

Isolated rat cortical progenitor cells are maintained in division in vitro by membrane-associated factors

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SUMMARY

Ventricular zone cells in the developing CNS undergo extensive cell division in vivo and under certain conditions in vitro. The culture conditions that promote cell division have been studied to determine the role that contact with cell membrane associated factors plays in the proliferation of these cells. Progenitor cells have been taken from the ventricular zone of developing rat cerebral cortex and placed into microwells. Small clusters of these cells can generate large numbers of neurons and non-neuronal progeny. In contrast, single progenitor cells largely cease division, approximately 90% acquiring neuron-like characteristics by 1 day in vitro. DiI-labeled, single cells from embryonic day 14 cortex plated onto clusters of unmarked progenitor cells have a significantly higher probability (approximately 3-fold) of maintaining a progenitor cell

phenotype than if plated onto the plastic substratum around 100 µm away from the clusters. Contact with purified astrocytes also promotes the progenitor cell phenotype, whereas contact with meningeal fibroblasts or balb3T3 cells promotes their differentiation. Membrane homogenates from cortical astrocytes stimulate significantly more incorporation of BrdU by E14 cortical progenitor cells than membrane homogenates from meningeal fibroblasts. These data indicate that the proliferation of rat cortical progenitor cells can be maintained by cell-type specific, membrane-associated factors.

Key words: rat, cortical progenitor cell, ventricular zone, CNS, cell division, in vitro culture

INTRODUCTION

The majority of CNS cells arise from the ventricular zone, a pseudo-stratified layer of columnar neural progenitor cells that lines the ventricles (Jacobson, 1991). The formation of a normal cerebral cortex depends on the generation, from these progenitor cells, of appropriate cortical cell types in appropriate numbers at set times in development. The mechanisms by which proliferation of ventricular zone cells is controlled are not entirely known. A number of soluble factors have been shown to stimulate division of embryonic CNS progenitor cells in vitro. For example, cells from the 10-day embryo (E10) murine telencephalon are stimulated to divide by basicFGF and fetal calf serum (FCS) (Kilpatrick and Bartlett, 1993) and older cortical cells (E13) from the rat divide in response to bFGF (Gensburger et al., 1987). EGF (Reynolds et al., 1992) and NGF in combination with bFGF (Cattaneo and McKay, 1990) stimulate division of embryonic rat striatal progenitor cells. Soluble factors released from embryonic striatal cells stimulate division of E13.5 rat septal cells (Temple, 1989). EGF, acidic and basicFGF and TGF α stimulate division of embryonic rat retinal cells (Anchan et al., 1991; Lillien and Cepko, 1992). Besides the action of soluble factors, there is evidence that cell contact may be important for maintaining division in ventricular zone cells.

Studies have shown that ventricular zone cells will maintain

division if kept as aggregates or explants but will largely cease division if plated as single, dispersed cells in monolayer cultures. This result has been reported for cultures of a number of CNS regions, including retina (Sheffield and Moscona, 1969; Watanabe and Raff, 1990; Sparrow et al., 1990), striatum (Reynolds et al., 1992), cerebellum (Gao et al., 1991), olfactory epithelium (Mahanthappa and Schwarting, 1993) and chick cerebral cortex (Barakat et al., 1982). In the last paper, it was shown that chick cortical ventricular zone cells plated at high density on a collagen substratum, which encouraged aggregate formation, divided more efficaciously than cells plated at the same density on poly-L-lysine, in which aggregates did not form. These data strongly suggest that cell contact is important in maintaining proliferation of chick cortical precursor cells. However, none of these experiments using aggregated cultures show directly that a membrane-associated factor has an effect on the proliferation of ventricular zone cells, leaving open the possibility that soluble factors released by the aggregates underlie the observed effects.

In the present study, we have addressed the role of cell-cell contact in controlling the division of rat cortical progenitor cells. We find that rat cortical cells, like chick cortical cells and other rat CNS progenitor cells, prematurely differentiate if grown in isolation as single cells, but continue to develop if grown as aggregates. Although it has been commonly assumed that many CNS progenitor cells need to be present in an

aggregate or explant to promote their division in culture, we find that small clusters of 4-8 cortical progenitor cells show a marked increase in proliferation compared to single CNS progenitor cells or pairs. We present evidence that contact with some cell types, but not others, promotes cortical progenitor cell division and that this effect is mimicked by membrane preparations from specific cell types, demonstrating a direct effect of membrane-associated factors on the division of E14 rat cortical ventricular zone cells.

MATERIALS AND METHODS

Culture conditions

The culture medium used for the single cell and cluster cultures was DMEM (Gibco) plus 10% FCS (Hyclone) that had been conditioned for 3-4 days by a mixed culture of neonatal cerebral cortical astrocytes and neonatal meningeal cells. This medium was harvested and 20% of T-stim (Collaborative) was added to it prior to use. 12 μ l of culture medium was added to poly-L-lysine-coated Terasaki culture wells, and cells were then plated into the wells. The culture medium used for the membrane preparation assay was DMEM (Gibco) plus B27 and N2 (Gibco) that had not been conditioned. All cells were grown at 35°C and 5% CO₂.

Preparation of cultures

E14 cerebral cortices were dissected and mechanically dissociated in either hibernation medium (HM) (Kawamoto and Barrett, 1986) plus 10% FCS, or culture medium. The resulting suspension of single cells and cell clusters was transferred to a bacteriological Petri dish and viewed with an inverted microscope. Cells were selected from the suspension using a pulled glass capillary and mouth suction and transferred to prepared Terasaki wells (Nunc).

Primary astrocyte cultures of greater than 95% purity (based on staining for GFAP) were prepared from 1 day neonatal Sprague Dawley rat cerebral cortices as described by Frangakis and Kimelberg (1984). Briefly, cortices were dissected, meninges were removed, and the cortices were enzymatically digested with 3-6 mg/ml dispase (Sigma). Cells were plated at a seeding density of $1-2 \times 10^4$ cells/cm². For plating as clusters, astrocytes were removed from culture flasks with 0.125% trypsin and 1 mM EDTA, washed in FCS-containing medium, transferred in suspension to a Petri dish and visualized with an inverted microscope. Astrocytes were picked up with a pulled glass capillary and blown out into prepared Terasaki wells to create clusters of cells. These cells were left overnight prior to plating onto the clusters single, DiI- or BrdU-labeled cells.

