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REGULAR ARTICLE

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Cytokine-induced conversion of mesencephalic-derived progenitor cells into dopamine neurons

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Abstract We have previously shown that a combination of the cytokines interleukin (IL)-1, IL-11, leukemia inhibitory factor (LIF), and glial cell line-derived neurotrophic factor (GDNF) can convert rat fetal (E14.5) mesencephalic progenitor cells into tyrosine hydroxylase (TH)-immunoreactive (ir) neurons in vitro. The experiments described here characterize the mesencephalic progenitor cells and their cytokine-induced conversion into dopamine (DA) neurons. For all experiments, we used bromodeoxyuridine (BrdU)-ir cultures of (E14.5) mesencephalic progenitor cells that had been expanded at least 21 days. We first demonstrated that IL-1 induced DA neuron conversion in mesencephalic progenitors, but not in striatal progenitors ($P<0.001$). Thus, these cells should be classified as lineage-restricted progenitors, and not omnipotent stem cells. To further characterize cell populations in these cultures, we used monoclonal antibodies against Hu (an early marker for neurons), growth-associated protein (GAP)-43 (a marker for neuronal process extension), TH (a marker for DA neurons), and glial fibrillary acidic protein (GFAP, a marker for astrocytes). We assessed (E14.5) mesencephalic progenitor cell cultures (plated at 125,000 cells/cm^2) incubated in the cytokine mixture (described above) or in complete media (CM, negative control). Following 7 days incubation, GFAP-positive cells formed a nearly confluent carpet in both types of cultures. However, numbers of Hu-ir and GAP-43-ir cells in the cytokine-incubated cultures far exceeded those in CM-incubated controls ($P=0.0003$, $P=0.0001$, respectively), while numbers of TH-ir cells were 58-fold greater in the cytokine-incubated cultures versus CM-incubated controls. The TH phenotype persisted for 7 days following withdrawal of the differentiation media. Numerous double-labeled cells that were BrdU-ir and also TH-ir, or Hu-ir and also TH-ir, were observed in the cytokine-incubated cultures. These data suggest that cytokines "drive" the conversion of progenitor cells into DA neurons.

Key words Transplantation · Parkinson's disease · CNS fetal development · CNS differentiation · Neurotrophic factors · Rat

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Introduction

Progenitor cells are pluripotent, self-renewing cells that are lineage restricted (Anderson 1989; McKay 1997; Ray et al. 1997; Stemple and Mahanthappa 1997). Thus, unlike omnipotent stem cells, which theoretically can differentiate into any cell type, progenitor cells can differentiate into only a restricted set of cell types (McKay 1997; Ray et al. 1997). Numerous studies have shown that self-renewing cells residing in the subependymal zone of the adult and fetal brain can migrate and then differentiate into neurons and glia (Cattaneo and McKay 1990; De Vitry et al. 1980; Fredriksen et al. 1988; Levison and Goldman 1993; Lois and Alvarez-Buylla 1993, 1994; Luskin 1993; Luskin and McDermott 1994; Morshead and van der Kooy 1992; Winkler et al. 1998). Hynes and colleagues (1995a,b) have shown that mesencephalic floor plate cells induce the differentiation of mesencephalic subependymal cells into a specialized phenotype, the dopamine (DA) neuron. These studies also showed that if mesencephalic subependymal cells were transplanted to a more rostral location, none of these subependymal cells differentiated into DA neurons (Hynes et al. 1995a,b), suggesting that local environmental factors play a role in the differentiation of mesencephalic subependymal cells into DA neurons. These data also support a hypothesis of lineage restriction, with respect to differentiation into a specialized cell type (the DA neuron) that

is specific to location along the rostral-caudal axis of the neural tube (Hynes et al. 1995b). Yet clearly this local environmental effect is constrained by the innate genetic predisposition of the mesencephalic subependymal cells to differentiate into neurons and glia (and not other cell types). Thus, these data demonstrate that local environmental factors and a genetic predisposition limiting differentiation to specific cell types together play a role in the differentiation of a subpopulation of the mesencephalic subependymal cells into DA neurons (Hynes et al. 1995a,b; Jessel and Dodd 1990; Kilpatrick and Bartlett 1993; McKay 1997; Richards et al. 1992; Temple 1989).

Recently, many researchers have cultured fetal and adult subependymal cells isolated from the striatum and mesencephalon using media supplemented with the mitogen epidermal growth factor (EGF) (Reynolds et al. 1992; Reynolds and Wiess 1992; Ptak et al. 1995; Svendsen et al. 1995). Subependymal cells cultivated in the EGF-supplemented media are self-renewing and capable of division for several months (Reynolds and Weiss 1996; Ptak et al. 1995; Svendsen et al. 1995). When grown in poly-l-lysine (PLL)-coated tissue culture wells in serum-containing media, the mesencephalic and striatal subependymal cells differentiate into neurons and glia. The cultured striatal subependymal cells differentiate into the glia and GABAergic neurons normally found in the striatum (Reynolds et al. 1992). However, cultured mesencephalic subependymal cells rarely differentiate into DA neurons (Svendsen and Rosser 1995). This finding suggests that additional environmental factors play a critical role in DA neuron differentiation. In support of this, we have recently shown that E14.5 rat mesencephalic subependymal cells can be induced to express the DA neuron phenotype by exposure to certain cytokines (Ling et al. 1998). When we incubated fetal mesencephalic subependymal cells in media containing interleukin-1 (IL-1), IL-11, leukemia inhibitory factor (LIF), glial cell-line derived neurotrophic factor (GDNF), striatal cell-conditioned media (15%) and flash-frozen mesencephalic cellular fragments (10%), the subependymal cells differentiated into cells that: (1) morphologically resembled mature DA neurons; (2) were immunoreactive to the DA neuron markers tyrosine hydroxylase (TH), dopa-decarboxylase (DDC), DA transporter (DAT), and DA neurotransmitter; but were (3) immunonegative to the noradrenergic (NE) neuron marker, DA-beta-hydroxylase (DβH). The converted TH-ir cells represented approximately one-half of the neurofilament-immunoreactive (NF-ir) cells. In addition, numerous glial fibillary associated protein (GFAP), and GAL-C-immunoreactive cells (markers for astrocytes and oligodendrocytes, respectively) were found in the cytokine-exposed cultures. These data suggest that hematopoietic cytokines, which regulate the differentiation and expansion of cells in the hematopoietic system, can perform a similar function in the central nervous system (CNS).

Although our recent experiments demonstrate that cytokines are capable of inducing DA neuron differentiation, many questions concerning the differentiation process remain. First, can cytokines induce the DA neuron phenotype in subependymal cells isolated from other CNS locations, or is the cytokine-induced DA neuron conversion unique to mesencephalic subependymal cells? If subependymal cells from many CNS locations can be converted into DA neurons following exposure to the same cytokines, this argues for classifying these subependymal cells as stem cells (Reynolds and Weiss 1996). If, on the other hand, only mesencephalic subependymal cells can be converted through cytokine exposure into DA neurons, then lineage restriction has already occurred, and these subependymal cells should be classified as progenitors. Second, do cytokines induce the conversion of the subependymal cells into neurons, or do the cytokines convert neurons into the DA neuron phenotype? Third, once cytokine exposure converts mesencephalic subependymal cells into DA neurons, will the converted cells revert to a non-DA phenotype following cytokine withdrawal? If the DA phenotype remains stable following cytokine withdrawal, this could argue in favor of cytokine involvement in the differentiation process, as these particular cytokines may be absent in the adult brain. The following set of experiments was designed to answer these three questions.

