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Cell-Cycle Kinetics of Neocortical Precursors Are Influenced by Embryonic Thalamic Axons

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Thalamic afferents are known to exert a control over the differentiation of cortical areas at late stages of development. Here, we show that thalamic afferents also influence early stages of corticogenesis at the level of the ventricular zone. Using an *in vitro* approach, we show that embryonic day 14 mouse thalamic axons release a diffusable factor that promotes the proliferation of cortical precursors over a restricted developmental window. The thalamic mitogenic effect on cortical precursors (1) shortens the total cell-cycle duration via a reduction of the G_1 phase; (2) facilitates the G_1/S transition leading to an increase in proliferative divisions; (3) is significantly reduced by antibodies directed against bFGF; and (4) influences the proliferation of both glial and neuronal precursors and does not preclude the action of signals that induce differentiation in these two lineages. We have related these *in vitro* findings to the *in vivo* condition: the organotypic culture of cortical explants in which anatomical thalamocortical innervation is preserved shows significantly increased proliferation rates compared with cortical explants devoid of subcortical afferents. These results are in line with a number of studies at subcortical levels showing the control of neurogenesis via afferent fibers in both vertebrates and invertebrates. Specifically, they indicate the mechanisms whereby embryonic thalamic afferents contribute to the known early regionalization of the ventricular zone, which plays a major role in the specification of neocortical areas.

Key words: development; cortex; proliferation; areal specification; mouse; ventricular zone

Cells of the cerebral cortex originate from the ventricular and subventricular zones of the embryonic telencephalon. The heterogeneous population of precursors lining the ventricular zone divide, migrate, and differentiate to form the cerebral cortex. Although many of the developmental events occurring during corticogenesis have been described (Angevine and Sidman, 1961; Smart, 1973; Smart and Smart, 1982; Rakic, 1988; Bayer and Altman, 1991), the contribution of early mechanisms that determine the phenotypes of cortical neurons and specify the identity of cortical areas still has to be resolved (McConnell, 1995).

The sensory periphery exerts an important control over the development of the immature cortical plate via thalamic afferents (O'Leary, 1989). Such afferent specification of cortex (Killackey, 1990) is in line with *in vivo* and *in vitro* experiments showing that thalamic afferents influence cell survival and differentiation (Repka and Cunningham, 1987; Windrem and Finlay, 1991; Lotto and Price, 1996; Price and Lotto, 1996; Zhou et al., 1999). However, there is also clear evidence that there is a specification of cortical neuron phenotype at the level of the ventricular zone before migration to the cortical plate (Arimatsu et al., 1992; Cohen-Tannoudji et al., 1994; Soriano et al., 1995; Levitt et al., 1997; Miyashita-Lin et al., 1999, Nakagawa et al., 1999).

Given the developmental impact of events in the ventricular zone, it is important to know whether they too are influenced by thalamic afferents. Environmental signaling during the final round of mitosis has been shown to be a key event in the specification of the future connectivity of cortical neuroblasts (McConnell and Kaznowski, 1991; Eagleson et al., 1997), and modulation of cell-cycle kinetics contributes to determining areal cytoarchitecture (Dehay et al., 1993; Polleux et al., 1997). There is indirect evidence that, in the primate, thalamic afferents contribute toward specifying the identity of cortical areas during very early stages of cortical development by regulating the rates of neurogenesis in the ventricular zone (Dehay et al., 1993, 1996). Thalamic afferents could influence rates of neuron production either by influencing cell death, which is prevalent in the ventricular zone (Blaschke et al., 1998; Haydar et al., 1999a), or by acting on cell-cycle parameters, in accordance with findings in lower vertebrates and invertebrates (Kollros, 1953, 1982; Williams and Herrup, 1988; Baptista et al., 1990; Selleck and Steller, 1991; Gong and Shipley, 1995).

