EFFECTS OF BASIC FIBROBLAST GROWTH FACTOR (bFGF) ON THE DEVELOPMENT OF MIDBRAIN DOPAMINE NEURONS *IN VITRO*

by

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ABSTRACT

EFFECTS OF BASIC FIBROBLAST GROWTH FACTOR (bFGF) ON THE DEVELOPMENT OF MIDBRAIN DOPAMINE NEURONS *IN VITRO*

by

Margaret M. Bouvier

Advisor: Dr. Catherine Mytilineou

Parkinson's disease is characterized by a loss of dopamine neurons in the substantia nigra leading to decreased dopamine in its target structure, the striatum. Drug therapy is commonly used to treat the symptoms of this disease, but it is complicated by side effects and limited efficacy. More recently, transplantation of substantia nigra primordia to the striatum has yielded encouraging preliminary results. This therapy would be improved by developing ways to expand *in vitro* the number of dopamine precursors, in order to generate large enough quantities of appropriate-aged, dopamine enriched tissue which would be available as needed for surgery.

The goal of this research was to explore the possibility of various factors to manipulate the proliferation and differentiation of dopamine precursors. Cultures were prepared from embryonic day 12 (E12) rat ventral mesencephalon, coinciding with the beginning of the birth of dopamine neurons of the substantia nigra. At low plating density in serum-free medium, dopamine precursors divide for approximately one day *in vitro*.

We report here that basic fibroblast growth factor (bFGF) expands the period of dopamine precursor division at least until day 8 in culture with the majority dividing on days 4-5, well beyond their normal division. This increase in cell division was accompanied by a delay in differentiation as compared to untreated control cultures. Upon differentiation, the dopamine neurons in the bFGF-treated cultures yielded high-affinity dopamine uptake values that were twenty times maximal control values.

Culturing E12 cells on a non-adhesive substrate generated a suspension of spheres, which is more suitable for transplantation. bFGF stimulated cell proliferation in these spheres; control cultures demonstrated limited division and survival. TH+ cells were not detected in spheres cultured for 5 days *in vitro* (DIV), but were found in spheres at DIV 7, 9, and 12. When DIV 12 spheres were attached to an adhesive substrate and further cultured, dopamine neurons migrated out of large spheres, forming extensive fiber networks. These large dopamine-containing spheres constituted approximately 25% of the spheres in a typical culture dish, and exhibited uptake values approximately 6 times higher than controls.

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*In memorium

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BACKGROUND

Development of Dopamine Neurons of Substantia Nigra

The first stage of embryonic development in mammals involves the division of the fertilized egg into a hollow sphere of cells called the blastula. The next step is gastrulation, in which a portion of the sphere begins to invaginate, ultimately resulting in the appearance of three germ layers surrounding a primitive gut. The inner layer is called the endoderm, which gives rise to the gut and associated organs. The middle layer, or mesoderm, gives rise to muscle, skeleton, connective tissue, and cardiovascular and urogenital systems. The outer layer, or ectoderm, forms the skin and nervous system. Neurulation proceeds as an anterior-posterior groove in the ectoderm. A thickening of the ectoderm on the dorsal side forms the neural plate, the margins of which fold in toward the midline to form the neural tube. At the rostral end of the tube, there are three vesicles called the prosencephalon, mesencephalon, and the rhombencephalon which give rise to the cerebral hemispheres, the midbrain, and to the lower brain stem and cerebellum respectively. These swellings occur because of rapid, disproportionate proliferation of cells in the neural tube.

The time of origin of the cells that give rise to the midbrain in the rat has been studied using ^{3}H-thymidine injections. Hanaway *et al.* (1970) found that the neurons that give rise to the substantia nigra are produced in the rostral portion of the ventral mesencephalon from embryonic day 11 (E11) to E15, with highest production at E14 and E15. Lauder and Bloom (1974) obtained the same results, but with highest production at E13. Altman and Bayer (1981) began by looking at E13, and found that substantia nigra neurons are produced at a fairly even rate from E13 to E15. This same study shows that the substantia nigra neurons originate in a medial portion of the neuroepithelium, posteroventral to the final settling site, and that the cells are distributed in an outside-in

pattern. No differences have yet been found between the neurogenesis of the pars compacta and that of the pars reticulata despite their functional and anatomical differences. However, the two components of the substantia nigra may be formed by two different cell lines that divide and migrate at the same time.

Immunocytochemical studies at the light microscope level have been done to examine the localization of the synthetic enzyme tyrosine hydroxylase (TH) in the rat brain at various stages of embryonic development (Specht *et al.*, 1981). This enzyme is a specific marker for catecholamine neurons in the developing and adult rat brain (Pickel *et al.*, 1981, 1975; Hokfelt *et al.*, 1976, 1977). It can also be detected biochemically in rat as early as E15 (Coyle and Axelrod, 1972). This cell specificity and early biochemical detection indicate that TH is an appropriate marker for a phenotypically distinct population of neurons during early differentiation.

TH is first seen at E12.5 where staining appears in the perikarya of four cell groups: one group in the prosencephalon, one group in the mesencephalon, and a rostral and a caudal group in the rhombencephalon. The mesencephalic cell group is the largest and most intensely stained. The cells appear as two anterior-posterior lines on each side of the midline, located rostral to the mesencephalic flexure. There are also short, positively stained fibers extending anterolaterally from these cells.

At E13.5 the double row of TH^+ cells in the mesencephalon have extended caudally, almost to the metencephalon. The cells are confluent across the midline at the level where the interpeduncular nucleus will form. The fibers first seen at E12.5 are now more numerous and extend into the diencephalon. At E14.5, the distribution of stained cells is more extensive. Neurons in the midline extend rostrolaterally, forming the neurons of the substantia nigra. There are a greater number of axons projecting from the ventrolateral cells which correspond to the nigrostriatal pathway and can be followed into the ventral lateral area of caudate-putamen (Anden *et al.*, 1964; Lindvall and Björklund, 1974; Moore *et al.*, 1971; Ungerstedt, 1971; Fallon and Moore, 1978).

By E18, all the TH^+ neurons have migrated away from the aqueduct of Sylvius and form two bands in the ventral mesencephalon. In the medial portion of these bands, the neurons are more densely packed and will give rise to the pars compacta. The nigrostriatal pathway now contains more fibers and can be followed for a greater distance. Together with other pathways, the nigrostriatal pathway projects rostrally through the lateral hypothalamus forming part of the medial forebrain bundle. From there the fibers of the nigrostriatal pathway enter the internal capsule and terminate in the ventral-lateral area of caudate-putamen, creating a fairly uniform innervation. At E21, the nigrostriatal innervation of the caudate putamen is more extensive, but is still only in the ventral-lateral region. This innervation progresses in a medial and dorsal direction.

Extrinsic Control of Neurogenesis

During neurulation the inner surface of the neural tube, called the ventricular zone, is the site of the division of neuronal precursors (Rakic, 1974). In this epithelium the single layer neuronal and glial precursors extend from the inner to the outer surface. Neuronal precursors divide in the ventricular zone then migrate along radial glia through the intermediate zone to the marginal zone, where they become post-mitotic, differentiated neurons. The peak time of neurogenesis does not coincide with the peak time of gliogenesis, which is mainly a postnatal process (Jacobson, 1978).

It is clear that, unlike the process of selective neuronal death, neurogenesis is not controlled by neuronal interaction with a target (Levi-Montalcini, 1949; Landmesser and Pilar, 1974; Kelly and Cowan, 1977; Oppenheim, 1981; Tanaka and Landmesser, 1986). Although it has been shown with chimeric mice that the number of cells in the central nervous system (CNS) can be controlled intrinsically in a cell autonomous fashion (Williams and Herrup, 1988), there are many examples of extrinsic control of neuronal proliferation. One example of a hormone that provides extrinsic control of neurogenesis

is thyroid hormone. The metamorphic changes that occur in amphibians are under the control of thyroid hormone (Allen, 1918, 1938). When the thyroid is removed, metamorphosis slows. Furthermore, when thyroxin is given, the larval stage ends prematurely. When thyroxin is given to tadpoles, there is an increase in cell division in the brain and spinal cord (Baffoni, 1957, 1959; Goldberg and Pollack, 1989). When thyroid hormone is administered to embryonic rat, brain development is accelerated as demonstrated by increased proliferation and shortening of the period of neurogenesis (Balazs *et al.*, 1971; Nicholson and Altman, 1972).

Insulin is also able to provide extrinsic control of neurogenesis. Insulin and insulin-like growth factor I (IGF-I) have been shown to stimulate neurogenesis in CNS cultures (Lenoir and Honegger, 1983; Aizenman and DeVellis, 1987; Raizada *et al.*, 1980) and cultures of embryonic rat sympathetic neuroblasts (DiCicco-Bloom and Black, 1988). Cultures of chick embryo sympathetic neurons demonstrate that neuronal proliferation depends on the presence of serum, but that insulin or IGF-I can replace the serum (Ernsberger *et al.*, 1989). IGF-I mRNA is expressed in the nervous system of E14 rat brain, a time which coincides with the production of neurons. The mRNA is also expressed *in vitro* in neurons and glia cultured from E17 rat brain (Rotwein *et al.*, 1988; Werner *et al.*, 1989). Insulin has been detected in brain cells by immunohistochemistry, radioimmunoassays, and *in situ* hybridization (Baskin *et al.*, 1987; Young, 1986). Radioreceptor assays of neural membranes and fetal brain cultures have shown that receptors for insulin and IGF-I are present in nervous tissue during the period of neurogenesis (Raizada *et al.*, 1980; Bassas *et al.*, 1985; DePablo *et al.*, 1985; Van Schravendijk *et al.*, 1986). The early expression of insulin and IGF-I and their receptors, coupled with the effects seen *in vitro*, suggest that they play a role in neural development.

Fibroblast growth factor (FGF) stimulates proliferation of neuronal precursors under serum-free conditions in cultures of E13 rat cerebral cortex (Gensburger *et al.*, 1987). Similar results were obtained with neuroepithelium cells from E10 mouse

(Murphy *et al.*, 1990). FGF receptors are present on CNS neurons (Walicke *et al.*, 1989). FGF and its receptor are expressed in the chick brain during the period of neurogenesis: FGF is seen *in vivo* in the nervous system of E3 chick embryos (Risau, 1986; Kalcheim and Neufeld, 1990); receptor levels in chick embryo are high from E2 to E6, and then decrease (Olwin and Hauschka, 1990); and the receptor in chick embryo is expressed preferentially in brain tissue at E17 to E19 (Olwin and Hauschka, 1990). In addition, bFGF mRNA (Drago *et al.*, 1991) and bFGF receptor mRNA (Reid *et al.*, 1990) are expressed in the neuroepithelium of E10 mouse. In the rat brain, bFGF mRNA is detected by *in situ* hybridization already at E16, which was the earliest time point studied. The same report showed that embryonic brain contained 1.5 to 2 times more bFGF mRNA than adult brain (Ernfors *et al.*, 1990). The *in vitro* effects and evidence for its appearance at the site and time of neurogenesis indicate that FGF is a mitogen in the developing CNS.

Epidermal growth factor (EGF) induces cell division of cultured sympathetic neuroblasts (DiCicco-Bloom *et al.*, 1990). EGF enhances proliferation of E18 or postnatal day 2 (P2) rat retinal neuroepithelial cells (Anchan *et al.*, 1991). Similar results were obtained with striatal embryonic progenitor cells (Reynolds *et al.*, 1992). In cultures of rat ventral mesencephalon at E16, EGF increases the survival of dopamine neurons (Casper *et al.*, 1991) and EGF stimulates the proliferation of neuronal precursors (Mytilineou *et al.*, 1992). EGF induces the proliferation of a multipotent progenitor cell from E14 mouse striatum (Reynolds *et al.*, 1992). This same study demonstrates EGF, transforming growth factor-α (TGF-α), and EGF receptor mRNAs in E14 striatum. These findings are in agreement with an earlier study, which demonstrates EGF and TGF-α mRNAs in E14 mouse (the earliest time point examined), with expression continuing into adulthood (Lazar and Blum, 1992). These data suggest that EGF is a mitogen in the developing CNS.

Regulation of Phenotypic Expression

Environmental Cues The influence of environment on phenotypic expression has been studied using microcultures of single sympathetic neurons (Furshpan *et al.*, 1976; Reichardt and Patterson, 1977; Potter *et al.*, 1981). In these studies, the neurons are capable of switching from adrenergic to cholinergic transmission when exposed to media that is conditioned by heart cells. Renfranz *et al.* (1991) examined the influence of environment using an immortalized embryonic precursor cell line derived from rat hippocampus. When the cells are implanted into either newborn hippocampus or cerebellum, the cells integrate and adopt the morphological traits of the neurons and glia of their host tissue.

Determination Studies of visual cortical neurons in the ferret have demonstrated that the commitment to a certain phenotype occurs close to the last cell division, prior to the migration of that cell away from the proliferative zone (McConnell, 1988). This transplantation study shows that the environment in which the neuron goes through its final mitotic division determines the cortical layer to which it will migrate. Autoradiographic studies indicate that different types of neurons become post-mitotic at different times during embryonic development (Altman, 1963, 1972 a,b,c; Altman and Das, 1965; Angevine, 1965; Rakic, 1974; Schlessinger *et al.*, 1975, 1978; Bayer, 1980; Altman and Bayer, 1990). These studies indicate that the embryo responds to temporally and spatially controlled cues to produce successive waves of committed, post-mitotic neurons which then migrate to their appropriate brain site and differentiate.

Differentiation Catecholamine synthesizing enzymes are expressed in the CNS only after final cell division of the precursors. In a study of the development of TH in embryonic rat brain, Specht *et al.* (1981) found labelled cells only in the intermediate zone, a region which contains only post-mitotic neurons (Sauer and Walker, 1959). They found no labelled cells in the ventricular zone. These results confirm earlier findings

using ^{3}H-thymidine autoradiography and immunocytochemistry to show a lack of DNA synthesis in TH^{+} neurons, and a concomitant lack of TH in dividing neurons (Rothman *et al.*, 1980).

Manipulation of Neurogenesis and Phenotype

Neurogenesis It is possible *in vitro* to expand the number of divisions of a neuronal precursor (Banker and Cowan, 1977; Temple, 1989). EGF allows precursors to survive and proliferate in E16 rat mesencephalon (Mytilineou *et al.*, 1992). bFGF, as well as EGF, has been shown to increase dopamine uptake and stimulate cell proliferation in cultures of E15-17 rat mesencephalon (Knusel *et al.*, 1990). Neuronal precursors from E13.5-14.5 rat striatum primordia proliferate in response to bFGF, a response that is potentiated by co-treatment with nerve growth factor (NGF) (Cattaneo and McKay, 1990). In E10 mouse neuroepithelium, bFGF induced proliferation of precursors is completely inhibited by blocking endogenous IGFs, and the proliferative effect of bFGF is markedly enhanced by the addition of IGFs (Drago *et al.*, 1991). This study demonstrates that bFGF is acting as a proliferative agent in concert with IGF, which is acting primarily as a survival agent.

It has been shown that some cells from the adult mouse striatum are induced to proliferate when cultured with EGF on a non adhesive substrate (Reynolds and Weiss, 1992). These proliferating spheres are grown for 6-8 days *in vitro* (DIV), and express nestin, an intermediate filament found in neuroepithelial stem cells. When replated on adhesive substrate, the cells differentiate into neurons and glia. The neurons stain for γ-aminobutyric acid (GABA) and substance P, but do not stain for other transmitters of the adult striatum. Another recent study shows that bFGF in the presence of serum stimulates the proliferation of cells derived from adult mouse brain (Richards *et al.*, 1992). When

bFGF and serum are removed and the cells are exposed to medium conditioned by an astrocytic cell line, they differentiate into neurons and glia.

Phenotype NGF and EGF have both been shown to induce TH in a rat pheochromocytoma cell line (Goodman *et al.*, 1980). TH expression is induced in striatal neurons that are co-cultured with mesencephalic glia (Beyer *et al.*, 1991). Adler and Hatlee (1989) studied the phenotype of cells born at E5 in the chick embryo retina. If these cells are isolated for culture at E6, they develop primarily into photoreceptors, and if they are isolated at E8, they develop into neurons, indicating that the cells respond to inducing signals from the *in vivo* microenvironment. In addition, sympathetic neurons express a cholinergic phenotype when exposed to certain agents (Johnson *et al.*, 1976; Patterson and Chun, 1977; Iacovitti *et al.*, 1985; Kessler, 1985). Conversely, parasympathetic neurons express an adrenergic phenotype when exposed to certain cues *in vitro* (Iacovitti *et al.*, 1985; Teitelman *et al.*, 1985) and *in vivo* (Coulombe and Bronner-Fraser *et al.*, 1986; Landis *et al.*, 1987).

Muscle-derived differentiation factor (MDF) affects the expression of transmitter-specific genes in cultures of brain neurons (Iacovitti *et al.*, 1989). This study shows that treatment of embryonic rat cerebral cortex with MDF yields a ten-fold increase in the level of TH mRNA. A later study shows that MDF can even induce TH in neurons that do not normally express it (Iacovitti, 1991). In this study, cells were receptive to the effects of MDF only during a critical period of development, a period which seems to correspond to the time just following peak withdrawal of that type of neuron from the mitotic cycle. The cells continue to express TH as long as they are exposed to MDF. If the MDF is withdrawn, cells stop expressing TH, but are able to express it again at later times upon exposure to MDF, indicating that the cells retain a biochemical "memory" of their exposure to MDF during the critical period.

I. CHARACTERIZATION OF CELLS CULTURED ON ADHESIVE SUBSTRATE

A) INTRODUCTION

During embryonic development, cells that give rise to the central nervous system proliferate in the ventricular zone of the neural tube. In the rat embryo, cells destined to become the substantia nigra are produced in the rostral portion of the ventral mesencephalon from embryonic day 11 (E11) to E15 (Hanaway *et al.*, 1972; Lauder and Bloom, 1974). Mesencephalic dopamine neurons begin to differentiate at E12.5 (Specht *et al.*, 1981) as demonstrated by the appearance of tyrosine hydroxylase (TH), the rate limiting enzyme in the synthetic pathway of dopamine. Although the division and differentiation of neuroepithelial cells follows a fixed schedule *in vivo*, it has been shown that *in vitro* the period of division can be expanded (Temple, 1989) and the process of differentiation can be altered (Banker and Cowan, 1977).