Meningeal fibroblasts of greater than 90% purity (judged by staining for fibronectin) were prepared from neonatal meninges using a similar method as for cortical astrocytes but 0.125% trypsin /1 mM EDTA was substituted for dispase in the enzymatic treatment prior to dissociation to a cell suspension. Meninges from 4 pups were pooled to seed one T-75 flask. Primary cultures were removed using trypsin/EDTA, then plated as clusters in Terasaki wells, as described for astrocytes.

Balb 3T3 cells were obtained from ATCC (CRL 1658) and cultured in DMEM with 10% ECS (Hyclone). Clusters of 3T3 cells were prepared in Terasaki plates using the same method as for clusters of astrocytes or meningeal cells.

DiI labeling of cells

DiI labeling of cortical progenitor cells was carried out based on the method of Honig and Hume (1986). Small pieces of E14 cortex were placed into a solution containing 0.5 ml of HM and 3 μ l of a DiI stock solution: 2.5 mg of DiI (Molecular Probes, D282) in 0.5 ml absolute ethanol. Cells were gently mechanically dissociated in this solution,

allowed to load for 30 minutes at room temperature and then washed with HM plus 10% FCS and gently spun out. The resulting cell pellet was brought up in medium for cell plating, as described above.

DiI-labeled cells were visualized using a Zeiss Axiovert 35 equipped with epifluorescence.

BrdU labeling of progenitor cells in vivo

Pregnant female rats with embryos at stages E13 or E13.5 were anaesthetized using CO₂ and injected intraperitoneally with 100 mg/kg BrdU (Sigma) in sterile saline. The injection was repeated 2-3 times at 6- to 8-hour intervals. At E14 or 14.5 the cortices were dissected and dissociated.

BrdU labeling of cells in vitro

BrdU was added to culture medium to a final concentration of 10 μ g/ml. After 24 hours, the cells were washed in HM and fixed for immunohistochemistry.

Immunohistochemistry

Staining for MAP-2 and GFAP

Cells in Terasaki wells were washed with Dulbecco's PBS with calcium and magnesium and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (4°C, pH 7.4) at room temperature for 30 minutes. After washing in PBS, the cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS, then incubated with polyclonal rabbit anti-MAP-2(1:500) (donated by Dr Izhak Fischer) and/or monoclonal mouse anti-GFAP (Sigma) (1:75), diluted with PBS containing 0.1% Triton X-100 and 2% normal goat serum (NGS) (Vector Labs), for 1 hour. The plates were washed three times with PBS, and incubated for 30 minutes with goat anti-rabbit Ig's FITC (1:75) and/or goat anti-mouse Ig's Rhodamine (1:50) (Tago) diluted with PBS containing 0.1% Triton X-100 and 10% NGS. After a final wash in PBS, PBS with 25 mg/ml NaI was added to the wells to reduce fading (Johnson et al., 1982).

Staining for MAP-2 while retaining DiI

Cells in Terasaki wells were washed with Dulbecco's PBS with calcium and magnesium and fixed with 4% paraformaldehyde, as described above. Because conventional methods of cell membrane permeabilization (Triton X-100, methanol, acetone) tended to destroy DiI labeling, we used freeze-thawing to permeabilize cells. After washing in PBS, fixed cultures were equilibrated in 30% sucrose in 0.1 M phosphate buffer at pH 7.4 for 30 minutes at room temperature. The culture plates containing sucrose were then frozen at -40°C and then thawed. The plates were washed three times with PBS and carried through the MAP-2 staining as above, omitting Triton X-100 from the diluent.

Staining for BrdU

BrdU immunohistochemistry was carried out as described by Gratzner (1982). Briefly, plates were fixed with acid alcohol (95% absolute ethanol, 5% glacial acetic acid, 4°C) for 30 minutes at 4°C. After washing, the DNA was denatured by incubation with 2 N HCl for 40 minutes. The plates were washed, incubated with mouse anti-BrdU (Becton Dickinson) (1:20 in PBS with 0.1% Triton X-100) for 1 hour at room temperature, washed, then incubated with goat anti-mouse Ig's (Tago) FITC diluted 1:50 in PBS with 10% NGS for 20 minutes at room temperature. When double labeling with MAP-2, the MAP-2 immunohistochemistry was carried as above, except without the paraformaldehyde fixation and with the substitution of goat anti-rabbit Ig's rhodamine for goat anti-rabbit Ig's FITC, prior to the exposure to 2 N HCl.

Vital dye staining

1 μ l of a 2 mM stock solution of the fluorescent dye Cell Tracker Blue, CMAC, (Molecular Probes) (10 mM cell tracker blue is made

in DMSO, add then diluted 1: 5 with HM to give a 2 mM stock) was added to each culture well containing 10 μ l of culture medium. The cultures were returned to the incubator for 30 minutes, after which the cells were washed in HM and fixed in acid alcohol for BrdU immunohistochemistry.

Preparation of membrane homogenates

The membrane preparation was based on the method of Lyon et al. (1987). Briefly, 75–100% confluent cultures of 2° astrocytes or 2° meningeal fibroblasts growing in T75 culture flasks (Corning) in DMEM plus 10% FCS are washed with PBS. This was aspirated off and 5 ml of homogenization buffer (50 mM Tris, pH 7.8, 10 mM MgSO₄ and 0.5 mM EDTA, ice cold) was added to the plates. Cells were scraped off the plates and homogenized in a dounce homogenizer. The resulting suspension was spun at 300 *g* for 10 minutes to remove large debris and then at 30,000 *g* for 45 minutes to pellet membrane particles. The pellet was washed in homogenization buffer and spun at 30,000 *g* for 45 minutes. The resulting pellet was resuspended in 200 μ l of PBS and bath sonicated in a glass test tube with a glass bead for 30 seconds. The concentration of membrane was assessed using the Pierce BCA protein assay.

RESULTS

Proliferation of E14 cortical progenitor cells increases with increasing cluster size

At E14 in the rat, the cerebral cortex consists largely of ventricular zone cells, with only a thin layer of differentiated cells in the emerging marginal zone (Bayer and Altman, 1991). If a pregnant rat is given three injections of BrdU (a DNA analog that is used as a marker of cell division), at 7-hour intervals starting at E13.5, the E14.5 cortex contains $83 \pm 7.7\%$ BrdU-positive cells, demonstrating that the majority of cortical cells at around E14 are still in division, and hence are 'progenitor cells'.