Materials and methods

Reagents used

Glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) were gifts from Amgen (Thousand Oaks, CA), and the monoclonal Hu antibody was a generous gift from Dr. Henry M. Furneaux, Sloan Kettering Institution (New York, NY). Epidermal growth factor (EGF) was purchased from Sigma Chemical (St. Louis, MO), and the remaining cytokines were purchased from R & D Systems (Minneapolis, MN). Dulbecco's modified Eagle's media (DMEM), F-12 nutrient mixture (F-12), and Hanks' buffered saline solution (HBSS), fetal calf serum (FCS) and B27 supplement were purchased from Gibco (Grand Island, NY). Normal horse serum (NHS), normal goat serum (NGS), penicillin-streptomycin (Pen-Strep), trypsin, deoxyribonuclease (DNase), transferrin, putrescine, sodium selenite, progesterone, insulin, glucose, glutamine, Triton X-100, 3-3' diaminobenzidine (DAB), nickel sulfate, imidazole (IA), sodium acetate, Trizma base, sodium and potassium phosphates were all purchased from Sigma Chemical (St. Louis, MO). The anti-rat monoclonal tyrosine hydroxylase (TH) primary antibody (1:5000) was purchased from Incstar (Stillwater, MN). The anti-rabbit polyclonal TH primary antibody (1:250) was purchased from Chemicon (Temecula, CA). The anti-mouse monoclonal growth-associated protein (GAP)-43 antibody (1:2000) was purchased from Boehringer Mannheim (Indianapolis, IN). The anti-mouse monoclonal glial fibrillary associated protein (GFAP) antibody (1:2000) was purchased from Sternberger Monoclonal (Baltimore, MD). The biotinylated anti-mouse secondary antibody (IgG), fluorescein (FITC)-avidin, Texas red-streptavidin, and avidin-biotin complex (ABC) were purchased from Vector Labs (Burlingame, CA). Neuropore used to prepare cultures for BrdU labeling was purchased from Trevigen (Gaithersburg, MD). BrdU labeling was performed using a kit (No. RPN20) from Amersham (Arlington Heights, IL).

Subependymal cell isolation, expansion, and preparation

The fetal mesencephalic subependymal cells (E14.5) were isolated according to methods previously described (Ptak et al. 1995; Svendsen et al. 1995). Striatal progenitor cells were isolated according to the methods of Reynolds and Weiss (1992). Mesencephalic and striatal cultures were expanded according to the methods described by

Svendsen et al. (1995). Briefly, the mesencephalic or striatal tissues were micro-dissected from fetal rats under sterile conditions, incubated in 0.1% trypsin for 30 min at 23°C, incubated in DNase (40 μg/ml) for 10 min at 37°C, gently triturated into a cell suspension using a fire-polished pipette, and plated into 25-cm^2 flasks (400,000 cells/ml) in 5 ml "expansion media" [F12/DMEM 1:3; 100 U penicillin/100 μg streptomycin (Pen-Strep)/ml; 2 ml B27 per 100 ml media; 20 ng/ml EGF] for 21 days. The yield from this procedure was approximately 50–100 "proliferation spheres" per cm^2. These cells have been passaged up to 4 months by feeding the cells once a week with expansion media, and gently triturating the spheres and reseeding the cells into new 25-cm^2 or 75-cm^2 flasks (filled with 10 ml expansion media) every 2 weeks.

Cytokine incubation of subependymal cells

Following incubation in expansion media, the proliferative spheres were triturated and the cells added (125,000 trypan blue-excluding cells/cm^2) to PLL-coated 48-well plates and incubated 7 days in either complete media, "cytokine-enriched" media, or "differentiation media." Complete media (CM) consisted of DMEM/F12 1:1, 10% FCS, and 1% Pen-Strep. Cytokine-enriched media consisted of CM and each of the cytokines listed in Table 1. The concentrations of cytokines used were 5 times the ED_{50} values recommended by the manufacturers (see Table 1). The differentiation media consisted of CM supplemented with the cytokines IL-1 (200 pg/ml), IL-11 (1 ng/ml), LIF (1 ng/ml), and GDNF (1 ng/ml), freeze-fractured mesencephalic membrane fragments (10%w/v) and striatal conditioned media (15%). We have previously demonstrated (Ling et al. 1998) that while IL-1 induces the DA neuron phenotype in mesencephalic subependymal cultures, a combination of IL-1, IL-11, LIF, and GDNF, mesencephalic fragments, and striatal conditioned media increased numbers of converted DA neurons, and also produced a mature morphology in the converted DA neurons. The mesencephalic membrane fragments were prepared as previously described (Ling et al. 1998) by plating out E14.5 mesencephalic primary cultures at 250,000 cells/cm^2. After 3 days, the complete media were removed and cultures were washed in CM, then placed in a –80°C freezer for 1 h and thawed at 23°C three successive times. The membrane fragments were collected and stored at –20°C until use. The striatal-conditioned media were collected after 72 h from E14.5 primary cultures of the lateral ganglionic eminence (striatal primordia) plated at 250,000 cells/cm^2 and grown in defined media [DM: DMEM/F12 1:1, 1% Pen-Strep., transferrin (100 μg/ml), putrescine (60 μM), sodium selenite (30 nM), progesterone (20 nM), insulin (25 μg/ml), glucose (0.63 g/100 ml), and glutamine (2nM)].

Immunocytochemistry

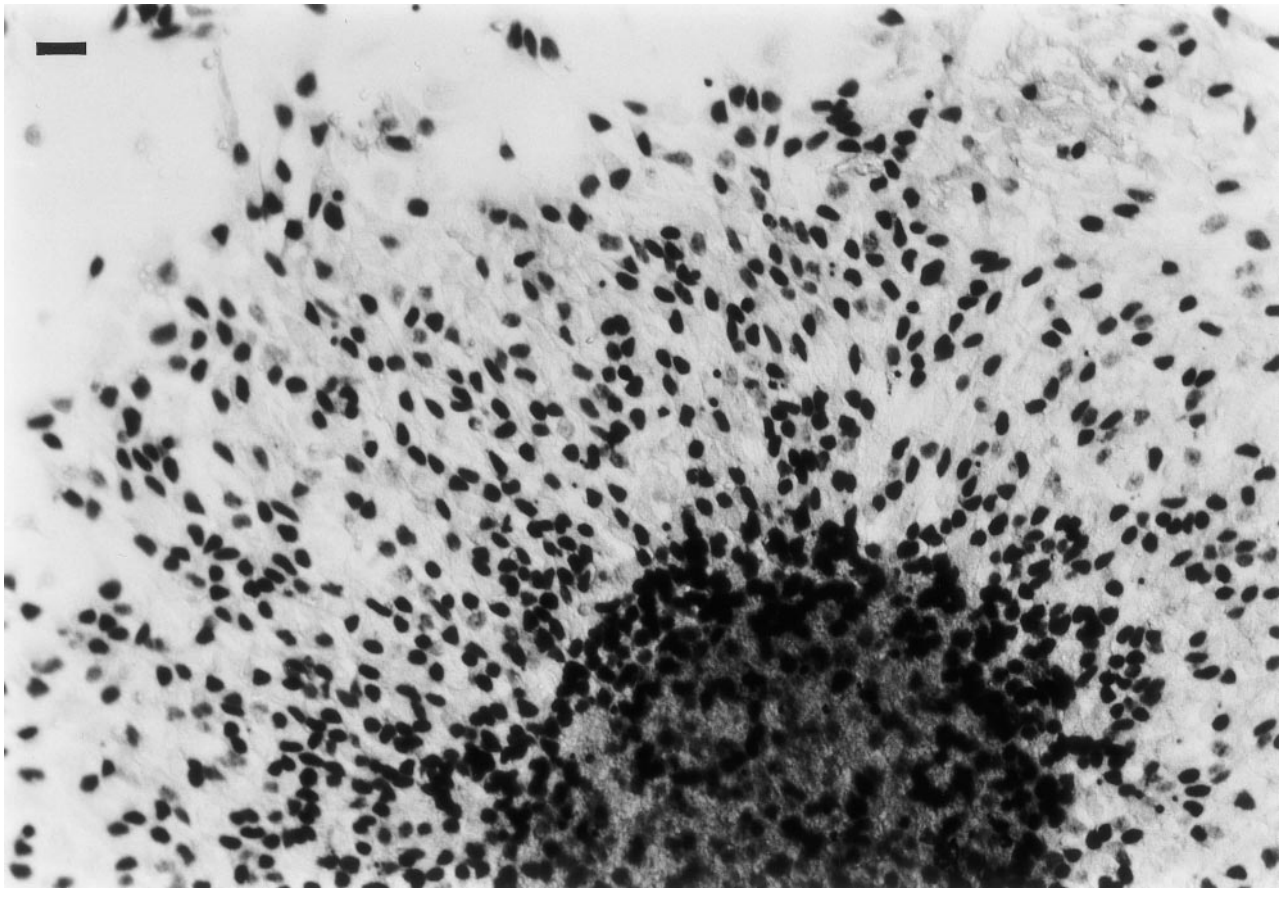
Following the prescribed incubation the cultures were fixed for 20 min and TH immunocytochemistry was performed according to methods described earlier (Ptak et al. 1995). Briefly, the cultures were fixed in 3.7% formalin/phosphate-buffered saline (PBS) solution for 20 min at 23°C, incubated 1 h in blocking solution consisting of Triton X-100 (0.2%)/normal horse serum (NHS:10%)/TRIS-buffered saline (TBS), and incubated in monoclonal anti-rat TH primary antibody (1:5000; Incstar) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG (0.5%), and incubated 1 h in avidin-biotin complex (ABC). The TH stain was developed using DAB (0.05%) and nickel sulfate (2.5%) during a 1-min incubation at room temperature. For Hu (1:4000) immunocytochemistry, the methods were the same. Hu is an RNA-binding protein that acts as a specific marker for neurons and for progenitor cells that are committed to express the neuronal phenotype (Barami et al. 1995; Marusich et al. 1994). For GFAP (1:2000) immunocytchemistry, the methods were the same, except 5% NHS was used in the blocker. GFAP is a marker for astrocytes. For (GAP)-43 (1:2000) immunocytochemistry, the methods were the same, except 5% NHS and no Triton was used in the blocker. GAP-43 is a marker for neurons extending processes. BrdU labeling was performed using Vector and Amersham reagents and a modified protocol from Amersham (Arlington Heights, IL) kit (RPN-20). BrdU labeling is a marker for cell proliferation. Double labeling with BrdU and polyclonal anti-rabbit TH antibody was used to assess whether progenitor cells have converted into TH neurons. Double labeling with Hu and polyclonal anti-rabbit TH antibody was used to assess whether TH-positive cells were also Hu-positive neurons.

