

Humoral and Contact Interactions in Astroglia/Stem Cell Co-cultures in the Course of Glia-Induced Neurogenesis

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ABSTRACT Astroglial cells support or restrict the migration and differentiation of neural stem cells depending on the developmental stage of the progenitors and the physiological state of the astrocytes. In the present study, we show that astroglial cells instruct noncommitted, immortalized neuroectodermal stem cells to adopt a neuronal fate, while they fail to induce neuronal differentiation of embryonic stem cells under similar culture conditions. Astrocytes induce neuron formation by neuroectodermal progenitors both through direct cell-to-cell contacts and via short-range acting humoral factors. Neuron formation takes place inside compact stem cell assemblies formed 30–60 h after the onset of glial induction. Statistical analyses of time-lapse microscopic recordings show that direct contacts with astrocytes hinder the migration of neuroectodermal progenitors, while astroglia-derived humoral factors increase their motility. In non-contact co-cultures with astrocytes, altered adhesiveness prevents the separation of frequently colliding neural stem cells. By contrast, in contact co-cultures with astrocytes, the restricted migration on glial surfaces keeps the cell progenies together, resulting in the formation of clonally proliferating stem cell aggregates. The data indicate that *in vitro* maintained parenchymal astrocytes (1) secrete factors, which initiate neuronal differentiation of neuroectodermal stem cells; and (2) provide a cellular microenvironment where stem cell/stem cell interactions can develop and the sorting out of the future neurons can proceed. In contrast to noncommitted progenitors, postmitotic neuronal precursors leave the stem cell clusters, indicating that astroglial cells selectively support the migration of maturing neurons as well as the elongation of neurites. © 2004 Wiley-Liss, Inc.

INTRODUCTION

Fate determination of multipotential stem cells is governed by signals derived from their microenvironment (Temple, 2001; Song et al., 2002; Horner and Palmer, 2003). The intimate stem cell niches are maintained and controlled by the concerted action of direct cell-to-cell and cell-to-extracellular matrix (ECM) contacts, together with soluble factors released from the neighboring cells (Panchision and McKay, 2002; Campbell, 2003; Doetsch, 2003).

Glial cells are among the major components of the cellular milieu surrounding neural stem cells in both

the developing and mature brain (Doetsch et al., 1997; Campbell and Götz, 2002; Doetsch, 2003; Alvarez-

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Buylla and Lim, 2004). Radial glial cells (i.e., cells recently shown to possess neural stem cell properties themselves) guide and regulate the migration of their own progeny—the neuronal precursors—during early cortical development and appear to provide site-specific cues for neuronal cell fate determination (Hatten, 1999; Malatesta et al., 2000; Noctor et al., 2001, 2002; Hall et al., 2003). Astrocytes within the adult subventricular zone and the subgranular zone of the hippocampus function both as neurogenic stem cells and as niche cells, providing a suitable microenvironment for the long-term maintenance of neurogenetic capacity and continuous generation of new granule cells (Doetsch et al., 1997; Seri et al., 2001; Doetsch, 2003; Alvarez-Buylla and Lim, 2004).

Although the extended and flexible developmental potential of the “radial neuroepithelial cells” (Götz and Steindler, 2003) or “multipotent astrocytes” (Steindler and Laywell, 2003) can no longer be disclaimed, the vast majority of astrocytes in the brain parenchyma display differentiated astroglial features rather than possessing multipotential stem cell properties (Lim and Alvarez-Buylla, 1999; Sanai et al., 2004). Whether most parenchymal astrocytes represent a terminally differentiated phenotype or a transient stage of astrocytic development is still under review.

Astrocytes were shown to promote the survival and maturation of young neurons and neuronal precursors by many investigators (e.g., Banker, 1980; Temple and Davis, 1994; Nedergaard, 1994). More recent reports have demonstrated that astrocytes induce neurogenesis both by adult neural (Lim and Alvarez-Buylla, 1999; Song et al., 2002) and by embryonic stem cells (Nakayama et al., 2003), *in vitro*. Although these observations confirm that astroglial cells have the potential to instruct noncommitted stem cells to adopt a neuronal fate, little is known about the factors responsible for the instructive effect.

In the present study, we examined contact cell-to-cell communication and humoral non-contact interactions between astrocytes and stem cells. As a highly reproducible *in vitro* model, primary murine astroglial cells were co-cultivated with noncommitted neuroectodermal cells of the one-cell-derived NE-4C cell line (Schlett and Madarász, 1997) and also with embryonic stem cells of the R1 and 1GFP/B2 clones (Nagy et al., 1993). All these stem cell lines are known to produce neurons upon appropriate induction.

The NE-4C cells (Schlett and Madarász 1997), derived from the fore- and midbrain vesicles of p53-deficient (Livingstone et al., 1992) 9-day-old mouse embryos, were shown to display several neural stem cell properties and to generate neurons and astroglial cells upon induction with all-*trans*-retinoic acid (RA) (Schlett and Madarász, 1997; Schlett et al., 1997; Tárnok et al., 2002; Demeter et al., 2004). Subclones of NE-4C cells with constitutive expression of histological marker proteins had been established and used for tracing inside the brain parenchyma after grafting (Madarász et al., 2000; Demeter et al., 2004). In the

present studies, the labeled NE-4C subclones were used to follow their fate in co-cultures with astrocytes.

Astrocytes induced neuronal differentiation of NE-4C neuroectodermal cells both in contact and in non-contact co-cultures, while they failed to instruct neuron formation by ES cells. The initial proliferation, time course of neuron formation, and motility of NE-4C cells, being at different stages of neural commitment, were investigated by computer-controlled time-lapse microscopy.

MATERIALS AND METHODS

Materials

Medium (MEM, DMEM), gentamycin, genetycin, trypsin, glutamine, F12, N2, poly-L-lysine, laminin, antibody to glial fibrillary acidic protein (GFAP), streptavidin-conjugated horseradish peroxidase (HRP), MTT, nonessential amino acid stock, β -mercaptoethanol, all-*trans*-RA, LIF, NBT, BCIP, and XGal were purchased from Sigma-Aldrich, Hungary; isopropanol from Merck, Austria; fetal calf serum (FCS), NeuroBasal Medium, ITS, B27 supplement, from Gibco-BRL-Life Technologies, UK; plasticware from Greiner, Hungary; antibody to neuron-specific β III-tubulin from ExBio, Czech Republic and Covance Research Products, Berkeley, CA, USA (Tu20 and TuJ1, respectively); antibody to NeuN from Chemicon, Temecula, CA; mouse neuron-specific M6 antibody from DSHB, Iowa City, IA, USA; biotin-conjugated anti-mouse IgG from Vector, Burlingame, CA, Vector, UK.

Astroglial Cultures

Cell suspensions of neonatal rat or mouse forebrains were prepared by enzymatic dissociation with 0.05% w/v trypsin in phosphate-buffered saline (PBS). Cells were plated onto poly-L-lysine-coated plastic surfaces at a cell density of $3\text{--}4 \times 10^5$ cell/cm². The cultures were grown in MEM supplemented with 10% FCS, 4 mM glutamine, and 40 μ g/ml gentamycin in humidified air atmosphere containing 5% CO₂, at 37°C. The culture medium was changed twice a week. The primary cultures were trypsinized weekly and replated onto poly-L-lysine-coated glass coverslips or into Petri dishes, according to the experimental design. Under these conditions, more than 95% of the cells were GFAP⁺ astrocytes with a slight contamination of Tuj1⁺ cells.

Neuroectodermal Progenitor Cells

NE-4C cells were maintained in MEM supplemented with 5% FCS, 4 mM glutamine, and 40 μ g/ml gentamycin, in humidified air atmosphere containing 5% CO₂, at 37°C. For maintenance, subconfluent cultures were regularly split by trypsinization (0.05% trypsin in

PBS) into poly-L-lysine-coated Petri dishes. For non-contact co-culture experiments, the cells were seeded onto glass coverslips coated with laminin (4 $\mu\text{g}/\text{ml}$).

GFP/NE-4C cells displaying high fluorescent intensity were assorted by sterile fluorescence-activated cell sorter (FACS) prior to experiments (Madarász et al., 2000; Demeter et al., 2004). Cells carrying heat-resistant placental alkaline phosphatase (PLAP) enzyme were identified by standard NBT and BCIP reaction, under alkaline conditions (pH = 9.5) (Madarász et al., 2000; Demeter et al., 2004). Before the addition of the substrates, the cultures were heated up to 65°C for 30 min to inactivate endogenous, heat-sensitive alkaline phosphatase activity. Both GFP/NE-4C and PLAP/NE-4C subclones were maintained in selection medium containing 200 $\mu\text{g}/\text{ml}$ genetycin.

Embryonic Stem Cells

The ES cell line R1 was established from (129/Sv \times 129/Sv-CP)F1 3.5-day blastocyst (Nagy et al., 1993). The 1GFP/B2 ES cell line was established by Julianna Kobilák et al. (submitted). Both R1 and 1GFP/B2 cell lines gave high-efficiency germline transmission by aggregating with CD1 outbreed 8-cell stage embryos. The cells were kept on mitomycin C inactivated primary embryonic mouse fibroblasts and were passed in 1 to 4 at 40% confluency. ES cells were grown in DMEM medium supplemented with 3.7 g/L NaHCO_3 , 50 $\mu\text{g}/\text{ml}$ streptomycin, 50 U/ml penicillin, 1 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, 0.1 mM nonessential amino acids, 1,000 U/ml of leukemia inhibitory factor (LIF), and 15% heat-inactivated FCS (Nagy et al., 2003).