Here, we show that mouse embryonic thalamic axons release a diffusable factor that promotes proliferation of cortical precursors. This temporally regulated mitogenic effect (1) shortens the total cell-cycle duration of cortical precursors via a reduction of the G_1 phase, and (2) facilitates the G_1/S transition of the cortical precursors, leading to an increase in proliferative divisions. Basic FGF might participate in the cell signaling underlying this mitogenic effect. We have investigated the relevance of these *in vitro* findings to normal development by showing that proliferation is enhanced in embryonic cortical organotypic slices when thalamocortical connections are conserved. These findings, pointing to the regulation of proliferation by thalamic afferents, indicate how

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these afferents can contribute to the regionalization of the ventricular zone and therefore the specification of neocortical areas.

MATERIALS AND METHODS

Dissection procedure

Embryos were removed by cesarean section from timed-pregnant mice (OF1 strain; Iffa Credo, L'Arbresle, France). The plug date was embryonic day 1 (E1). Fetal brains were removed under sterile conditions in iced HBSS containing 10 mM HEPES. The cerebral hemispheres were detached by medial longitudinal section. The neopallium, including the ventricular zone, the intermediate zone, and the cortical plate, was isolated. Dorsal thalamic nuclei were dissected out.

Culture preparation

Cortical cells underwent enzymatic dissociation (trypsin 0.2%; 3 min at 37°C). Trypsin activity was stopped by washing in Glasgow Modified Essential Medium (GMEM; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS). Cells then underwent a mechanical dissociation and were centrifuged for 5 min at 4°C and resuspended in GMEM + 10% FCS. Viability was estimated by trypan blue exclusion assay, and cells were counted under a hemocytometer. Cells were seeded at a density of 4×10^5 cells per 14 mm diameter polylysine–laminin-coated glass coverslip and were cultured in 500 μl of GMEM + 10% FCS. The medium was renewed every 4 d. Cell viability was evaluated by means of the trypan blue assay.

Thalamus-conditioned medium (TCM) was prepared from thalamic explants. Explants from E14 or E15 thalamus were obtained from several embryos from the same litter, pooled, and cut into 200 μm pieces with a tissue chopper. The explants were cultured in 500 μl of GMEM on polylysine–laminin-coated glass coverslips (diameter, 14 mm) for 2 d *in vitro* (DIV). The amount of explants per coverslip corresponded to that obtained from the dorsal thalamus of one embryo. After 48 hr, the culture medium was collected, filtered, and frozen at −80°C. In some experiments, we examined the effect of concentrated TCM using Centricon YM-3, 3,000 MW cutoff filters.

When the effect of E18 thalamus was tested, E18 thalamus explants were growth-inactivated by gamma irradiation (137 Cs, 45 Grays) (CIS bio international, Saclay, France) for 30 min before the culture to prevent any putative effect of proliferating glial cells that could be present in the thalamus at this later stage (Kilpatrick et al., 1993). This treatment did not affect cell viability. We verified that this irradiation protocol, when applied to E14 thalamic explants, did not interfere with the mitogenic effect.

Cortical cultures were grown for 48 hr (2 DIV) in 500 μl of GMEM + 10% FCS or TCM + 10% FCS before cell-cycle parameters were assayed. The 500 μl of TCM were prepared from explants corresponding to the dorsal thalamic nuclei of one embryo (see above). Monoclonal antibody directed against bFGF (F 6162; Sigma, St. Louis, MO) was used at 60 ng/ml.

Western blotting

Cell lysates from E14 dorsal thalamic nuclei, mouse adult cortex (positive control), and mouse embryonic stem cells (negative control) were prepared as follows. Cells (10^6) were lysed in 100 μl of lysis buffer (66 mM Tris-HCl, pH 6.8, 1.25% SDS, and 175 mM 2-mercaptoethanol). Samples were analyzed on 7.5% polyacrylamide gel, followed by immunoblotting on nitrocellulose membranes in 12.5 mM Tris-HCl, 100 mM glycine, 0.05% SDS, and 20% methanol. Membranes were blocked for 2–4 hr in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, and 2% dry milk; washed three times for 10 min in 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20; and incubated for 1 hr with HRP-conjugated secondary antibody 1:5000 (Amersham, Arlington Heights, IL). HRP activity was revealed with the ECL detection kit (Amersham). All incubations with antibodies were performed using Biocomp Navigator (Serlabo, France). The primary antibody was anti-GFAP (Sigma).