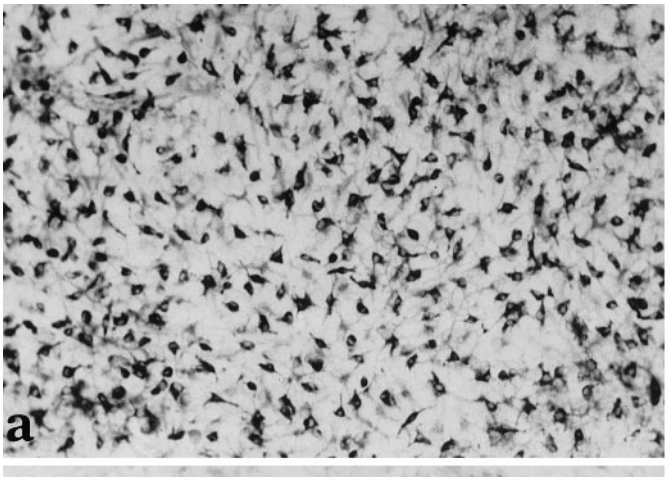
For BrdU and TH double labeling, the cultures were fixed as described above. To prepare the formalin-fixed cultures for BrdU labeling, the cells were incubated 5 min in 70% EtOH, 10 min in PBS, and 10 min in 0.2% Triton/TBS. The cells were then incubated in a 50:50 solution of the monoclonal BrdU antibody (1:100) and Neuropore (Trevigen) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG:0.5%, and incubated 1 h in Texas red streptavidin (1:100). Following three washes in PBS, the cultures were incubated 1 h in a blocking solution consisting of Triton X-100 (0.2%)/NGS(5%)/TBS, and incubated in the polyclonal anti-rabbit TH primary antibody (1:250; Chemicon) overnight at 4°C. The cultures were incubated 1 h in anti-rabbit IgG:0.5%, and incubated 1 h in FITC-avidin (1:300).

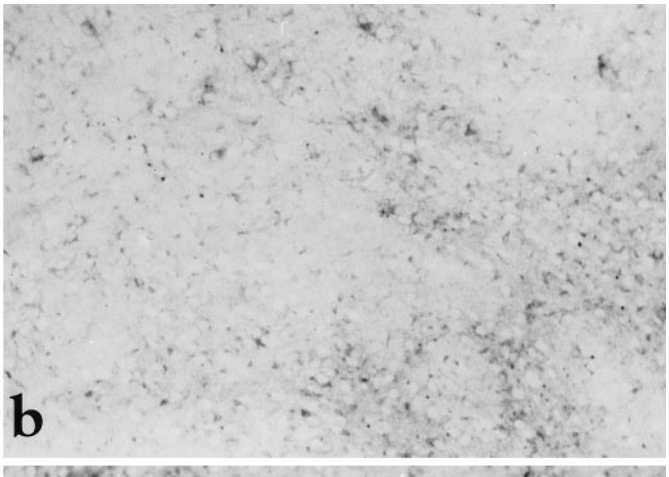
For Hu and TH double labeling, the cultures were fixed as described above, incubated 1 h in blocking solution consisting of Triton X-100 (0.2%)/NHS(10%)/TBS, and incubated in monoclonal anti-rat Hu primary antibody (1:4000) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG(0.5%), and incubated 1 h in Texas red streptavidin (1:100). Then following three washes in PBS, the cultures were incubated 1 h in a blocking solution consisting of Triton X-100 (0.2%)/NGS(5%)/TBS, and incubated in polyclonal anti-rabbit

Table 1 Effect of cytokines in conversion of progenitors into DA neurons [*"M"* mesencephalic progenitors (125,000 cells/cm^2) incubated 7 days in complete media supplemented with the designated cytokine (see below), *"S"* striatal progenitors (125,000 cells/cm^2) incubated 7 days in complete media supplemented with the designated cytokine, *IL-1* interleukin-1 (200 pg/ml), *IL-2* (2 ng/ml), *IL-3* (1 ng/ml), *IL-4* (5 ng/ml), *IL-6* (200 pg/ml), *IL-9* (100 pg/ml), *IL-11* (1 ng/ml), *CSF* colony-stimulating factor (1 ng/ml), *TGFβ* transforming growth factor beta (1 ng/ml), *TGFα* (1 ng/ml), *LIF* leukemia inhibitory factor (1 ng/ml), *EGF* epidermal growth factor (100 ng/ml), *SCF* stem cell factor (40 ng/ml), *βFGF* beta fibroblast growth factor (100 ng/ml), *EPO* erythropoietin (0.5 U), *INFγ* interferon gamma (100 ng/ml), *TNFα* tumor necrosis factor alpha (200 pg/ml), *GDNF* glial-cell-line-derived neurotrophic factor (1 ng/ml), *BDNF* brain-derived neurotrophic factor (1 ng/ml), *Zero* nothing added, +++ cells positive for the dopamine neuron marker TH are present, – no TH immunoreactivity]

Cytokine progenitors	"M"	"S"
IL-1	+++	–
IL-2	–	–
IL-3	–	–
IL-4	–	–
IL-6	–	–
IL-9	–	–
IL-11	–	–
CSF	–	–
TGFβ	–	–
TGFα	–	–
LIF	–	–
EGF	–	–
SCF	–	–
βFGF	–	–
EPO	–	–
INF-γ	–	–
TNFα	–	–
GDNF	–	–
BDNF	–	–
Zero	–	–



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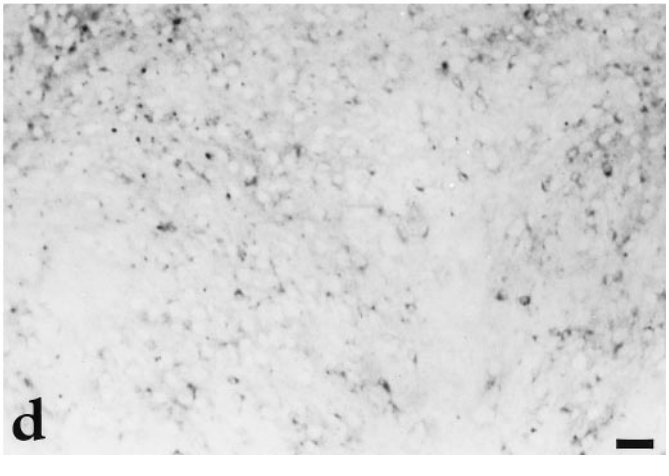
b


c
d

◀ **Fig. 1** Bromodeoxyuridine (*BrdU*) antibody staining of mesencephalic progenitor sphere plated onto poly-L-lysine (*PLL*)-coated wells and incubated 24 h in complete media. ×320. *Bar* 15 μm

TH primary antibody (1:250; Chemicon) overnight at 4°C. The cultures were incubated 1 h in anti-mouse IgG:0.5%, and incubated 1 h in FITC-avidin (1:300). Following three washes in NaCl (0.15 M)/bicarbonate (0.1 M), the fluorescent stains were visualized using an Olympus Fluoview Confocal microscope.