Contact Co-cultures

Single-cell suspensions of NE-4C cells were plated on top of confluent astroglial monolayers at a cell density of 10^4 or 10^3 cell/ cm^2 , respectively. Before establishment of the co-cultures, astroglial cells were treated with cytosine arabinofuranoside (CAR, 10 μm , 24 h) to prevent glial proliferation. Co-cultures were grown in either MEM containing 5% FCS (MEM 5% FCS) or NeuroBasal Medium containing B27 supplement and 2 mM glutamine (NB-B27). In some experiments, MEM supplemented with F12 and N2, or MEM supplemented only with insulin-transferrin-selenite (ITS), was used. The first change of medium was performed after NE-4C cell attachment to the glial surface (within 30–60 min after seeding). The medium was changed three times a week. Contact co-cultures of astrocytes and embryonic stem (ES) cells were established in the same way as neural stem cell/astroglia co-cultures. Single-cell suspensions of ES cells were plated on top of the glial monolayer in a density of 10^3 cell/ cm^2 . The astroglia–ES co-cultures were maintained without

LIF, either in serum containing (5% FCS) or in defined (NB-B27) medium, for 7 or 14 days.

Non-contact Co-cultures

Glial conditioned (48-h) medium (GCM) was collected from astroglial monolayers grown in 90-mm-diameter dishes, either in MEM supplemented with 5% FCS or in NB-B27. The GCM was supplemented with fresh culture medium (in 1:1) and was transferred immediately onto NE-4C cells plated 24 h earlier. GCM treatment of NE-4C cells was repeated every second day. Non-contact co-cultures of neural stem cells with living astrocytes were established in the following way. NE-4C cells were grown on laminin-coated coverslips for 24 h. Astrocytes were maintained for 14 days in 90-mm dishes. Four days before the experiment, cell-free areas were prepared in the confluent astroglial monolayer with a cell scraper. The small coverslips carrying NE-4C cells were affixed to the cell-free patches with a small amount of silicon cloning grease.

To detect the migratory activity of NE-4C cells in non-contact co-cultures, we used two different experimental systems. One system was similar to that described above. Briefly, NE-4C cells were seeded onto poly-L-lysine-coated glass coverslips 3 h before the onset of the experiments, at 10^4 cell/ cm^2 density. The coverslips were then affixed into the cell-free areas of the astroglial cultures (scraped 48 h earlier) just before starting the time-lapse recordings. In the second experimental system, we seeded NE-4C cells (10^4 cell/ cm^2) directly onto the scraped astrocytic cultures. NE-4C cells settled onto the cell-free areas were monitored.

Immunocytochemistry and Determination of the Neuron Number

Cells grown on poly-L-lysine- or laminin-coated glass coverslips were fixed with 4% paraformaldehyde in PBS for 20 min, at room temperature. The cells were permeabilized with Triton X-100 (5 min, 0.1% v/v in PBS). Nonspecific antibody binding was blocked by incubation with 5% FCS in PBS at room temperature, for 1 h. Antibodies to GFAP (monoclonal mouse; 1:2,500), to neuron-specific β III-tubulin (Tu20, TuJ1, mouse monoclonal antibodies; 1:2,000) and M6 mouse neuron-specific antibody (1:500) were diluted with MEM-FCS and were used overnight, at +4°C. Second-layer antibodies to anti-mouse IgG conjugated with biotin were added in a dilution of 1:1,000, for 1.5 h at room temperature. The immunoreactions were visualized by standard streptavidin-peroxidase/diaminobenzidine (DAB) or the fluorescent avidin-TRITC or FITC reactions (1-h incubation).

The stained preparations were evaluated by using normal light (Leitz Laborlux), fluorescence (Zeiss Axioptot) and confocal laser scanning (Olympus BX61-FV300) microscopes. The number of neurons was de-

terminated by counting neuron-specific β III-tubulin-immunostained cells in 60 microscopic fields or on 60 photomicrograms taken from each co-culture. Averages and standard deviations were calculated from data obtained from three or four equally treated sister cultures. Experimental series were repeated minimum three times.

Cell Viability

The overall viability of cells in non-contact cultures was determined according to Mosmann (1983). Briefly, cells grown on poly-L-lysine-coated coverslips were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in a final concentration of 25 mg/ml. After a 2-h incubation, the cells and the Formazan crystals were dissolved in acidic (0.08 M HCl) isopropanol and transferred onto 96-well plates. The optical density (OD) was determined at a measuring wavelength of 570 nm against 630 nm as the reference, using an SLT 210 enzyme-linked immunosorbent assay (ELISA) reader.

Time-Lapse Microscopy

Time-lapse recordings were performed on either a computer-controlled Leica DM IRB or an Olympus CKX41 inverted microscope equipped with 10 \times , 20 \times objectives and an Olympus Camedia 4040z digital camera. Cell cultures were kept at 37°C in a humidified 5% CO₂ atmosphere within a custom-made microscope stage incubator as described previously (Czirók et al., 1998; Schlett et al., 2000). Phase-contrast images were acquired every 5 min for 3–6 days. Fluorescent images were captured manually. Analyses was performed both in contact and in non-contact co-cultures.

Cell Positions and Motility

The position of selected cells both in contact and in non-contact co-cultures was tracked manually on every second image (i.e., every 10 min in real time) with a precision of ~ 5 μ m, comparable to the average cell diameter (20–25 μ m). This procedure resulted in cell trajectories, $x_a(t)$, where the various cells are identified by the index a , and time is denoted by t . Cell motility was characterized by $d_a(\tau)$, the average distance of migration during a period of length τ (Stokes et al., 1991), calculated for each cell a , and a wide range of τ as $d_a(\tau) = \langle |x_a(t + \tau) - x_a(t)| \rangle_t$. For a given value of τ , the average $\langle \cdot \cdot \cdot \rangle_t$ includes each possible time point t for which both $x_a(t + \tau)$ and $x_a(t)$ is known. In a similar fashion, an entire cell population is characterized by $d(\tau) = \langle |x_a(t + \tau) - x_a(t)| \rangle_{a,t}$, where averaging extends over each cell of the population in addition to t . The functional form of d is quite characteristic of the underlying motion. In the case of a mathematical random

walk, d grows as a square root of τ , while for a highly persistent straight motion, d is proportional to τ .

To quantitate the velocity reduction of NE-4C cells caused by encountering astroglial cells in some of the non-contact co-cultures, first we determined the precise time of the establishment of stem cell/glia cell contacts. For these stem cells, reaching the glial border, the average velocity was determined for periods both before and after contacting glial cells.

Daughter Cell Separation

After a cell division, the progeny of the dividing cell was tracked, resulting in positions $x_a(t)$ and $x_b(t)$. The time of division was then recorded as t_{ab} . Their distances $D_{ab}(t)$ were calculated as $D_{ab}^2(t) = [x_a(t) - x_b(t)]^2$ for various time points t . To characterize the typical course of daughter cell separation in the observed population, we determined the average distance $D(t) = \langle D_{ab}(t - t_{ab}) \rangle_{ab}$ for each pair (a,b) of daughter cells.

RESULTS

To examine the influence of astroglial cells on the fate of neural stem cells, immortalized, neuroectoderm-derived multipotential cells (NE-4C cell line, mouse; Schlett and Madarász, 1997) were co-cultured with primary neonatal murine astrocytes. Astrocytes and neural stem cells were co-cultivated in two different culture systems. In contact co-cultures, NE-4C neural stem cells were seeded on top of a confluent monolayer of astroglial cells; in non-contact co-cultures, stem cells communicated with glial cells only through the culture medium. Co-cultures were maintained either under serum-free conditions or in the presence of 5% FCS. There were no differences in the recorded parameters between the cultures maintained in any of the three chemically defined media: Neurobasal-B27, MEM-F12-N2, and MEM-ITS. In addition to primary astrocytes, C6 rat glioma, U87 human glioma cell lines, primary mouse fibroblasts, and 3T3 cells were also tested with respect to their ability to induce neuronal differentiation.

Astroglia-induced neurogenesis by NE-4C neural stem cells was compared with the glia-initiated neuron formation by two different embryonic stem cell (ES) clone [(R1 Nagy et al., 1993) and GFP-expressing 1GFP/B2] under the same culture conditions. Both ES clones were shown to display broad developmental potential and have been used to produce chimera animals.

To trace NE-4C cells within the co-cultures, we used subclones constitutively expressing either green fluorescent protein (GFP) or placental alkaline phosphatase (PLAP) (Madarász et al., 2000; Demeter et al., 2004). The induction of NE-4C cells with 10^{-7} M all-trans-RA resulted in abundant neuron production (Fig. 1A). The expression of GFP or PLAP did not interfere with the RA-induced neuron formation (see Demeter et al., 2004).