We examined the influence of contact between E14 cortical progenitor cells on their development in vitro by comparing the fates of single cells and small clusters of 2–38 cells grown under otherwise identical culture conditions. Gentle mechanical trituration of E14 cortical cells gives a suspension of single cells and cell clusters. We transferred the cells to microculture wells by micromanipulation, plating one cell or one cluster per Terasaki well. Single cells grown in isolation were found to have a low growth potential under these conditions. Over the first 24 hours in culture 50% of single cells divide, but the majority of these give small clones of 2–4 neuron-like cells by 1 day in vitro. Only 2% of single cells divide further to give 5 or more progeny, 15 being the maximum observed. The survival of single cells is high for the first 24 hours in culture, around 80%, but then drops to 63% at 48 hours and 22% at 4 days in vitro (DIV). Cell pairs behave similarly to single cells, the majority dividing once or not at all, and while over 80% of cells derived from pairs survive the first 24 hours in culture, only 35% survive to 4 DIV. Clusters of 3–4 cells have a higher growth potential than single cells or cell pairs, usually showing proliferation for the first 3 DIV, but then the culture typically decays. In contrast, clusters of 5–38 cells, can produce large, well-sustained cultures of cells that contain neurons and non-neuronal cells and continue to grow for up to several weeks. These results demonstrate that as cluster size increases the proliferation and survival of E14 cortical cells in vitro also increases.

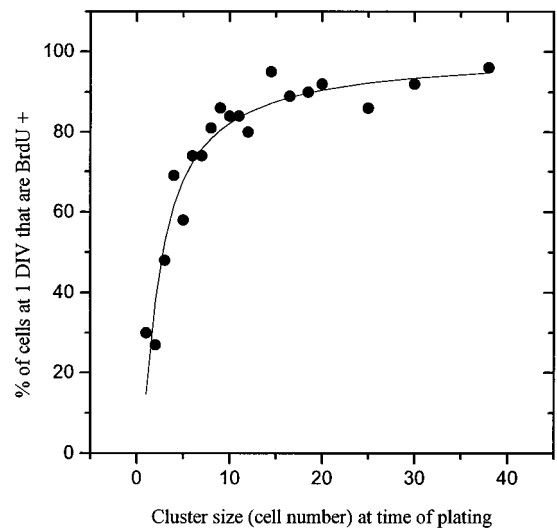


Fig. 1. Single cells and clusters of 2–38 cells were plated as one cell or one cluster per Terasaki well in the presence of BrdU, an indicator of cell division. After 24 hours the cultures were fixed and stained with an antibody to BrdU. The number of BrdU-positive cells was expressed as a percentage of viable cells present in the wells after 24 hours. Cell viability was assessed by morphology and in some cases by MAP-2 staining. This percentage is shown on the y axis. The size of the cell cluster at the time of plating is shown on the x axis. The curve represents the line of best fit through the data, calculated from the equation $F = Ae^{K/x}$ where x is cluster size, K is 1.87 ± 0.28 and A is 99.6 ± 2.8 .

In order to investigate how increasing cluster size affects division of E14 cortical cells we plated single cells and variable-sized clusters in the presence of BrdU. We recorded the number of cells present in each well on the first day of plating, and after 24 hours we fixed the cultures and stained them for BrdU incorporation. The results of this experiment are shown in Fig. 1. In this figure the percentage of cells incorporating BrdU is plotted against the number of cells initially plated in the wells. We found that single cells and cell pairs behaved similarly: less than 30% of the cells at 1 day have incorporated detectable BrdU. The percentage of cells incorporating BrdU increases with cluster size, so that approximately 50% of the cells derived from 3-cell clusters contain BrdU at 1 DIV, compared with approximately 80% of cells derived from 8-cell clusters. The curve plateaus at around 90% of cells derived from clusters of 14–38 cells showing BrdU incorporation at 1 DIV. These data show that increasing cluster size stimulates the division of cortical progenitor cells in an exponential manner. The fact that the percentage incorporation of BrdU in Fig. 1 plateaus at around 90% shows that the majority of cells, rather than a subpopulation, are able to respond to the effects of an increase in cluster size.

Examples of BrdU uptake into clusters of different sizes are shown in Fig. 2. A small cluster of three cells, shown in Fig. 2A,B, has not divided over the 24 hours of culture and does not show BrdU incorporation (Fig. 2B). Note that the cells in Fig. 2A are process-bearing and neuron-like. A larger cluster that was made up of 8 cells at the time of plating is shown in Fig. 2C and D. All the cells in the larger cluster have incorporated BrdU and these cells have a flattened morphology.

We conducted control experiments to test whether the behavior of E14 cortical cells was related to their ease of dissociation: cells that easily fell apart to give singles being already destined to differentiate, whereas cells that remained clustered being destined to continue division. We selected clusters from our cell suspensions and re-triturated them to yield single cells and smaller clusters. We knew that the starting clusters would have shown a high degree (more than 90%) of cell division. When these clusters were dissociated further, the single cells were found to behave indistinguishably from single cells obtained from the initial cell suspension: less than 30% take up BrdU over the first 24 hours in culture and 85% (± 14 s.d.) expressed MAP-2, a microtubule-associated protein present in neurons, after 1 DIV. This suggests that the effect of cluster size on division is not related to the ease of dissociation of the cells.

Dissociation of E14 cortical cells leads to premature cell differentiation

When E14 cortical cells are plated onto poly-L-lysine-coated plastic, two basic phenotypes are present at 1-2 days of culture. One type is flattened, smaller and less spread than typical cultured astrocytes or fibroblasts. These cells were negative for expression of MAP-2, glial fibrillary acidic protein (GFAP) and fibronectin (FN), assessed by immunohistochemistry. Over 90% of these flattened cells incorporate BrdU over the first 24 hours in culture. The other major cell type is a process-bearing cell that stains for MAP-2. None of the process-bearing cells are positive for GFAP or FN, and these cells do not appear to incorporate BrdU in our culture system. These process-bearing cells have the characteristics of a neuronal phenotype.

