformed Rat-1 fibroblasts (Rat-1-EJ). DNA amplification was performed with pairs of specific oligonucleotide primers after synthesis of cDNA. The resulting DNA was fractionated in an agarose gel that was used for Southern blot analysis with NDF- or β-actin-specific radioactive oligonucleotide probes. (*B*) Effect of NDF on DNA synthesis. Mixed neuron/glia cultures were grown as monolayers in 24-well dishes and incubated for 24 hr in serum-free medium. The indicated concentrations of NDF were then added and incubation was continued at 37°C for additional 48 hr. The last 4 hr of incubation were in the presence of [*methyl*-^{3}H]thymidine, and the incorporation of radioactivity into acid-precipitable macromolecules was determined. Each data point is the mean ± SD of a triplicate determination. The experiment was repeated three times. Parallel stimulation with fetal bovine serum resulted in a 13-fold increase in DNA synthesis. (*C*) Effect of NDF on cell viability. Primary cultures were treated with NDF as in *B*, and the relative numbers of metabolically active cells were determined by MTT assay. Light absorbance at 540–630 nm, which parallels the extent of cell proliferation and viability, is shown as mean ± SD for six wells. The experiment was repeated four times.

godendrocyte-specific molecule (myelin basic protein), antibodies to GFAP indicated that native NDF elevated the expression of this glial marker 4- to 6-fold after a 3-day incubation (data not shown). Astrocyte maturation was promoted also by four recombinant NDFs (β1, β3, α1, and α2) and by serum (7- to 8-fold increase in GFAP).

NDF Protects Astrocytes from Cell Death *in Vitro*. The limited effect of NDF on DNA synthesis and its strong maturation-promoting effect on embryonic astroglia suggested that the factor may prolong survival of brain cells *in vitro*. The MTT assay was used to measure cell viability over time, including the start point before any treatment was given. Long-term incubation of primary cultures of brain cells under serum-free conditions resulted in a gradual decrease in cell number and gave ≈40% cell death by day 9. By contrast, maintaining the culture for the same period of time in the presence of NDF resulted in a 50–60% increase in cell numbers. To visualize cell death we used DAPI staining. This dye homogeneously labels cell nuclei, but in cells that undergo apoptotic death it yields figures of condensed and then fragmented nuclei (21). The latter pattern characterized monolayers that were cultured for 9 days in the absence of added factors, unlike native NDF- or serum-treated cells, which displayed mostly intact large nuclei (Fig. 4). Immunohistochemical analysis of the same primary cultures with anti-GFAP and anti-GAP-43 antibodies not only indicated that the surviving cells were mostly astrocytes but also revealed that treatment with NDF induced the appearance of an elongated and branched cellular morphology that differed from the shape of serum-treated astrocytes. Quantification of the effect of NDF on cell survival showed that the β1 isoform of the factor was more efficient than the α1 isoform (57% and 32% cell survival, respectively, at 2 ng/ml, compared with 94% survival in the presence of serum). In conclusion, NDF specifically promoted survival of astrocytes *in vitro*, and this was accompanied by maturation and distinct morphological changes.

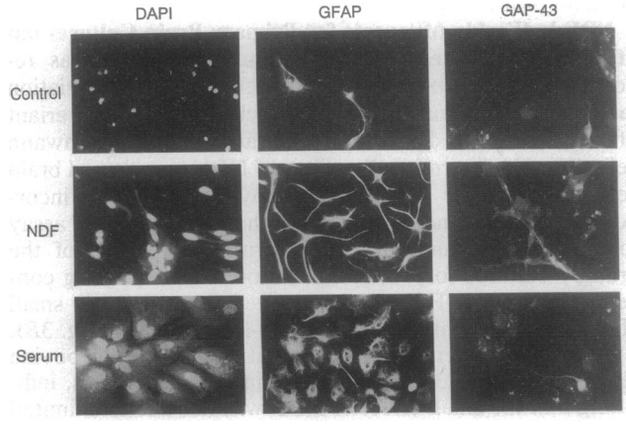

FIG. 4. Effect of NDF on astrocyte survival. Primary cultures were established and starved for serum factors as described in the legend to Fig. 3*B*. Separate cultures were then treated for 9 days with NDF (10 ng/ml) or fetal bovine serum (10%) or were left untreated (Control). Incubation was terminated by cell fixation and staining with either the DNA-intercalating dye DAPI, antibodies to GFAP, or antibodies to GAP-43. The immunostained monolayers were further reacted with fluorescently labeled secondary antibodies. (×75.)