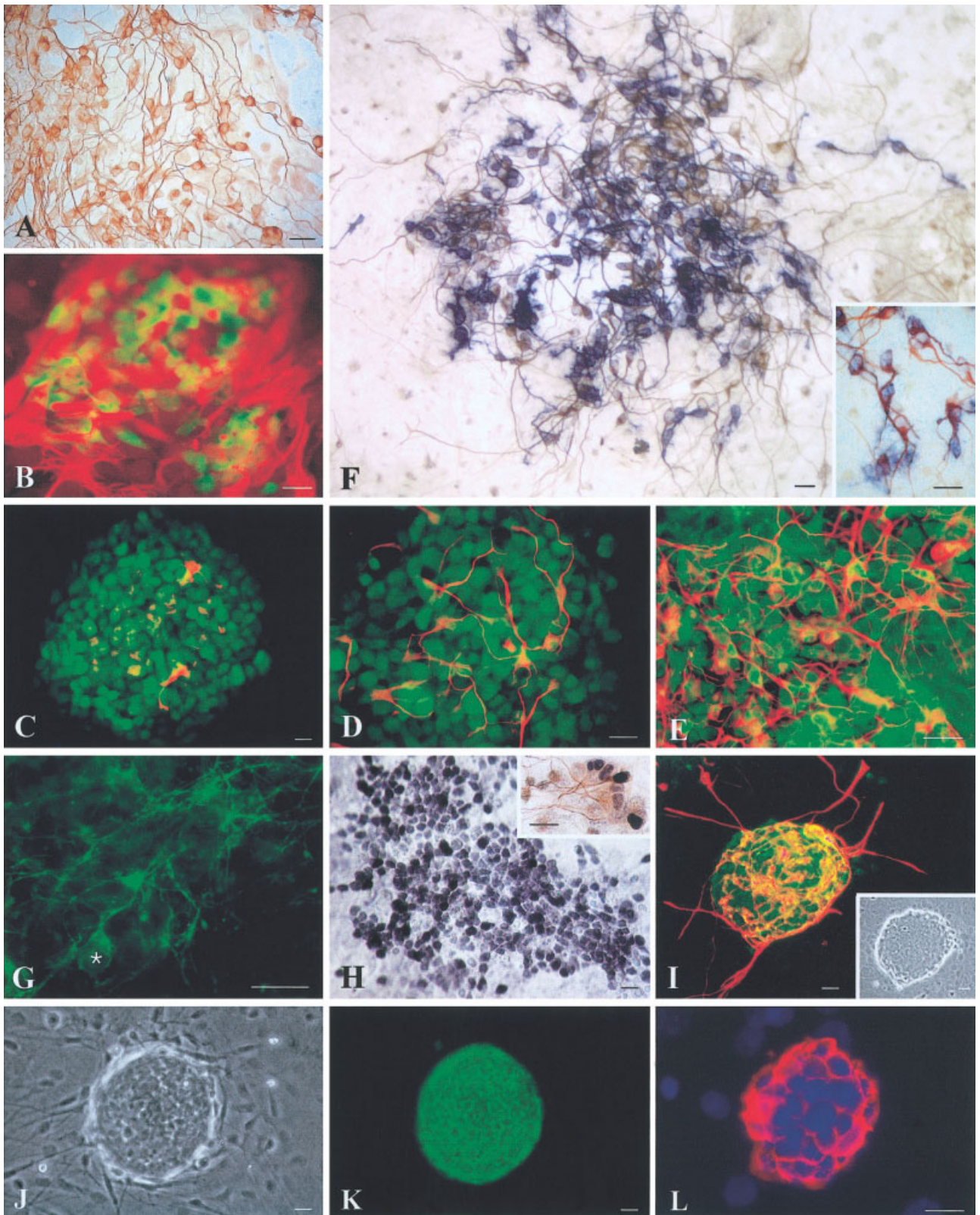


Figure 1.

Astrocytes Induce Neuron Formation by NE-4C Neuroectodermal Stem Cells in Contact Co-cultures

Single-cell suspensions of PLAP/NE-4C or GFP/NE-4C cells were seeded onto monolayers of astrocytes at a relatively low density (10^3 – 10^4 cell/cm²), resulting in a high astroglia/progenitor ratio. Exogenous RA was not added to the co-cultures. The neuronal differentiation of NE-4C cells was monitored daily by phase-contrast microscopy and by immunocytochemical analyses of fixed preparations. Co-localization of GFP, or the products of PLAP with neuron specific class III-type β -tubulin (Figs. 1C–F and 4C) or neurofilament (Fig. 1I) demonstrated the NE-4C origin of the neurons.

During the first few days after seeding, the neural stem cells formed clusters and produced quickly expanding aggregates. The cell assemblies were encircled by astroglial processes displaying intense GFAP immunostaining (Fig. 1B). By the third day, cells with neuronal features appeared inside the aggregates among nondifferentiated cells (Figs. 1C–I and 4C). The total number of neuron-specific β III-tubulin-positive cells rapidly increased between the days 3 and 7 (Fig. 2A). The average density of neurons reached the value found in all-*trans*-RA-induced monocultures of NE-4C cells (see Schlett et al., 1997). From day 5 on, NE-4C neurons formed loose networks on the surface of astrocytes (Fig. 1F) reminiscent of that seen in RA-induced monocultures (Fig. 1A).

The time course of co-culture development was similar in mouse astroglia/mouse NE-4C and rat astroglia/mouse NE-4C co-cultures. In the case of rat astrocytes, the formation of NE-4C neurons was demonstrated by the presence of the mouse-neuron specific antigen M6 (Fig. 1G).

NE-4C-derived neurons were not evenly distributed in the co-cultures, but were accumulating in distinct foci. By the end day 7 in co-culture, the local density of neurons was as high as 100,000–200,000 neuron/cm² ($142,691 \pm 43,738$ neuron/cm², $n = 3$) inside the cell assemblies. If co-cultures were grown in serum-free medium, nearly 100% (99 ± 2) of the NE-4C clusters developed into neurogenetic foci (Fig. 2B). In the presence of 5% serum, however, neurons were found only in $36 \pm 12\%$ of the clusters by the end of the first week (Fig. 2B).

The growth of NE-4C neural stem cells was supported by both astroglia and primary fibroblasts. However, fibroblasts did not induce neuron formation: neuron-specific β III-tubulin-expressing cells did not appear in any of the fibroblast/NE-4C co-cultures grown in serum containing medium and only sporadically in serum-free medium (Fig. 2B). C6 rat and U87 human glioma cells were more efficient in inducing NE-4C-derived neuron formation than 3T3 cells, although their inductive capacity was smaller than that of primary astrocytes (Fig. 2C). Aged primary astrocytes (i.e., cultured for more than 2 months) retained the ability to induce neuron formation by NE-4C cells (data not shown).

Our data clearly show that astrocytes or glia-derived cells induce abundant neuron formation of immortalized neural stem cells if cell-to-cell contacts are formed between astrocytes and stem cells. The rate of neuron formation is dependent on the presence, or absence, of serum factors (Fig. 2B).

To see whether astrocytes derived from distinct brain regions can differently modulate the neuron formation by neural stem cells, we prepared co-cultures of NE-4C cells with perinatal astrocytes obtained from murine cortex, diencephalon, mesencephalon, and cerebellum. The number of NE-4C-derived neurons was significantly higher in all astroglial co-cultures than that in noninduced NE-4C monocultures (Schlett and Madarász, 1997) or in co-cultures with fibroblasts (see Fig. 2B). The inductive capacities of different glial co-cultures varied considerably (Fig. 2D). The variations, however, were not consistent if we compared the results of distinct experimental series. Therefore, we cannot state that astrocytes from any of the four investigated brain areas provide more support for neuron formation than the others. Figure 2D presents the results of a representative experiment.

Astrocytes Also Induce Neuron Formation by NE-4C Cells in Non-contact Co-cultures

To investigate the contribution of glia-derived soluble factors to the neuronal differentiation of NE-4C neural stem cells, NE-4C cells were grown in the presence of glial conditioned medium (GCM). Conditioned media were taken from confluent 3–6-week-old astroglial cultures, as well as from primary fibroblast cul-

Fig. 1. (Overleaf.) NE-4C neural stem cells differentiate into neurons if grown in contact co-cultures with astrocytes. Mouse embryonic stem (ES) cells, however, form compact aggregates and do not show signs of differentiation under the same culture conditions. **A:** Retinoic acid (RA) induced NE-4C cells in monocultures, stained for neuron-specific β III-tubulin on day 7 following RA treatment. **B–I:** NE-4C neural stem cells in co-cultures with astrocytes, stained for different neuronal markers between days 4–7 of co-culture maintenance. The co-cultures were not treated with RA. **B:** Clusters of nondifferentiated GFP/NE-4C cells are surrounded by GFAP⁺ (red) astroglial cells. **C–E:** Neuron-specific β III-tubulin (red/orange) expressing GFP/NE-4C neuronal precursors (green) on day 4 (C), day 5 (D), and day 6 (E) day of co-culture development. The number of young neurons grows day by day. **F:** Loose neuronal network (brown: N-tubulin) of

PLAP/NE-4C cells (blue) on day 7 in co-culture, reminiscent of that seen on image A). Inset shows double labeled cells at higher magnification. **G–I:** Mouse neuron-specific antigen (M6) (G), NeuN (H), and Neurofilament-M (I) expressing NE-4C cells on astroglial monolayer on day 6. M6 staining was performed in co-cultures established with rat astrocytes. G: Asterisk marks a cell body. H: Inset shows N-tubulin (brown) and NeuN (dark blue) co-expressing cells at higher magnification. I: Inset is the phase-contrast image of the stained cell assembly, sitting on a glial monolayer. **J–L:** Mouse embryonic stem (ES) cells in co-cultures with astrocytes. J,K: ES cells of the 1GFP/B2 ES clone on the surface of astrocytes at day 14 in co-culture. L: SSEA-1 (red) staining of a nondifferentiated ES cell aggregate. The nuclei of ES cells are marked with Hoechst 33258 (blue). Scale bars = 25 μ m.

tures (FCM) for control. CMs were diluted with fresh culture medium at 1:1 and were transferred immediately onto NE-4C cells.

The relative viability of NE-4C neural progenitors was not enhanced by the presence of GCM (Fig. 3A). However, a significant increase was observed, if the cells were grown in a common fluid environment with living astrocytes in non-contact co-cultures, where the communication between the two cell types was allowed solely by diffusion (Fig. 3A). Living fibroblasts and, to a lesser extent, fibroblast CM, also supported the growth and survival of NE-4C cells (Fig. 3A).

Non-contact co-culturing with fibroblasts or addition of GCM did not induce neuronal differentiation of NE-4C cells. A significant increase in neuron formation was observed, however, if NE-4C cells were grown in a common fluid environment with living astrocytes (Fig. 3B). The density of NE-4C neurons decreased sharply with the distance from astroglial cells (Fig. 3C,D).

The data indicate that astroglial cells produce neuronal cell fate commitment initiating soluble factor(s). The short distance of action suggests that these factor(s) are either rapidly degraded or neutralized near the release sites.