We have observed many cases in which small clusters of flattened cells have yielded process-bearing, neuron-like progeny with time in culture. Fig. 3A-C shows a photographic series of the development of a cell cluster that was around 8 cells at the time of plating. Note that after 24 hours in culture (Fig. 3A), the cluster is made up solely of flattened cells. By 3 days, process-bearing, neuron-like cells are visible (Fig. 3B) and these cells mature with time in culture, so that by 12 days many of them have complex processes and phase-bright soma (Fig. 3C). The neuronal cells overlie a monolayer of astrocytic glia, defined by staining with antibodies to GFAP. Based on these results we have concluded that the initial flattened

cells in our early cultures that are negative for MAP-2, GFAP and FN, and that incorporate BrdU, are cortical progenitor cells that, as a population, give rise to neurons as well as glia.

The incidence of progenitor cells and neuron-like cells at one day of culture is related to cluster size. Although 50% of single cells divide over the first 24 hours of culture, the majority of these cells give rise to differentiated progeny by 1 DIV - 88% of single cells or their progeny having a process-bearing, MAP-2-positive morphology and only 12% a flattened, MAP-2-negative, GFAP-negative, progenitor cell morphology. In contrast, clusters that consisted of 8-38 cells at the time of plating give rise to cultures at 24 hours that contain only 7% process-bearing, MAP-2-positive cells and 93% flattened, MAP-2-negative, GFAP-negative, progenitor cells. The peak of neuronal differentiation in larger clusters of 8-38 cells occurs 2-5 days after plating. Thus single cells acquire characteristics of differentiation earlier in the culture period than do cells in clusters of 8-38 cells.

Contact with selected cell types can promote or repress the progenitor cell phenotype

Having shown that separating cortical progenitor cells essen-

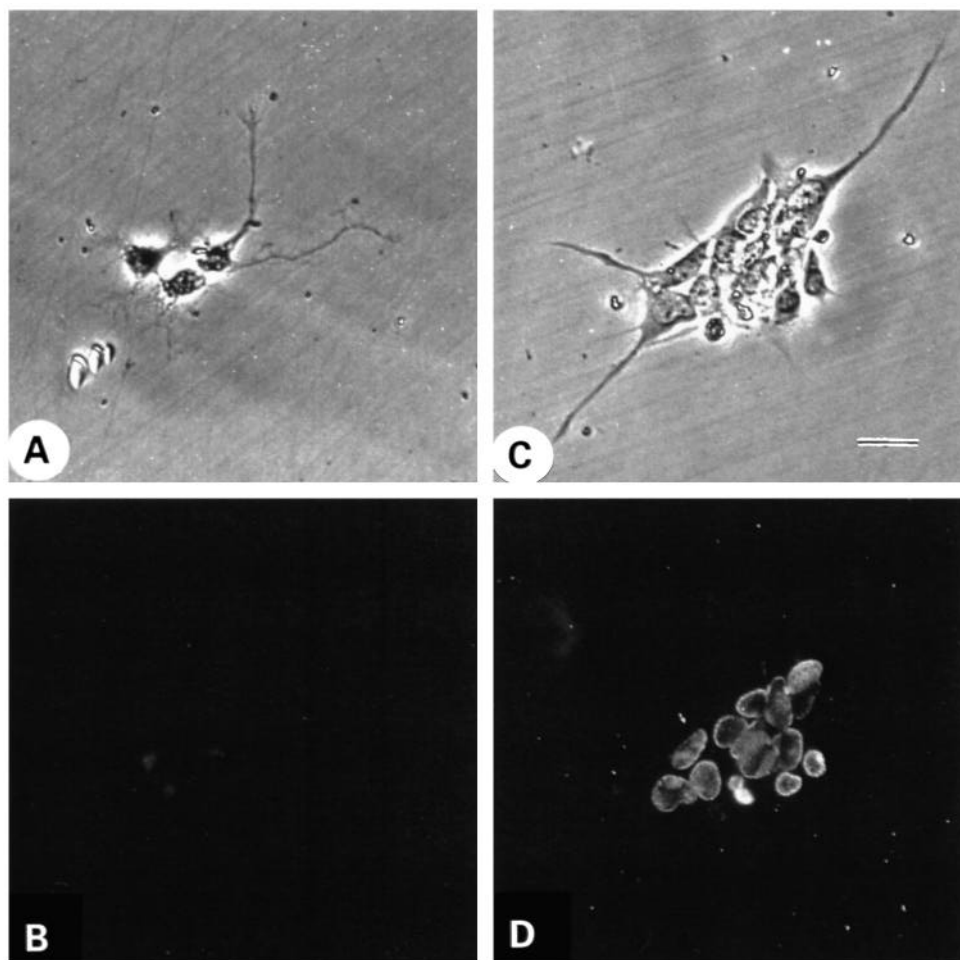


Fig. 2. (A) A phase micrograph of a cluster of 3 cells that did not take up BrdU, revealed by immunohistochemical labeling with a fluorescein-conjugated antibody to BrdU (B). Note that the cells have a process-bearing, neuron-like phenotype. (C) A phase micrograph showing a larger cluster that was 8 cells at the time of plating. Note that the majority of nuclei show uptake of BrdU (D) and that the cells have a flattened, rather than a process-bearing morphology. Scale bar, 15 μ m.