Bromodeoxyuridine labeling

Bromodeoxyuridine (BrdUrd) was added to the medium for either brief [2–3 hr in the case of labeling index (LI) measurements] or prolonged exposures, in the case of cumulative labeling. The cultures were washed with phosphate buffer before being fixed with 70% alcohol at −20°C.

BrdUrd cumulative labeling. BrdUrd cumulative labeling (Nowakowski et al., 1989) was performed in GMEM + 10% FCS and in TCM + 10% FCS-grown cultures at 2 DIV, and coverslips were fixed after appropriate exposure times with 70% ethanol (−20°C). The culture medium containing 20 μg/ml BrdUrd was renewed every 12 hr.

Percentage of labeled mitoses. At 2 DIV, cultures grown in GMEM + 10% FCS and in TCM + 10% FCS were exposed for 1 hr to BrdUrd, rinsed, and finally fixed after various survival times with ethanol (70%) at −20°C. For both techniques, a minimum of two coverslips were analyzed for each time point.

For the percentage of labeled mitoses (PLM) experiments (Quastler and Sherman, 1959), statistical significance was tested by means of an F test applied to the ascending slope of the curve. For BrdUrd cumulative labeling, the statistical differences between slopes were tested by means of an F test, combined with a bootstrap analysis (implemented with Matlab software) that makes it possible to determine whether the intersection of the two slopes is on the *x*-axis.

Immunocytochemistry

Proliferating cell nuclear antigen/BrdUrd double-labeling. After fixation with 70% alcohol at −20°C, the coverslips were incubated first for 20 min in TBS + 0.6% H_2O_2, and then for 20 min in normal goat serum. Proliferating cell nuclear antigen antigen (PCNA) was revealed according to the following three-step immunostaining procedure: mouse anti-PCNA (DAKO, PC10, 1:75 in TBS) (Dako, High Wycombe, UK) for 30 min at room temperature (RT), biotinylated goat anti-mouse (1:400 in TBS; Dako) for 30 min at RT, and peroxidase-conjugated streptavidine (1:500 in TBS; Dako). Peroxidase activity was revealed by incubating the coverslips in DAB (1 mg/ml in 0.05 M Tris; Sigma) for 5 min and then adding 3% H_2O_2 for 10 min. DNA denaturation was subsequently performed by 2N HCl for 30 min, followed by a wash in borate buffer, pH 8.5. Mouse anti-BrdUrd (1:10; Bioscience) was incubated overnight at 4°C. Labeling was revealed by a final incubation in FITC rabbit anti-mouse (1:100; Dako) antibody or Cy3 rabbit anti-mouse (1:400, 1 hr) (Interchim, Montlucon, France).

Cells in S phase at the time of the pulse were positively stained for BrdUrd. Precursors were identified by means of PCNA labeling. Cell nuclei were counterstained with Hoechst (1 ng/ml) (Molecular Probes, Eugene, OR). Coverslips were examined using an oil objective microscope (50× or 100×) under UV light to detect FITC (filter 450–490 nm) and Hoechst (filter 355–425 nm). PCNA labeling revealed by DAB was observed under white illumination. Coverslips were scanned at regular spacing with a grid corresponding to a field of 0.128 mm^2. From 100 to 150 fields were observed per coverslip. A minimum of two coverslips were observed for each condition.