Astrocytes Do Not Initiate Differentiation of Embryonic Stem Cells

To compare neural stem cells with embryonic stem cells in terms of the astroglia-induced neurogenesis, we plated a single-cell suspension of R1 or 1GFP/B2 mouse ES cells on the top of astroglial monolayers. Similar results were obtained with both ES clones: the single embryonic stem cells proliferated and formed dense aggregates within a few days. Most of the aggregates retained their compact structure throughout the two week period of co-culturing (Fig. 1J–L). During this period little outmigration and only sporadic neuronal differentiation could be observed and the ES cells retained the expression of stage-specific embryonic antigen-1 (SSEA-1) (Fig. 1L). The percentage of the neurogenic ES aggregates was as low as 0.64% in serum containing medium ($n = 4$) and 3.18% in serum-free medium ($n = 4$). In parallel assays on ES monocultures, standard RA treatment (10^{-7} M) resulted in abundant neuron formation by ES cells.

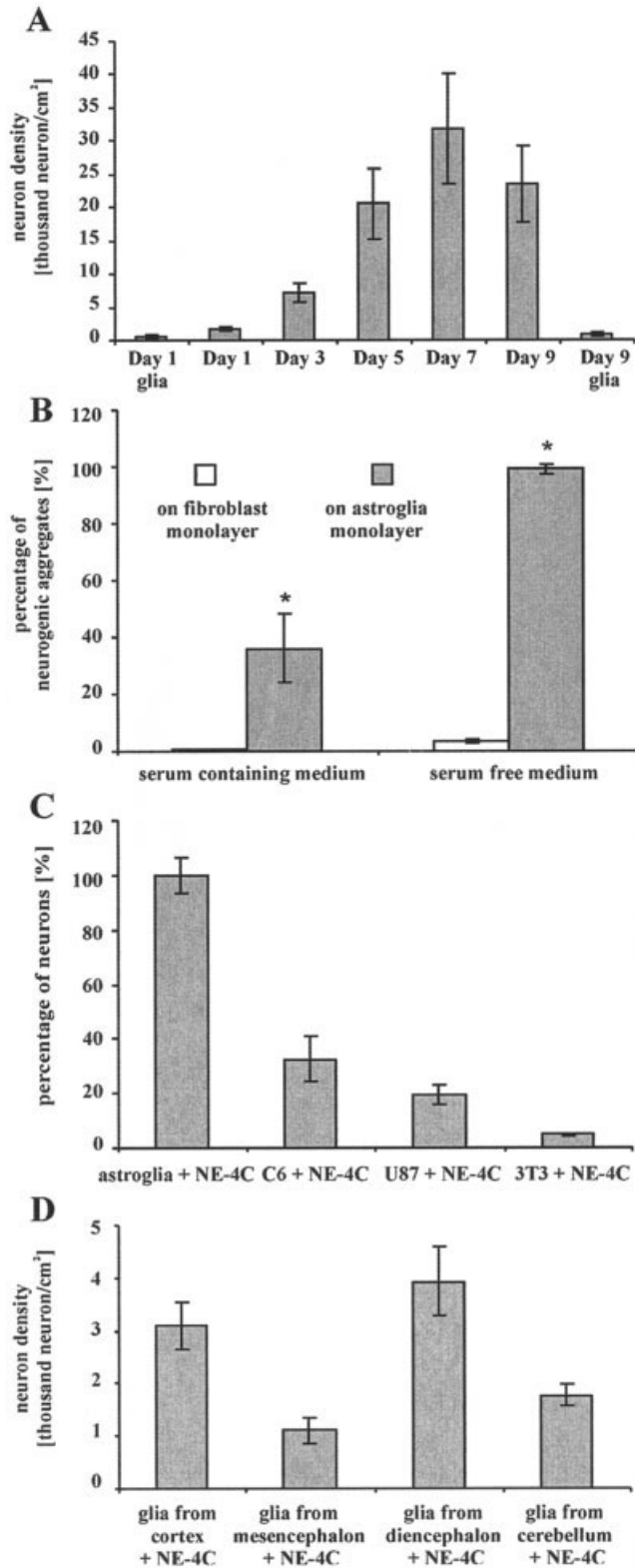


Fig. 2. Neuronal differentiation of NE-4C immortalized neural stem cells in various contact co-cultures. Young neurons were identified with antibodies to neuron-specific β III-tubulin in all experiments presented. **A**: Neuron density in astroglia/NE-4C co-cultures at different times from the onset of a representative experiment. Day 1 glia and day 9 glia represent data taken from astroglial monolayers. The cultures were grown in serum-free medium ($n = 3$, for each time). **B**: Percentage of neurogenic aggregates in astroglia/NE-4C and fibroblast/NE-4C contact co-cultures. The cultures were grown either in serum-containing or in serum-free medium ($n = 4$). $P < 0.05$. **C**: Percentage of neurons in contact co-cultures with primary rat astrocytes ($n = 3$), C6 rat glioma ($n = 2$), U87 human glioma ($n = 3$), and 3T3 cells ($n = 3$). Data obtained from astroglia/NE-4C co-cultures represent 100%. **D**: Neuron density in different astroglia/NE-4C co-cultures. The cultures were grown in serum containing medium ($n = 3$).

Contacts With Astrocytes Hinder, While Secreted Glial Compounds Enhance, the Motility of Noncommitted Neuroectodermal NE-4C Cells

To study the method of neurogenic aggregate formation and the development of the neurogenic cell assemblies, we made long-term time-lapse recordings and analyzed cell movements on the consecutive images. GFP-labeled neuroectodermal stem cells settled within ~30 min after seeding and were nearly evenly distributed on the surface of the confluent glial monolayer. According to the area calculations, the spacing of the NE-4C neural stem cells was ~100 μm —a distance long enough to ensure insular settlement but short enough to be overcome by cell migration (Fig. 4A).

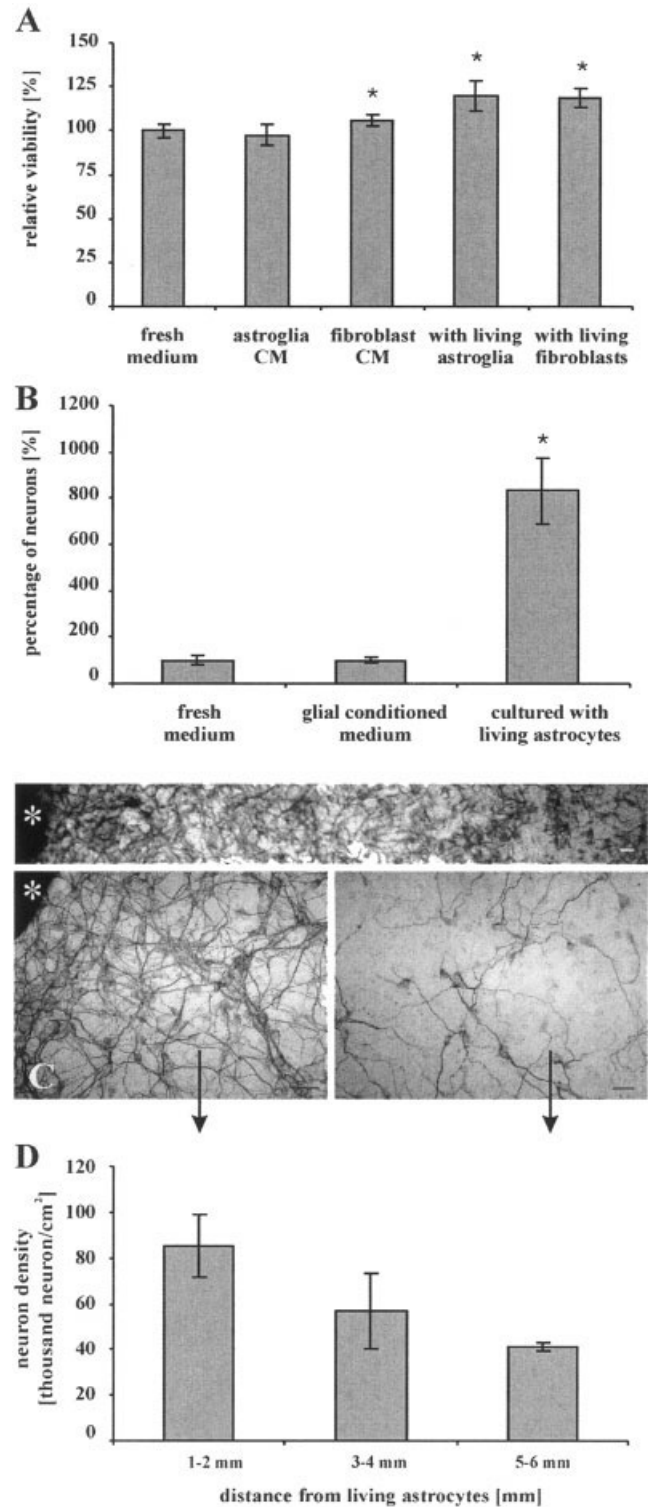
To trace the migratory routes, we determined the positions of individual cells by tracking the cell bodies on consecutive frames (in every 10 min) of the time-lapse recordings. The individual cell trajectories showed that, in contrast to the pronounced motile activity on poly-L-lysine-coated surfaces, NE-4C cell did not move far from the initial attachment site on astrocytes (Fig. 5A,B). The time-displacement functions calculated from the position data clearly showed that the migration of the neural stem cells was hindered on the surface of astrocytes (Fig. 5C). The average neural stem cell velocity was $12.2 \pm 2.8 \mu\text{m/h}$ in the monocultures and $4.7 \pm 1 \mu\text{m/h}$ in the contact co-cultures during the first two days after seeding.