Experiment 1

Mesencephalic subependymal cells cultivated at least 3 weeks in expansion media, were labeled 24 h with BrdU, then plated 24 h on poly-L-lysine (PLL)-coated 48-well Costar plates, fixed and incubated with the monoclonal BrdU antibody, and BrdU immunocytochemistry was performed. This experiment was performed to demonstrate the presence, or absence, of proliferating cells in the mesencephalic subependymal cell cultures used in the experiments described below.

Experiment 2

Striatal and mesencephalic subependymal cultures that had been incubated in expansion media for at least 21 days were plated onto PLL-coated 48-well Costar plates, and cultured under identical conditions in the "cytokine-enriched" media. Nineteen different cytokines were evaluated (see Table 1). Following 7 days incubation in the cytokine-enriched media, the cultures were fixed and the conversion of subependymal cells was assessed by counting numbers of TH-ir cells. This experiment was performed to determine if cytokines could induce DA neuron differentiation in striatal subependymal cell cultures, as well as in mesencephalic subependymal cell cultures.

Experiment 3

Hu, GAP-43, TH, and GFAP immunoreactivity of mesencephalic subependymal cultures was assessed 7 days after plating EGF-stimulated proliferative spheres into PLL-coated 48-well Costar plates. Hu immunocytochemistry was used to assess numbers of subependymal cells capable of differentiation that were committed to the neuronal phenotype. GAP-43 immunocytochemistry was used to assess numbers of neurons extending processes. TH immunocytochemistry was used to assess numbers of subependymal cells converted into DA neurons. The cultures were incubated in either differentiation media (see above) or CM (control) for 7 days and then fixed. Four computer-generated random fields of each well were identified on a reticle grid, and the number of Hu-immunoreactive (Hu-ir) cells was assessed and compared across treatment groups. In sister cultures on the same 48-well Costar plates, GAP-43, TH, and GFAP immunoctyochemistry was performed and the immunoreactive cell counts were similarly gathered. These assessments were performed to determine the relative cell counts of each of the four labeled cell types.

Experiment 4

To demonstrate the conversion of progenitors into TH neurons, cultures of mesencephalic subependymal cells were double-labeled using BrdU and TH antibodies. To demonstrate that TH-positive cells were neurons, these same cultures were double-labeled using Hu and TH antibodies. Proliferating mesencephalic subependymal cells were plated on PLL-coated wells and incubated in either differentiation media (see above) or CM (control) for 7 days, then fixed and double labeled using BrdU and TH antibodies, or fixed and double labeled with Hu and TH antibodies as described above. An Olympus Fluoview Confocal microscope was used to evaluate double-labeling with the fluorescent-tagged antibodies.

◀ **Fig. 2** TH immunoreactivity of a mesencephalic progenitor sphere plated onto PLL-coated wells (125 000 cells/cm^2) and incubated 7 days in CM supplemented with (**a**) and without (**c**) IL-1 (200 pg/ml). Striatal progenitors plated using the same procedure and incubated with (**b**) and without (**d**) IL-1. ×250. *Bar* 20 μm

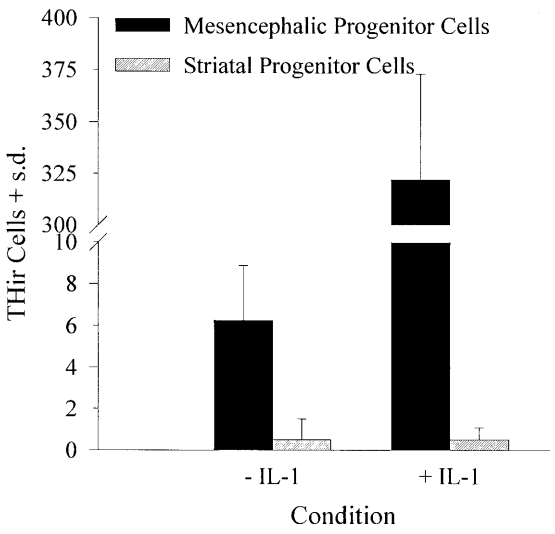


Fig. 3 Comparison of the TH-ir cell counts from the conditions depicted in Fig. 2. Mesencephalic and striatal progenitor cells were exposed to IL-1 (+IL-1; 200 pg/ml) in complete media or to complete media alone (–IL-1)

Experiment 5

Proliferating mesencephalic subependymal cells that had been incubated in expansion media for at least 21 days were plated on PLL-coated wells and incubated in the differentiation media (see above) for 7 days, after which cultures were incubated another 7 days in CM without cytokines. A set of sister cultures was incubated for 14 days in CM only. Following fixation numbers of TH-ir cells were assessed to determine whether continuous cytokine exposure was needed to maintain the DA neuron phenotype.

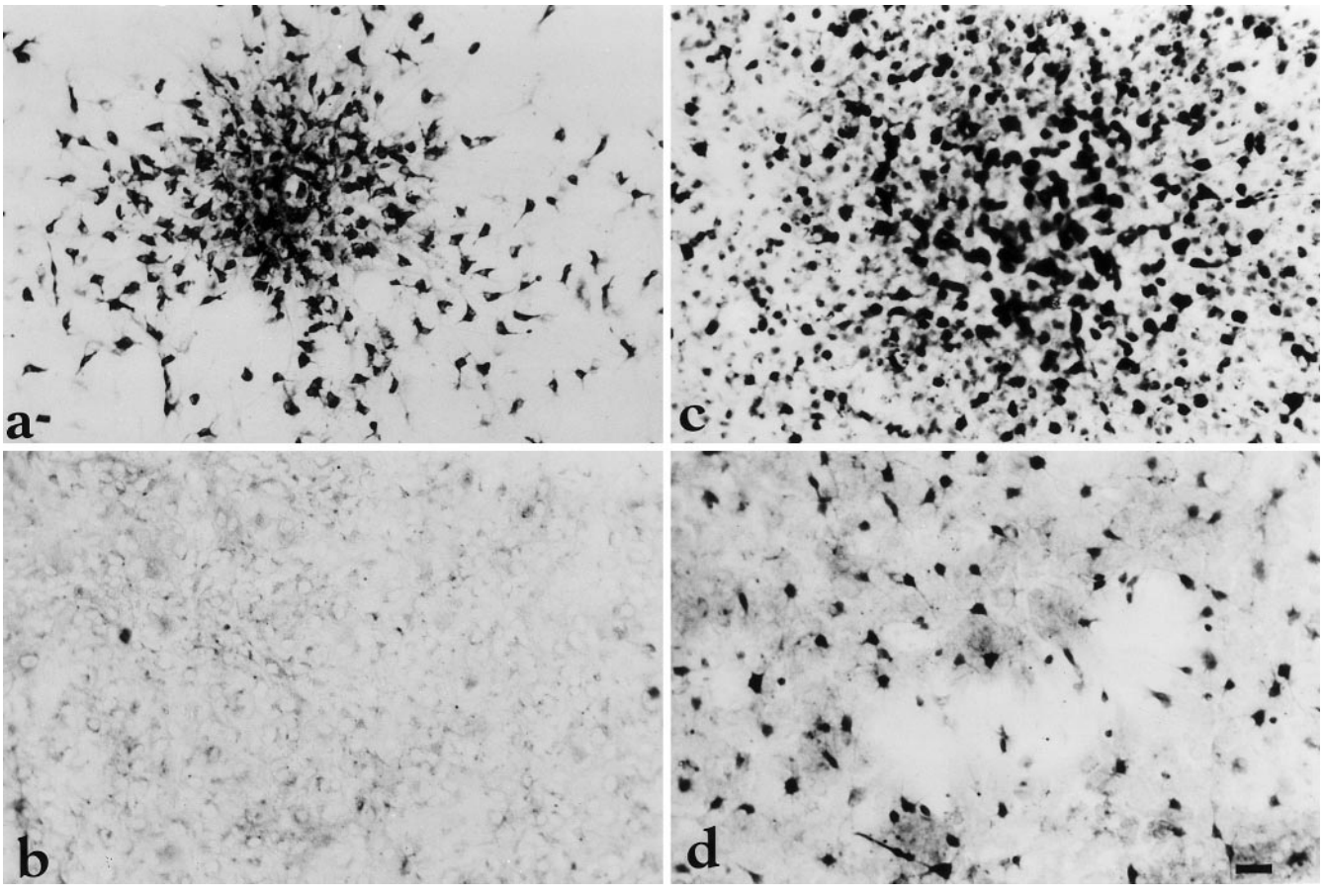
Results

BrdU labeling and immunocytochemistry of proliferating subependymal cells: experiment 1

Numerous cells in the proliferating sphere of subependymal cells were BrdU-positive, as indicated with the black DAB-nickel stain (Fig. 1). All of the spheres plated contained numerous BrdU-positive cells although not all of the cells in each sphere were labeled.