Several growth factors have been shown to affect the survival and division of neuroepithelial cells *in vitro*. For example, epidermal growth factor (EGF) allows the survival and proliferatation of precursor cells from E16 rat mesencephalon (Mytilineou *et al.*, 1992; Casper *et al.*, 1991) and adult mouse striatum (Reynolds and Weiss, 1992). EGF, as well as basic fibroblast growth factor (bFGF), stimulates cell proliferation and increases dopamine uptake in cultures of E15-17 rat mesencephalon (Knusel *et al.*, 1990; Park and Mytilineou, 1992). bFGF has been shown to improve the growth and survival of dopamine neurons *in vitro* (Ferrari *et al.*, 1989; Engele and Bohn, 1991). A number of studies indicate that bFGF is mitogenic to neuronal precursors (Gensburger *et al.*, 1987; Murphy *et al.*, 1990; Ray *et al.*, 1993) and the mRNA for the bFGF receptor is found in the epithelium lining the ventricles of the rat embryonic central nervous system (Wanaka

et al., 1991), where mitotic precursor cells are found. In addition, other growth factors in conjunction with bFGF have been shown to stimulate proliferation of neuronal precursors *in vitro* in a number of systems. For example, neuronal precursors from E14 rat striatum proliferate in response to bFGF and nerve growth factor (NGF) (Cattaneo and McKay, 1990). bFGF and insulin-like growth factors (IGFs) induce proliferation of precursors in E10 mouse neuroepithelium (Drago *et al.*, 1991), and E10 mouse mesencephalon can be induced to proliferate with bFGF and serum (Kilpatrick and Bartlett,1993).

In the present study we investigated the regulation of division and differentiation of dopamine precursors in the ventral mesencephalon. In order to start with the maximum number of dopamine precursors, we used ventral mesencephalon from E12 rat, the developmental stage that corresponds to the beginning of the birth of the dopamine neurons of the substantia nigra. Cells were cultured in serum-free media in the presence of various growth factors in an attempt to stimulate the division of neuroepithelial cells. We report that bFGF extends the time over which dopamine precursors divide *in vitro*. The delay in differentiation and continuance of cell division results in a larger number of dopamine neurons, with bFGF-treated cultures achieving high-affinity dopamine uptake values approximately 20 times higher than values from untreated control cultures.

B) MATERIALS AND METHODS

1. Preparation of cell cultures

Pregnant Sprague-Dawley rats at 12 days gestation (embryonic day 12 = E12; Taconic Farms, Taconic, NY) were sacrificed, and the embryos (crown-rump length = 8mm) removed. The ventral mesencephalon was dissected out (see Fig. 1) without the membrane coverings and pooled on ice in Ca^{++} and Mg^{++} free phosphate-buffered saline (PBS). Tissue was suspended in a chemically defined medium, which was composed of a basal medium (Eagle's MEM and HAM F-12 nutrient mixture 1:1, Gibco, Grand Island, NY) containing glucose (33 mM), glutamine (2 mM), and HEPES buffer (15 mM), as well as the supplements of N2 described by Bottenstein and Sato (1979): transferrin, 100 μg/ml; insulin, 25 μg/ml; progesterone, 20 nM; putrescine, 60 μM; sodium selenite, 30 nM. The tissue was gently triturated through a fire-polished pipet, and the resulting cell suspension was plated at 18,000 cells per cm^2 in 35-mm plastic dishes (Falcon, Lincoln Park, NJ) pre-coated with polyornithine (100 μg/ml, Sigma, St. Louis, MO). The final volume in the dishes was 1.5 ml, and 1 ml was replaced with fresh media three times a week. The cultures were maintained in an incubator at 37°C, in an atmosphere of 95% air and 5% CO_2, 100% relative humidity. Growth factors were added at the time of plating and at each feeding thereafter. bFGF (human recombinant; Synergen, Boulder, CO), EGF (mouse submaxillary gland; Collaborative Research, Lexington, MA), and IGF-I (human recombinant; Gibco, Grand Island, NY) were used at a concencentration of 20 ng/ml. Glial cell line-derived neurotrophic factor (human recombinant GDNF; Synergen, Boulder, CO) was used at a concentration of 1 ng/ml.

2. ^{3}H-Thymidine Incorporation

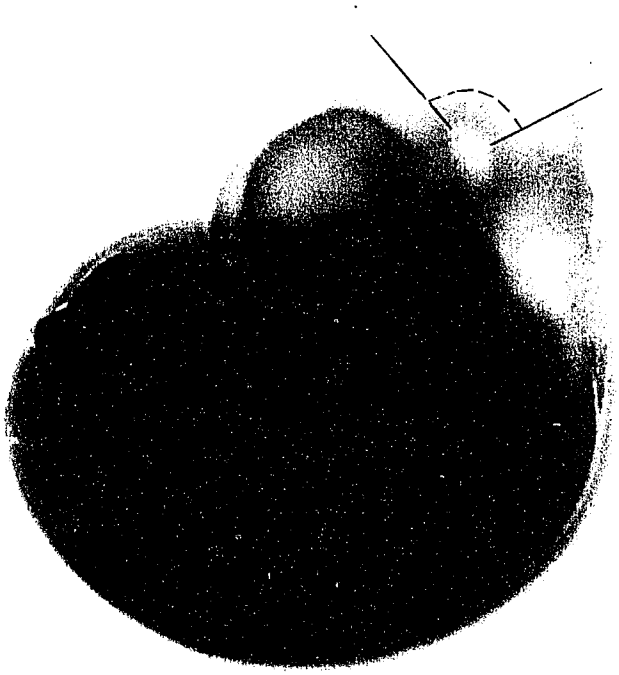


Figure 1: Photomicrograph of an E12 rat embryo. Black lines indicate the location of the rostral and caudal incisions and the dotted line the incision made to isolate the ventral mesencephalon. Crown-rump length is typically 7-8mm.

^{3}H-Thymidine incorporation was measured by a modification of the method described by Simpson *et al.* (1982). Cultures were exposed to 2 μCi/ml ^{3}H-thymidine (New England Nuclear, Boston, MA) for 24 hours at 37°C, in the presence of growth factors as appropriate. Cultures designated as blanks received 1000-fold cold thymidine in addition to the radioactively labelled thymidine. After washing 3 times with basal media, the cultures were treated with 10% trichloroacetic acid (TCA) for 30 minutes at 4°C, to remove free ^{3}H-thymidine. The cultures were then rinsed three times with 10% TCA, and once with 95% ethanol. The incorporated ^{3}H-thymidine was solubilized with 0.5M NaOH for 30 minutes at 37°C, which was then neutralized with 1M HCl. 1 ml of this solution was added to 10 ml of scintillation cocktail (Ecoscint; National Diagnostics, Mannville, NJ) and counted in a scintillation spectrometer.

3. **Immunocytochemistry**

Cultures were fixed in 10% formalin/0.03% glutaraldehyde in 0.1M sodium acetate buffer, pH 6.5, for 10 min. followed by 10% formalin/0.03% glutaraldehyde in 0.1M sodium phosphate buffer, pH 8.5, for 1 hour at room temperature (O'Malley *et al.*, 1991). Neurons were labelled with tau (polyclonal from Sigma, St. Louis, MO) which *in vitro* labels the soma and processes (Kosik and Finch, 1987). Astrocytes were labelled with antibodies to glial fibrillary acidic protein (GFAP; polyclonal from Accurate, Westbury, NY), and dopamine neurons were labelled with antibodies to tyrosine hydroxylase (TH; monoclonal from INCStar, Stillwater, MN; polyclonal from Eugene Tech, Richfield Park, NJ). Cells were permeabilized with 0.2% Triton X-100 for 30 minutes, and reacted with antibodies at the recommended dilutions overnight at 4°C. They were visualized with the peroxidase- or alkaline phosphatase-coupled avidin-biotin

staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with diaminobenzidine (DAB) or Vector Red as a chromagen.

To label dividing cells, cultures were pulsed with 5-bromo-2'-deoxyuridine (BrdU; Amersham, Arlington Hts., IL) for 24 hours at 10μM. Cells were fixed in 90% ethanol, 5% glacial acetic acid, and 5% water, reacted with antibodies to BrdU (monoclonal; Amersham), and visualized with the peroxidase-coupled staining kit (Cell Proliferation Kit, Amersham) with DAB as a chromagen. Double labelling was achieved using the antibodies described above, visualized with the alkaline phosphatase-coupled avidin-biotin staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with Vector Red as a chromagen. Cells were then photographed.

A different TH/BrdU staining protocol was used on cultures where number of double labelled cells was quantitated. Cultures were pulsed for 24 hours with 1 μM BrdU at DIV 1-2, 4-5, or 8-9. Cultures were then grown until DIV 12, and fixed with 4% paraformaldehyde for 30 minutes. Cells were treated with 2N HCl, then reacted with BrdU overnight (mouse monoclonal from Becton-Dickinson, San Jose, CA). Staining proceeded using the peroxidase-coupled avidin-biotin staining kit (Vector Labs) with DAB as a chromagen with cobalt chloride and nickel ammonium sulfate for intensification. Cells were double labelled with TH (monoclonal from INCStar, Stillwater, MN) and visualized using the peroxidase-coupled avidin-biotin staining kit with a peroxidase red chromagen (Vector Labs). Counting of double labelled cells was achieved using a microscope eyepiece with a reticle.

4. ^{3}H-Dopamine Uptake

Cultures were incubated for 30 minutes at 37°C with ^{3}H-dopamine (0.5 μCi/ml, 40 Ci/mmol, New England Nuclear, Boston, MA) in a Krebs Ringer phosphate buffer (0.1M) with added EDTA (1.3 mM), glucose (5.6 mM) and ascorbic acid (0.2 mg ml^{-1}).

Cells were then rinsed twice with buffer, incubated for 30 minutes at 37°C with 95% ethanol, which was then added to 10 ml of scintillation cocktail (Ecoscint; National Diagnostics, Mannville, NJ) and counted. Counts were corrected for non-specific uptake using cultures that were treated with the dopamine uptake blocker mazindol (10 μM; Sandoz Pharmaceuticals, Hanover, NJ).

C) RESULTS

1. Effect of bFGF on the division and differentiation of embryonic mesencephalic cells.

The effect of bFGF on the proliferation of cells derived from E12 rat ventral mesencephalon was examined. ^{3}H-Thymidine incorporation during a 24-hour period was used as an index of total cell division. Preliminary experiments using 0.1, 1.0, 10, and100 ng/ml bFGF showed that the effect peaked at approximately 10-20 ng/ml. All subsequent experiments were done using 20 ng/ml bFGF added three times a week. Figure 2 shows that incorporation in control cultures was highest after 1-2 days *in vitro* (DIV), and then declined until DIV 8-9, indicating a limited mitotic activity of mesencephalic cells during the first few days *in vitro*. The presence of bFGF in the cultures resulted in a significant increase in ^{3}H-thymidine incorporation over control values at all time points examined. Incorporation in the bFGF-treated cultures continued to increase throughout 13 days *in vitro*, with the highest rate of increase occurring between DIV 1-2 and DIV 4-5 (2.3-fold increase).

Immunocytochemistry was performed on DIV 5, 9 and 13, to determine the phenotype of the cells that were generated by bFGF-induced cell proliferation. Antibodies against the intermediate filament glial fibrillary acidic protein (GFAP) were used as markers for astrocytes. Neurons were labelled with antibodies against the microtubule-associated protein tau, which *in vitro* is present in the soma and processes (Kosik and Finch, 1987). After 5 days *in vitro* very few GFAP-positive astrocytes could be observed in cultures treated with bFGF (Fig. 3A). This finding indicates that the 2.3 fold increase in ^{3}H-thymidine incorporation between DIV 1-2 and 4-5 (Fig. 2) was not due to proliferation of differentiated astrocytes. The presence of differentiated glia would not be

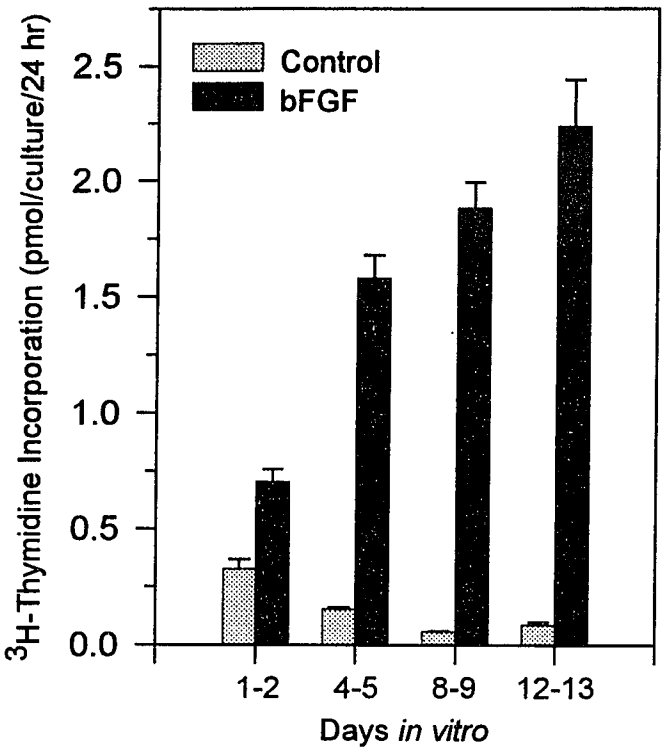
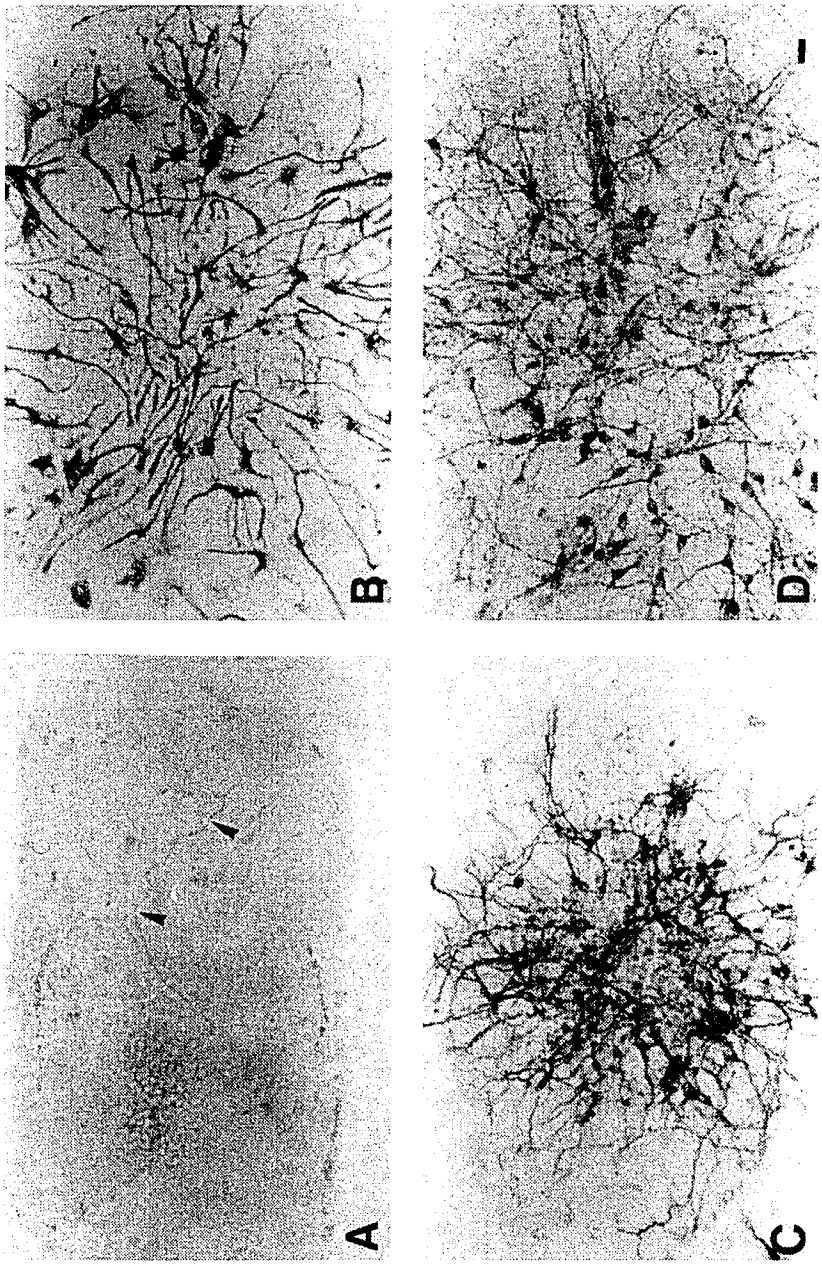


Figure 2. Thymidine incorporation in control and bFGF (20 ng/ml) treated cultures exposed to ^{3}H-thymidine (2 μCi/ml) for 24 hours at various times *in vitro*. The bars represent the means ± SEM of 10-20 samples per group from 5 separate experiments. bFGF-treated cultures incorporated significantly higher amounts of ^{3}H-thymidine at all time points ($p<0.001$; unpaired t-test).

Figure 3. Photomicrographs from bFGF-treated cultures immunostained with antibodies to GFAP (A and B) or tau (C and D). Only very few faint GFAP-positive processes are present on DIV 5 (A; arrowheads), but many astrocytes are labelled with GFAP antibodies on DIV 9 (B). Tau-positive process-bearing neurons aggregated in a cluster on DIV 5 (C). The clusters increase in size by DIV 9. Bar = 25 μm.