GFAP and MAP2 immunohistochemistry. GFAP and MAP2 were revealed according to the following two-step procedure. Coverslips were rinsed three times in TBS + 0.5% Triton X-100 and then incubated in a mixture of ethanol (95%) and acetic acid (5%) for 20 min. After three rinses in TBS, coverslips were incubated for 20 min in normal goat serum and rinsed three times before proceeding to primary antibodies incubation as follows. Rabbit anti-GFAP (1:100 in DAKO-diluent) from Sigma (G9269) and mouse anti-MAP2 (1:100 in DAKO-diluent) from Sigma (M4403) were simultaneously incubated overnight at 4°C. After three TBS rinses to reveal GFAP labeling, goat anti-rabbit-Cy2 (1:200 in DAKO-diluent) (Interchim) was incubated for 1 hr at RT. After three rinses, coverslips were further incubated with goat anti-rabbit-Cy3 (1:200 in DAKO-diluent) (Interchim) for 1 hr at RT to reveal MAP2 labeling. Coverslips were counterstained with Hoechst (1 μg/ml). All experiments were performed at least in duplicate, and a minimum of two coverslips were examined for each condition.

RESULTS

***In vitro* assay of cortical precursor proliferation**

A stable *in vitro* system that permits the accurate quantification of the proliferative activity of cortical precursors was developed. Dissociated neuroblast cultures were prepared from the cerebral wall of the mouse at E14, a stage when thalamic axons are just arriving in the vicinity of the cortex (Bicknese et al., 1994; Cohen-Tannoudji et al., 1994; Polleux et al., 1996).

GMEM supplemented with 10% FCS was used to grow cortical precursors to optimize proliferation rates (Smith et al., 1988). Under these conditions, cortical precursors show a high rate of proliferation, and rates of cell death are <5% (Guibert et al., 1995). This was not the case in Sato medium (Darmon et al.,

1981) in which mouse cortical precursors show a reduced proliferative activity as well as increased levels of cell death.

To characterize the proliferative activity of the culture, we identified the growth fraction (GF), i.e., the proportion of the cycling precursors using immunostaining with an antibody directed against PCNA (Fig. 1*A*). PCNA is a 36 kDa nonhistone protein that is involved in DNA replication and functions as a cofactor for DNA polymerase delta (Bravo et al., 1981). Among the different proliferation-associated antigens, PCNA expression is a faithful marker of cycling cells (Bolton et al., 1994). The PCNA expression level is cell-cycle dependent, being upregulated during G_1, S, G_2, and M phases and markedly depressed during G_0 (Bolton et al., 1994). Flow cytometry analysis shows that in the fixation conditions used in the present study (i.e., ethanol at −20°C), PCNA expression is optimally detected during all phases of the cell cycle (Sasaki et al., 1993; Teague and el-Naggar, 1994).

In a first instance, we have confirmed that under the experimental conditions used in this study, PCNA expression is restricted to cycling neuroblasts. This is shown in Figure 1*A–C*, which shows that after fixation with ethanol (70%) at −20°C, PCNA-positive nuclei are restricted to the germinal zones of the brain. In the cortex, the computation of the GF (PCNA-positive cells with respect to the total number of cells in the germinal zones) returns values between 96 and 98% at E14 and E15. This is in agreement with numerous previous findings in which the GF has been estimated by means of tritiated thymidine or BrdUrd cumulative labeling *in vivo*. Using ^{3}H-thymidine labeling, Reznikov and van der Kooy (1995) report GF values of 99.99% in E14 and E15 lateral and dorsal rat neocortex. Using BrdUrd cumulative labeling and fluorescence-activated cell sorting analysis, Miller and Nowakowski (1991) report GF values of 90% in E13 rat. In the mouse, Takahashi et al. (1993, 1995) report GF values ranging from 95 to 99%.

Under certain conditions, prolonged BrdUrd exposure (at least equal to the cell-cycle duration) labels all cohorts of precursors going through S phase and accurately identifies the GF (Fig. 2). The proportion of PCNA-positive cells (64.8%) is similar to the proportion of BrdUrd-positive cells (64%) after prolonged exposure (e.g., 40 hr), proving that PCNA labeling accurately measures the GF under the experimental conditions used in this study (Fig. 2*A*). This is further confirmed by the observation that 99.3% of PCNA-positive cells are also BrdUrd positive after a 40 hr BrdUrd exposure (Fig. 1*D*,*E*). The proportion of BrdUrd-positive cells that are PCNA negative (i.e., the cells that have incorporated BrdUrd and subsequently left the cell cycle) was of the order of 2% after a 40 hr exposure (Fig. 2*A*), indicating that most precursors are undergoing symmetric proliferative divisions under our experimental conditions. Furthermore, we have specifically addressed whether PCNA expression is downregulated after cell-cycle exit by examining PCNA and MAP2 colocalization (Fig. 1*G–I*). Of a total of 7148 MAP2-positive cells, <0.01% are PCNA positive (Fig. 2*A*). This indicates that PCNA expression is rapidly downregulated in the postmitotic neuron, as has been shown in other cell types (Sasaki et al., 1993), and that therefore, under the present conditions, PCNA immunolabeling accurately identifies the pool of cycling cells.