We monitored the migration of NE-4C cells in non-contact co-cultures as well. At 48 h before the onset of the time-lapse recordings, we scraped small parts of the glial cultures. Astrocytes at the wound edge proliferated and moved toward the gap (see Környei et al., 2000). NE-4C cells were seeded onto scraped glial cultures at a density of 10^4 cell/cm^2 . The average velocity of NE-4C cells in the astrocyte-free areas was higher ($20.2 \pm 2.8 \mu\text{m/h}$) than that in monocultures seeded at similar density ($12.2 \pm 2.8 \mu\text{m/h}$). NE-4C cells displayed a substantial velocity loss when docked to astroglial cells ($13 \pm 2 \mu\text{m/h}$). The average velocity after contact necessarily reflects the glial velocity (Környei et al., 2000; $12 \mu\text{m/h}$) at the wound, as well. The time-lapse recordings demonstrated that most of the NE-4C cells remained either attached to or in the vicinity of the initial contact with the glial cells.

Fig. 3. Neuronal differentiation of NE-4C immortalized neural stem cells in non-contact co-cultures. Neurons were identified with antibodies to neuron-specific $\beta\text{III-tubulin}$. **A**: Relative viability of NE-4C cells grown for 48 h either in glia- or fibroblast-conditioned media or in the presence of living astrocytes or fibroblasts ($n = 6$) (100% = the viability in serum containing fresh medium). $P < 0.05$. **B**: Percentage of NE-4C neurons in cultures treated with glia-conditioned medium or grown together with living astroglial cells. $P < 0.001$. **C,D**: The decreasing density of neurons as a function of the distance from living astrocytes was demonstrated by immunocytochemical staining of neuron-specific $\beta\text{III-tubulin}$ -expressing cells. **C** (top): low-magnification overview, left bottom panel: higher magnification picture taken from a field next to astrocytes; (bottom): a field far from astrocytes. *Edge of the coverslip. Scale bar = 25 μm .

NE-4C Cells Form Aggregates in the Presence of Astroglial Cells

In monocultures of noninduced NE-4C cells the daughter cells departed from each other after each division (Movie 1, Fig. 5D [All supplementary movie



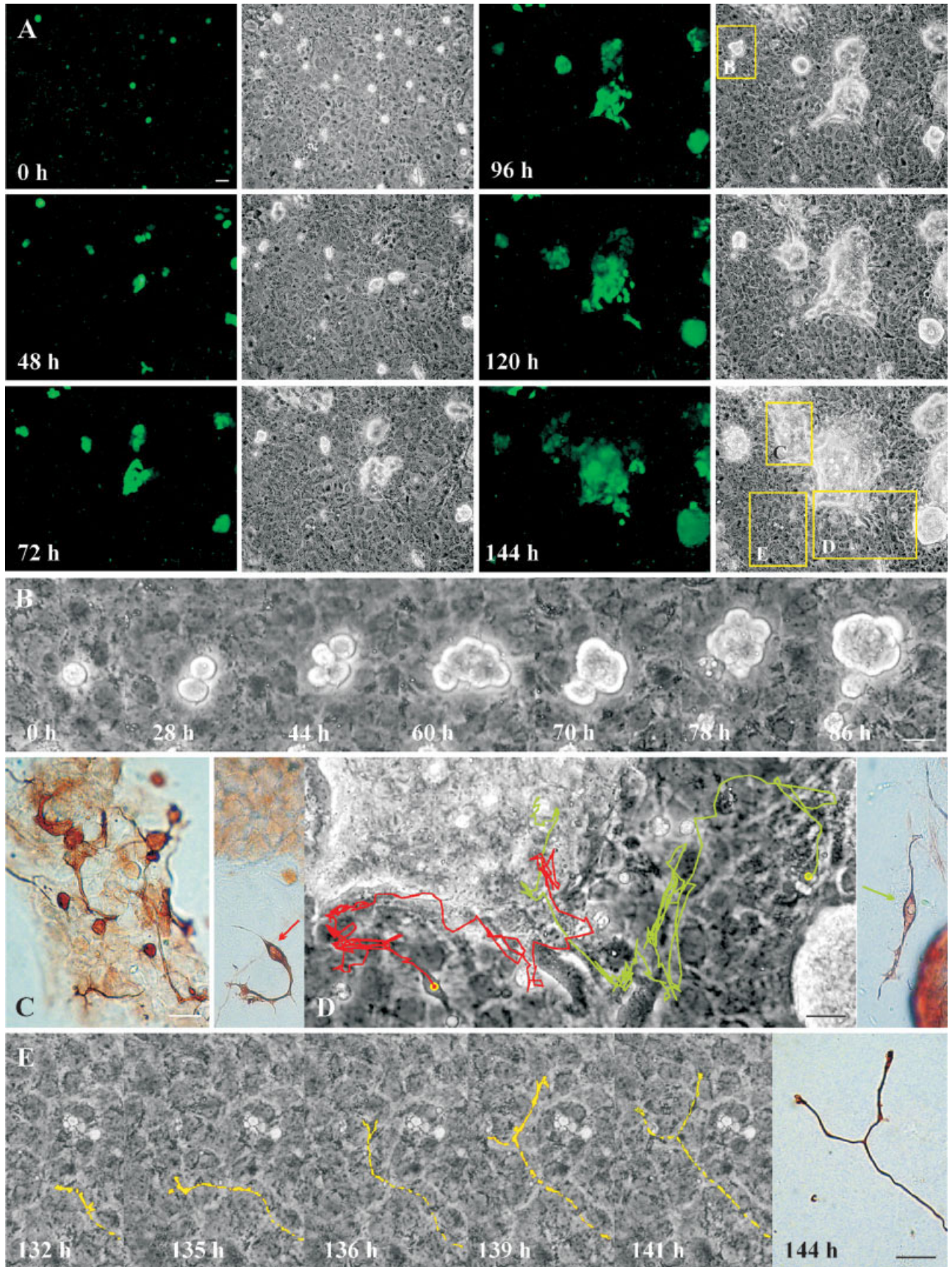


Figure 4.

clips available at <http://www.interscience.wiley.com/jpages/0894-1491/suppmat/index.html>). On the surface of astrocytes, however, the progenies of individual stem cells did not separate from each other in the course of repeated cell divisions (Movie 2). Within the first 40 h, the average distance between mother and daughter cells remained around 20 μm (Fig. 5D) indicating a close localization of the cells. The clonal proliferation led to the formation of compacted multicellular aggregates (Fig. 4B; Movie 2).

Aggregate formation by NE-4C cells was observed in non-contact co-cultures with astrocytes, as well (Fig. 6; see Movie 6). In this respect, there was no difference between co-cultures established by seeding stem cells directly onto scraped astrocytes, or onto coverslips inserted into astrocytic cultures. The aggregation was manifested in a marked decrease in the separation-distance of daughter cells after cell division. To demonstrate this effect, we assigned the observed cell division events into two groups. One group consisted of cell divisions occurred during the first 36 h of non-contact co-culturing. Cell divisions at later time points, when the aggregation of cells became evident (Fig. 6), consisted of the second group. For both groups, we determined the average distance of the daughter cells. The resulting curves (Fig. 5E) indicate that while daughter cells are moving away from each other rapidly at the onset of culturing (0–36 h), after 36 h of co-culturing they persist in close contacts (average distance $\sim 20 \mu\text{m}$, corresponding to the average cell diameter; see also Fig. 6 and Movie 6). In the corresponding (0–48 h) period, noninduced NE-4C cells in monoculture grew as evenly distributed monolayers, where aggregate formation did not occur (Schlett et al., 2000) (see Movie 1).

The time elapsed between consecutive cell divisions decreased during the initial phase of aggregate formation in the contact co-cultures [21 ± 5.2 h between the first and second divisions; 16.2 ± 1.6 h between the second and third divisions]. Some smaller, nonsignificant decrease was also observed in the monocultures of NE-4C cells [16.6 ± 4.4 h between the first and second divisions; 14.8 ± 2.1 h between the second and third divisions]. Attachment onto astrocytes seemed to delay the moment of the first cell division. In non-contact co-cultures, the average duplication time [10.5 ± 2 h] decreased significantly, in comparison with NE-4C monocultures [15.2 ± 3.2 h], indicating the immediate action of some soluble, mitogenic factors.

Migration of Neuronal Precursors Is Supported by Astroglial Cells

Characteristic changes in the morphology of neural stem cells were not revealed during the period of aggregate formation and growth comprising the first few days of co-cultivation (Fig. 4B; Movie 2). The progenies did not display neuronal characteristics, they did not leave the aggregates, nor were the processes growing out. By days 3–4, however, process-bearing cells appeared within the aggregates (Figs. 1C–E and 4C) or at the edges of the larger clusters (Fig. 4D). The process outgrowth was quick (see Movie 3) and reversible.

Some cells displaying polarized shape and extending processes left the aggregates by rapid translocation of the cell body (Movie 4; Fig. 4D). These cells later elongated long, neurite-like processes (Movie 5; Fig. 4E). Neuronal precursors gradually lost their previous preference of adhering to progenitors: in the first phase of their outmigration, they often followed the aggregate boundary (see Movie 4). According to the time-lapse recordings, the outmigrating cells did not divide and were later identified as neuronal precursors by staining for neuron-specific β III-tubulin (Fig. 4D,E). By days 5–6, a large number of neuron-specific tubulin-positive cells were revealed either in loose neuronal networks formed on the surface of astrocytes (Fig. 1F) or inside the NE-4C aggregates (Figs. 1D,E and 4C). Thus, the time-lapse data show that while astrocytes do not support the migration of proliferating progenitors, they provide migratory surfaces for committed neuronal precursors.