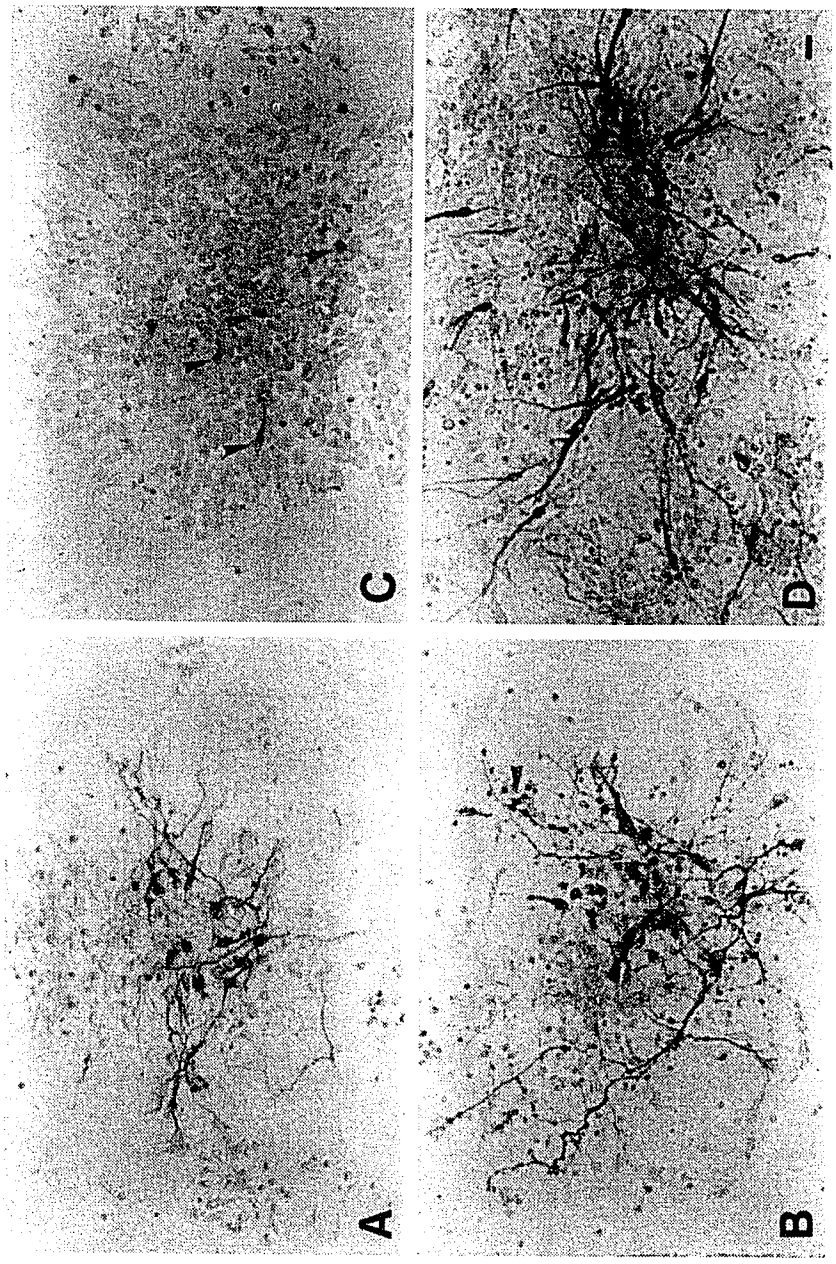

A
B
C
D

expected in the first four days in cultures prepared from E12 rat embryos, since glial differentiation *in vivo* begins at E16 and dissociated cultures taken after E10 show a parallel development *in vitro* (Abney *et al.*, 1981). Nonetheless, the number of GFAP-positive astrocytes increased greatly in bFGF-treated cultures by DIV 9 (Fig. 3B), and even further by DIV 13 (not shown). By contrast, in control cultures virtually no GFAP-positive astrocytes could be found at DIV 5, 9 or 13, as expected given that a low plating density and serum-free media do not support the growth of astrocytes (Walicke and Baird, 1988). Tau-positive neurons mainly in the form of clusters could be seen on DIV 5 in cultures treated with bFGF (Fig. 3C) and the size of these tau-positive clusters increased with time, indicating an increase in the overall number of differentiated neurons (Fig. 3D). In control cultures, tau-positive neurons were also found primarily in clusters, but their size was smaller than those treated with bFGF and the size did not appear to increase over time. In addition, beginning on DIV 9 in control cultures some clusters containing tau-positive neurons appeared to be in the process of degeneration. Degenerating clusters were not detected in the bFGF-treated cultures, indicating a survival effect of bFGF.

The presence of dopamine neurons was visualized by staining with antibodies against TH. In control cultures at DIV 5, there were a number of strongly stained cells (Fig. 4A). In contrast, bFGF-treated cultures at DIV 5 had only a few very immature TH-positive cells (Fig. 4C) suggesting that bFGF delayed the differentiation of dopamine neurons. By DIV 9 there was growth of TH-positive processes in control cultures but many of the TH-positive cells began to show signs of degeneration (Fig. 4B), indicating limited survival of this cell type. On the other hand, much larger clusters with many well differentiated TH-positive cells could be observed on DIV 9 in cultures treated with bFGF (Fig. 4D and 4E).

Because of the clustering and overlapping of the cells, counting of the number of TH-positive neurons in the cultures was not feasible. However, the presence of dopamine

Figure 4. Dopamine neurons from control (A and B) and bFGF-treated cultures (C, D and E) labelled with antibodies to TH. In control cultures, TH labelled neurons are present on DIV 5 and their processes increase in length and complexity by DIV 9. Indication of degenerating processes is also seen at this time (arrowhead). Very few, immature TH-positive neurons are found in bFGF-treated cultures on DIV 5 (arrowheads). By DIV 9, the number of TH-positive neurons and their processes have increased greatly (D). A micrograph taken at lower magnification (E) shows the size of the cluster and the extent of process outgrowth of TH-positive neurons. Magnification for A, B, C and D, bar=25 μm; for E bar = 100 μm.


C
D
A
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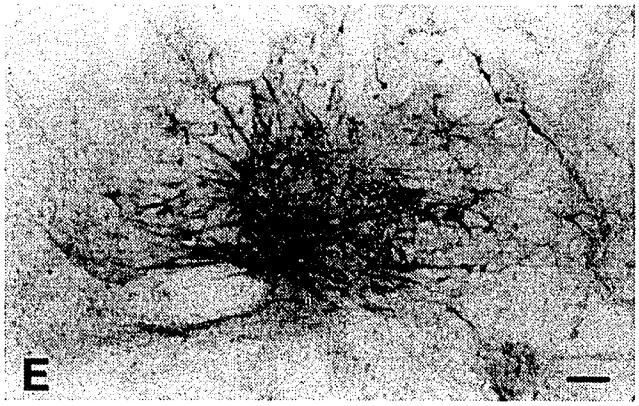

E

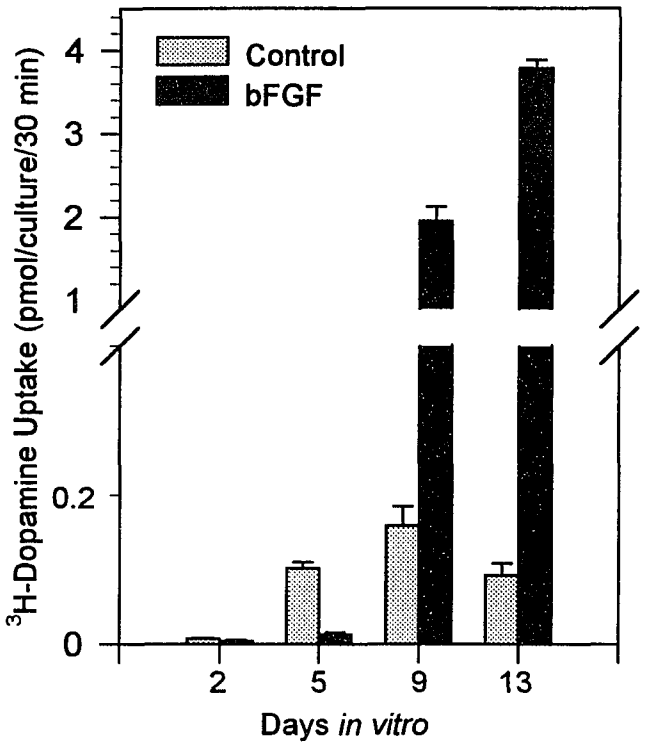
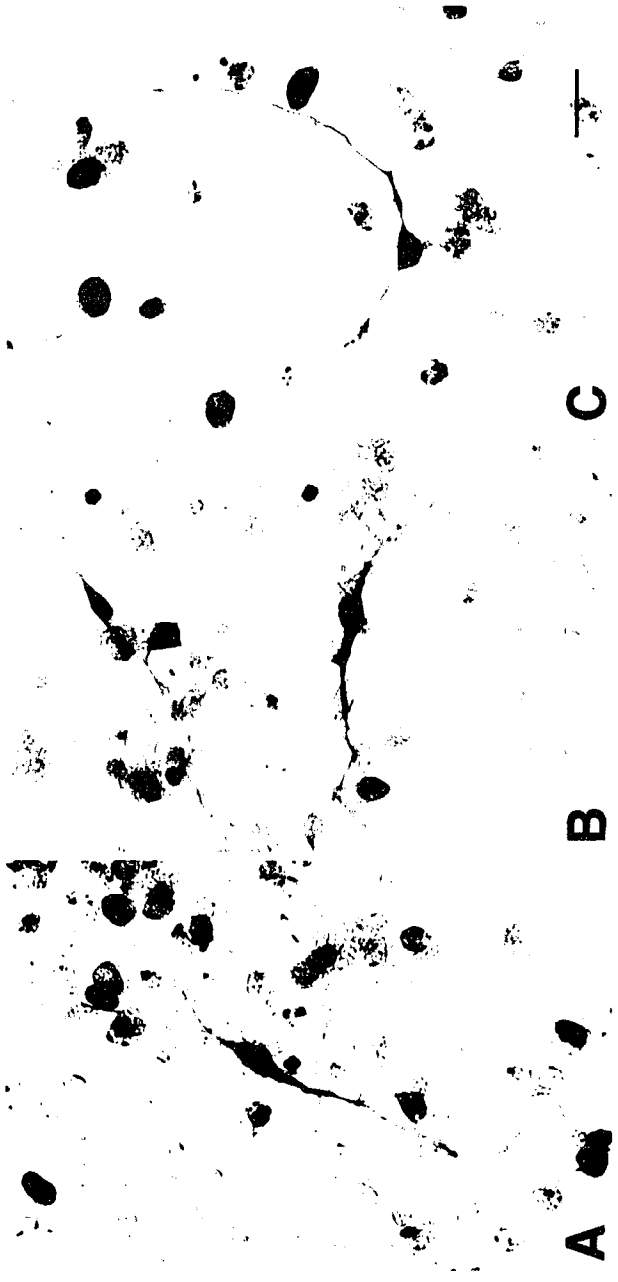


Figure 5. ^{3}H-Dopamine uptake in control and bFGF-treated cultures during development *in vitro*. The bars represent the means ± SEM of 4 samples per group (representative of 3 experiments). ^{3}H-Dopamine uptake in bFGF-treated cultures at DIV 5 was significantly lower that in the controls ($p<0.001$), while on DIV 9 and 13 it was significantly higher ($p<0.001$; unpaired t-test).

neurons and the extent of neuritic growth was quantitated by measuring ^{3}H-dopamine uptake. Uptake in control cultures increased between DIV 2 and 9 (Fig. 5), but it was reduced on DIV 13, probably as a result of the deterioration of the overall cell survival in these cultures. The bFGF-treated cultures had lower uptake levels than the controls at DIV 2 and 5 (Fig. 5), which agreed with immunocytochemical results showing delayed differentiation of the TH-positive neurons on DIV 5 (Fig. 4). Between DIV 5 and 9 there was a 150-fold increase in ^{3}H-dopamine uptake in the bFGF-treated cultures, which again confirmed the results obtained by TH-immunocytochemistry showing increased number and maturation of dopamine neurons. A further but slower increase in dopamine uptake occurred on DIV 13, reaching a value approximately 20 times the maximal control value.

The increase in the number of TH-positive neurons observed between DIV 5 and DIV 9 in the bFGF-treated cultures could be the result of proliferating dopamine precursors. To determine whether precursors of dopamine neurons were dividing in cultures treated with bFGF, we labelled dividing cells *in vitro* by pulsing the cultures with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) for 24 hours on DIV 1-2, 4-5, 8-9 and 12-13. The cultures were grown further to allow differentiation of the pulsed cells and then processed for double labelling with antibodies against BrdU in conjunction with TH. Cells double labelled for BrdU and TH were observed in cultures pulsed on DIV 1-2, DIV 4-5, and DIV 8-9 and allowed to grow until DIV 12 (Fig. 6). Of the cells that were TH+ at DIV 12, the percent that were double labelled at each time point was quantitated (Table 1). 31.5% of the cells were dividing at DIV 1-2. The BrdU label in these cells was not very strong (Fig. 6A), suggesting dilution of the label due to continuous cell division. The largest number of double labelled cells were seen in cultures pulsed on DIV 4-5, where 74.5% of the TH-positive cells had some degree of BrdU labelling in the nucleus (Fig. 6B). Double labelled cells could also be seen in cultures pulsed on DIV 8-9 (Fig. 6C), but their number had decreased to 8.7%. No double labelled cells could be observed

Figure 6. Photomicrographs from cultures labelled with antibodies to BrdU (black) and TH (red). BrdU labelled nuclei indicate that the cells were in the S phase of the mitotic cycle during the time of BrdU exposure (A: DIV 1-2; B: DIV 4-5 and C: DIV 8-9). All cultures were fixed on DIV 12. Double labelling with BrdU and TH indicates that these dopamine neurons differentiated from precursors that were still dividing during BrdU exposure. Two TH-positive neurons on the top part of 6B have no BrdU label.
Bar = 25 μm


C
B
A

Percent of Dividing Dopamine Precursors at Specific Time Points

DIV 1-2	DIV 4-5	DIV 8-9
31.5 ± 1.8	74.5 ± 2.7	8.7 ± 1.7
N=5	N=5	N=5

Table 1: Cultures were pulsed for 24 hours with 1 μM BrdU at DIV 1-2, DIV 4-5, or DIV 8-9. Cultures were then grown until DIV 12, when they were fixed and processed for TH/BrdU double label immunocytochemistry. There were between 510 and 574 TH+ cells in each dish. The number of double labelled cells was counted and expressed as a percentage of the total number of TH+ cells. Numbers represent the mean ± SEM. The largest number of dopamine precursors was dividing on DIV 4-5.

when cells were pulsed on DIV 12-13 and fixed on DIV 16. This staining indicates that dopamine precursors in the bFGF-treated cultures were dividing at least up until DIV 8.

Double labelling with antibodies to BrdU and tau or GFAP was also performed in cultures pulsed on DIV 1-2, 4-5, 8-9 and 12-13, to determine whether neuronal and astroglial precursors were dividing across all time points. Cultures pulsed with BrdU on DIV 1-2 and fixed on DIV 12 had many tau-positive neurons double labelled with BrdU, but no double labelled astrocytes. When the BrdU pulse was on DIV 4-5, the number of double labelled tau-positive neurons was greater than those pulsed on DIV 1-2. Some GFAP-positive astrocytes were also double labelled at this time. Double labelled neurons were less frequently observed when BrdU was added on DIV 8-9 or 12-13. In contrast, about 50% of the GFAP-positive astrocytes were double labelled when the cultures were pulsed with BrdU on DIV 8-9 or 12-13, suggesting active glial proliferation at these time points.

2. Influence of other growth factors on the division and differentiation of embryonic mesencephalic cells.

The effect of the continuous presence of other growth factors on the development of mesencephalic cells was examined. In addition to bFGF, both EGF and IGF-I have been shown to be mitogenic to mesencephalic precursors (Mytilineou *et al.*, 1992; Lenoir and Honegger, 1983), and GDNF has been shown to promote the survival and differentiation of dopamine neurons in rat embryonic midbrain cultures (Lin *et al.*, 1993). The effect on total cell division of these factors, either alone or in conjunction with bFGF, was examined using ^{3}H-thymidine incorporation at DIV 4-5 (Fig. 7). Only bFGF increased the rate of cell division in E12 mesencephalic cultures. Treatment with the other growth factors yielded values that resembled those from controls, and did not modify the bFGF effect in co-treated cultures. The effect of the continuous presence of

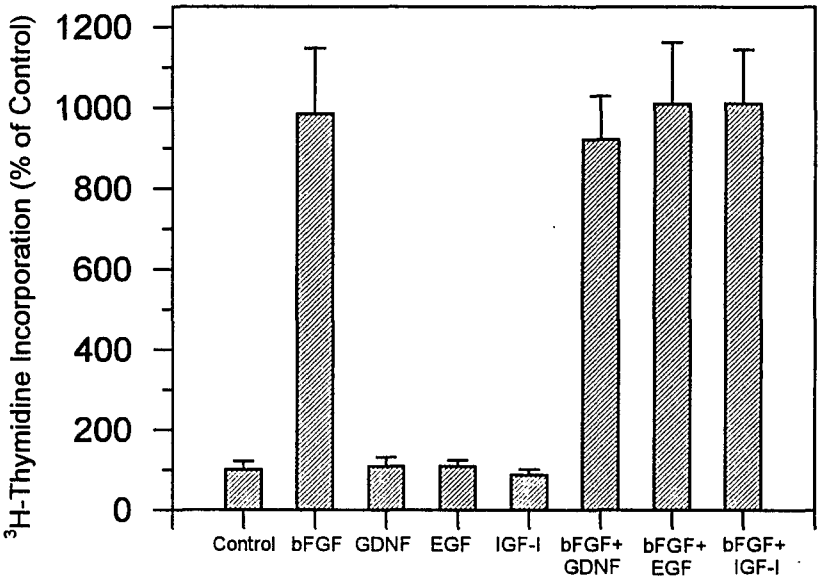


Figure 7. Effect of various growth factors on ^{3}H-thymidine incorporation in E12 mesencephalic cultures during DIV 4-5. Bars represent the means ± SEM of 12-13 samples from 3 separate experiments. Only bFGF was effective in stimulating cell proliferation in the cultures ($p<0.001$; Analysis of variance followed by Tukey test). Co-treatment with bFGF and the other growth factors did not modify the effect of bFGF.

Figure 8. Effect of growth factors on the development of ^{3}H-dopamine uptake in E12 mesencephalic cultures. The bars represent the means ± SEM of 10-14 samples from 3 separate experiments. Treatment with bFGF resulted in significantly lower uptake values on DIV 5 compared to the controls ($p<0.001$) and higher on DIV 9 ($p<0.001$; Analysis of variance followed by Tukey test). This effect was not modified by co-treatment with other growth factors. GDNF, EGF and IGF-I were ineffective in modifying ^{3}H-dopamine uptake on DIV 5 or 9.