DISCUSSION

The multiple transcripts of NDF can be divided into two groups—namely, mesenchymal isoforms and neural variants (22). Although the former group displays greater structural heterogeneity, transcripts that are specific to the nervous system are more abundant, implying that the latter system is a major site of NDF action. The present study concentrated on neural NDFs with a dual aim: (*i*) to characterize the patterns of distribution of all NDF isoforms by using immunological and molecular reagents that do not discriminate between the various isoforms and (*ii*) to study the effects of NDF *in vitro* on brain-derived primary cultures, which are best suited to the analysis of glial cells. Our major findings can be summarized as follows. Similar to the multiple-site distribution of the neurotrophic factors NGF and BDNF (23), widespread expression of NDF was observed, and it displayed regional and temporal specificity. *In vitro*, NDF was found to be a poor mitogen for brain cells. However, it exerted a remarkable survival effect that appeared to be specific for astrocytes.

In agreement with previous mapping of the distribution of NDF transcripts in a 14.5-day mouse embryo (9), the protein product was found to be present in 17-day rat embryo in components of the limbic system, such as septum and hypothalamus, as well as in some regions that are presumably involved in the control of motor activity, such as putamen, globus pallidus, and basal nucleus of Meynert. Although these regions continued to display NDF immunoreactivity also through adulthood, changes in the pattern were observed. In general the immunoreactivity decreased or vanished (e.g., putamen), but some regions displayed higher levels in adult (e.g., cerebellum and to some extent hypothalamus). *In situ* hybridization that localized all the various NDF transcripts matched the immunological mapping, except for the hippocampus, where relatively high mRNA levels were observed (mainly in CA1, CA2, and CA3) but little immunoreactivity was detectable (data not shown). The overall correlation between the locations of NDF transcripts and their protein product suggests that NDF may not be transported to remote sites after synthesis and processing of its transmembrane precursor.

It is relevant to address the possibility that NDF expression correlates with specific brain functions or with regions that use certain neurotransmitters. NDF expression was mapped to many regions that belong to the cholinergic system, such as medial septal nucleus, medial habenular nucleus, and the paraventricular and supraoptic nuclei. This observation is in accordance with the action of ARIA, which is one of the nerve-specific isoforms of NDF, on cholinergic synapses in the peripheral nervous system (13). However, neurons that apparently use either glutamate (e.g., cerebellar granular cells and neurons in the entorhinal cortex) or γ-aminobutyric acid (e.g., cerebellar Purkinje cells and neurons in the medial septal nucleus) as neurotransmitters also contain the factor.

The status of NDF/heregulin as a mitogen or as a differentiation factor is an open issue. In the present study we addressed this question by using primary cultures of rat brain cells and several independent bioassays. It was found that native and recombinant NDFs exerted only a limited mitogenic effect that was at least 20-fold smaller than the maximal proliferative potential of the primary cultures (Fig. 3*B*). In contrast with the small increase in DNA synthesis, both α and β isoforms of the factor promoted a significant survival effect that predominantly affected astrocytes (Fig. 4). Quantitatively the survival effect was comparable to the response to whole serum. However, the surviving astrocytes displayed a multipolar mature morphology that differed from the cellular form of serum-treated cultures (Fig. 4). It is, therefore, conceivable that NDF can accelerate maturation of embryonic brain astrocytes and maintains this type of glial cell *in vivo*. This hypothesis is consistent with the finding that the expression of NDF precedes the major burst of glial growth and differentiation that occurs in late mid-gestation. In addition, the observation that NDF has little if any mitogenic activity on cultured brain cells may be related to early observations that were made with crude or partially purified preparations of glial growth factors from bovine pituitary or brain (24, 25). These preparations were not mitogenic to cultured Schwann cells unless added in the presence of serum factors. Like NDF, glial growth factors, which are most likely a mixture of neural isoforms of NDF, specifically affected astrocytes but not oligodendrocytes and microglia (24). The effects of ARIA on muscle cells—namely, induction of increased synthesis of acetylcholine receptors and sodium channels and acceleration of synapse formation (13)—may be considered as differentiation processes. Taken together, these observations indicate that NDF does not function as a mitogen on primary brain cultures, but as a survival and maintenance factor. Nevertheless, on the basis of the available data it may be inferred that the factor may act as a potent mitogen for certain cell types, but only in cooperation with still-unknown serum polypeptide factors.