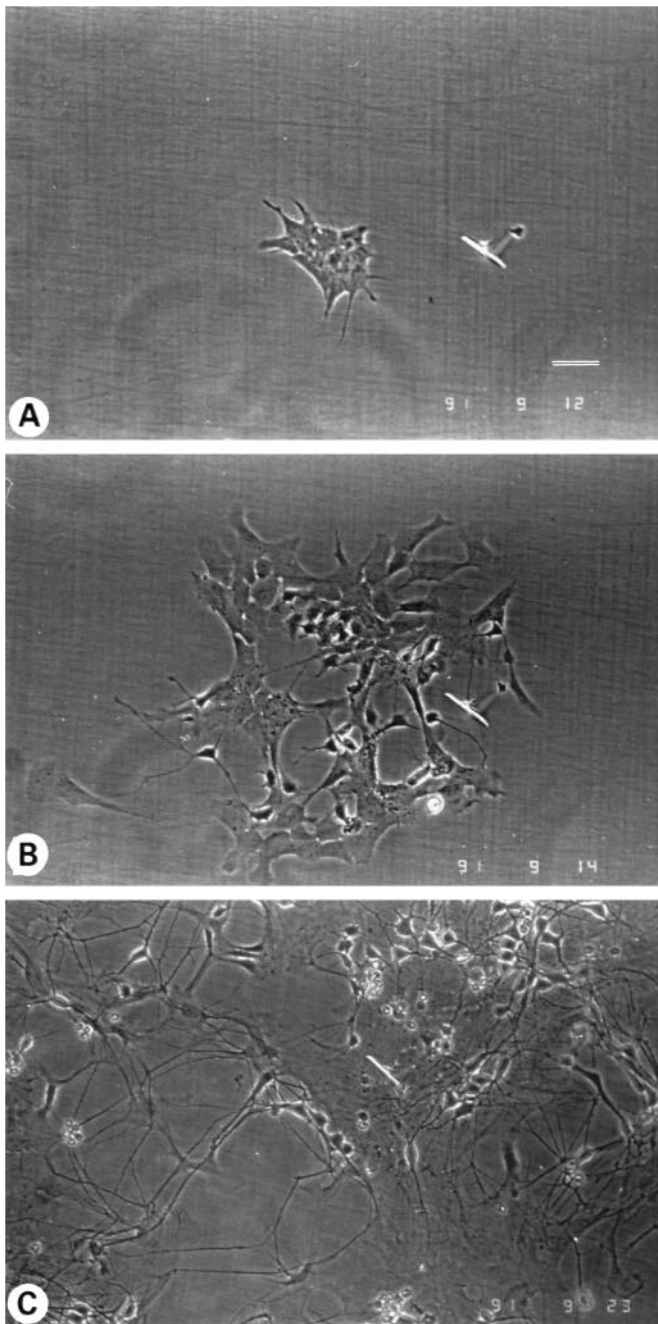


Fig. 3. Development of a small cluster of E14 cortical progenitor cells into a mixed culture of neurons and glia. (A) 1 DIV: cluster of flattened cortical cells. (B) 3 DIV: note the appearance of phase-dark, process-bearing, immature neuron-like cells. (C) 12 DIV: the culture now consists of complex neurons overlying a monolayer of glia. Note that there are different neuronal morphologies in the culture. Scale bar, 30 μ m.

tially leads to cessation of division and premature differentiation, we decided to assess what factors may be important for their division by exposing single cells, which would largely cease division if cultured in isolation, to different culture environments to examine which condition might allow them to maintain proliferation in vitro. Different culture environments

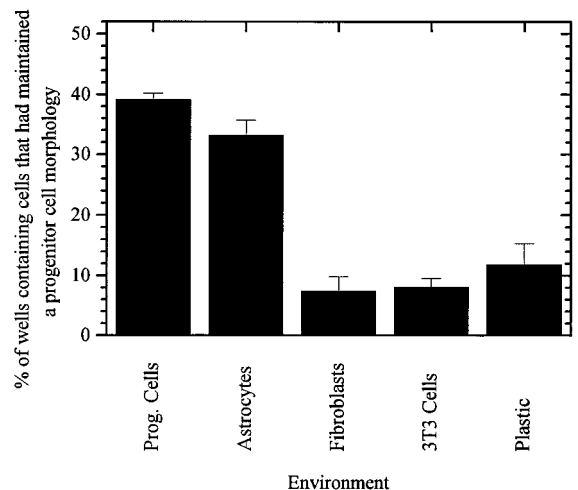


Fig. 4. Single E14 cortical cells were labeled with DiI or BrdU and plated on top of clusters of cells of different types. The clusters consisted of unlabeled E13–14 cortical progenitor cells, or neonatal cortical astrocytes, or neonatal meningeal cells or balb 3T3 cells. In some cases the single cell was plated on poly-L-lysine-coated plastic in wells that did not contain other cells. After 24 hours the cultures were fixed and stained for MAP-2 using a technique that also preserved the DiI labelling (see methods section). Where BrdU was used, the fixed cells were stained for MAP-2 and BrdU. The fates of the single labeled cells were assessed by counting the percentage of wells that, after 24 hours, contained cells with the flattened MAP-2-negative phenotype characteristic of progenitor cells. This percentage is shown on the y axis of the bar graph and is related to the different culture environments on the x axis. The viability of single labeled cells was not significantly different in the different culture environments and viability varied between 74% and 85%. Error bars=s.e.m.

were created by plating small clusters of E13–E14 cortical progenitor cells, neonatal cortical astrocytes, neonatal meningeal fibroblasts or balb 3T3 cells (a mouse fibroblast cell line) into the center of Terasaki wells. E14 cortical cells were dissociated, labeled with DiI and placed into the different culture environments: one labeled cell was placed on top of each cluster and in some cases a second cell was placed onto the plastic substratum around 100 μ m from the edge of the cell cluster. Single labeled cells were also placed onto the plastic base of Terasaki wells that did not contain other cells. The DiI-labeled, E14 cortical cells were picked from the suspension at random and then plated into different culture environments. The effect of the culture environments was assessed by analysing the morphology and MAP-2 expression of the labeled cells after 24 hours of culture. The results of these experiments are summarized in Fig. 4.

We found that after 24 hours in culture, 39% (± 2 s.d.) of wells contained cells with the flattened, MAP-2-negative, progenitor cell morphology when the labeled cells were grown contacting E13–E14 cortical cell clusters, compared to 33 \pm 5.7% when contacting astrocytes, 7.5 \pm 5.2% when contacting fibroblasts, 8.1 \pm 3% when contacting 3T3 cells and 11.8 \pm 7.7% when contacting plastic (see Fig. 4). Analysis of variance using the Newman-Keuls test shows that there is a significant difference, at the 99% confidence level, between the