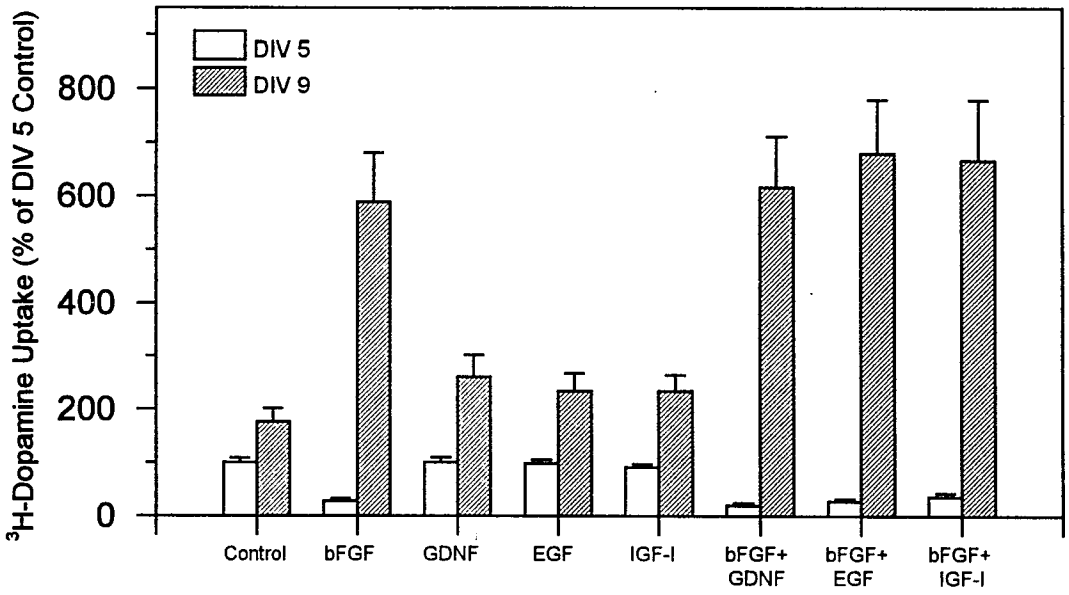

DIV 5
DIV 9
^{3}H-Dopamine Uptake (% of DIV 5 Control)
800
600
400
200
0
Control
bFGF
GDNF
EGF
IGF-I
bFGF+ GDNF
bFGF+ EGF
bFGF+ IGF-I

Table 2. Effect of neuronal differentiation factors on the development of dopamine neurons in E12 mesencephalic cultures treated with bFGF for 5 DIV.

Differentiation factors added on DIV 5	^{3}H-Dopamine Uptake on DIV 9 (pmol/culture/30 min)	
	(+) bFGF	(-) bFGF
None	1.73 ± 0.11	1.76 ± 0.12
Retinoic Acid (0.1 μM)	2.30 ± 0.22	1.82 ± 0.08
GDNF (1 ng ml^{-1})	2.03 ± 0.20	1.96 ± 0.20
cAMP (1 mM)	1.83 ± 0.21	2.01 ± 0.13

Values are means ± SEM from 4 cultures per group. There was no significant difference between the groups. Cultures were treated with 20 ng/ml bFGF until DIV 5 and then the differentiation factors were added in the presence or absence of bFGF. ^{3}H-Dopamine uptake in cultures treated with bFGF for 5 DIV was 0.69 ± 0.16 pmol/culture/30 min.

GDNF, EGF, and IGF-I on the ^{3}H-dopamine uptake in these cultures at DIV 5 and 9 was also examined (Fig. 8). None of the growth factors alone were able to mimic the ability of bFGF to either delay differentiation at DIV 5 or yield high uptake values at DIV 9. The growth factors were also unable to modify the bFGF effect in co-treated cultures.

The ability of various factors to affect the differentiation of dopamine precursors generated by treatment with bFGF was also examined (Table 2). In addition to GDNF, retinoic acid and dibutyryl cyclic adenosine monophosphate (dbcAMP) were used because they have been shown to stimulate neuronal differentiation in rodent embryonic cultures (Ved and Pieringer, 1993; Sklair-Tavron and Segal, 1993). Cultures were grown for 5 days in the presence of bFGF (at which time ^{3}H-dopamine uptake was measured to obtain a baseline value of dopamine neuron development). bFGF was then washed off the cultures, and either retinoic acid (0.1μM), dbcAMP (1mM), GDNF (1ng/ml) or no differentiation factor was added in the presence or absence of bFGF. ^{3}H-dopamine uptake was performed on all cultures at DIV 9. The data show that none of the factors was able to significantly increase the dopamine uptake in these cultures, nor were they able to modify the bFGF effect in co-treated cultures. Interestingly, there was also no significant difference in the development of dopamine neurons on DIV 9 between cultures treated continuously with bFGF and cultures treated with bFGF only until DIV 5. This finding indicates that the effect of bFGF on dopamine neurons occurs in large part during the first five days in culture.

D) DISCUSSION

The dopamine neurons of the rat substantia nigra are generated from dividing neuroepithelial cells in the ventral mesencephalon during a span of about 5 days, between E11 and E15 (Hanaway *et al.*, 1972; Lauder and Bloom, 1974; Altman and Bayer 1981). Expression of phenotypic markers begins after the final mitosis (Rothman *et al.*, 1980; Specht *et al.*, 1981) with TH being detectable first at E12.5 (Specht *et al.*, 1981). Differentiation of dopamine neurons proceeds quickly and axonal processes have already assembled in the medial forebrain bundle and reached the ventral-lateral portion of the neostriatum by E14.5 (Specht *et al.*, 1981).

It has previously been shown that bFGF and serum can increase mitosis during the normal period of cell division of dopamine precursors (about one day *in vitro*) from E12 rat embryos (Mayer *et al.*, 1993). We now report that the period of proliferation of dopamine precursors can be extended to at least 8 days *in vitro* by the continuous exposure of E12 ventral mesencephalic cells to bFGF in serum-free medium. Incorporation of the thymidine analogue BrdU followed by double labelling with antibodies to BrdU and TH demonstrated that precursors of dopamine neurons divide for at least 8 days *in vitro*. In the embryo this would correspond to E20, well beyond the end of the birth period of dopamine neurons of the substantia nigra. Quantitation of these double labelled cells indicated that, of the cells that were TH+ at DIV 12, approximately three-quarters of them had been dividing at DIV 4-5. The stimulation of cell division by bFGF is accompanied by an initial delay in the differentiation of dopamine neurons. When no growth factors were added, maturation of dopamine neurons by DIV 2 and continuing through DIV 9 was indicated by increased ^{3}H-dopamine uptake, and by increased process outgrowth in TH immunolabelled cells. By comparison, in the presence of bFGF the numbers of TH-positive neurons, their process outgrowth, and their ability to

accumulate ^{3}H-dopamine were minimally developed on DIV 2 and DIV 5. Between DIV 5 and DIV 9, however, differentiation began to occur at a very rapid rate in bFGF-treated cultures, causing the appearance of many TH-positive neurons by DIV 9 with well developed processes and a ^{3}H-dopamine uptake level which reached 150 times that of DIV 5. These results indicate that bFGF can delay the terminal differentiation of dopamine precursors and prolong their period of mitosis *in vitro*, with the consequence of an increased number of differentiated dopamine neurons at later time points.

The stimulation of proliferation by bFGF did not appear to be restricted to dopamine precursors. Neurons labelled with antibodies to tau increased in cultures treated with bFGF and a large proportion of the cells that were tau-positive at DIV 12 had incorporated BrdU during the first five days in culture. It is known that the cells destined to become the GABA neurons of the substantia nigra pars reticulata have the same birthdates and originate from the same area of the neuroepithelium as the dopamine neurons of the pars compacta (Hanaway *et al.*, 1972; Altman and Bayer, 1981). Therefore it is likely that both types of precursors are present in our cultures and are dividing in response to bFGF, since there is evidence that bFGF is mitogenic to GABA precursors (Deloulme *et al.*, 1991) and that bFGF increases the survival and neurite outgrowth of GABA neurons in cultures from E14 rat mesencephalon (Ferrari *et al.*, 1989; Ferrari *et al.*, 1991).

Stimulation of cell proliferation by bFGF during the first days in culture involved mainly neuronal precursors. Although bFGF is a potent mitogen of astrocytes (Kniss and Burry, 1988; Park and Mytilineou, 1992), staining with the astrocytic marker GFAP showed no differentiated astrocytes in the cultures treated with bFGF during the first 5 days *in vitro*. Furthermore, in cultures pulsed with BrdU on DIV 1-2 and fixed on DIV 12, no BrdU labelled GFAP-positive astrocytes could be found, suggesting that the cells dividing on DIV 1-2 did not include precursors of astrocytes. Some double labelled astrocytes could be observed when BrdU was added on DIV 4-5, but numerous

proliferating astrocytes incorporated BrdU on DIV 8-9 and 12-13. Whether bFGF stimulated the proliferation of glial precursors or differentiated astrocytes at these later time points cannot be determined from these data. However, a large number of differentiated astrocytes were already present on DIV 9, and these cells were probably responding to the mitogenic effects of bFGF.

The stimulation of precursor cell division followed by increased differentiation of dopamine neurons was specific to bFGF. Although both EGF and IGF-I can stimulate the proliferation of neuronal precursors (Reynolds and Weiss, 1992; Mytilineou *et al.*, 1992; DiCicco-Bloom *et al.*, 1990), they showed no mitogenic effect on E12 mesencephalic cells, and were not able to modify the mitogenic effects of bFGF. It is possible that receptors for these growth factors are not present or are not biologically active on these cells during this stage of development. GDNF, a recently characterized growth factor (Lin *et al.*, 1993) with potent neurotrophic activity for dopamine neurons, was also unable to stimulate cell proliferation. Furthermore, EGF, IGF-I and GDNF failed to mimic the bFGF effect on differentiation of dopamine precursors and were unable to modify the bFGF effect in co-treated cultures.

It is of interest that the rapid rate of differentiation and growth of dopamine neurons, which occurs between DIV 5 and DIV 9, coincides in time with the appearance of astrocytes in the cultures. As stated, bFGF is mitogenic to astrocytes (Kniss and Burry, 1988). In turn it is well established that astrocytes possess neurotrophic activity (Banker, 1980; Müller and Siefert, 1982; Kadle *et al.*, 1988) and promote the survival and development of dopamine neurons (Beyer *et al.*, 1991; O'Malley *et al.*, 1994). It has also been shown that treatment of cortical astrocytes with bFGF induces the secretion of molecules which stimulate the differentiation of mesencephalic dopamine neurons (Gaul and Lubbert, 1992). It seems quite possible that the bFGF treatment is causing division of astrocytes, and that the resulting large number of astrocytes is inducing the differentiation of dopamine neurons.

Recently it has been shown that the induction of dopamine neurons in the ventral mesencephalon occurs through contact with the floor plate (Hynes *et al.*, 1995), and the authors suggest that the induction of neurons in this area may be the result of both diffusible and contact-dependent signals. Within the context of our results, it is possible that in addition to stimulating division of dopamine precursors, bFGF may be stimulating the production of a dopamine neuron inducer, which would also contribute to the increase in dopamine neurons seen at later time points.

Neuronal precursors generated during the first 5 days of treatment with bFGF differentiated into dopamine neurons even if bFGF was removed on DIV 5. This suggests that the actions of bFGF, which resulted in an increased number of differentiated dopamine neurons on DIV 9, occurred in large part during the first five days. This conclusion is also supported by the finding that the largest number of dividing dopamine precursors was found on DIV 4-5. One explanation for this finding is that an initial exposure to bFGF is sufficient to set in motion the extended proliferation of these cells. Alternatively, perhaps the appearance of mature astrocytes between DIV 5 and 9 is stimulating the differentiation of dopamine precursors, gradually bringing the period of division to an end.

Whether all dopamine neurons present in the bFGF-treated cultures on DIV 9 were generated from precursor cells dividing *in vitro* cannot be determined from our data. Some precursor cells which had undergone their last division *in vivo* could have been maintained in an undifferentiated state by bFGF during the first 5 days *in vitro*, and bFGF could be supporting their survival. However, as shown by the incorporation of BrdU and the quantitation of dividing dopamine precursors over time, a large number of the dopamine neurons underwent their last division *in vitro*. Thus either precursors were already committed to the dopamine phenotype or culture conditions were favorable for the expression of dopaminergic traits, since commitment to phenotype can be determined

by both intrinsic programs and epigenetic signals (Temple, 1989; Luskin *et al.*, 1993; Hynes *et al.*, 1995).

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopamine neurons of the substantia nigra pars compacta. A form of therapy for this disease is the transplantation of embryonic mesencephalic cells into the denervated striatum, although the limited efficacy of this treatment is indicative of the need for further research in this field (for a review, see Björklund, 1991). The requirement to have available enough fetal tissue of the appropriate developmental stage at the time of transplantation and the poor survival of the grafted cells within the host brain are major difficulties with this treatment. The *in vitro* manipulation of dopamine precursors could result in a cell population appropriate for transplantation. This would create a window of time between collection of fetal tissue and grafting. Furthermore, a cell population rich in dopamine precursors may survive and integrate better than differentiated neurons.

II. CHARACTERIZATION OF CELLS CULTURED ON NON-ADHESIVE SUBSTRATE

A) INTRODUCTION

The transplantation of embryonic tissue into human brain has proven to be a promising strategy for the treatment of Parkinson's disease as well as other neurodegenerative disorders (Freed *et al.*, 1990; Freed *et al.*, 1992; Lindvall *et al.*, 1990; Spencer *et al.*, 1992). In order to become a more viable clinical therapy, certain practical problems must be overcome such as the heterogeneity of the transplanted tissue, the possibility of infectious agents within the tissue, and the limited supplies of these embryonic cells (for further discussion, see Björklund 1993). For the treatment of Parkinson's disease, the ventral mesencephalon is transplanted into the striatum of the diseased brain to replace lost dopamine. Limited supplies of appropriate tissue have proven to be especially challenging, since dopamine neurons constitute only a few percent of the cells in the embryonic ventral mesencephalon, and cell survival within grafts is commonly only about 5-10%. The transplantation of genetically modified cells has been an active area of research (Renfranz *et al.*, 1991; Snyder *et al.*, 1992), but genetically modified tissue can show poor survival within the host brain, and often does not stably express the desired protein at high enough levels. In addition, this tissue can be tumorigenic.

Another strategy for transplantation is to use progenitor cells from the central nervous system. For this therapy, it would be useful to be able to isolate the appropriate progenitor cell and to expand its proliferation *in vitro* prior to transplantation. This tissue may survive better in the host than genetically modified non-brain cells, and would not be expected to be tumorigenic. The use of cultured progenitor cells would be an

improvement over fresh embryonic tissue in that one would have time before the transplant surgery to properly screen for infectious agents. In addition, culture conditions could be established to increase the amount of tissue so that fewer embryos are needed, and one could perhaps establish conditions to increase the amount of the target precursor.

We now know that the brain possesses much more plasticity than was once thought. It has even been shown that with the appropriate culture conditions and growth factors, one can generate dividing precursors from the adult mammalian brain (Reynolds and Weiss, 1992). In one attempt to study dopamine precursors, porcine mesencephalic cells were isolated at a very young age and cultured in suspension, then transplanted into lesioned rats. These cultured dopamine neurons developed along the same time course and in the same numbers *in vitro* as they would have *in vivo*, and when transplanted survived and decreased rotational behavior almost as well as fresh embryonic transplants (Spector *et al.*, 1993).

We have previously shown that in monolayer cultures of E12 rat ventral mesencephalon, treatment with bFGF can expand the period of dopamine precursor division from about 1 day to at least 8 days *in vitro*, resulting in larger quantities of dopamine neurons (see Chapter I). In the present study, we applied that finding to cultures grown in suspension, which are more appropriate for transplantation. We report that treatment with bFGF resulted in spheres of dividing cells, and that at later time points colonies of dopamine neurons were found in large-sized spheres. These large spheres had a mean area of 1.26 mm^2, and constituted about one quarter of the spheres in a typical culture dish. Typically about half the processes and cell bodies in these large spheres were dopaminergic. The high-affinity dopamine uptake value in the spheres was 6 times higher that of controls. We believe that we have established conditions to produce tissue that is more appropriate for use in transplantation in Parkinson's disease than the currently used paradigm employing fresh embryonic tissue.

B) MATERIALS AND METHOD

1. Preparation of cell cultures

Primary neuronal cell cultures were prepared as described in Chapter I with the following exceptions: Cells were plated on dishes that were not pre-coated with polyornithine, in order to prevent cell attachment. The final volume in the dishes was 1.5 ml, and starting 5 days after plating 1 ml of media was replaced with fresh media three times a week. This replacement was accomplished by allowing spheres to settle in a dish, and carefully drawing off exactly 1 ml from the top of the liquid. Cultures were maintained in a modular incubator chamber (Vangard International Inc., Neptune, NJ) which was purged with 5% CO_2/ 95% air, then placed on a rotating shaker in a 37°C oven in order to further discourage cell attachment. Growth factors were added at the time of plating and three times a week thereafter. bFGF (human recombinant; Synergen, Boulder, CO), and EGF (mouse submaxillary gland; Collaborative Research, Lexington, MA) were used at a concentration of 20 ng/ml.

2. ^{3}H-Thymidine incorporation

Cultures were exposed to 2 μCi/ml ^{3}H-thymidine (New England Nuclear, Boston, MA) for 24 hours at 37°C, in the presence of growth factors as appropriate. Cultures designated as blanks received 1000-fold cold thymidine in addition to the radioactively labelled thymidine. At the end of the culture period, the media was pipetted onto a filter, and vacuum filtered. Dishes were rinsed twice with balanced salt solution (BSS) which was also added to the filter. The filters were washed twice more with BSS, then rinsed four times with 10% trichloroacetic acid (TCA) in order to solubilize the free ^{3}H-

thymidine. Filters were dissolved in 10 ml of scintillation cocktail (Ecoscint; National diagnostics, Mannville, NJ) and counted in a scintillation spectrometer.

3. Cell counts

Suspension media from a culture dish was collected in a test tube, centrifuged, and rinsed several times with bicarbonate buffer containing EDTA (Versene). Tissue was then incubated in Versene containing 0.04% trypsin for 15 minutes at room temperature. The trypsin solution was inactivated using an equal volume of serum-containing media. The tissue was gently triturated, and cells were counted using a hemocytometer.