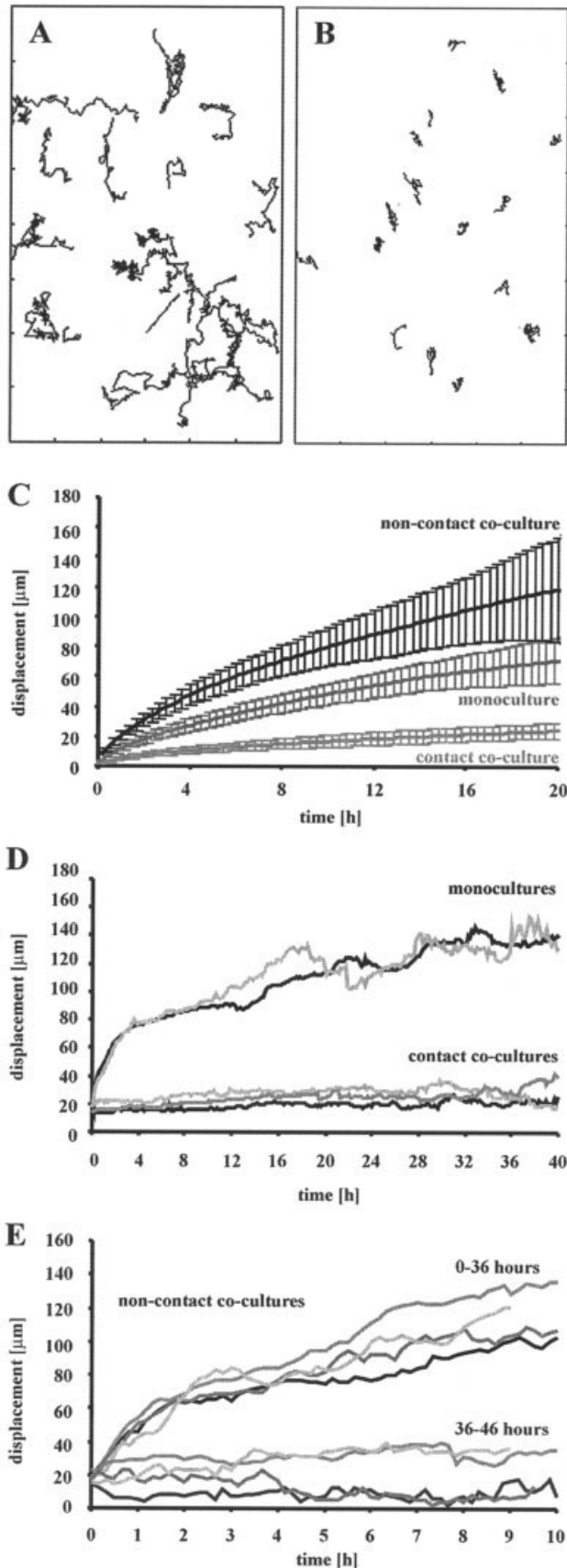
DISCUSSION

In order to investigate the astroglia-induced neurogenesis, we established co-cultures of astrocytes and NE-4C one-cell-derived neuroectodermal stem cells. In the present study, we show that primary neonatal astrocytes induce multitudinous neuron formation by noncommitted NE-4C cells without any exogenous inducers.

NE-4C cells (Schlett and Madarász, 1997) were shown to display several neural stem cell properties. Noninduced NE-4C cells express nestin intermediate filament proteins (Schlett and Madarász, 1997). Upon induction with all-*trans*-RA, they differentiate into neurons and astroglia in a progressive process, while

Fig. 4. (Overleaf.) Time-lapse images taken from different periods of a GFP/NE-4C/astroglia contact co-culture grown in serum-free medium. The time-lapse recordings are presented as supplementary movies (Movies 1–5). **A:** Overview of the development of GFP-labeled NE-4C cell aggregates in a co-culture. Serial fluorescent and phase-contrast images were taken from the same field in the first 144 h after plating the GFP/NE-4C cells onto astroglial monolayer. **B:** Aggregate formation by a NE-4C neuroectodermal stem cell on the surface of astrocytes (see Movie 2). **C:** Neuron-specific tubulin-positive cells inside an aggregate marked by a quadrangle on image A). **D:** The red and green lines on the phase-contrast image show the trajectories of

two neuronal precursors (see Movie 4). The traced cells left the aggregate, wandered on the surface of astrocytes and did not divide during the 60-h period of the recording. The cells were subsequently identified as neuronal precursors by neuron-specific β III-tubulin immunostaining (left and right). The migratory routes were recorded from the moment of birth of the “green” neuron (80 h after seeding the NE-4C cells onto astroglia). **E:** Elongation of a neurite of an NE-4C neuron on the surface of astroglial cells (see Movie 5). The neurite was shown to express neuron-specific β III-tubulin (last panel). Scale bars = 25 μm .



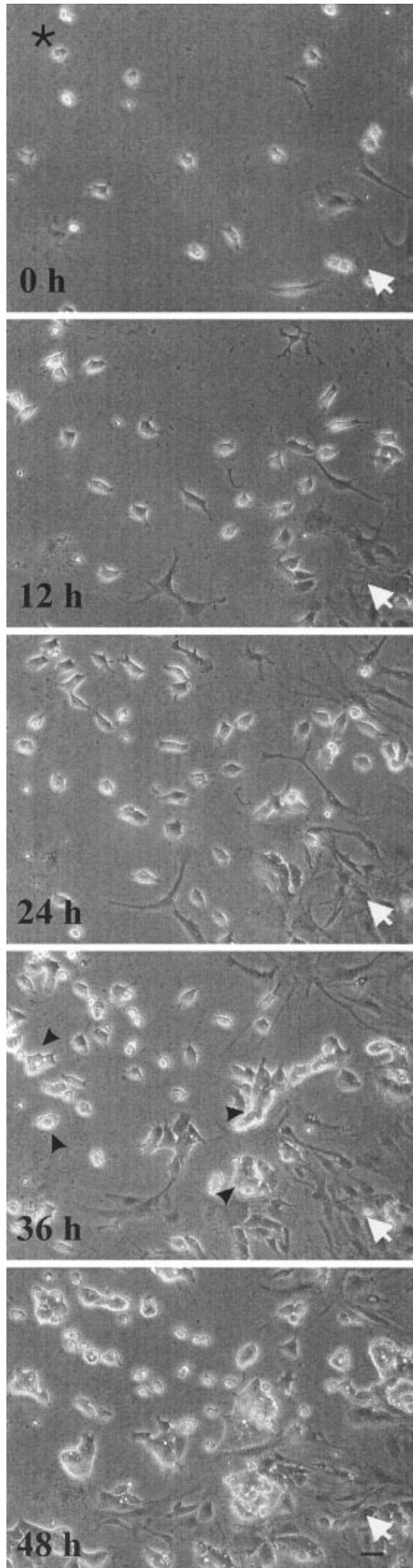
persistently producing nondifferentiated, multipotent cells (Schlett and Madarász, 1997; Schlett et al., 1997, 2000; Tárnok et al., 2002). If implanted into the embryonic brain, NE-4C cells integrate into the host tissue and produce morphologically differentiated neurons (Madarász et al., 2000; Demeter et al., 2004). Our highly reproducible model of astroglia-induced neurogenesis allowed the targeted investigation of successive events. In the present study, we focused on the initial steps of astroglia-induced neuron formation.

Specificity of Astroglial Induction

Astrocytes were shown to produce a variety of growth factors (e.g., fibroblast growth factor type 2 [FGF-2], epidermal growth factor [EGF], platelet-derived growth factor [PDGF]) stimulating the viability and proliferation of many distinct cell types (Gomez-Pinilla et al., 1992; Ridet et al., 1997; Reuss and Unsicker, 2000). However, we observed a growth-inducing effect of primary fibroblasts as well, indicating that facilitation of neural stem cell growth is not an exclusive property of astroglia. Although direct (^3H -thymidine or BrdU incorporation) assays on proliferation were not performed, according to phase-contrast microscopic observations, fibroblasts appeared to display an equal or little bit more effective growth inducing activity than the astrocytes. This finding was also supported by the fact that fibroblast-conditioned media enhanced the relative viability of NE-4C cells, while glial CM was not effective in that sense.

Although fibroblasts promoted NE-4C cell proliferation, they did not initiate the formation of neuron-specific β III-tubulin-expressing neuronal precursors. In contrast, astrocytes provided an instructive environment for neuronal differentiation of NE-4C cells. These observations are in accord with data presented on the neuronal cell fate inducing activity of subventricular zone-derived neonatal and adult astroglia (Lim and Alvarez-Buylla 1999; Song et al., 2002). Our findings clearly show that enhancement of the rate of stem cell growth is not sufficient for qualitative changes; i.e., some astroglia-specific inductive factors are responsible for the switch to a neuronal cell fate.

Fig. 5. Migratory activity of NE-4C neural stem cells in monocultures and in co-cultures with astrocytes. **A:** The 24-h migratory routes of NE-4C cells on poly-L-lysine-coated culture dishes. **B:** On astroglial monolayer. The trajectories are shown in fields of $800\ \mu\text{m} \times 600\ \mu\text{m}$ ($n = 30$ in both cases). **C:** Average displacements of NE-4C cells in monocultures, in contact- and in no-contact co-cultures with astrocytes were plotted as a function of time ($n = 2$; $n = 3$). **D:** Average distances between the two daughter cells of a cell division as a function of time. Each line represents data obtained from independent experiments (NE-4C monocultures and NE-4C/astroglia contact co-cultures). The periodic fluctuations in the distances between NE-4C cells in monocultures are due to a quick and rectilinear separation of the progenies. **E:** Average distances between daughter cells born during the 0–36-h and 36–48-h periods of co-culturing. The lines represent data obtained from 4–4 fields from two independent experiments (NE-4C/astroglia non-contact co-cultures). Cells were grown in serum-free medium in all experiments.



We found that primary astrocytes induced neuron formation by the mouse-derived neuroectodermal cells, regardless of whether they were derived from rats or mice. Moreover, C6 rat or U87 human glioma cells supported neuron formation by NE-4C neural progenitors. The data indicate that some neuronal differentiation initiating signals are conserved among various mammalian species.