Cytokine-induced DA neuron conversion in mesencephalic and striatal subependymal cells: experiment 2

The ability of 19 different cytokines to convert E14.5 mesencephalic and E14.5 striatal fetal subependymal cells to cells immunoreactive for TH is shown in Table 1. While IL-1 (200 pg/ml) converted mesencephalic subependymal cells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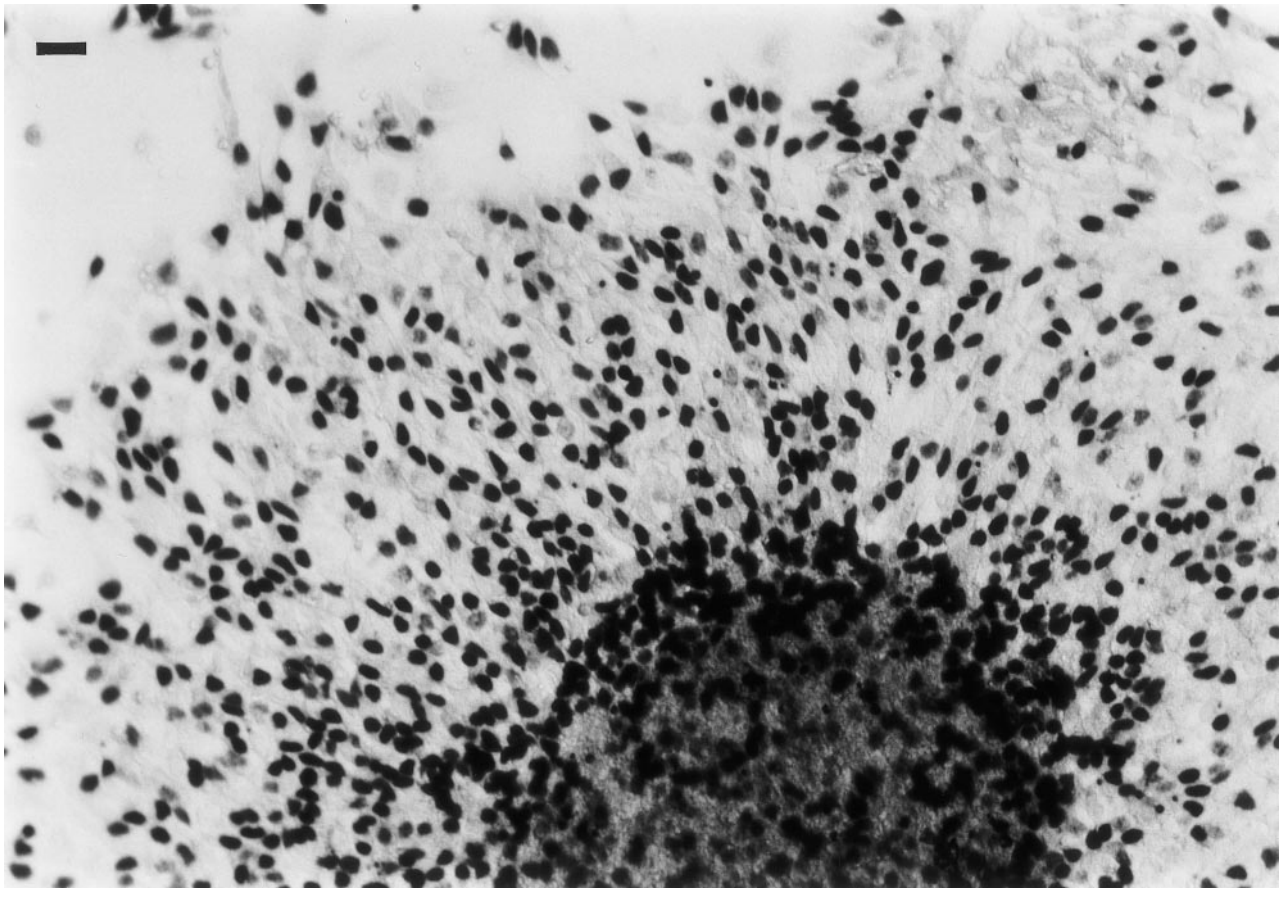

◀ **Fig. 4** TH immunoreactivity of mesencephalic progenitor spheres exposed to differentiation media (**a**) or complete media (**b**) for 7 days. Hu immunoreactivity in sister cultures exposed to differentiation media or complete media for 7 days is shown in **c** and **d**, respectively. ×250. *Bar* 20 μm

into primitive TH-ir cells (Figs. 2a, 3; $F_{1,15}$=164.36; P<0.001), none of the striatal cultures incubated in the cytokines had more than two TH-ir cells (Figs. 2b, 3). In the absence of cytokines, no more than two TH-ir figures were observed in the mesencephalic and striatal progenitor cultures (Fig. 2c,d, respectively; Fig. 3).

Hu, GAP-43, TH and GFAP immunoreactivity in subependymal cells: experiment 3

In mesencephalic cultures incubated 7 days in either the differentiation media (described above), or CM without cytokines, numbers of TH-ir, Hu-ir, and GAP43-ir cells were counted. Mesencephalic progenitor cell cultures incubated 7 days in the differentiation media exhibited a dramatic maturation in cells having a neuronal phenotype (Fig. 4a), compared with cultures incubated in CM without cytokine supplementation (Fig. 4b). TH-ir cells exposed to the cytokines were far more numerous and exhibited a cell body more typical of DA neurons from primary cultures. Moreover, most of the cells contained processes. Although numerous Hu-ir cells were present in cultures incubated in CM only (Fig. 4d), the number of Hu-ir cells increased dramatically in the presence of cytokines (Fig. 4c). A similar trend was observed when GAP43-ir cell counts were assessed (Fig. 5a,b). Thus, the number of GAP43-ir cells was dramatically increased and their morphology was more variegated compared with cultures incubated with CM only. The cell count data from these cultures reflected the impression of the photomicrographs (Fig. 6). Thus, the number of TH-ir cells was increased 58-fold in the cytokine-incubated cultures relative to CM-incubated controls ($F_{1,8}$=76.77, P>0.001), while the Hu-ir and GAP43-ir cell counts were increased 6-fold and 13-fold, respectively (Fig. 6; $F_{1,8}$=55.81, P=0.0003; $F_{1,8}$=95.29, P=0.0001, respectively). TH-ir cell counts represented on average 45% of the Hu-ir and GAP43-ir cell counts. Unfortunately, the GFAP-ir cells formed a confluent carpet of astrocytes in all cultures and were too numerous to count although it did appear that the differentiation media did affect their morphology (cf. Fig. 5c with Fig. 5d).