4. ^{3}H-dopamine uptake

Cultures were grown as spheres for 12 days. They were then attached to adhesive substrate and cultured for another 7 days to allow cell differentiation and analysis of phenotype. Cultures were incubated for 30 minutes at 37°C with ^{3}H-dopamine (0.5 μ Ci/ml, 40 Ci/mmol, New England Nuclear, Boston, MA) in a Krebs Ringer phosphate buffer (0.1M) with added EDTA (1.3 mM), glucose (5.6 mM) and ascorbic acid ().2 mg/ml). Tissue was scraped off the dish into the Krebs solution, which was added to a filter and vacuum filtered. The scraping was repeated, then the filters were rinsed five times with fresh Krebs solution. Filters were dissolved in 10 ml of scintillation cocktail (Ecoscint; National Diagnostics, Mannville, NJ) and counted. Counts were corrected for non-specific uptake using cultures that were treated with the dopamine uptake blocker mazindol (10 μM; Sandoz Pharmaceuticals, Hanover, NJ).

5. Measurement of sphere sizes

bFGF-treated cultures were grown as spheres for 12 days. They were then attached to adhesive substrate and cultured for another 7 days to allow cell differentiation and analysis of phenotype. The area of each sphere in one typical culture dish was determined using a microscope eyepiece with a reticle. Area was calculated using πr^2 and inserting the following numbers: (π)*(longest diameter÷2)*(shortest diameter÷2). The areas of the spheres fell into three distinct groups of sizes, and the mean area of each group was determined, ± standard error of the mean (SEM).

6. Immunocytochemistry of spheres after attachment to adhesive substrate

bFGF-treated cultures were grown as spheres for 12 days. They were then attached to adhesive substrate and cultured for another 7 days to allow cell differentiation and analysis of phenotype. Cultures were fixed in 10% formalin/0.03% glutaraldehyde in 0.1M sodium acetate buffer, pH 6.5, for 10 min. followed by 10% formalin/0.03% glutaraldehyde in 0.1M sodium phosphate buffer, pH 8.5, for 1 hour at room temperature (O'Malley *et al.*, 1991). Neurons were labelled with tau (polyclonal from Sigma, St. Louis, MO) which *in vitro* labels the soma and processes (Kosik and Finch, 1987) or neuron specific enolase (NSE; polyclonal from Polysciences, Warrington, PA). Astrocytes were labelled with antibodies to glial fibrillary acidic protein (GFAP; polyclonal from Accurate, Westbury, NY), and dopamine neurons were labelled with antibodies to tyrosine hydroxylase (TH; monoclonal from INCStar, Stillwater, MN; polyclonal from Eugene Tech, Richfield Park, NJ). Cells were permeabilized with 0.2% Triton X-100 for 30 minutes, and reacted with antibodies at the recommended dilutions overnight at 4°C. They were visualized with the peroxidase- or alkaline phosphatase-coupled avidin-biotin staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with diaminobenzidine (DAB) or Vector Red as a chromagen.

To label dividing cells, cultures were pulsed with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU; Amersham, Arlington Hts., IL) for 24 hours at 10μM. Spheres were triturated, plated on polyornithine-coated dishes, and cultured for an additional 7 days to allow cell differentiation. Cells were fixed in 90% ethanol, 5% glacial acetic acid, and 5% water, reacted with antibodies to BrdU (monoclonal; Amersham), and visualized with the peroxidase-coupled staining kit (Cell Proliferation Kit, Amersham) with DAB as a chromagen. Double labelling was achieved using the antibodies described above, visualized with the alkaline phosphatase-coupled avidin-biotin staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with Vector Red as a chromagen.

7. Immunocytochemistry on sections of gelatin-embedded spheres

Suspension culture media was collected in a tube and centrifuged. The tissue pellet was rinsed in phosphate buffered saline (PBS) then fixed in 4% paraformaldehyde for 30 minutes. After several rinses in PBS, a small amount of 8% gelatin was added to the pellet and allowed to solidify. This gelatin block was then placed in an embedding mold filled halfway with 12% gelatin, then covered with more 12% gelatin so that the pellet was suspended in the middle of the mold. Once this solidified, the block was placed in 4% paraformaldehyde overnight to harden, then sectioned at 30 or 40 μ on a vibratome.

Free-floating sections were stained using the antibodies described above. Sections were blocked in 3% bovine serum albumin (BSA) and reacted with antibodies overnight at 4°C. Reactions were visualized using the peroxidase-coupled avidin-biotin staining kit (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with DAB as a chromagen. All incubation solutions were made with 0.1% triton. When staining was complete, tissue was mounted and counterstained with cresyl violet.

To label dividing cells within spheres, cultures were pulsed with 10μM BrdU for 24 hours. Staining was accomplished using a modification of the method described by Tischler *et al.* (1992). Spheres were fixed for 10 minutes in 90% ethanol followed by 30 minutes in 4% paraformaldehyde. After blocking in 3% BSA, sections were rinsed in PBS, then reacted overnight in PBS containing antibodies to BrdU (mouse monoclonal from Becton-Dickinson, San Jose, CA) and DNAse I (Sigma, St Louis, MO). Staining proceeded using the peroxidase-coupled avidin-biotin staining kit (Vector Labs), and 0.1% triton was added to all incubation solutions. DAB was used as a chromagen, with cobalt chloride and nickel ammonium sulfate used for intensification. After staining, tissue was mounted and counterstained with cresyl violet.

C) RESULTS

1. Effect of bFGF on cell division in spheres of embryonic mesencephalic cells

Cells derived from E12 rat ventral mesencephalon were plated in the presence of bFGF on dishes containing no adhesive substrate, and maintained throughout the culture period on a rotating shaker. This process generated a suspension of spheres, each containing numerous cells (Fig. 9). The effect of bFGF on total cell division in these spheres was examined using ^{3}H-thymidine incorporation during a 24-hour period. Figure 10a shows that incorporation peaked in control cultures at DIV 1-2, then declined throughout the culture period, indicating a limited mitotic activity of these cells. In the bFGF-treated cultures, ^{3}H-thymidine incorporation was higher than controls beginning at DIV 4-5, and at each time point thereafter. Incorporation in bFGF-treated cultures increased over time, with the highest increase occurring between DIV 7-8 and DIV 11-12. The increase in total cell number over 12 days in culture was approximately 47-fold (Fig. 10b).

Dividing cells within the spheres were identified by pulsing cultures with BrdU on DIV 4-5, 6-7, 8-9, and 11-12, then embedding, sectioning, and staining.. Spheres that were pulsed up until DIV 6-7 appeared to have dividing cells in discrete circles primarily toward the center of the sphere (Fig. 11a). This finding indicates that there are spherical masses at the center of the spheres that are dividing. In addition to this location, by DIV 8-9 dividing cells were also detected along the extreme periphery (Fig. 11b).

The sizes of the spheres typically fell into three distinct groups. To quantitate this observation the area of each sphere in one typical culture dish was determined. bFGF-treated cultures were grown as spheres for 12 days, then attached to adhesive substrate

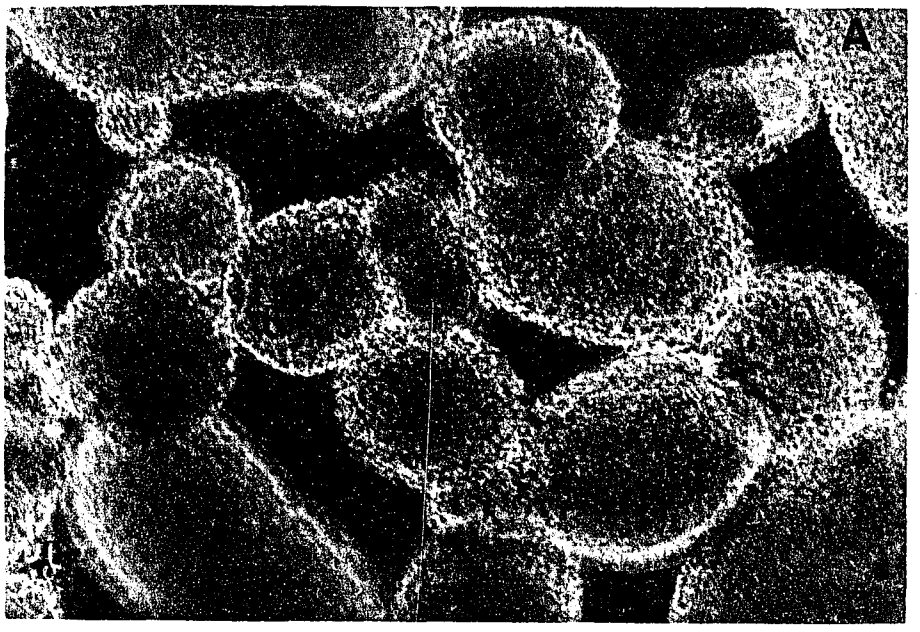


Figure 9. bFGF-treated spheres were grown in suspension for 8 days on non-adhesive substrate (non-coated dishes), then collected and fixed with paraformaldehyde. Phase micrograph with green interference filter. X75

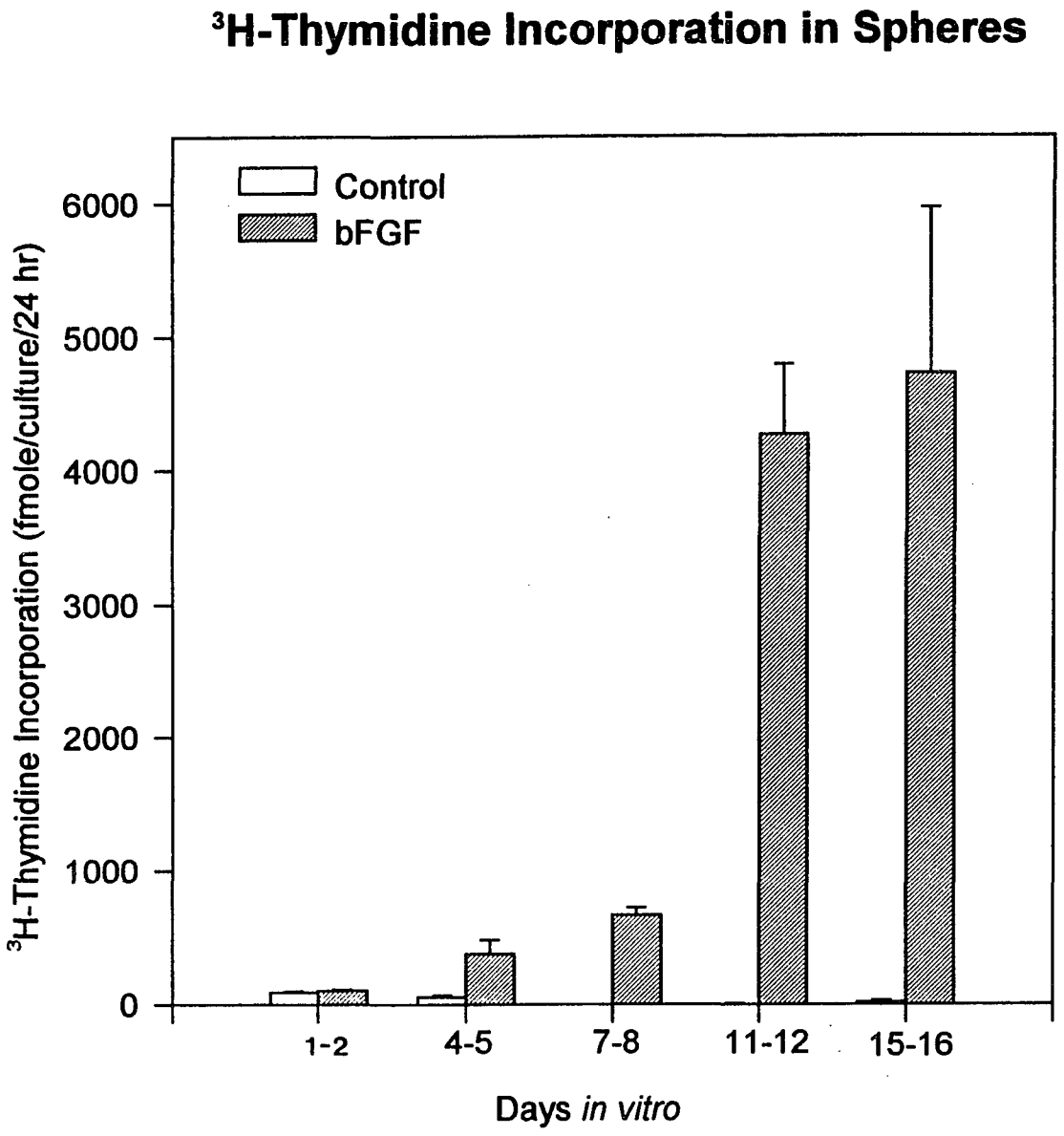


Figure 10a. Thymidine incorporation in control and bFGF (20 ng/ml) treated cultures exposed to ^{3}H-thymidine (2 μCi/ml) for 24 hours at various times *in vitro*. The bars represent the mean ± SEM. bFGF-treated cultures incorporated higher amounts of ^{3}H-thymidine at DIV 4-5, and at each point thereafter.

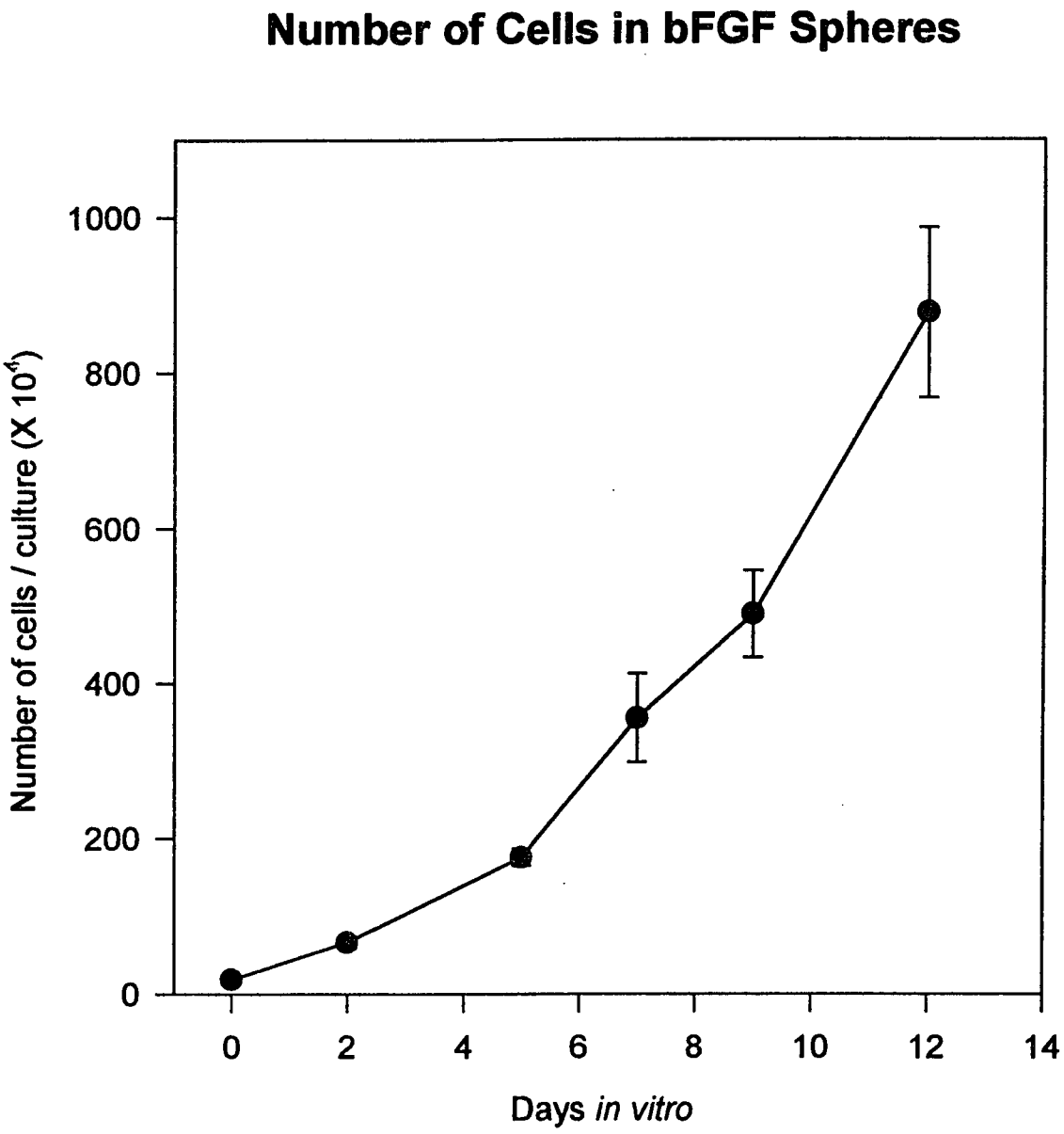


Figure 10b. Increase in total cell number over time in bFGF-treated cultures. Points represent the mean of three dishes ± SEM. Increase in total cell number over 12 days in culture was approximately 47-fold.