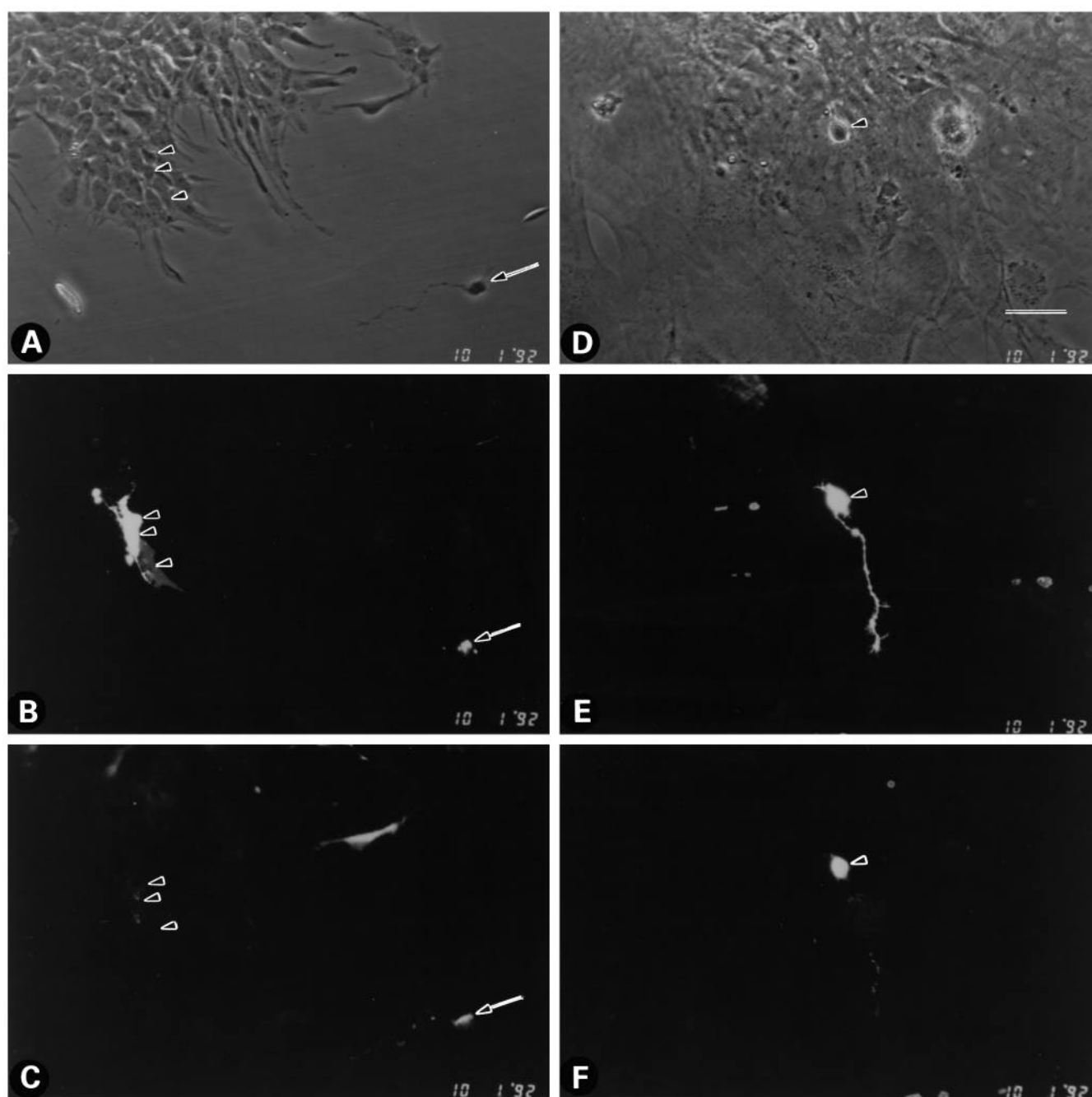


Fig. 5. The behavior of single DiI-labeled E14 cortical progenitor cells exposed to different culture environments for 24 hours. (A) A phase micrograph of a progenitor cell cluster that had a single DiI-labeled E14 cortical cell plated onto it 24 hours previously and a single DiI-labeled cortical cell that was plated close to, but not in contact with, the cluster. After 1 DIV the single cell plated in contact with the progenitor cell cluster has divided to give three cells that have the flattened, progenitor cell morphology, and that are DiI labeled (B) and MAP-2-negative (C) (three arrowheads in A-C). In contrast, a single cell plated initially around 100 μ m from the same cluster has differentiated into a process-bearing, MAP-2-positive, neuron (single arrow in A-C). (D) A phase micrograph of a cluster of fibroblasts that had a single DiI-labeled cortical cell plated in contact with it 24 hours previously. The single cell has differentiated into a MAP-2-positive, process-bearing neuron (arrowhead in D-F). Scale bar, 30 μ m.

behavior of single, DiI-labeled, E14 cortical cells grown on E13-14 cortical cell clusters and those grown in contact with plastic, fibroblasts or 3T3 cells. There was no statistical difference between the behavior of single labeled cells grown on

E13-14 cortical cells and those grown on neonatal astrocytes, or between those growing on fibroblasts and those growing on 3T3 cells. In addition, there was no statistical difference between the behavior of single labeled cells that were plated

on the poly-L-lysine-coated plastic close to, but not contacting, clusters of cells and those that were plated in wells not containing other cells.

To test whether DiI was affecting the behavior of the cells, this experiment was repeated using cells that had been labeled by exposure to BrdU in utero. In these control experiments, the pregnant female rat was given three injections of BrdU, 7 hours apart starting at E13. The embryonic cortices were removed at E14 and dissociated to give single cells. 80-90% of the cells in an acute cell suspension were found to be BrdU labeled. Single cells were randomly picked from the suspension and transferred to different culture environments, as described above. After 24 hours, the cultures were fixed and stained for BrdU incorporation. There was no difference between the behavior of cells labeled with DiI and those labeled with BrdU.

The behavior of DiI-labeled E14 cortical cells grown under different culture conditions is further illustrated in Fig. 5. Fig. 5A-C shows the results of plating single DiI-labeled E14 cortical cells in contact with, or in close proximity to, a cluster of E14 cortical progenitor cells. Two single, DiI-labeled E14 cortical progenitor cells had been plated 24 hours previously into this well, one cell onto the unlabeled progenitor cell cluster and one approximately 100 μ m away from the cluster. The single cell that contacted the cluster divided overnight to give three cells, all of which have a flattened, MAP-2-negative, progenitor cell-like morphology (three arrowheads, Fig. 5A-C). In contrast, the single cell plated onto the plastic nearby the cluster differentiated into a process-bearing, MAP-2-positive cell (arrows in Fig. 5A-C). Fig. 5D-F shows the results of plating a single, DiI-labeled cell in contact with a cluster of fibroblasts. After 24 hours the cortical cell has not divided and has a MAP-2-positive, process-bearing, neuron-like form.