Cell density (CD) measurements at different time points were used to characterize the proliferative behavior of cortical precursors in the present culture conditions (Fig. 2*B*). In E14 and E15 cultures, 70–80% of the cells are PCNA positive at 1 DIV. This proportion remains fairly stable for up to 3 DIV and then decreases. Computation of the numbers of newly generated cells (precursors plus postmitotic cells) over time in culture shows that the doubling time of the precursor population is of the order of 30 hr, indicating a mean cell-cycle duration of 30 hr in the first 2 d. This duration is in agreement with the recently reported values of 25 hr in E15 mouse cortical slices (Haydar et al., 1999b). In the present *in vitro* conditions, there is a lengthening of the mean cell-cycle duration to a value of 43 hr at 4 DIV. *In vivo*, the mean cell-cycle duration is considerably shorter (15 hr) but shows a comparable 30% increase over the same period (Takahashi et al., 1995).

The analysis of the variation of the relative proportions of PCNA-positive cells and postmitotic cells within the population of newly generated cells with time indicates changes in the mode of division (Fig. 2*C*). During the first 24 hr, precursors produce 10 times more precursors than postmitotic cells, indicating that proliferative divisions are prevalent at this stage. From DIV 3 to 4, precursors produce only six times more precursors than postmitotic cells, showing that there is a significant decrease in the prevalence of proliferative divisions and an increase in the incidence of differentiative divisions generating postmitotic cells. Again, the decrease in the incidence of proliferative divisions *in vitro* mirrors a similar decrease reported *in vivo* (Takahashi et al., 1995).

Serial examination of the cultures over several days revealed the appearance of morphologically differentiated neurons and glia (Fig. 1*I*). Few cells were found to be GFAP positive in the E14 or E15 cultures at 1 DIV [in agreement with the observation of Temple and Davis (1994)], and this number increased substantially over time (Fig. 3*D*). The number of MAP2-positive (Fig. 3*C*) cells is substantially higher than the number of GFAP-positive cells, confirming that most precursors differentiate into postmitotic neurons. These results indicate that the signals that regulate proliferation and differentiation of cortical precursors into the neuronal and glial lineages continue to operate in the culture conditions implemented in this study, with a temporal schedule reminiscent of that observed during corticogenesis *in vivo*.

Thalamic axons influence the proliferative behavior of cortical precursors

E14 thalamic explants were first grown in GMEM on polylysine–laminin-coated slides. After 2–4 DIV, most of the explants exhibited substantial outgrowth of putative axons extending several hundred micrometers. Control experiments showed that thalamic cells do not migrate out of the explants along the axons. Cortical precursors were then plated in GMEM + 10% FCS onto the coverslips with the thalamic explant. The medium was renewed every 12 hr, thereby preventing the buildup of mitogenic factors in the bulk of the medium and making it possible to detect localized effects. After 2–3 DIV, there was a significant increase in cell density in the immediate vicinity of axon terminals (Fig. 4*A–D*). Because this could result from an increase in the frequency of proliferative divisions or survival, or both, we assessed GF in the vicinity of axonal terminals compared with locations at >60 μm from the terminals (Fig. 4*E*,*F*). This shows that embryonic thalamic neurons release via their axons a mitogenic factor, which leads to an increase in the proportion of daughter cells that remain in the cell cycle.