Figure 11. Photomicrographs of sections of bFGF-treated spheres pulsed with BrdU. Sphere pulsed with BrdU on DIV 6-7 (A) shows dividing cells in discrete circles primarily toward the center of the sphere. Sphere pulsed with BrdU on DIV 8-9 (B) shows that dividing cells now also appear along the periphery. X185

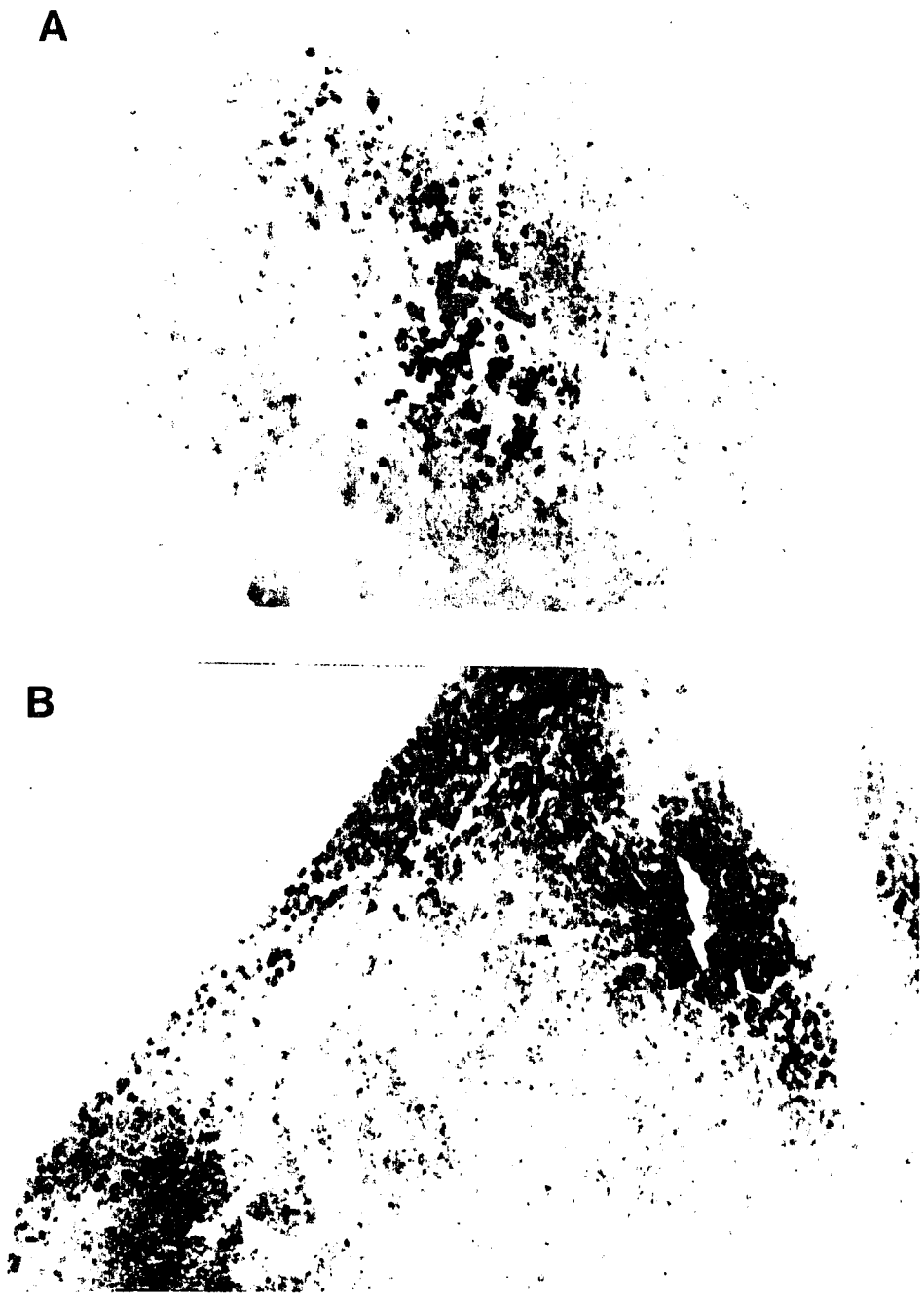
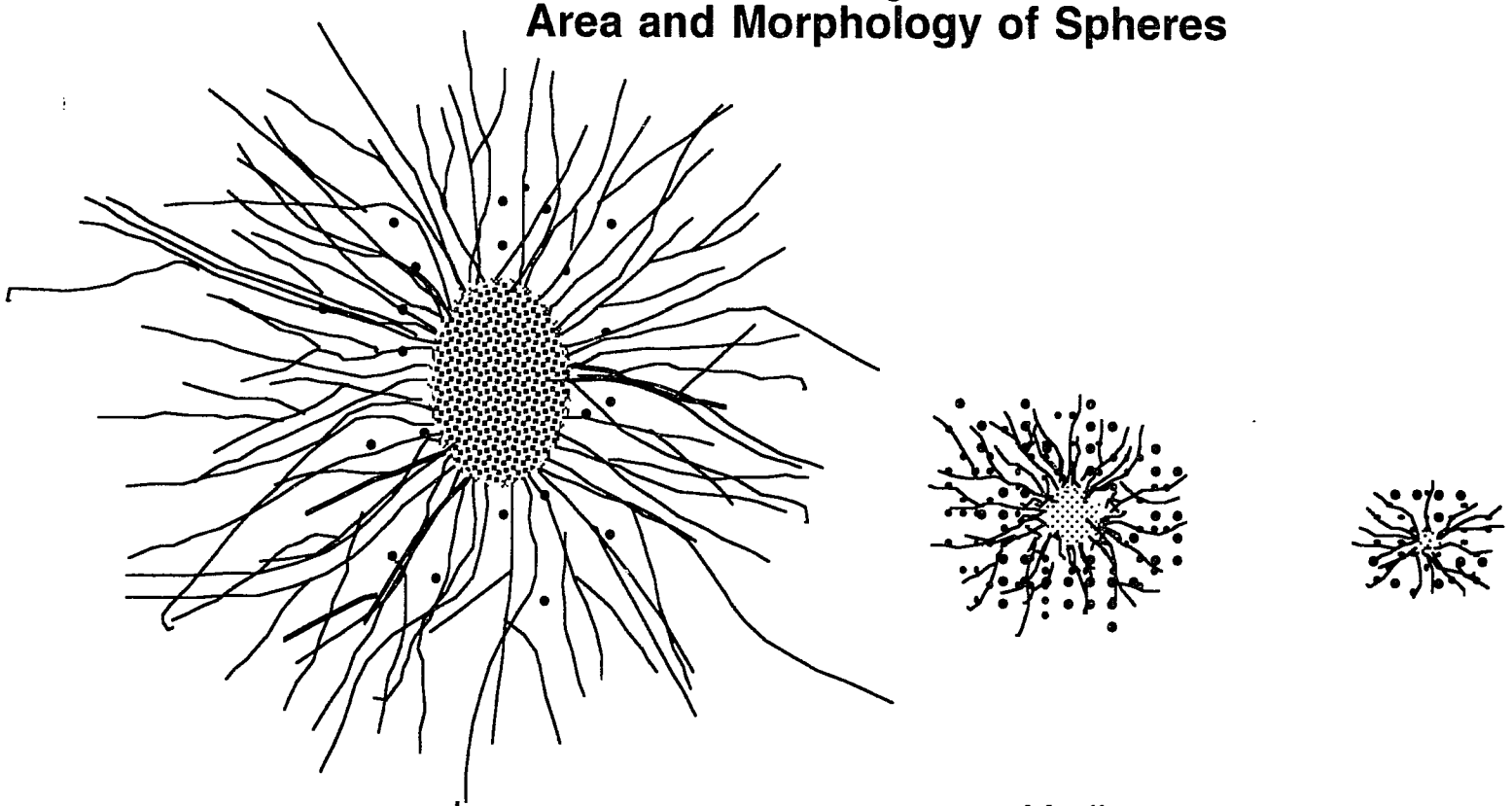

A
B

Figure 12. Area and morphology of spheres. Numbers represent mean area ± SEM of all the spheres in one typical culture dish. Percentages are out of a total of 43 spheres in the dish. Large Spheres were oblong, dense with tightly packed cells, showed limited cell migration and a long and extensive network of processes. They were 5.7 times larger than medium-sized spheres. Medium Spheres were round, less dense with cells, and showed extensive cell migration and fewer processes than large spheres. They were 3.9 times larger than small spheres. Small Spheres were round and showed extensive cell migration, with the fewest processes. These spheres were the most numerous (44%).

Figure 12

Area and Morphology of Spheres



Large
1.26 mm^2 ± 0.16
(26%)

Medium
220 μm^2 ± 22
(30%)

Small
57 μm^2 ± 13
(44%)

and cultured for another 7 days to allow cell differentiation. Area was measured using a microscope eyepiece with a reticle. The results are shown in Figure 12. The spheres fell into three disparate groups based on their area. The numbers within each group clustered tightly, as evidenced by the small SEM for each group.

The large spheres had a mean area of 1.26 mm^2, making them 5.7 times larger than the medium-sized spheres. These large spheres were generally oblong and dense with tightly packed cells. They demonstrated limited cell migration, though there was an extensive network of processes emanating from these spheres. Large spheres constituted 26% of the spheres in the dish.

Medium-sized spheres had a mean area of 220 μm^2, making them 3.9 times larger than small spheres. These spheres were generally round, and less densely packed with cells than large spheres. They demonstrated extensive cell migration, though there were fewer processes extending out of medium-sized spheres than were seen in the large spheres. These spheres constituted 30% of the spheres in the dish.

Small spheres had a mean area of 57 μm^2 and were generally round. They demonstrated extensive cell migration, though these spheres exhibited the fewest processes extending out of them as compared to medium and large spheres. Small spheres were the most numerous, constituting 44% of the spheres in the dish.

2. Effect of bFGF on the development of neurons and astrocytes in spheres.

Cultures were grown as spheres for 12 days. They were then attached to adhesive substrate and cultured for another 7 days to allow cell differentiation and analysis of phenotype. Figure 13 shows a control (non bFGF-treated) sphere after attachment. The spheres in these dishes were smaller and less numerous than in bFGF-treated dishes. Upon attachment they initially extended processes, but there was virtually no cell

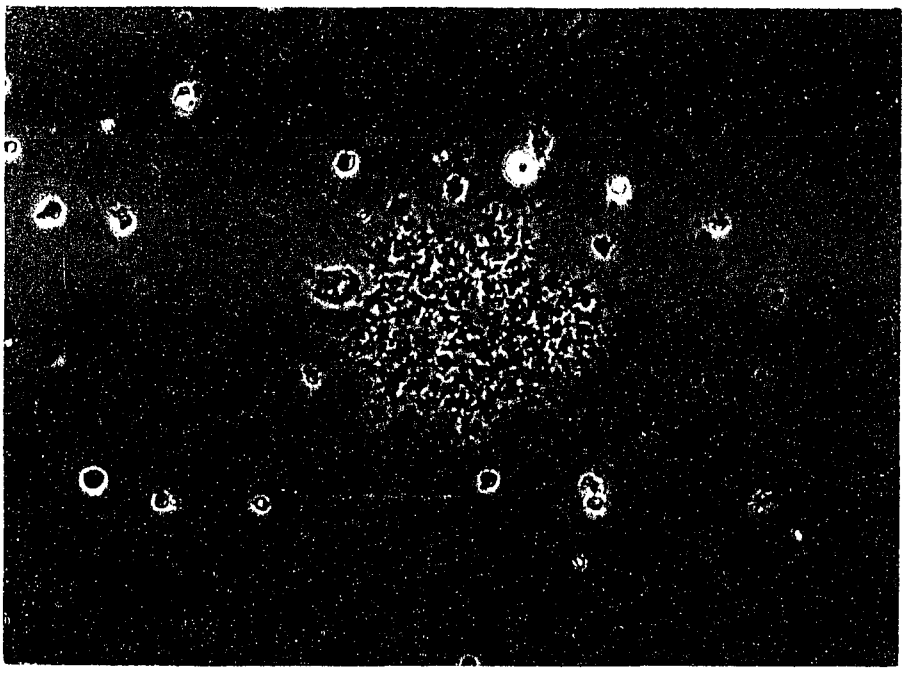


Figure 13. Phase micrograph of a sphere in a control culture (no growth factors), which was grown for 12 days in suspension then replated on adhesive substrate for an additional 7 days. These spheres were smaller and less numerous than bFGF-treated spheres. They did not stain for neuronal and astrocytic markers, indicating limited cell survival.

Figure 14. NSE-labelled cells grown for 12 days in suspension, then replated on adhesive substrate for an additional 7 days. NSE+ cell bodies and processes were seen migrating out of medium-sized spheres. NSE+ processes were also seen migrating out of large spheres (not shown). X185

migration. After 7 days there appeared to be some deterioration of the cultures, and they did not stain for neuronal and astrocytic markers, indicating limited cell survival.

When bFGF-treated spheres were grown for 12 days and further cultured on adhesive substrate, NSE+ processes extended out of large-sized spheres. NSE+ cell bodies and processes were also seen migrating out of medium-sized spheres (Fig. 14). Neuronal precursors had been dividing during the culture period, as demonstrated by pulsing spheres on DIV 1-2 or DIV 4-5 with BrdU, then double labelling with tau and anti-BrdU antibodies (Fig. 15).

To examine the development and distribution of astrocytes, spheres were embedded at DIV 5, 7, 9, and 12, then sectioned and stained for GFAP. No astrocytes were detected at DIV 5 or 7, and very few had appeared by DIV 9. By DIV 12 there were many astrocytes (Fig. 16). In the larger tissue sections, the astrocytes appeared almost entirely at the periphery of the circle. In smaller sections of tissue, astrocytes appeared throughout the circle.

When DIV 12 spheres were plated on adhesive substrate and further cultured, the small-sized spheres appeared to consist primarily of astrocytes (Fig. 17a). Medium-sized spheres exhibited some migration of astrocytes (Fig. 17b), though the neuronal migration in these spheres extended beyond the migration of astrocytes (Fig. 18a and 18b). Few astrocytes were detected migrating out of large-sized spheres.

3. Effect of bFGF on the development of dopamine neurons in spheres.

To examine the development and distribution of dopamine neurons, spheres were embedded at DIV 5, 7, 9, and 12, then sectioned and stained for TH. Mature dopamine neurons were not detected in DIV 5 spheres. At DIV 7, there appeared small nests of TH+ cells within the spheres, typically consisting of a few dozen cells (Fig. 19a). By DIV 9 the nests had become larger and typically contained more numerous TH+ cells (Fig. 19b). A

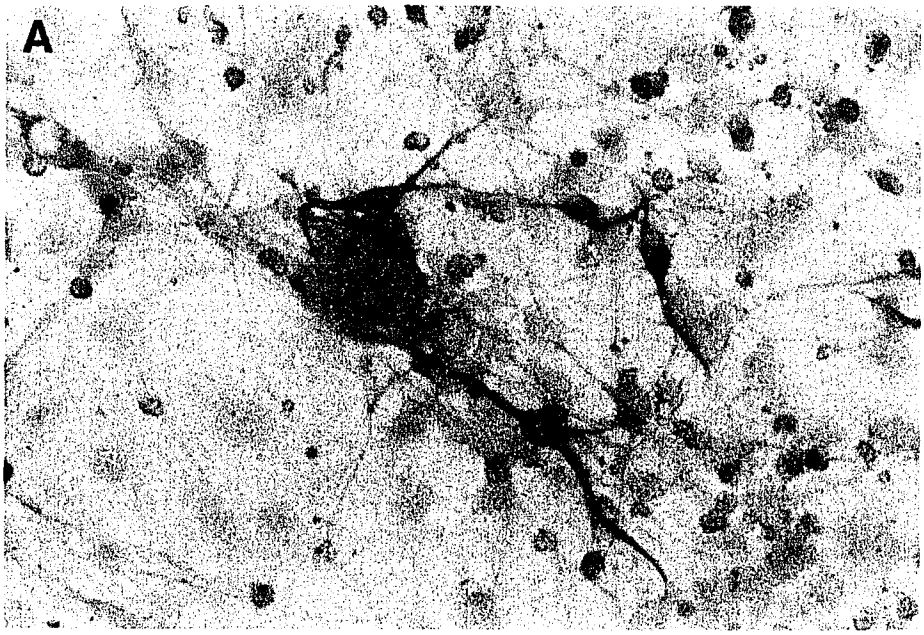


Figure 15. BrdU (black) and tau (red) double labelling demonstrating division of neuronal precursors in bFGF-treated spheres. Spheres were pulsed with BrdU on DIV 1-2 (A) or DIV 4-5 (B), cultured until DIV 12, then triturated and plated on adhesive substrate, where they were further cultured.

B

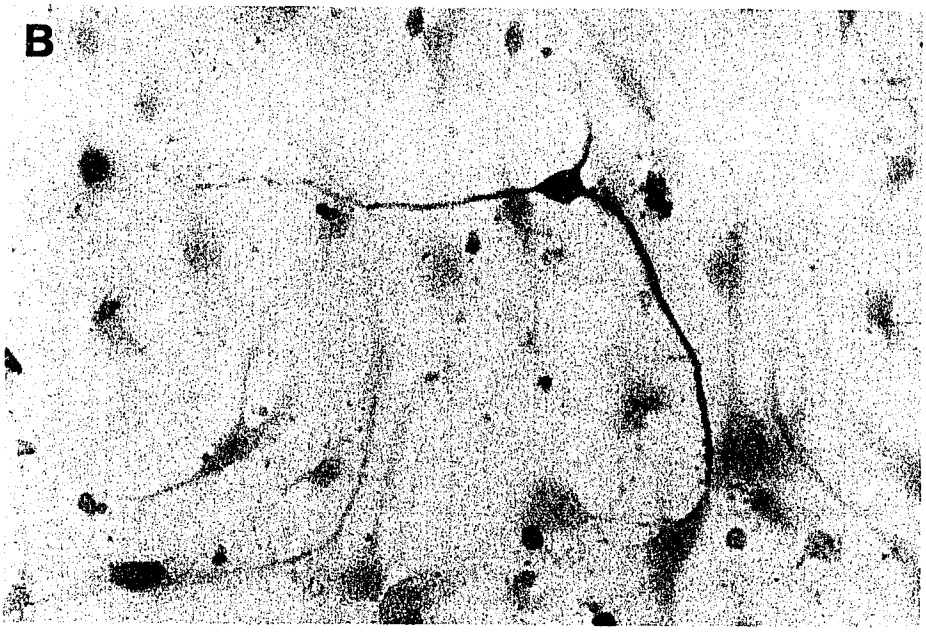


Figure 16. Two photographs of GFAP labelled cells in sections of bFGF-treated DIV 12 spheres. In larger tissue sections, astrocytes appeared almost entirely at the periphery of the circle. In smaller sections of tissue, astrocytes appeared throughout the circle. X185