◀ **Fig. 5** Sister cultures of the cells seen in Fig. 4 are shown in **a–d**. GAP43 immunoreactivity in mesencephalic progenitors incubated 7 days in differentiation media (**a**) or in complete media (**b**). GFAP immunoreactivity antibody staining in differentiation media or in complete media is shown in **c** and **d**, respectively. ×250. *Bar* 20 μm

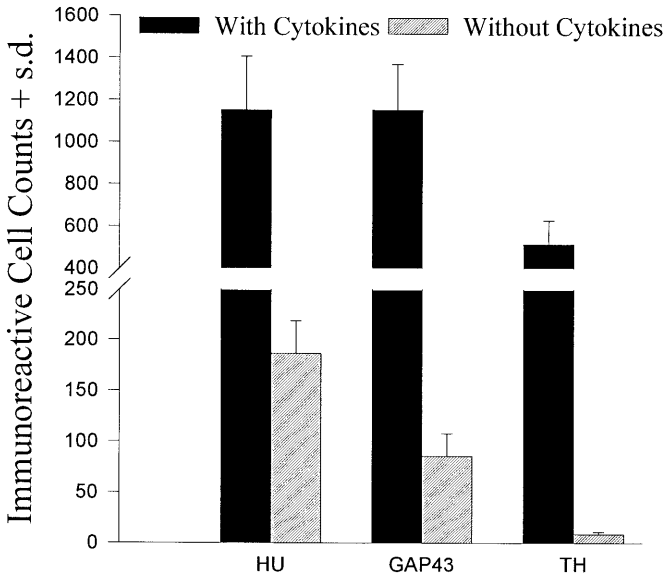

Fig. 6 Comparison of the numerical results depicted in Figs. 4 and 5 showing HU, GAP43, and TH cell counts in differentiation media (with cytokines) and CM (without cytokines)

Double-labeling with BrdU and TH immunocytochemistry, Hu and TH immunocytochemistry: experiment 4

Double labeling of progenitors with BrdU and TH fluorescent immunocytchemistry (Fig. 7a–c) demonstrated the conversion of mesencephalic progenitors into TH neurons. Most of the TH-ir cells were also BrdU-ir while many BrdU-ir cells were not TH-ir. The failure to detect BrdU immunoreactivity in all TH-ir cells likely reflects the incomplete incorporation of the BrdU label into all cells during the incubation period (note the incomplete labeling observed in Fig. 1). In contrast, double labeling was not seen in control cultures incubated in CM, as TH labeling was not present in these cultures (data not shown). Double labeling with Hu and TH fluorescent immunocytochemistry (Fig. 7d–f) demonstrated that all TH-ir cells were Hu-ir neurons. The number of Hu-ir cells that were not double labeled with TH appeared to reflect about one-half of the total Hu labeled cell population. This is in agreement with the results from the sister culture study reported in Fig. 6. Hu and TH double labeling was rarely seen in the CM-incubated controls (data not shown), as TH-ir cell numbers were extremely low in these control cultures (see Fig. 6).

Stability of cytokine-induced DA neuron conversion: experiment 5

Cells incubated in differentiation media for 7 days, followed by incubation in CM without cytokines for 7 days, maintained the expression of TH-ir cells. The cells appeared to maintain a neuronal morphology similar to that seen in cultures incubated in differentiation media for 7

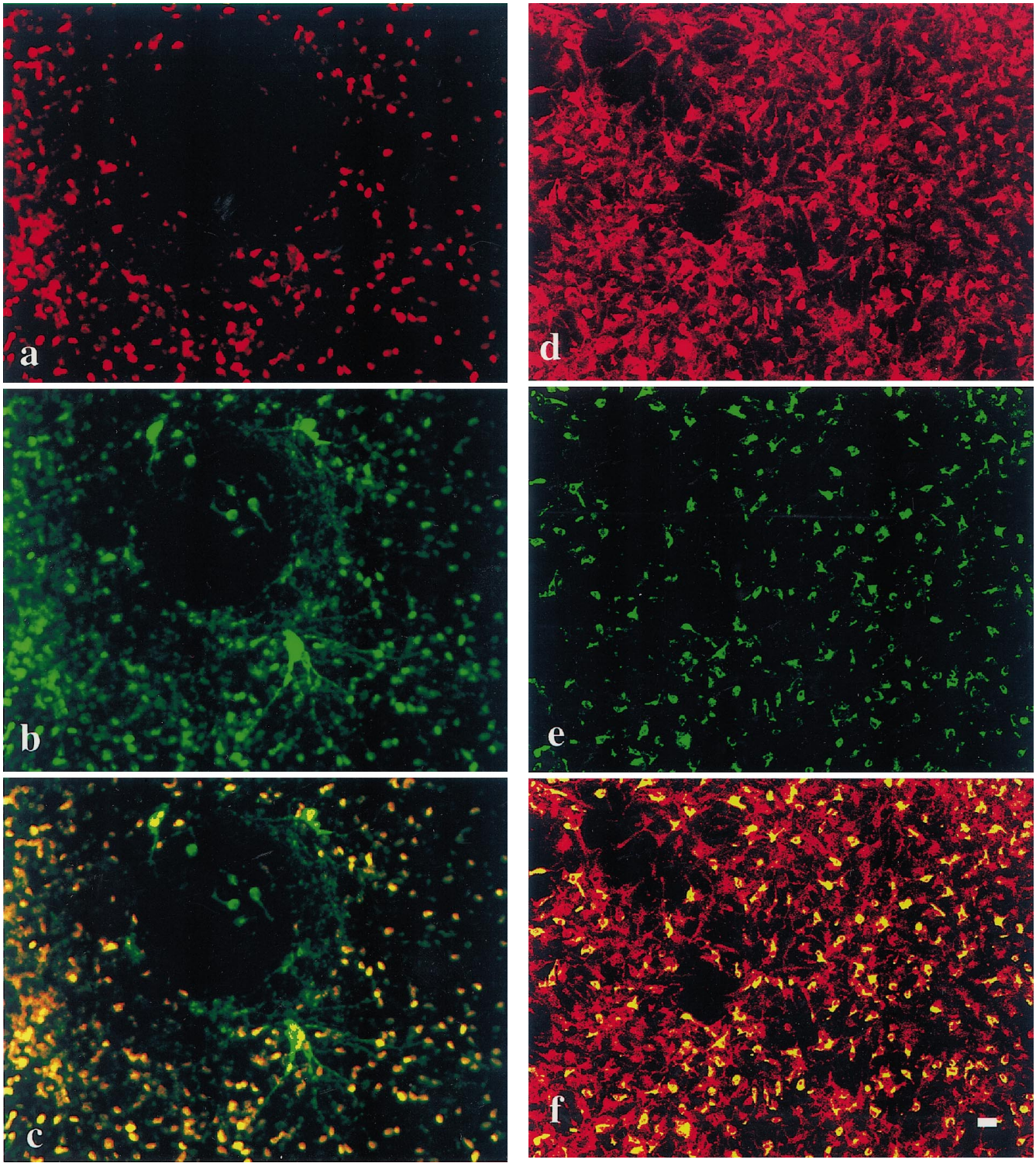

Fig. 7 Confocal images of BrdU and TH antibody staining of mesencephalic progenitor spheres plated onto PLL-coated wells (125,000 cells/cm^2) and incubated 7 days in differentiation media supplemented with cytokines [BrdU-labeled cells are red (**a**), TH-labeled cells are green (**b**), and BrdU- and TH-double-labeled cells are yellow (**c**)]. Confocal images of Hu and TH antibody staining in sister cultures of mesencephalic progenitor spheres are also shown (**d–f**) [Hu-labeled cells are red (**d**); TH-labeled cells are green (**e**); Hu- and TH-double-labeled cells are yellow (**f**)]. ×200. *Bar* 15 μm

days (cf. Fig. 8 with Fig. 4a). However, the 14-day-old TH-ir cells appeared to be slightly larger and did not have more extensive processes than cells exposed to differentiation media for only 7 days. Numbers of TH-ir cells incubated 7 days in differentiation media followed by 7 days in CM without cytokines far exceeded numbers of TH-ir cells incubated 14 days in CM without cytokines (Fig. 9;