Membrane preparations from selected cell types can maintain division of isolated cortical progenitor cells

Although these experiments strongly suggest that a factor or factors associated with the surface of some cell types is key for maintaining E14 cortical ventricular zone cells in division, the results are also compatible with the action of a short-lived soluble factor that cannot traverse the approximately 100 μ m to act on a cell close to, but not in direct contact with, a cluster of 'active' cells, such as E14 progenitor cells or astrocytes. In order to address the question of membrane-associated factors versus soluble factors more directly, we made membrane preparations from different cell types and assessed their ability to maintain single, E14 progenitor cells in division.

Membranes were prepared, based on the method of Lyon et al. (1987), from cortical astrocytes and meningeal fibroblasts. The membrane homogenates were sonicated prior to being added to Terasaki wells at different dosages. E14 cortical ventricular zone cells were dissociated and plated into the Terasaki wells at very low density, around 20-50 cells per well. The cells were added to the wells just after the membrane homogenate was added and for these experiments the culture medium used did not contain serum and was not conditioned by other cultures. After 12 hours in culture, 10 μ g/ml BrdU was added to each well and the cells were incubated for a further 24 hours. The number of viable cells per well was then assessed using the fluorescent viability dye Cell Tracker Blue. This dye was especially useful when cells were plated in the presence of high

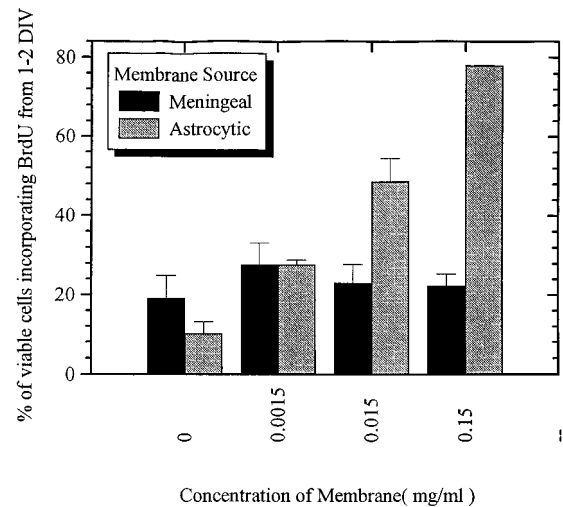


Fig. 6. Bar graph showing the percentage of viable E14 cortical cells that had incorporated BrdU when cultured in the presence of different concentrations of astrocyte or meningeal membranes. Single E14 cortical cells were plated at low density, 20-50 cells per Terasaki well, in the presence of different concentrations of either astrocytic or meningeal membrane homogenates. Cells were exposed to BrdU from 1-2 days in vitro and the percentage of viable cells (stained with cell tracker blue) that had incorporated BrdU was assessed. The percentage of viable cells at low membrane concentrations was not statistically different between the astrocyte and meningeal conditions. As membrane concentrations increased, cell viability also increased, for both membrane types tested. However, the number of viable cells present in the presence of high concentrations of astrocytic membrane was higher than the number present in the presence of high concentrations of meningeal membrane, most likely due to differences in cell division, as reflected by BrdU uptake. Error bars=s.e.m.

concentrations of membrane, which obscured the appearance of the cells making viability difficult to assess by phase contrast microscopy. After viability staining, cells were fixed and processed for BrdU immunohistochemistry. The results of this experiment are shown in Fig. 6. In this figure the percentage of viable E14 cortical cells that had incorporated BrdU is plotted against membrane concentration for astrocyte and meningeal membranes. The astrocyte membranes stimulated division in a dose-dependent manner. Around 25% of the single E14 cortical cells incorporated BrdU at the lowest astrocyte membrane concentration tested, 0.0015 mg/ml of membrane protein, and nearly 80% of cells had incorporated BrdU at the highest astrocyte membrane concentration tested, 0.15 mg/ml. In contrast, the addition of meningeal membranes had no significant effect on division of E14 cortical cells over the same membrane protein concentration range.

DISCUSSION

In vivo studies reveal that the E14 rat cerebral cortex is composed almost entirely of cells that are in division (progenitor cells). In contrast, when E14 cortical cells are cultured as single cells in individual Terasaki wells, the majority divide once or not at all. Approximately 90% of live cells that derive

from single cells express MAP-2 and are process-bearing after 24 hours of culture. Dissociation of E14 cortical cells into small clusters, however, allows their continued division and the development of neuronal and glial cell types; this resembles the behavior of cortical ventricular zone cells *in vivo*. Thus dissociation of E14 cortical cells into single cells induces them to differentiate prematurely.

The behavior of rat embryonic cortical cells is consistent with that of a number of other CNS progenitor cells. For example, cell dissociation has been shown to lead to cessation of division and premature differentiation of oligodendrocytes (Raff et al., 1985) and retinal cells (Reh and Kljavin, 1989). Previous studies have also shown a high incidence of cell division in explant or aggregate cultures from cerebellum, striatum, retina and chick cortex (see introduction). In most of these cases, the aggregates or explants consisted of hundreds of cells. In assessing how small a cluster was needed to support rat cortical progenitor cell division, we were surprised to find that relatively small clusters of 4-8 cells can show markedly increased growth *in vitro* compared to single cells and cell pairs. It has been reported that retinal cells behave similarly to these cortical progenitor cells in that they cease division if dissociated from one another but keep on dividing if cultured as small groups of around 4 cells (Reh and Kljavin, 1989). Both retina and cerebral cortex derive from a flattened, pseudostratified ventricular layer of progenitor cells; and one might speculate that a similar mechanism sustains division of both cortical and retinal progenitor cells in culture. For example, a critical number of cells may be required to provide soluble mitogenic factors that sustain division. Alternatively, a critical number of cells might be required to provide necessary contact between cell surfaces. We investigated whether cell contact was key to the continued division of CNS progenitor cells, or whether close cell apposition (implying the action of soluble factors) was sufficient.

When single, DiI-labeled, E14 cortical progenitor cells are plated in contact with clusters of similar E13-14 cortical progenitor cells they have a significantly higher probability of maintaining the progenitor cell phenotype than if plated onto the plastic substratum close to, but not in contact with, the cell clusters. The majority of single cortical cells plated on plastic close to progenitor cell clusters prematurely differentiate as they do if plated into a well that does not contain other cells. This suggests that cell contact is key for maintaining the progenitor cell phenotype.