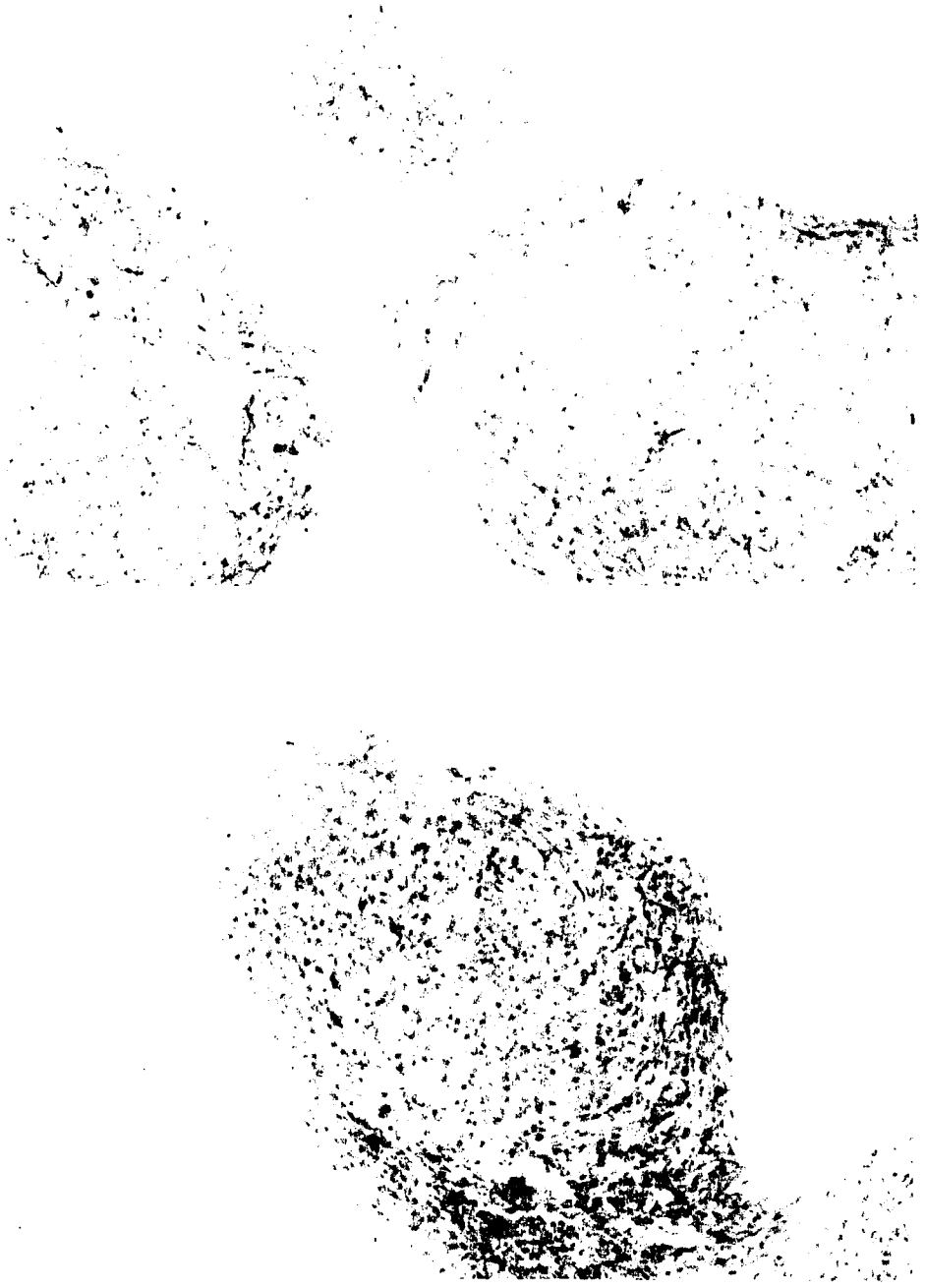


Figure 17. GFAP-labelled cells from bFGF-treated spheres grown for 12 days in suspension, then attached to adhesive substrate for an additional 7 days. Small-sized spheres appeared to consist primarily of astrocytes (A). Medium-sized spheres exhibited limited migration of astrocytes (B). Few astrocytes were detected migrating out of large-sized spheres (not shown). X185

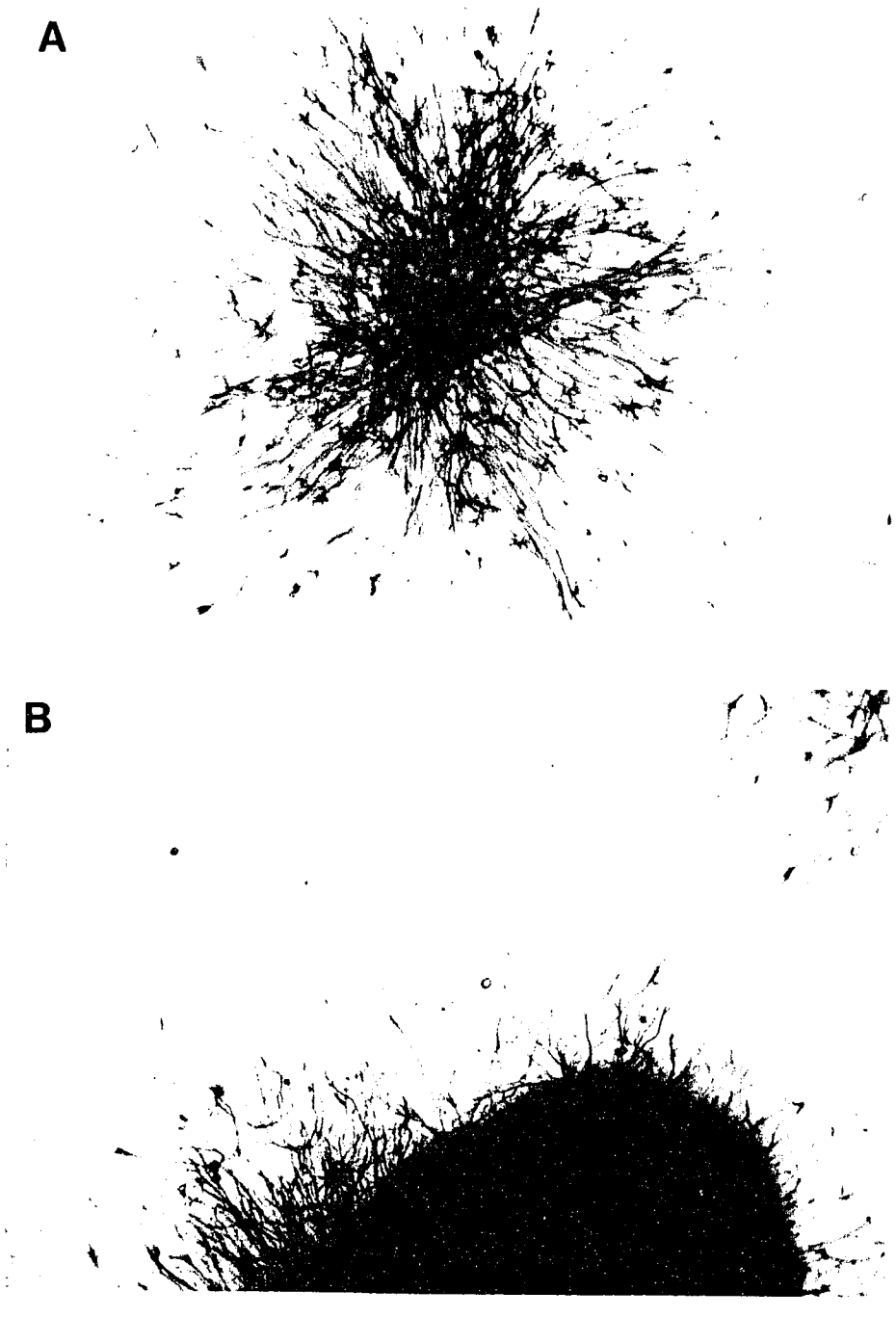

A
B

Figure 18. Photomicrograph of GFAP-labelled cells from bFGF-treated spheres grown for 12 days in suspension, then replated on adhesive substrate for an additional 7 days. There is limited migration of astrocytes out of medium-sized spheres (A). A phase micrograph of the same field shows that neuronal fiber outgrowth extended beyond astrocyte outgrowth in medium-sized spheres (B). X185

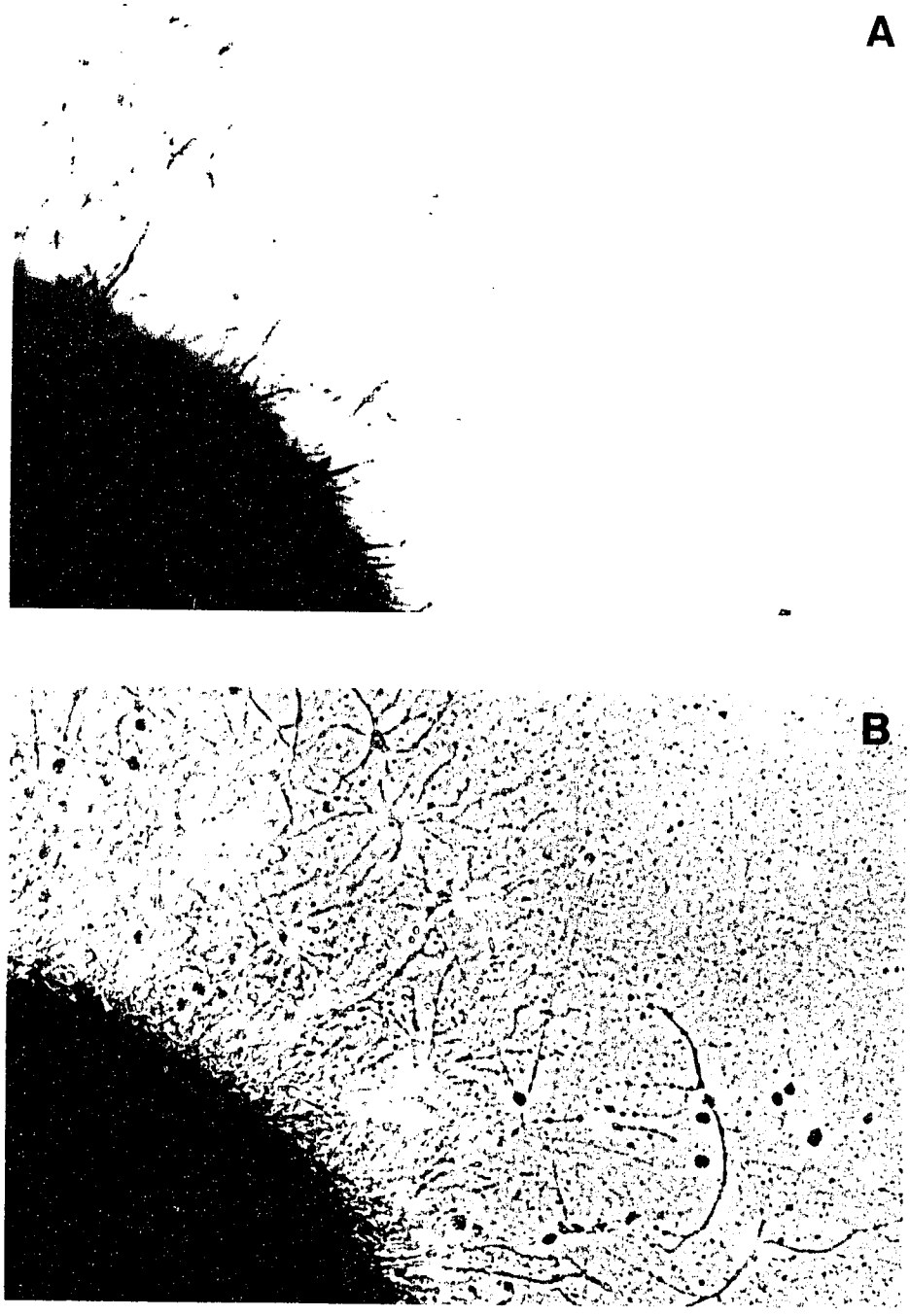

A
B

Figure 19. TH labelled cells in sections of bFGF-treated spheres. Tissue containing TH+ cells appeared to consist of several round colonies of cells: the TH+ cells appeared as a circle of stained cells, or at the periphery of a nissl-stained circle. In DIV 7 spheres (A), there were small nests of TH+ cells, typically consisting of a few dozen cells. In DIV 9 spheres (B), colonies of TH+ cells were larger and contained more numerous cells. X185


A

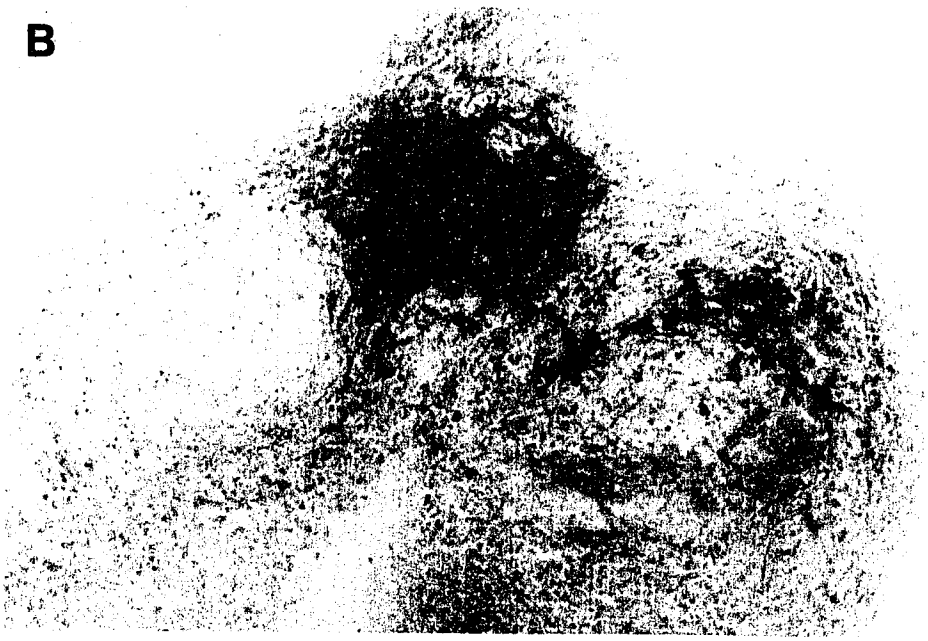
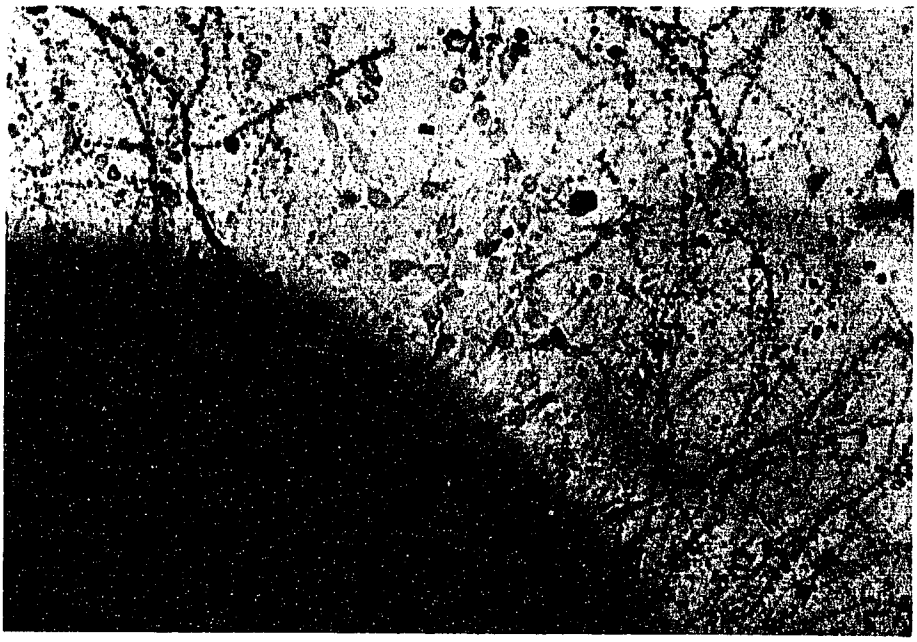
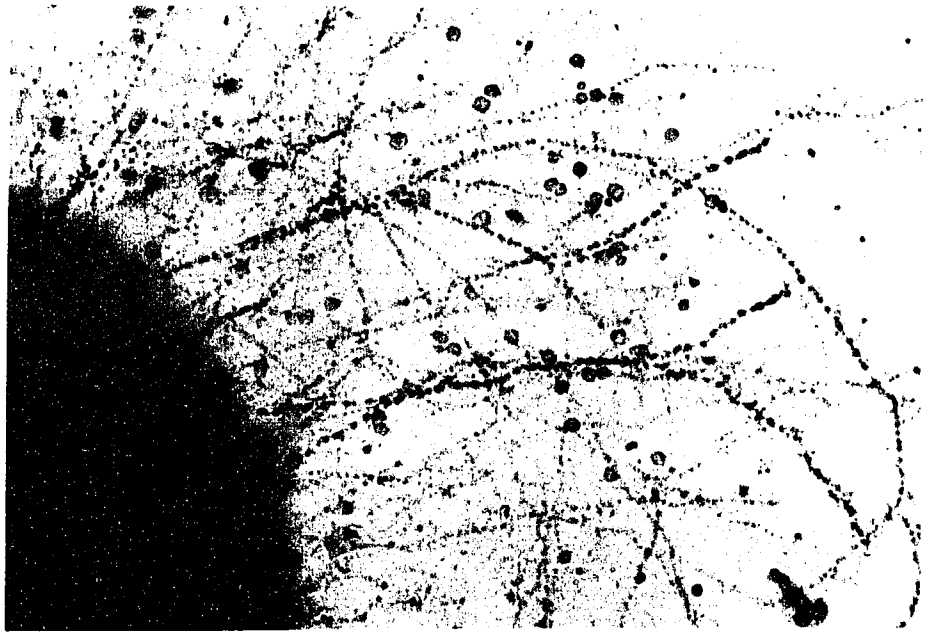

B

Figure 20: Four photographs of TH labelled cells from bFGF-treated spheres grown for 12 days in suspension, then replated on adhesive substrate for an additional 7 days. TH+ cell bodies and an extensive network of TH+ fibers emanated from large-sized spheres. TH+ fibers and cell bodies were not detected in medium and small-sized spheres. X185





given tissue section that contained TH+ cells appeared to consist of several round colonies of cells. For example, the TH+ cells themselves frequently appeared as a tight circle of stained cells (Fig. 19b), or the TH+ cells appeared at the periphery of nissl-stained circles (Fig. 19a and 19b). Occasionally, these unstained circles appeared as rosettes of growth, giving a tumor-like appearance indicative of tissue that is growing faster than its surrounding tissue (Fig. 19a).