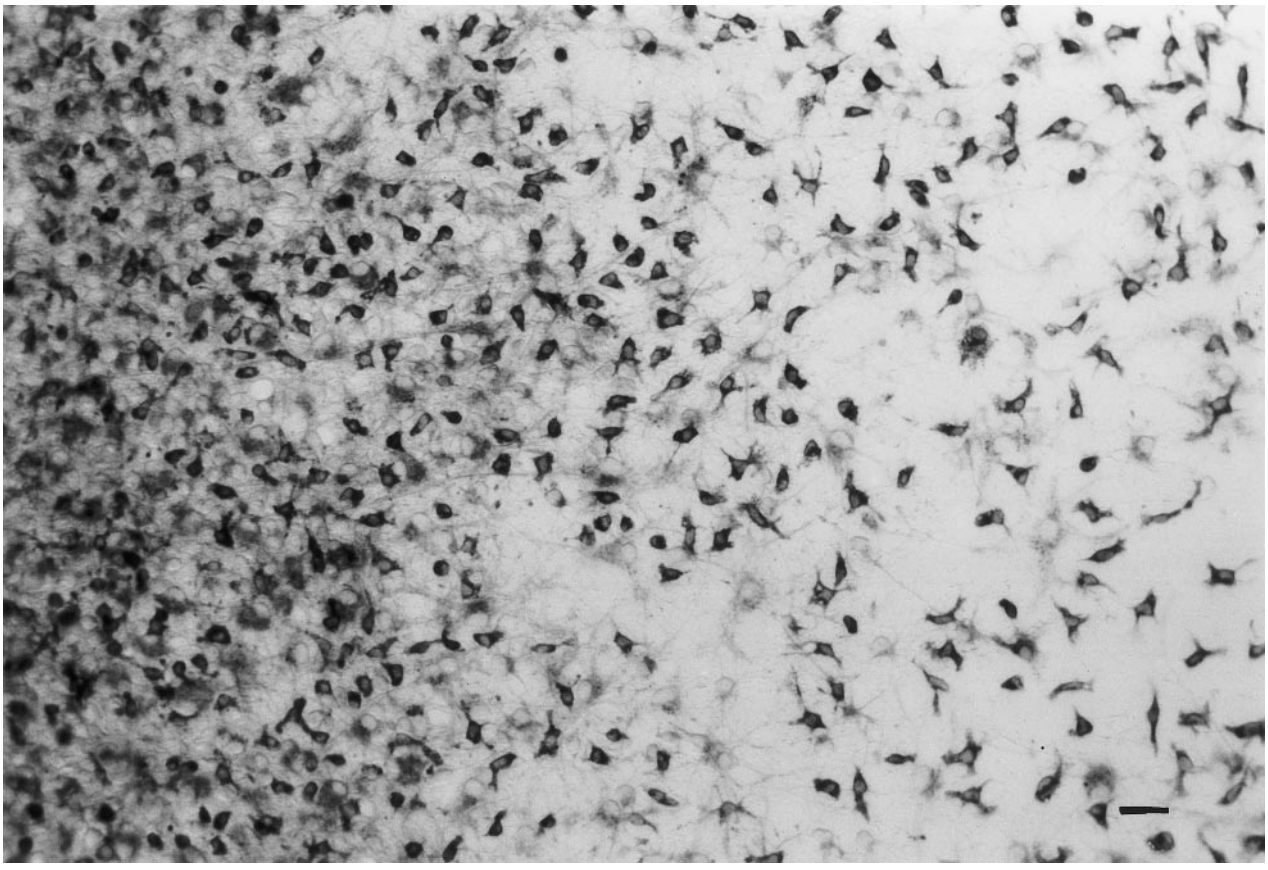

Fig. 8 TH immunoreactivity in a 14-day-old culture of mesencephalic progenitor cells incubated 7 days in differentiation media followed by 7 days incubation in CM. ×250. *Bar* 20 μm

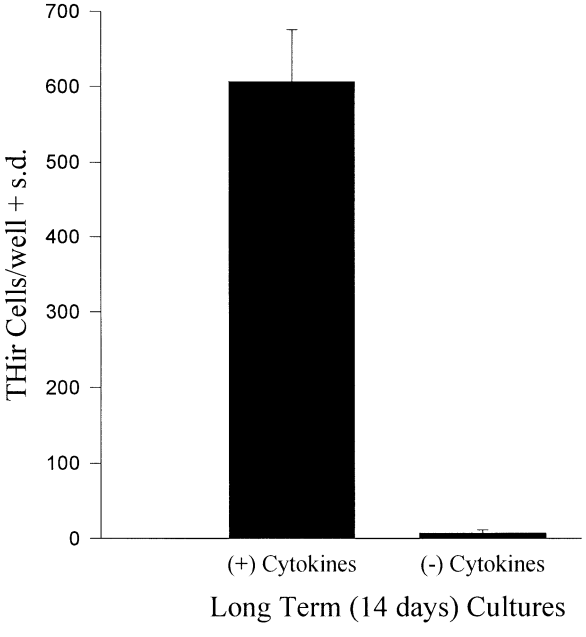

Fig. 9 Numerical results of TH-ir cell counts from cultures depicted in Fig. 8 incubated in differentiation media for 7 days followed by 7 days in CM (+cytokines) relative to cultures incubated in CM only (–cytokines) for 14 days

$F_{1,7}$=302.57; P<0.001). Moreover, the overall cell counts of these converted 14-day-old cultures were similar to those seen in cultures incubated only 7 days in the differentiation media (compare TH-ir counts in Figs. 6 and 9).

Discussion

The numerous BrdU-ir cells observed in the fetal mesencephalic spheres in experiment 1 demonstrated that active proliferation was occurring in the cultures since this label can only be incorporated into actively dividing cells. Moreover, since the proliferating spheres utilized in that study were passaged at least 4 times and expanded 21 days prior to plating, it is unlikely that primary differentiated cells could have survived. These data suggest that the source of cells for all subsequent experiments was actively dividing cells which could be classified as either stem or progenitor cells. We previously demonstrated that IL-1 along with other cytokines induced the expression of TH-ir cells in E14.5 mesencephalic subependymal cultures. Given that IL-1 influences the differentiation of mesencephalic subependymal cells, it might be suggested that IL-1 would exert a similar effect on all subependymal cells. However, this does not appear to be the case, since IL-1 failed to induce conversion of E14.5 striatal subependymal cells into TH-ir cells in experiment 2. These data demonstrate that striatal subependymal and mesencephalic subependymal

cells respond differently to the same cytokine, and this finding is consistent with the notion that during fetal development, lineage restriction with respect to differentiation occurs along a rostral-caudal axis of the neural tube (Yamada et al. 1993; Placzek et al. 1993; Hynes et al. 1995a,b). In other words, since striatal and mesencephalic subependymal cells responded differently to the same environmental factor, i.e., IL-1, lineage restriction in these cells has already occurred. Therefore, subependymal cells isolated from the E14.5 striatum and mesencephalon should be classified as progenitor cells, and not omnipotent stem cells (Anderson 1989; McKay 1997; Ray et el. 1997; Stemple and Mahanthappa 1997). Since we have also demonstrated that the mesencephalic subependymal cells are self-renewing and pluripotent, these lineage-restricted mesencephalic subependymal cells meet the three major criteria for their classification as progenitor cells.

The influence of IL-1 on neuronal differentiation is plausible given that many hematopoietic cytokines including the interleukins are present in fetal brain parenchyma during neuronal maturation (Hynes et al. 1995a,b; Jessell and Dodd 1990; Kilpatrick and Bartlett 1993; McKay 1997; Temple 1989). The trophic effects of IL-1 on DA and other catecholaminergic neurons also have been documented both in in vitro and in vivo experiments (Akaneta et al. 1995; Nakao et al. 1994). Thus, similar to their effects on hematopoietic cell development, cytokines such as IL-1 likely play a role in CNS development and differentiation. However, we also included IL-11, LIF, and GDNF in our differentiation media because we previously showed that this mixture of cytokines increased numbers of converted TH-ir cells, and further these cells exhibited greater maturity in their neuronal morphology (Ling et al. 1998). In addition, freeze-fractured mesencephalic membrane fragments as well as media from striatal cultures were also included in the differentiation media. We included these because we have previously demonstrated that extracellular matrix proteins and an unknown trophic activity found in striatal conditioned media enhanced process development and cell body maturation in the converted DA neurons (Ling et al. 1998). Although the possibility cannot be completely ruled out that the mesencephalic membrane fragments or striatal conditioned media are critical to the conversion process, this seems unlikely since the addition of membrane fragments only enhanced the morphological development of cells when IL-1 was present (Ling et al. 1998), and we have previously shown that progenitor cells grown in co-culture with E14.5 striatal cultures failed to convert to DA neurons (Ptak et al. 1995). This suggests that the added cytokines, and in particular IL-1, play a central role in the conversion process. Although the relative contribution of each cytokine during conversion of mesencephalic progenitors into DA neurons is currently being evaluated in our laboratory, it is clear that IL-1 is a critical element since we have never observed significant numbers of TH-ir cells in progenitor cultures that did not contain IL-1. This might suggest that IL-1 induces the expression of specific receptors, which allow the progenitor cells to become responsive to the additional factors present in the differentiation media. For example, IL-1 may increase numbers of gp130-linked receptors, thereby increasing the progenitor cell response to LIF and IL-11 (Hirano et al. 1994). In addition, IL-1 may increase numbers of ret receptors, thereby increasing their response to GDNF (Durbec et al. 1996; Jing et al. 1996).