We found that neonatal cortical astrocytes that have been in culture for 1-3 weeks are similar to E13-14 cortical cell clusters in their ability to prevent premature differentiation of single, DiI-labeled E14 cortical cells (Fig. 4). Cultured cortical astrocytes also sustain division of other CNS progenitor cells, including those from the olfactory epithelium (Pixely, 1992) and the O-2A progenitor cell (Noble and Murray, 1984; Raff et al., 1985). However, in the case of the O-2A progenitor cell the key effect is not due to cell contact but to a soluble mitogen (PDGF) released from astrocytes (Raff et al., 1988; Richardson et al., 1988). In the case of E14 cortical progenitor cells, actual contact with astrocytes appears to be required to maintain cell division because single cells plated on the plastic close to, but not in contact with an astrocyte cluster preferentially differentiate (not shown). (Note also that these experiments were conducted in medium conditioned by astrocytes).

Contact with cortical astrocytes or their membranes has been shown to inhibit the division of another type of CNS progenitor cell, cerebellar external granular layer cells (Gao et al., 1991), a different result to the one we observe. This raises the possibility that different types of CNS progenitor cells may respond differently to similar cell-surface environments. Alternatively, this may reflect a difference in the surface characteristics of cortical astrocytes grown from a minor population of cells *in vitro* (Frangakis and Kimelberg, 1984) and cerebellar astrocytes isolated by percoll purification of a cell suspension (Gao et al., 1991).

Not all cell types can promote the progenitor cell phenotype. Contact between labeled E14 cortical cells and meningeal fibroblasts or balb 3T3 cells promotes their differentiation into process-bearing, MAP-2-positive, neuron-like cells. Fibroblast cell types produced slightly more pronounced differentiation than poly-L-lysine-coated plastic (Fig. 4). Thus the characteristics that maintain the progenitor cell phenotype are specific to a subset of cells, rather than being a general feature of all cell types.

Although these results strongly suggest that cell-surface associated factors are key for maintaining E14 rat cortical progenitor cells in division, the data are also compatible with the action of a short-lived soluble molecule that cannot traverse the approximately 100 μ m distance that separates the single cells from the cluster. To assess the role of cell contact more directly we tested the ability of membrane preparations from astrocytes or meningeal cells to maintain division of dissociated E14 cortical cells growing at very low density. We found that astrocytic membranes stimulated division of cortical cells in a dose-dependent manner. We also have evidence that membranes from E14 cortical cells stimulate division of isolated cortical ventricular zone cells (not shown). The experiments using membrane homogenates were conducted in the absence of conditioned medium or serum. The stimulation of division that was observed under these conditions may suggest therefore that the membrane homogenate has a mitogenic effect itself. Alternatively, the membrane factors may have a permissive role, allowing the action of other soluble factors, either present in the culture medium or present *in vivo* and still influencing the cells in these short-term cultures. It is possible then, that sustained growth of cortical progenitor cells for longer periods requires additional soluble factors. Meningeal membranes do not appear to stimulate cortical ventricular zone cell division over the membrane concentrations tested. Taken together, these data suggest that a membrane-associated component made by specific cell types has a stimulatory effect on the division of E14 rat cortical cells.

The membrane-associated factors that maintain the cortical progenitor cell phenotype and prevent premature differentiation have not been biochemically identified. An important functional characteristic of these factors is that increasing the cluster cell number from 2-8 has a profound effect on cortical progenitor cell division *in vitro*, revealed by an exponential relationship between BrdU incorporation and cluster size (Fig. 1). This exponential relationship is consistent with a simple underlying biochemical mechanism, and suggests a mechanism based on a co-operative interaction between the clustered cells.

The E14 cortical progenitor cell is a flattened cell, smaller than typical astrocytes or fibroblasts, that is negative for MAP-

2, GFAP and FN. These E14 cortical progenitor cells have a similar morphology to other CNS progenitor cells in culture, including those from septal region (Temple, 1989), striatum (Cattaneo and McKay, 1990) and retina (Anchan et al., 1991). By following the fates of E14 cortical cells daily, we were able to show that the flattened cortical cells divide and give rise to process-bearing, neuron-like cells and GFAP-positive astrocytes. There are no obvious morphological differences between flattened progenitor cells that give rise to neurons and those that give rise to glial cells. We do not know if a single E14 cortical progenitor cell can give rise to both neurons and astrocytes in this culture system, whereas there is evidence for a common neuron-astrocyte precursor in the E14 septal region (Temple, 1989) and striatal region (Reynolds et al., 1992). Retroviral lineage studies have provided evidence that at around E14 in the rodent the majority of cortical cells are already fated to give a neuronal or a glial cell type (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988). However, there is a minor population of cells that can produce both neurons and glial cells (Luskin et al., 1988; Williams et al., 1991; Walsh and Cepko, 1992). It is possible that different progenitor cell types respond differently to cell contact. In Fig. 1, it appears that as cluster size increases division of almost all the cells can be stimulated. Hence in this assay the effect of contact may be on all ventricular zone cells regardless of type, although minor cell populations may be excluded. In contrast, in Fig. 4 only around 40% of the cells are maintained as progenitor cells over 1 day in vitro. Although this relatively low percentage could reflect the technical difficulty of the experiment, it leaves open the possibility that the different culture environments tested are selecting amongst subpopulations of progenitor cells. This interesting possibility will be clarified by an understanding of the progenitor cell types present in the cortical ventricular zone, and we are currently assessing the fates of single embryonic cortical cells to address this issue.

When E14 cortical cells are induced to differentiate prematurely by dissociation, the majority give rise to cells with neuronal characteristics; no GFAP-positive cells have been recorded. It is possible that cortical cells fated to give glial cells at E14 are rare or that they die, when cultured in isolation, before they can express glial markers such as GFAP. Alternatively, one might speculate that cells destined to become glial cells in vivo develop neuronal features when stimulated to differentiate early, over a time-period in which the predominant cell type produced is neuronal, just as retinal cells stimulated to differentiate early give rise to age-appropriate cells (Reh and Kljavin, 1990).

In conclusion, we have shown that membrane-associated factors present on selected cell types can maintain E14 rat cortical progenitor cells in division. Thus, contact between cortical cells in the ventricular zone may be key to maintain the proliferation of these cells, hence regulating the overall numbers of cortical cells. As cortical cells migrate from the ventricular zone, changes in their cell contacts may trigger differentiation events involved in the formation of specialized neural cell types.

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