When DIV 12 spheres were plated on adhesive substrate and further cultured, TH+ cell bodies and an extensive network of TH+ fibers emanated from large sized spheres (Fig. 20). TH+ fibers and cell bodies were not detected in medium- or small-sized spheres. The majority of large spheres contained TH+ cells. In one typical culture dish, 12 out of a total of 43 spheres were large (28%), and of those 12 large spheres 10 of them were rich in TH+ cells. ^{3}H-Dopamine uptake values were obtained for control and bFGF-treated spheres. Cultures were grown for 12 days, then attached to adhesive substrate and cultured for an additional 7 days to allow cell differentiation. The results in Table 3 show that uptake values in bFGF-treated spheres were more than six times higher than control values.

4. Effect of EGF on spheres.

The effect of epidermal growth factor (EGF) on spheres of E12 ventral mesencephalic cells was compared to that of bFGF. EGF has been shown to stimulate proliferation of neuronal precursors (Mytilineou *et al.*, 1992) and to increase survival of dopamine neurons from E16 rat ventral mesencephalon (Casper *et al.*, 1991). The effect of EGF, bFGF, and EGF + bFGF on cell division was examined and compared to untreated control cultures. Table 4 shows the results of ^{3}H-thymidine incorporation on DIV 11-12 and DIV 23-24. The EGF-treated spheres showed limited cell division at DIV 12, but by DIV 24 cell division had increased. At DIV 24, cell division in bFGF-treated

cultures had decreased from their DIV 12 values. EGF was unable to modify the effect of bFGF in co-treated cultures. When DIV 24 EGF-treated spheres were plated on adhesive substrate and further cultured, neurons and astrocytes migrated out (Fig. 21), though no TH+ cells were detected.

[^{3}H]-Dopamine Uptake in DIV 12 Spheres (fmols/culture/30 minutes)

Control	bFGF
96.3±23.7	583.8±45.1
N=4	N=6

Table 3: Spheres were grown in the presence or absence of bFGF for 12 days. They were then attached to adhesive substrate and cultured for anther 7 days to allow cell differentiation, at which time uptake values were obtained. Values represent the mean ± SEM. bFGF values were more than 6 times higher than control values.

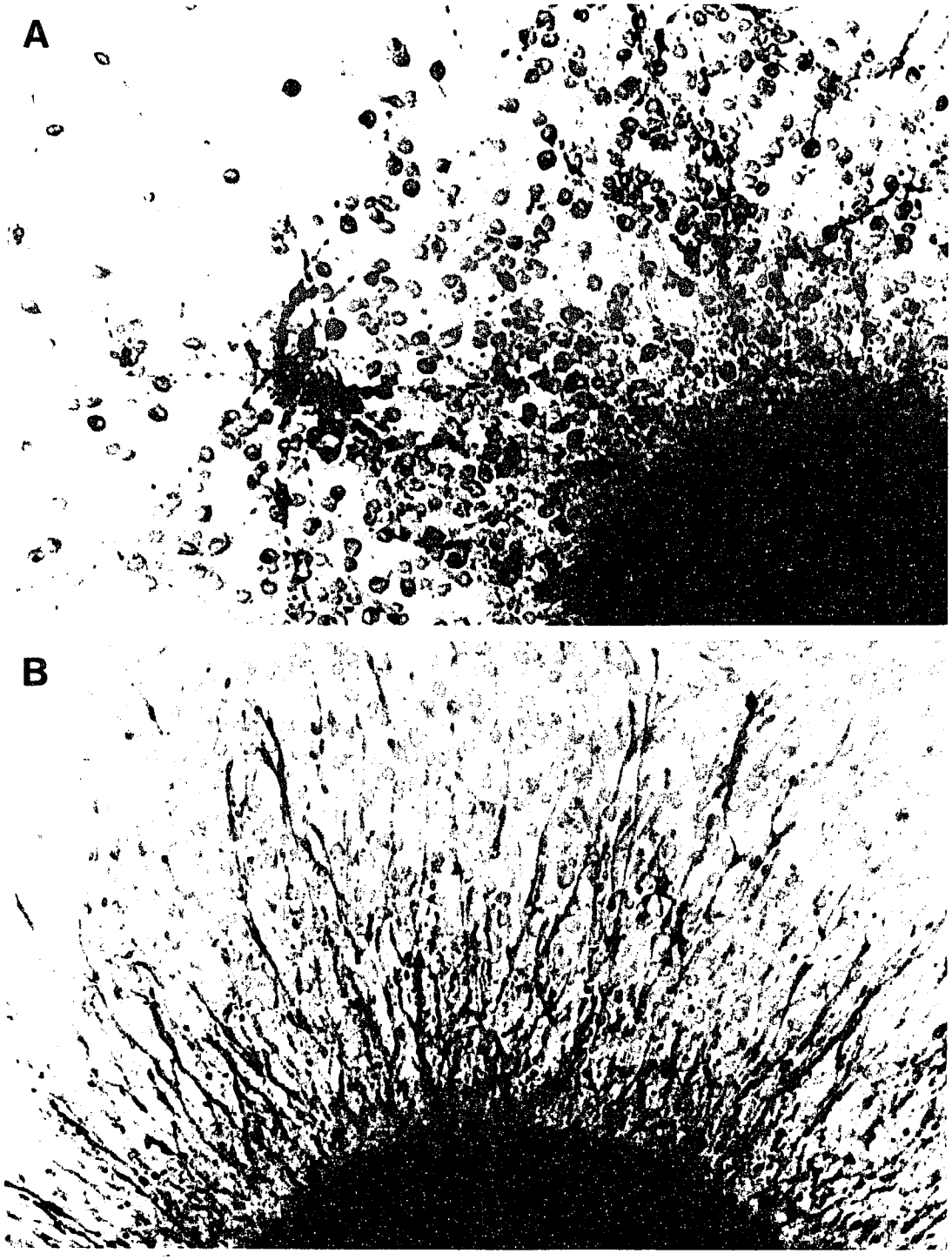
Table 4: Proliferation in DIV 12 and DIV 24 spheres treated with bFGF, EGF, or both. EGF showed limited cell division at DIV 12, but by DIV 24 cell division had increased. Cell division in bFGF-treated cultures had decreased by DIV 24 from their DIV 12 values. EGF was unable to modify the effect of bFGF in co-treated cultures.

PROLIFERATION OF MESENCEPHALIC CELLS IN NON-COATED DISHES

	[^{3}H]THYMIDINE INCORPORATION (fmols/culture/24 hrs)			
	Control	**bFGF**	**EGF**	**bFGF+EGF**
DIV 11-12	**16.0 ± 1.2** N=9	**210.3 ± 7.9[a]** N=8	**19.8 ± 6.1** N=8	**241.3±17.3[a]** N=9
DIV 23-24	**15.0 ± 0.6** N=10	**125.5 ± 8.8[a]** N=10	**224.9±11.8[a,b]** N=10	**112.9 ± 1.7[a]** N=10

[a]Significantly different from Control $p<0.001$; [b]different from bFGF and bFGF+EGF $p<0.001$; Analysis of variance followed by Tukey post-hoc test.

Figure 21: EGF-treated spheres grown for 24 days in suspension, then replated on adhesive substrate for an additional 7 days. Tau+ (A) and GFAP+ (B) cells and processes migrated out of spheres. No TH+ cells were detected. X210


A
B

D) DISCUSSION

We have previously shown that bFGF expands the period of division of dopamine precursors from approximately 1 day to at least 8 days *in vitro* (Chapter I). This increase in division was accompanied by a delay in differentiation, with the eventual consequence of a larger quantity of dopamine neurons. In the present study we sought to examine this effect on tissue cultured in suspension, which is a more appropriate form for transplantation. We cultured E12 ventral mesencephalon on dishes that lacked an adhesive coating, and maintained them on a rotating shaker in order to discourage cell attachment. This process generated spheres of cells. Cultures that were not treated with growth factors exhibited little cell division and limited survival. Spheres treated with bFGF exhibited robust cell division across 12 days of culture, resulting in spheres that were distinctly large, medium, or small.

When these 12 day spheres were plated on adhesive substrate and further cultured, dopamine neurons were found migrating exclusively out of large spheres. They were not detected in medium or small spheres. Large spheres had a mean area of 1.26 mm^2, and constituted about one quarter of the spheres in a typical culture dish. Virtually all of them contained dopamine neurons, and contained them in large numbers, with typically about half the processes and cell bodies migrating out being dopaminergic. In addition to dopamine neurons, there were many non-dopaminergic neurons that migrated out of these spheres, though astrocytic migration was limited. Medium-sized spheres consisted primarily of neurons, with some migration of astrocytes, and small spheres consisted primarily of astrocytes, though some neurons were also detected.

Sections of spheres containing dopamine neurons at DIV 7, 9, and 12 consisted of several round colonies of cells, with dopamine neurons either forming a discrete round colony, or appearing at the periphery of a nissl stained rosette. BrdU labelling at these

time points showed that dividing cells also appeared in discrete circles within the spheres. We propose that these spheres consist of a mosaic of clones, arising from several dividing precursor cells that were still attached to each other at the time of plating. Each round clone contains dividing cells at the center, with differentiated cells migrating out and appearing at the periphery. This scenario would represent a spherical approximation of that which normally occurs in the ventricular zone of the embryo, where dividing cells are in the lumen and neurons leave the mitotic cycle to migrate out along radial glia. It has been shown that dissociated neural precursor cells from E10 mouse can reconstruct neural tube-like structures when cultured in a collagen gel matrix (Tomooka *et al.*, 1993).

BrdU staining revealed that dividing cells began appearing at the periphery of spheres at DIV 9. These dividing cells were probably astrocytes, which also began appearing at DIV 9, typically along the periphery. Neuroepithelial cells destined to become astrocytes may have migrated out from the center of the round colonies, through the neurons, to become astrocytes residing at the periphery of the sphere. In smaller sections of tissue, astrocytes were not detected at the periphery, but rather were found uniformly throughout the tissue. This tissue may represent sections of small-sized spheres, which are composed primarily of astrocytes. Conversely, they may constitute the extreme edge of a larger sphere, where only peripheral tissue would be represented.

About half the neurons in the large-sized spheres were not dopaminergic, and occasionally there was a large sphere that contained no dopamine neurons. It is possible that these cells are GABAergic. It is known that the GABAergic neurons of the substantia nigra pars reticulata originate from the same part of the neuroepithelium and migrate out at the same time as the dopamine neurons of the pars compacta (Hanaway *et al.*, 1972; Altman and Bayer, 1981). It is therefore likely that our cultures contain significant numbers of GABAergic neurons. In addition, it has been shown that bFGF is a mitogen to GABA precursors (Deloulme *et al.*, 1991) and that bFGF is neurotrophic to GABA neurons cultured from E14 rat mesencephalon (Ferrari *et al.*, 1989; Ferrari *et al.*, 1991).

A study by Abney *et al.* (1981) showed that in rat brain cultures isolated after E10, the time course of the development of astrocytes and various subclasses of neurons *in vitro* parallels their *in vivo* developmental time course. Dopamine neurons are normally born between E11 and E15. In our spheres isolated from E12 rat, dopamine neurons do not appear until DIV 7, corresponding to E18-19 in the embryo. Therefore dopamine neurons do not develop on schedule in our cultures. In addition, astrocytes do not appear until DIV 9, corresponding to E20-21 in the embryo, which is past the point that they normally appear. Therefore bFGF appears to be delaying the differentiation of both dopamine neurons and astrocytes in our cultures.

The appearance of astrocytes after the differentiation of dopamine neurons would indicate that the effect of bFGF on dopamine precursors in these spheres is not glial mediated. The neurotrophic effect of bFGF on E18 rat hippocampal cultures has been shown to be a direct effect, independent of glia (Walicke and Baird, 1988). Recently, a method was developed to generate purified cultures of dopamine neurons by injecting a fluorescent dye into the embryonic striatum, from where it would retrogradely label nigral dopamine neurons,. then isolating these cells using a cell sorter. There was a direct and robust trophic effect of bFGF on these purified dopamine neurons (Romero *et al.*, 1994). Conversely, the trophic effects of bFGF on dopamine neurons from E14.5 rat mesencephalon have been shown to be glial mediated (Engele and Bohn, 1991). We cannot rule out the possibility that astrocytic precursors may play a role in the bFGF effects on our cultures.

The identity and patterning of cell types in the neural tube can arise by induction of neurons via diffusible factors, homeogenetic signals, or direct contact with other tissue (Placzek *et al.*, 1993; Yamada *et al.*, 1993). Recently, it was shown that midbrain dopamine neurons can be induced by contact with the floor plate (Hynes *et al.*, 1995). In light of these findings, it seems possible that in our cultures, bFGF may be acting in part

by increasing tissue that can induce the dopamine neuron phenotype either directly or through diffusible factors.

The purpose of culturing tissue on non-adhesive substrate on a rotating shaker was to generate suspension cultures, which would be more appropriate for transplantation. In addition, it was thought that a lack of adhesive substrate on which to attach would maintain the cells in a proliferative state for a longer period of time. Indeed, there was twice as much ^{3}H-thymidine incorporation in DIV 11-12 spheres than there was in monolayer cultures on DIV 12-13 (see Chapter I). However, the appearance of dopamine neurons in the two culture paradigms was roughly the same, occurring between DIV 5 and 9. Therefore, the delay in differentiation of dopamine neurons did not seem to be enhanced by denying the cells an adhesive substrate. Moreover, the ^{3}H-dopamine uptake value in DIV 13 monolayer cultures was more than 6 times higher that of DIV 12 spheres. Perhaps the differentiative capability of dopamine neurons in spheres would be improved by providing the spheres with an adhesive substrate on DIV 7 or 9, when dopamine neurons are beginning to differentiate.

It seems surprising that EGF did not have a mitogenic effect on DIV 12 spheres. EGF and transforming growth factor α (TGF-α) mRNAs have been detected in E14 mouse brain (Lazar and Blum, 1992), and EGF has neurotrophic and mitogenic effects on mesencephalic cells from E16 rat (Casper *et al.*, 1991; Mytilineou *et al.*, 1992). However, when E14 mesencephalic cultures are treated with EGF, they show no neurotrophic effect at 10 days, and a pronounced neurotrophic effect at 20 days, despite the fact that astrocytes constitute less than 0.1% of the cells in these 20 day cultures (Ferrari *et al.*, 1991). Our cultures show a similar pattern, being unresponsive to EGF at DIV 12, and becoming responsive at DIV 24. The inability of cells to initially respond to EGF may be the result of the altered development of this tissue. It is possible that by isolating the tissue at such an early age, we have delayed the appearance of the cell machinery needed to respond to EGF, or that we have delayed the differentiation of a cell type that responds

to EGF, such as astrocytes. The lack of dopamine neurons in EGF-treated spheres may also be a result of isolating the tissue from such an immature embryo. Perhaps the information needed to induce the dopaminergic phenotype had not yet appeared, and was not subsequently provided in these cultures. If this is the case, it would mean that the addition of bFGF to our cultures was sufficient to induce the dopamine phenotype or allow for the survival of dopamine precursors. This would also help explain the inability of EGF to modify the effect of bFGF in co-treated cultures.

We believe that we have established conditions to produce tissue that is more appropriate for use in transplantation in Parkinson's disease than the currently used paradigm employing fresh embryonic tissue. Further experiments involving the transplantation of this tissue into striatum of lesioned animals would explore the ability of this tissue to integrate into the host striatum, differentiate into dopamine neurons, secrete dopamine, and ameliorate behavioral deficits.

CONCLUSION

The substantia nigra is a midbrain structure which derives from the mesencephalon. The substantia nigra has two parts, the pars compacta which is situated dorsomedially and the pars reticulata which is situated ventrolaterally. The pars compacta contains neurons whose cell bodies display strong dopamine fluorescence (Dahlström and Fuxe, 1964.) The axons of these neurons project to the caudate nucleus and putamen, forming the nigrostriatal pathway (Anden *et al.*, 1965; Bedard *et al.*, 1969; Moore *et al.*, 1971). Neuronal degeneration and depigmentation in the substantia nigra, leading to decreased dopamine in the caudate nucleus and putamen, are the characteristics of Parkinson's disease. Akinesia, rigidity, and tremor caused by this depletion are the symptoms of a person suffering from Parkinson's disease.

The treatment of choice for Parkinsonism is L-dopa in combination with peripheral dopa decarboxylase inhibitors, and often dopamine agonists, anticholinergics or more recently the MAO B inhibitor deprenyl. However, the efficacy of this treatment varies in different patients and creates severe side effects after prolonged use.

Animal models of Parkinsonism have been established in which there is a deficiency of nigrostriatal dopamine due to treatment with either 6-OHDA or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). These animal models have been used to explore the possibility of using brain grafts as a replacement therapy for Parkinson's disease. Fetal mesencephalic or adrenal medullary grafts can restore lost function in experimental animals with nigrostriatal dopaminergic denervation (see review by Björklund *et al.*, 1987). This functional recovery in animal models has encouraged the use of transplants for treatment of parkinsonian patients (Backlund *et al.*, 1987; Sladek and Gash, 1988; Lindvall *et al.*, 1990; Freed *et al.*, 1990; Madrazo *et al.*, 1990).

The embryonic cells used for transplantation are usually young, differentiated neurons, which seem to survive best. Therefore, one difficulty with this treatment is the need to have available enough fetal tissue of the appropriate developmental stage at the time the transplantation is to occur. It currently requires on the day of surgery between four and ten fresh human embryos of the same gestation for a single transplant. Another difficulty with brain grafts is the poor survival of the transplanted cells within the host brain.

The goal of this research was to explore the possibility to manipulate the proliferation and differentiation of dopamine precursors in vitro. We used the novel approach of culturing very immature cells from the ventral mesencephalon of E12 rat embryos, a time which coincides with the beginning of the birth of dopamine neurons of the pars compacta. We report that by treating these young precursors with bFGF, one can expand the period over which they divide from approximately 1 day to at least 8 days *in vitro*. This increase in cell division resulted in the eventual formation of larger colonies of dopamine neurons in bFGF-treated cultures. We have therefore established conditions to expand *in vitro* a cell population for transplantation. This approach would eliminate the need to have available large quantities of appropriate aged, fresh embryonic tissue on the day of transplant surgery. In addition, this cell population is enriched in dopamine precursors, which when transplanted may survive and integrate better than differentiated neurons. Finally, our culture conditions establish a window of time between collection of fetal tissue and grafting, during which tissue could be properly screened for infectious agents, and collected and cryopreserved for subsequent use.

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