Regardless of the mechanism(s) involved, it is important to note that the relative percentage of TH-ir cells present in these cultures (~25%) was significantly higher than the 1–5% normally found in primary mesencephalic cultures. Since it has been demonstrated that progenitor cells differentiate into neurons and glia (Ray et al. 1997), the preferential enrichment of TH-ir cells in the converted cultures suggests that the differentiation media drive the conversion of progenitors into DA neurons. The studies with the Hu antibody presented here begin to address this issue.

The Hu antibody labels an RNA-binding protein found only in neurons and in progenitor cells destined to become neurons (Barami et al. 1995). It is expressed in neuronal precursors during their residence in the subependymal zone prior to migration and is considered the earliest known marker for the neuronal phenotype (Marusich and Weston 1992; Barami et al. 1995). In our mesencephalic progenitor spheres, a few Hu-ir cells were apparent within 3 h of plating in CM without cytokines (data not shown). It is possible the FCS-supplemented media very quickly induced expression of the neuronal phenotype or, alternately, the Hu-positive cells were present in the expansion cultures prior to plating. Unlike the round Hu-ir cells observed 3 h after plating, Hu-ir cells observed following 7 days incubation in CM (containing 10% FCS) exhibited a distinct neuronal morphology. This implicates FCS in neuronal maturation. However, incubation in the differentiation media increased numbers of Hu-ir cells in the cultures sixfold and suggests that the cytokines increased the number of progenitor cells which converted into neurons, approximately one-half of which were also TH-ir cells, as shown by the cell counts and the double-labeling studies. Thus, the differentiation media not only increased the number of progenitors converting into neurons, but also increased the number of neurons developing into DA neurons. As innumerable astrocytes were observed in the cultures, a ratio of Hu-ir to GFAP-ir cells could not be determined. Therefore, we could not establish if the differentiation media increased Hu-ir cells while decreasing GFAP-ir cells. This would have supported the notion that the differentiation media were "driving" the conversion of progenitors into neurons. However, it was clear that the differentiation media did increase the percentage of neurons converting into TH-ir cells, since these cell counts increased 58-fold relative to cultures incubated in CM (only), whereas the Hu-ir cell counts increased only sixfold. Thus, the differentiation media clearly increased the conversion rate of progenitor cells to TH-ir cells, and this highly increased conversion rate could not be accounted for by the observed preferential conversion of progenitors into Hu-ir cells.

Incubation in differentiation media appeared to increase the morphological maturation of neuronal cells in the mesencephalic progenitor cultures. While increased TH-ir cell counts were observed in cultures incubated in the differen-

tiation media, greater numbers of cells with processes were also observed. GAP43-ir cell counts were approximately 45% of the Hu-ir cell counts observed in sister cultures incubated with CM. Since GAP43 is considered a marker for neurons extending processes (Meiri et al. 1988), only about half of Hu-ir cells were likely to extend processes when the progenitor cells were incubated in CM with 10% FCS. However, addition of the differentiation media to sister cultures generated numbers of Hu-ir cells that were similar to numbers of GAP43-ir cells. This suggests that most of these neurons were extending processes. Although a similar study could not be performed on the astrocytes, it did appear that with incubation in CM, the astrocytes formed a confluent carpet with short thin ramifications. In contrast, with incubation in differentiation media, the astrocytes exhibited thick, dense ramifications. Thus, it appears that the differentiation media not only influenced the relative numbers of different cell types in progenitor cultures, it also influenced the morphological maturation of these cell types. It is possible that the mesencephalic membrane fragments included in the differentiation media were wholly responsible for these effects. Mesencephalic membrane fragments are thought to provide additional extracellular matrix proteins to the cultures, and these proteins may influence cell maturation (see Ling et al. 1998).

In experiment 4, we used double-labeling to characterize the converted TH-ir cells. While in cytokine-incubated progenitor cultures we observed numerous BrdU-ir cells that were also TH-ir, very few double-labeled cells were seen in sister cultures incubated in CM without cytokines. This suggests that the cytokine-enriched differentiation media converted mesencephalic progenitor cells into DA neurons. However, not all TH-ir cells were BrdU-ir, which might suggest that some of the TH-ir cells were not derived from a progenitor cell. Since the cultures were expanded for 21 days and passaged at least 4 times, it is highly unlikely that these cultures could have included primary neurons that survived the passaging process. In addition, as seen in Fig. 1, it is clear that the BrdU incubation procedure used did not label all the cells. This is often the case given the short duration of exposure to the label. Thus, it is quite possible that the TH-ir cells that did not double label for BrdU simply reflected that subpopulation of cells that did not originally incorporate the label. The double-labeling experiments also demonstrated that all TH-ir cells were also Hu-ir following exposure to differentiation media whereas cultures exposed to CM contained few and in most cases no cells with this double label. Based on these results we therefore conclude that the TH-ir cells seen in cultures exposed to media containing cytokines reflect a phenotypic conversion of progenitor cells into DA neurons.

A relevant issue is stability of the DA neuron phenotype once the cytokine-induced conversion of progenitor cells has occurred. Aside from the practical aspects, stability of the converted phenotype would lend insight into the mechanism underlying the conversion process. If the converted phenotype was dependent upon continuous exposure to cytokines, this would suggest the involvement of a translational event. However, if exposure to the differentiation media resulted in a more permanent expression of TH, transcriptional events are more likely involved. The results from experiment 5 demonstrated that 7 days following withdrawal of the cytokine-supplemented media, the TH-ir phenotype was still present. Moreover, the number of TH-ir cells present was similar to that seen immediately following 7 days incubation in the differentiation media. Thus, it is unlikely that the cytokines simply increased the levels of TH, thereby allowing detection by the immunoassay. Rather, it appears that elements in the differentiation media turned on the expression of TH through a transcriptional event. In addition, it appears that the various elements in the differentiation media were not responsible for maintaining the viability of the progenitor cells since the cell counts observed 7 days following differentiation media withdrawal were similar to those seen after 7 days exposure to the differentiation media. Thus, elements in the differentiation media primarily regulated the differentiation process. It was surprising to note, however, that the morphology of the cells in the cultures withdrawn from the differentiation media for 7 days was similar to that seen in the cultures after 7 days. Because of the presence of FCS in the CM used during the withdrawal phase of this experiment, we expected to see continued morphological development and process extension as is typically seen in primary mesencephalic cultures. The fact that this was not seen may be explained by the absence of trophic factors such as GDNF or, alternatively, that the cytokines used to convert these progenitor cells were not able to completely induce the typical DA neuron phenotype that is responsive to FCS. Be that as it may, the likely involvement of cytokines in a transcriptional event involved with neuronal differentiation has implications for our understanding of the relative roles of genetics and environment in the development of neuronal phenotypes.

Our data suggests an interplay between genetic predisposition and environmental factors. While elements in the differentiation media appeared to drive the conversion of progenitors into DA neurons, these cells exhibited a genetic predisposition to differentiate into neurons and glia. In fact, regardless of the source of the subependymal cells (striatal or mesencephalic), the “default” program appeared to be a combination of neurons and glia. However, none of the striatal progenitor cells converted into DA neurons by a differentiation media that did convert mesencephalic progenitors into DA neurons. This implicates a genetic predetermination consistent with a lineage restriction along the rostral-caudal axis of the neural tube (Hynes et al. 1995b). Such a predisposition cannot, however, be expressed without exposure to a specific environmental signal which, in the case of mesencephalic progenitor cells, appears to involve IL-1. Given the appropriate predisposition and environmental signal, mesencephalic progenitors can then become responsive to other environmental signals such as IL-11 or GDNF. Thus, it is a unique combination of predetermination and environment that results in the expression of the DA neuron. Several investigators have observed similar findings using other types of progenitor cells (see Bele et al. 1995; Birling and Price 1998; Shetty and Turner 1998) and Qian et al. (1997) have also shown that the genetic predisposition influencing the response to environmental signals changes with embryonic development age, implicating a

cascade of signaling events that regulate the expression of a neuronal phenotype. If this is indeed the case, then mesencephalic subependymal cells taken from early or older embryos might respond differently to environmental signals such as IL-1.

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