Influence of TCM on cell-cycle kinetics

To characterize mitogenic effects, we developed an assay based on the use of TCM. It was necessary to ensure that the thalamic

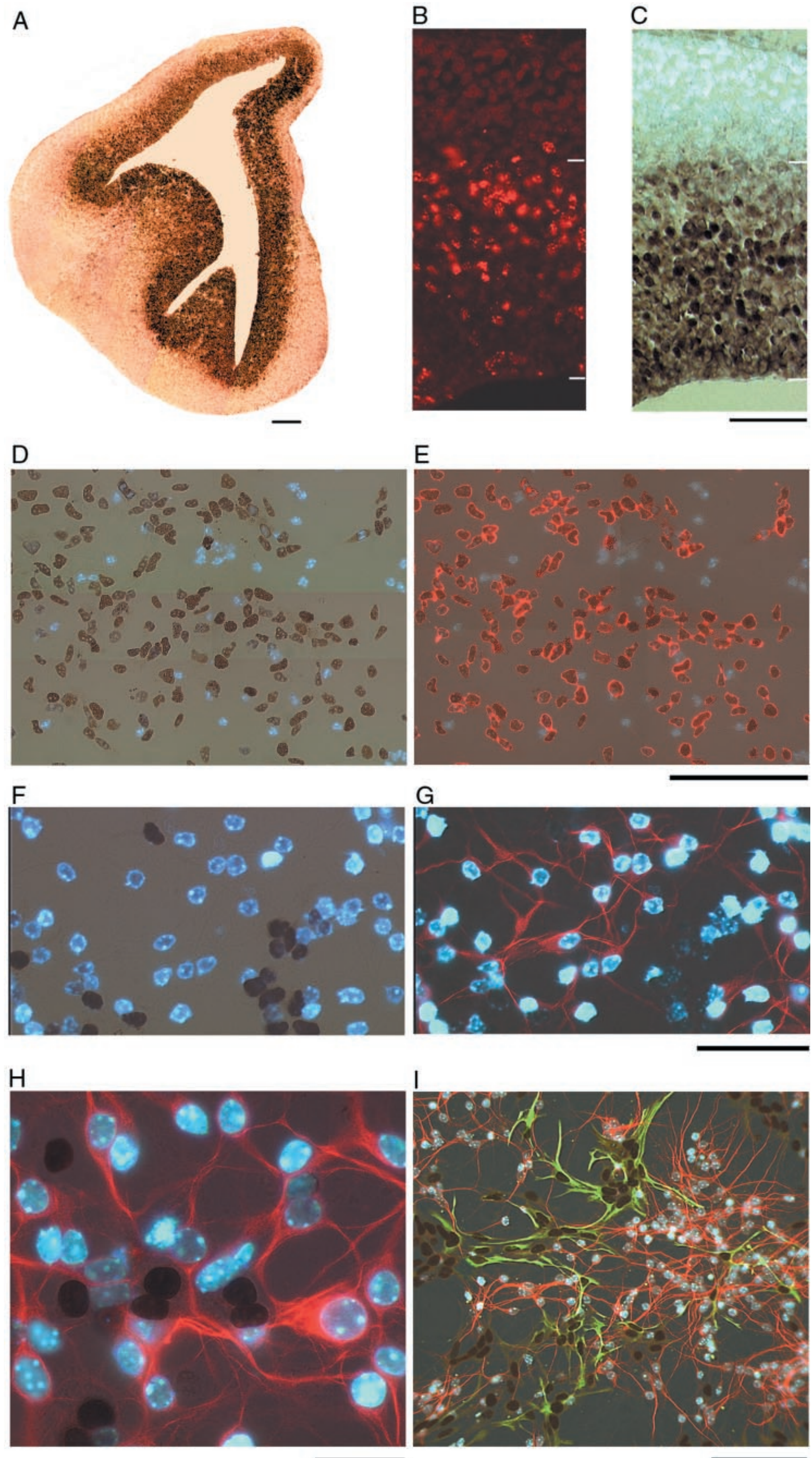


Figure 1. *A*, PCNA expression in the telencephalic wall of an E14 hemisphere mouse that has been fixed with ethanol (70%) at −20°C. PCNA-immunopositive nuclei have been revealed by DAB staining and appear *dark brown*. This shows that PCNA-positive neuroblasts are restricted to the proliferative zone. Scale bar, 100 μm. *B*, A high-power microphotograph of E14 dorsal cortex showing the distribution of cells in S phase (after a BrdUrd injection and 2 hr survival time). BrdUrd-positive cells are immunostained with Cy3 and appear *red* when viewed under fluorescent illumination. The majority of labeled cells are located in the top part of the ventricular zone. *C*, Corresponds to the same section as in *B*. PCNA-immunopositive cells are revealed by DAB staining, and the section has been counterstained with bisbenzimide. This shows that PCNA-immunopositive cells are restricted to the germinal zones. Scale bar, 50 μm. *D*, PCNA-positive precursors visualized by DAB immunochemistry (*brown nuclei*) after 2 DIV. Postmitotic cells are stained by bisbenzimide (*blue fluorescence*). *E*, BrdUrd-positive nuclei (*red fluorescence*) after a prolonged BrdUrd exposure (same field as *D*). Scale bar, 100 μm. *F–H*, Double immunostaining for MAP2 and PCNA showing the absence of colocalization of these two markers. *F*, PCNA+ precursors are immunostained with DAB (*brown*); nuclei of postmitotic cells are stained with bisbenzimide. *G*, Same field as *F*, showing MAP2 immunostaining (*red fluorescence*). Scale bar, 50 μm. *H*, High-power magnification of PCNA+ precursors (*brown*) and MAP2 immunolabeling. Note that MAP2-positive cells are PCNA negative. Scale bar, 20 μm. *I,* Immunostaining with MAP2 (*fluorescent red*), PCNA (*brown*), and GFAP (*green*). E15 cultures, 3 DIV. All GFAP-positive cells are PCNA positive. Scale bar, 100 μm.

cultures used for generating TCM were free of glia because glial cells release a number of factors that exert mitogenic, differentiation, and survival effects (Kilpatrick et al., 1993) that *in vivo* would not be in a position to influence the developing cortex.