To investigate the regional heterogeneity of glial neurogenesis supporting effect, we used neonatal astrocytes derived from the cortex, diencephalon, mesencephalon, or cerebellum. Although all these astroglial cells induced abundant neuron formation by NE-4C cells, we did not find consistent differences in the neurogenesis-inducing potential of astrocytes derived from the different brain areas.

It is likely, however, that the potential of astrocytes to induce stem cell-derived neurogenesis declines with aging, and the regional differences become more characteristic. Indeed, neonatal astrocytes were shown to be more effective in supporting neuron formation by adult stem cells than adult-derived astroglia (Song et al., 2002). Also, astrocytes taken from adult neurogenic tissue (hippocampus) were shown to support stem cell-derived neurogenesis, in contrast to astrocytes derived from the non-neurogenic regions (spinal cord) (Song et al., 2002). The phenomenon seems to be related to the finding that astrocytes outside the neurogenic regions do not appear to be neurogenic under normal *in vivo* conditions (Alvarez-Buylla and Lim, 2004, Sanai et al., 2004).

Our results suggest that the potential of perinatal and mainly parenchymal astroglial cells to induce neuron formation is maintained in culture and is fairly uniform throughout the developing brain. In order to conduct more precise studies on the impact of different astroglial populations on neural stem cell differentiation, we have recently monitored the regional identity of astrocytes during extended *in vitro* maintenance and have correlated it to their neural induction capacity (Varga et al., 2004).

Astroglia–Embryonic Stem Cell Interactions

In contrast to NE-4C neuroectoderm-derived cells, glial cells did not support the neuronal differentiation of the initially single, totipotent ES cells, at least during a period of 14 days. We cannot claim that ES cells would not become capable of neuron production in later phases of glia/ES coexistence. Protocols for neural differentiation of mouse ES cells usually require extended

Fig. 6. Time-lapse images taken from a NE-4C/astroglia non-contact co-culture grown in serum-free medium. The time-lapse recording is presented as supplementary Movie 6. Note that the aggregate formation (black arrows) of NE-4C cells starts around 36 h and occurs in both astrocyte-free and astrocyte-covered areas. Astrocytes can be recognized by their flattened shape. Asterisks, center of the cell-free area; white arrowheads, major migratory direction of astroglial cells.

TABLE 1. Summary of Data Provided on Astroglia-Induced Neurogenesis

Stem cells	Astroglial induction of neuronal differentiation			
	Contact interactions	Effect	Humoral interactions	Effect
Adult hippocampal stem cells (Song et al., 2002)	On live glia	↑	In shared fluid environment with live glia	↑
Neonatal and adult SVZ stem cells (Lim and Alvarez-Buylla, 1999)	On fixed glia	↑	In shared fluid environment with live glia	∅
	On live glia	↑		
NE-4C neuroectodermal cells (present work)	On killed glia	∅	In concentrated GCM	∅
	On glia membrane	∅	In shared fluid environment with live glia	↑
On live glia	↑			
Mouse and primate ES cell colonies (Nakayama et al., 2003)	On live glia	↑	In GCM	∅
Dissociated mouse ES cells (present work)	On live glia	∅	In GCM	↑
			nd	nd

↑, increased rate of neurogenesis; ∅, unchanged rate of neurogenesis; GCM, glial conditioned medium; SVZ, subventricular zone; EC, embryonic stem; nd, not detected.

in vitro maintenance, and the formation of multicellular assemblies appears to be a prerequisite for the later differentiation (Rathjen et al., 2002). Data provided by Nakayama et al. (2003) showed that aggregate formation and pre-differentiation of totipotent embryonic stem cells rendered these cells responsive to astroglia-derived neurogenetic signals.

In contrast to ES cells, the nestin-positive NE-4C cells (Schlett et al., 1997), derived from the neuroectoderm of E9 embryonic brain vesicles, responded readily to the signals provided by astrocytes. Their origin and stage of differentiation may explain their responsiveness to the astroglial instructive signals. In accordance with accumulating data (Zhang et al., 2001; Wichterle et al., 2002; Bjorklund et al., 2002), we assume that stem cell populations should reach a yet nondefined stage of cell fate commitment in order to respond to tissue-specific differentiation signals.

Contribution of Humoral Factors and Contact Interactions to Astroglia-Induced Neuronal Cell Fate Decision

Available data, even if obtained on different stem cells and under different culture conditions, indicate that astrocytes can regulate stem cell-derived neurogenesis through contact interactions (Table 1). The contribution of humoral communication to this activity is less clear (Lim and Alvarez-Buylla, 1999).

In our experiments, 1:1 diluted glial conditioned medium did not induce neuronal differentiation of NE-4C cells. In contrast, the immediate humoral communication with living astrocytes led to abundant neuron formation. The action of some glia-derived soluble factors was also demonstrated by shortening of the duplication time of NE-4C cells in shared fluid environment with astrocytes. In both contact and non-contact types of co-cultures, the vicinity of living astrocytes provoked the formation of stem cell aggregates, an inevitable early step of neuronal differentiation (Schlett et al., 2000; Tárnok et al., 2002).

In non-contact co-cultures established by inserting high-density NE-4C cultures on coverslips into astrocytic cultures, the rate of neuronal differentiation was inversely proportional to the distance between astrocytes and stem cells. The observation indicates that some released factors play an essential role in glia-induced neuron formation, and their concentration declines in a short range. The secreted compounds might be degraded, bound by local matrix and/or cell surface molecules, or simply overdiluted by the disproportionately large extracellular fluid compartment. The gradient formation, in contrast, implies a continuous production of these factors by astrocytes. For the time being, it remains to be determined whether astrocytes in monocultures produce similar factors or whether the production of inductive molecules is initiated upon interaction with stem cells.

During the initial period of co-culturing, striking differences were observed between the motile activity of the stem cells grown either in contact or in non-contact co-cultures. The vicinity of glial cells increased the motility of NE-4C cells, as long as they did not come into immediate contact with astrocytes. Direct contact with astrocytes, in contrast, reduced the motility significantly. Several growth-inducing factors, released by astrocytes, were shown to enhance the motility of various cell types (e.g., Szebenyi and Fallon, 1999). We report that some secreted, yet nonidentified, glial factors enhance the in vitro motility of neuroectoderm-derived neural progenitor cells too. Among the released factors, astroglia-derived ECM molecules must also be taken into account. Such molecules might provide both attachment surface and matrices for immobilizing growth/induction-promoting factors.

The time-lapse recordings revealed that in contact co-cultures, single NE-4C cells attached to, but did not spread on, astrocytes. Also, NE-4C cells showed hardly any active migration on the surface of glial cells, but divided and formed clonal clusters. In agreement, NE-4C cells in non-contact co-cultures displayed a substantial velocity loss upon encountering astrocytes. The restricted migration, accompanied by local prolif-

eration, resulted in the formation of NE-4C clusters. The cluster formation resembles the aggregation of cells during the initial period of RA-induced neurogenesis in monotypic NE-4C cultures (Schlett et al., 2000; Tárnok et al., 2002).

While noninduced NE-4C cells migrate randomly on pLL-coated surfaces (Czirók et al., 1998) and display random collisions and separations, RA-induced NE-4C cells “stick” together upon encountering each other, forming aggregates (Schlett et al., 2000). Detailed analyses (Tárnok et al., 2002) showed that cell surface properties change dramatically in response to RA. As a result, homotypic cell-to-cell contacts are formed, which are indispensable for the initiation of neuronal differentiation (Bittman et al., 1997; Schlett et al., 2000; Tárnok et al., 2002; Chojnaki et al., 2003; Grandbarbe et al., 2003). The initial aggregate formation by NE-4C cells in the vicinity of living astrocytes may be a consequence of some rapid changes in their adhesive properties and might indicate an early response to astroglia-derived inducing factors.

The restricted migration of noncommitted cells on astrocytes also indicates that the set of adhesion molecules on NE-4C cells is not compatible with the adhesive surfaces provided by the astroglial environment. A large body of published data (Alvarez-Buylla and Garcia-Verdugo, 2002; Thomas et al., 1996; Lois et al., 1996; Wichterle et al., 1997) demonstrate that the migration of neuroblasts is restricted to, and permitted only along, some defined “migratory pathways.” The great mass of the astroglia-rich brain parenchyme represents a nonpermissive environment for neuroblast migration. Our previous observations also show that noncommitted NE-4C cells segregate from the host tissue and form separate clusters if implanted into the newborn or adult (but not into the embryonic) mouse brain (Madarász et al., 2000; Demeter et al., 2004).

Taken together, the nonpermissive astroglial surfaces in concert with preferential homotypic contacts can lead to aggregate formation by NE-4C neural stem cells. It appears that astrocytes can crowd the cells into a microenvironment in which stem cell/stem cell interactions can develop, and the future neurons can be sorted out by mechanisms such as lateral induction/inhibition (Artavanis-Tsakonas et al., 1995; Chojnaki et al., 2003; Grandbarbe et al., 2003). Indeed, the microenvironment of the aggregates was the scene for the appearance of neuron-specific β III-tubulin-expressing cells.

Crowding NE-4C cells into compact assemblies either by forced aggregation (Tárnok et al., 2002) or by fibroblast-induced growth, however, was not sufficient for the initiation of neuron formation. Astrocytes, therefore, not only ensure the required local stem cell density, but actively contribute to the neuronal fate decision. Some short-range-acting soluble factors, which were shown to induce neuron formation in dense NE-4C cultures, might serve such tasks. Based on the similarities between RA- and glia-induced neurogen-

esis, we presume that retinoids might be among the glia-derived inductive factors.