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1. Aaronson, S. A. (1991) *Science* **254**, 1146–1153.
2. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61**, 203–212.
3. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben-Levy, R. & Yarden, Y. (1992) *Cell* **69**, 205–216.
4. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Ben-Levy, R., Luo, Y. & Yarden, Y. (1992) *Cell* **69**, 559–572.
5. Peles, E., Ben-Levy, R., Tzahar, E., Naili, L., Wen, D. & Yarden, Y. (1993) *EMBO J.* **12**, 961–971.
6. Plowman, D. G., Culouscou, J.-M., Withney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G. & Shoyab, M. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1746–1750.
7. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, M., Wood, W. I., Goeddel, D. V. & Vandlen, R. L. (1992) *Science* **256**, 1205–1210.
8. Bacus, S. S., Gudkov, A. V., Zelnick, C. R., Chin, D., Stern, R., Stancovski, I., Peles, E., Ben-Baruch, N., Farbstein, H., Lupu, R., Wen, D., Sela, M. & Yarden, Y. (1993) *Cancer Res.* **53**, 5251–5261.
9. Orr-Urtreger, A., Trakhtenbrot, L., Ben-Levy, R., Wen, D., Rechavi, G., Lonai, P. & Yarden, Y. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1867–1871.
10. Peles, E. & Yarden, Y. (1993) *BioEssays* **15**, 815–824.
11. Wen, D., Suggs, S. V., Karunagaran, D., Liu, N., Cupples, R. L., Luo, Y., Jansen, A. M., Ben-Baruch, N., Trollinger, D. B., Jacobson, V. L., Meng, T., Lu, H. S., Hu, S., Chang, D., Yanigahara, D., Koski, R. A. & Yarden, Y. (1994) *Mol. Cell. Biol.* **14**, 1909–1919.
12. Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., *et al.* (1993) *Nature (London)* **362**, 312–318.
13. Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S. & Fischbach, G. D. (1993) *Cell* **72**, 801–815.
14. Eilam, R., Malach, R., Bergmann, F. & Segal, M. (1991) *J. Neurosci.* **11**, 401–411.
15. Hogan, B., Constantini, F. & Lacy, E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
16. McCarthy, K. D. & de Vellis, J. (1980) *J. Cell Biol.* **85**, 890–902.
17. Barde, Y. A., Edgar, D. & Thoenen, H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1199–1203.
18. Pandiella, A., Beguinot, L., Velu, T. J. & Meldolesi, J. (1988) *Biochem. J.* **254**, 223–228.
19. Mosman, T. (1983) *J. Immunol. Methods* **65**, 55–63.
20. Jin, J. J., Nikitin, A. Y. & Rajewsky, M. F. (1993) *Cell Growth Differ.* **4**, 227–237.
21. Lazebnik, Y. A., Cole, S., Cooke, C. A., Nelson, W. G. & Earnshaw, W. C. (1993) *J. Cell Biol.* **123**, 7–22.
22. Ben-Baruch, N. & Yarden, Y. (1994) *Proc. Soc. Exp. Biol. Med.*, in press.
23. Ernfors, P., Wetmore, C., Olson, L. & Parson, H. (1990) *Neuron* **5**, 511–526.
24. Brockes, J. P., Lemke, G. E. & Balzer, D. R. J. (1980) *J. Biol. Chem.* **255**, 8374–8377.
25. Raff, M., Abney, E., Brockes, J. P. & Hornby-Smith, A. (1978) *Cell* **13**, 813–822.