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

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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 basic-FGF 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 basic-FGF and TGFα 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 found 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 µl 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 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. When double labeling with MAP-2, the MAP-Ig's Rhodamine for goat anti-rabbit Ig's FITC, prior to the exposure to 2 immunohistochemistry was carried as above, except without the permeabilization (Triton X-100, methanol, acetone) tended to destroy the 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.**

**BrdU labeling of progenitor cells in vivo**

Pregnant female rats with embryos at stages E13 or E13.5 were anesthetized 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.

**Preparation of clusters**

Small pieces of E14 cortex were prepared in Terasaki plates using the same method as for clusters of cortical astrocytes or meningeal cells.

**Preparation of cultures**

Preparation of cultures was carried through the MAP-2 staining as above, omitting Triton X-100 from the diluent.

**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 in 0.1 M phosphate buffer (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 Izak 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 BrdU**

BrdU immunohistochemistry was carried out as described by Gratzner (1982). Briefly, plates were fixed with acid alcohol (95% 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 µl 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 mL 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 mL 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±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 microwell plates by micromanipulation, plating one cell or one cluster per Terasaki well. 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.](image-url)
Proliferation of cortical progenitor cells

In the study, we assessed what factors might be important for the proliferation of single cortical cells by placing them in different culture environments. Different culture environments were created by plating small clusters of E13-E14 cortical progenitor cells, neonatal cortical astrocytes, neonatal meningeal cells or Balb 3T3 cells. In some cases, single cells were placed on poly-L-lysine-coated plastic wells that did not contain other cells. After 24 hours, the cultures were fixed and stained for MAP-2 to assess the morphology and fate of the single labeled cells. The results showed that the percentage of wells containing cells with the flattened, MAP-2-negative phenotype varied significantly among the different culture environments. Error bars represent ±s.e.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 µm.

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 placed on poly-L-lysine-coated plastic wells that did not contain other cells. After 24 hours, the cultures were fixed and stained for MAP-2 to assess the morphology and fate of the single labeled cells. The viability of single labeled cells was not significantly different in the different culture environments and viability varied between 74% and 85%. Error bars represent ±s.e.m.
behavior of single, Dil-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 µm 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 µm 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 µg/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 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 dis-
sociation 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 Kjavin, 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 dis-
sociated from one another but keep on dividing if cultured as
small groups of around 4 cells (Reh and Kjavin, 1989). Both
retina and cerebral cortex derive from a flattened, pseudostrat-
ified 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, Dil-labeled, E14 cortical progenitor cells are
plated in contact with clusters of similar E13-14 cortical prog-
enitor 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 prog-
enitor 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,
Dil-labeled E14 cortical cells (Fig. 4). Cultured cortical astro-
cytes 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; Richard-
son 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 preferen-
tially differentiate (not shown). (Note also that these experi-
ments 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 progeni-
tor 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. Alter-
natively, this may reflect a difference in the surface character-
istics 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 character-
istics 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 μm 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 dissoci-
ated 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 exper-
iments 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 differen-
tiation 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). Rétroviral 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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