In Contrast to Noncommitted Progenitors, Postmitotic Neuronal Precursors Migrate Readily on the Surface of Astrocytes

In contrast to noncommitted cells, postmitotic neuron-specific β -III tubulin-expressing precursors left the aggregates, migrated, and elongated processes on the surface of astrocytes. The data show that astroglial cells provide selective support for attachment and migration of developing cells depending on their stage of neural differentiation. ES cells or noncommitted proliferating neuroectodermal cells receive nonpermissive migratory signals, while the migration and process elongation of postmitotic neuronal precursors are supported by perinatal astrocytes, at least in vitro.

Besides their basic cell and neurobiological interests, cellular interactions between stem cells and astrocytes have an impact on the elaboration of potential methods for brain cell replacement or gene delivery. Intracerebral cell grafts are implanted into astroglia-rich environments, where the interaction with parenchymal astrocytes appears to be crucial with respect to stem cell survival, proliferation, and/or tissue-type differentiation (Demeter et al., 2004; Emsley et al., 2004). Communication of stem cells with astrocytes should be carefully analyzed in order to find the right developmental stage of stem cells and the appropriate physiological state of astrocytes for any sort of future neural stem cell therapy.

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REFERENCES

- Alvarez-Buylla A, Garcia-Verdugo JM. 2002. Neurogenesis in adult subventricular zone. *J Neurosci* 22:629–634.
- Alvarez-Buylla A, Lim DA. 2004. For the long run: maintaining germinal niches in the adult brain. *Neuron* 41:683–686.
- Artavanis-Tsakonas S, Matsuno K, Fortini E. 1995. Notch signaling. *Science* 268:225–323.
- Banker GA. 1980. Trophic interactions between astroglial cells and hippocampal neurons in culture. *Science* 209:809–810.
- Bittman K, Owens DF, Kriegstein AR, LoTurco JJ. 1997. Cell coupling and uncoupling in the ventricular zone of developing neocortex. *J Neurosci* 17:7037–7044.

- Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O. 2002. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc Natl Acad Sci USA* 99:2344–2349.
- Campbell K. 2003. Signaling to and from radial glia. *Glia* 43:44–46.
- Campbell K, Götz M. 2002. Radial glia: multi-purpose cells for vertebrate brain development. *Trends Neurosci* 25:235–238.
- Chojnacki A, Shimazaki T, Gregg C, Weinmaster G, Weiss S. 2003. Glycoprotein 130 signaling regulates Notch1 expression and activation in the self-renewal of mammalian forebrain neural stem cells. *J Neurosci* 23:1730–1741.
- Czirók A, Schlett K, Madarász E, Vicsek T. 1998. Exponential distribution of locomotion activity in cell cultures. *Phys Rev Lett* 81:3038–3041.
- Demeter K, Herberth B, Duda E, Domonkos Á, Jaffredo T, Herman JP, Madarász E. 2004. Fate of neural stem cells implanted into the adult, newborn and embryonic forebrain. *Exp Neurol* 188:254–267.
- Doetsch F. 2003. A niche for adult neural stem cells. *Curr Opin Genet Dev* 13:543–550.
- Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. 1997. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci* 17:5046–5061.
- Emsley JG, Arlotta P, Macklis JD. 2004. Star-cross'd neurons: astroglial effects on neural repair in the adult mammalian CNS. *Trends Neurosci* 27:238–240.
- Gomez-Pinilla F, Lee JW, Cotman CW. 1992. Basic FGF in adult rat brain: cellular distribution and response to entorhinal lesion and fimbria-fornix transection. *J Neurosci* 12:345–355.
- Götz M, Steindler D. 2003. To be glial or not—how glial are the precursors of neurons in development and adulthood? *Glia* 43:1–3.
- Grandbarbe L, Bouissac J, Rand M, Hrabe de Angelis M, Artavanis-Tsakonas S, Mohier E. 2003. Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. *Development* 130:1391–1402.
- Hall A, Mira H, Wagner J, Arenas E. 2003. Region-specific effects of glia on neuronal induction and differentiation with a focus on dopaminergic neurons. *Glia* 43:47–51.
- Hatten ME. 1999. Central nervous system neuronal migration. *Annu Rev Neurosci* 22:511–539.
- Horner PJ, Palmer T.D. 2003. New roles for astrocytes: the nightlife of an “astrocyte.” *La vida loca!* *Trends Neurosci* 26:597–603.
- Környei ZS, Czirók A, Vicsek T, Madarász E. 2000. Proliferative and migratory responses of astrocytes to in vitro injury. *J Neurosci Res* 61:421–429.
- Lim DA, Alvarez-Buylla A. 1999. Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. *Proc Natl Acad Sci USA* 96:7526–7531.
- Livingstone LR, White A, Sprouse J, Livanos E, Jacks T, Tlsty TD. 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* 18:923–935.
- Lois C, Garcia-Verdugo JM, Alvarez-Buylla A. 1996. Chain migration of neuronal precursors. *Science* 271:978–981.
- Madarász E, Demeter K, Herberth B, Herman JP, Domonkos Á, Kúsz E, Duda E. 2000. Fate of embryonic neural progenitor cells in the forebrain of adult mice. *Eur J Neurosci* 12(suppl):292.
- Malatesta P, Hartfuss E, Götz M. 2000. Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* 127:5253–5263.
- Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63.
- Nagy A, Rossant J, Nagy R, Abramow-Newerly W, Roder J. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proc Natl Acad Sci USA* 90:8424–8428.
- Nagy A, Gerstensten M, Vintersten K, Behringer R. 2003. Manipulating the mouse embryo. Cold Spring Harbor Press, NY: Cold Spring Harbor Laboratory Press.
- Nakayama T, Momoki-Soga T, Inoue N. 2003. Astrocyte-derived factors instruct differentiation of embryonic stem cells into neurons. *Neurosci Res* 46:241–249.
- Nedergaard M. 1994. Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science* 25:1768–1771.
- Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR. 2001. Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409:714–720.
- Noctor SC, Flint AC, Weissman TA, Wong WS, Clinton BK, Kriegstein AR. 2002. Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J Neurosci* 22:3161–3173.
- Panchision DM, McKay RD. 2002. The control of neural stem cells by morphogenic signals. *Curr Opin Genet Dev* 12:478–487.
- Rathjen J, Haines BP, Hudson KM, Nesci A, Dunn S, Rathjen PD. 2002. Directed differentiation of pluripotent cells to neural lineages: homogeneous formation and differentiation of a neuroectoderm population. *Development* 129:2649–2661.
- Reuss B, Unsicker K. 2000. Survival and differentiation of dopaminergic mesencephalic neurons are promoted by dopamine-mediated induction of FGF-2 in striatal astroglial cells. *Mol Cell Neurosci* 16:781–792.
- Ridet JL, Malhotra SK, Privat A, Gage FH. 1997. Reactive astrocytes: cellular and molecular cues to biological function. *Trends Neurosci* 20:570–577.
- Sanaï N, Tramontin AD, Quinones-Hinojosa A, Barbaro NM, Gupta N, Kunwar S, Lawton MT, McDermott MW, Parsa AT, Garcia-Verdugo JM, Berger MS, Alvarez-Buylla A. 2004. Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature* 427:740–744.
- Schlett K, Madarász E. 1997. Retinoic acid induced neural differentiation in a neuroectodermal cell line immortalized by p53 deficiency. *J Neurosci Res* 47:405–415.
- Schlett K, Herberth B, Madarász E. 1997. In vitro pattern formation during neurogenesis in neuroectodermal progenitor cells immortalized by p53-deficiency. *Int J Dev Neurosci* 15:795–804.
- Schlett K, Czirók A, Tárnok K, Vicsek T, Madarász E. 2000. Dynamics of cell aggregation during in vitro neurogenesis by immortalized neuroectodermal progenitors. *J Neurosci Res* 60:184–194.
- Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A. 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci* 21:7153–7160.
- Song H, Stevens CF, Gage FH. 2002. Astroglia induce neurogenesis from adult neural stem cells. *Nature* 417:39–44.
- Steindler DA, Laywell ED. 2003. Astrocytes as stem cells: nomenclature, phenotype, and translation. *Glia* 43:62–69.
- Stokes CL, Lauffenburger DA, Williams SK. 1991. Migration of individual microvessel endothelial cells: stochastic model and parameter measurement. *J Cell Sci* 99:419–430.
- Szebenyi G, Fallon JF. 1999. Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytol* 185:45–106.
- Tárnok K, Pataki A, Kovács J, Schlett K, Madarász E. 2002. Stage-dependent effects of cell to cell contacts on in vitro induced neurogenesis by immortalised neuroectodermal progenitors. *Eur J Cell Biol* 81:403–412.
- Temple S. 2001. The development of neural stem cells. *Nature* 414:112–117.
- Temple S, Davis AA. 1994. Isolated rat cortical progenitor cells are maintained in division in vitro by membrane-associated factors. *Development* 120:999–1008.
- Thomas LB, Gates MA, Steindler DA. 1996. Young neurons from the adult subependymal zone proliferate and migrate along an astrocyte, extracellular matrix-rich pathway. *Glia* 17:1–14.
- Varga BV, Herberth B, Bence M, Hádinger N, Madarász E. 2004. Expression of neurogenic and region specific genes during the in vitro induced neurogenesis of NE-4C cloned neuroectodermal cells. Meeting abstract, International Society for Developmental Neuroscience, Edinburgh, August 3–8.
- Wichterle H, Garcia-Verdugo JM, Alvarez-Buylla A. 1997. Direct evidence for homotypic, glia-independent neuronal migration. *Neuron* 18:779–791.
- Wichterle H, Lieberam I, Porter JA, Jessell TM. 2002. Directed differentiation of embryonic stem cells into motor neurons. *Cell* 110:385–397.
- Zhang SC, Wernig M, Duncan ID, Brustle O, Thomson JA. 2001. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. *Nat Biotechnol* 19:1129–1133.