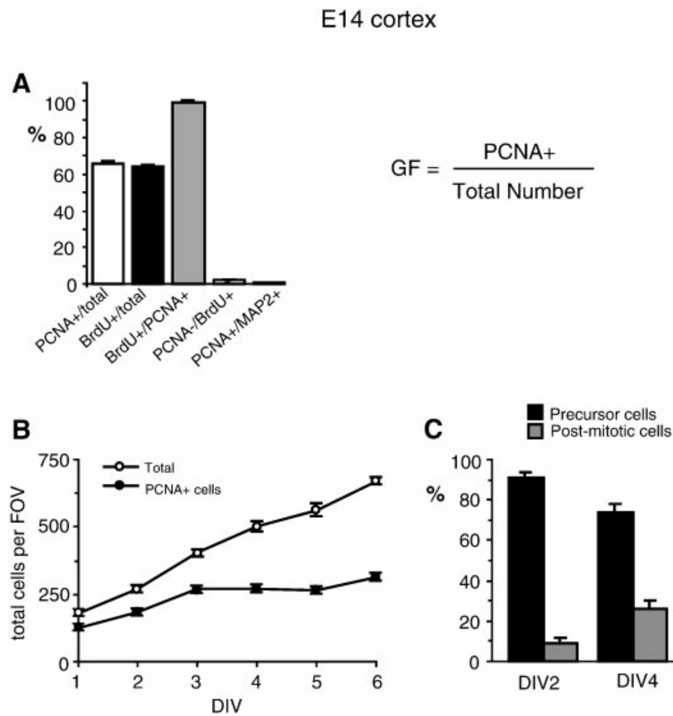


Figure 2. Characterization of cell cultures and quantification of the GF. *A*, Percentage of cycling cells, identified by PCNA immunostaining, corresponds to the GF. In a cell population undergoing proliferative divisions, prolonged (40 hr) BrdUrd exposure (at least equal to the cell-cycle duration) labels all cohorts of precursors going through S phase and identifies the GF. The GF values returned by the cumulative BrdUrd method (64%) are nearly identical to those returned by the percentage of PCNA+ cells (64.8%) in the same population. The ratio PCNA−/BrdUrd+ measures the fraction of BrdUrd+ cells that quit the cell cycle. The percentage of MAP2+ cells that also express PCNA approaches zero, indicating that PCNA expression is downregulated in postmitotic neurons. *B*, *C*, Characterization of E14 cortical cultures growing in GMEM + 10% FCS . *B*, Increase per field of view (*FOV*) in total cell number and in PCNA+ cells. The total population doubles during the first 48 hr. *C*, Proportions of precursor and postmitotic cells in the population of newly generated cells.

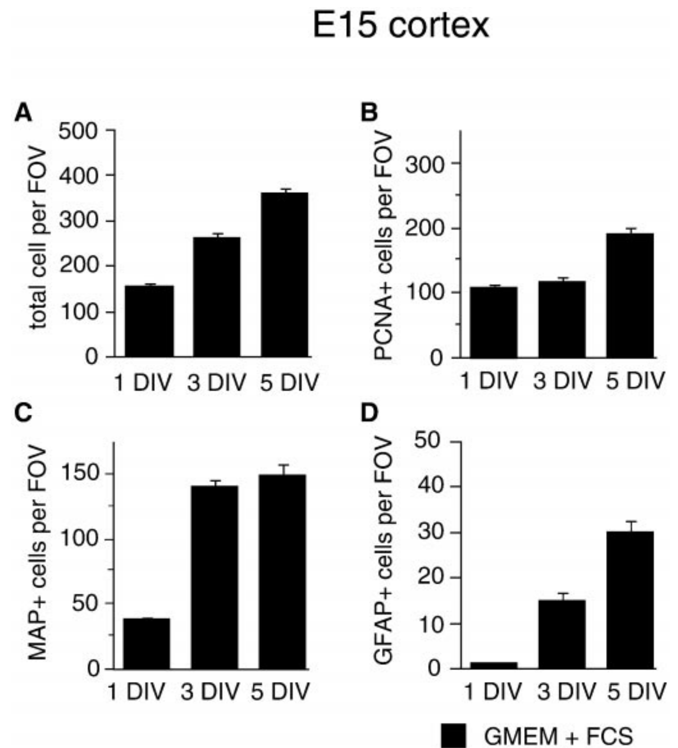


Figure 3. Variations of different cell types per field of view (*FOV*) over time in an E15 culture. *A*, Variations in total number of cells; *B*, variations in PCNA+ cells; *C*, variations in MAP2+ cells; and *D*, variations in GFAP+ cells.

TCM was prepared by growing E14 thalamic explants for 2 DIV in GMEM without serum. At E14, neurogenesis is terminated in the dorsal thalamus, and the use of GMEM ensures that no cell proliferation is induced, as confirmed by the absence of ^{3}H-thymidine incorporation (data not shown). Under these culture conditions, there are numerous axonal processes growing out of the thalamic explants, and no cells are labeled by GFAP. The absence of glial cells in the E14 thalamic explants was confirmed by GFAP immunoblotting (Fig. 4*G*).

The influence of TCM on cortical precursors was tested over the first 2 DIV. TCM was not found to influence the viability of cortical cultures (data not shown). In all experiments, TCM leads to a significant increase in cell density in the cortical cultures. To test whether the mitogenic effect of TCM was dose dependent, we have examined the influence of different dilutions of TCM on cell density in cortical cultures (see Fig. 6*A*). The results show that the mitogenic effect is decreased twofold with a 30% dilution of TCM. It is almost completely abolished when dilution is superior to 50%. This indicates that, within the range of dilutions tested, the mitogenic effect of TCM is proportional to the concentration of the active factor(s). It also indicates that the concentration of active factor(s) in undiluted TCM is close to the threshold under which the mitogenic effect starts to be detected. Hence, it is likely that the concentration of TCM would lead to stronger mitogenic effects.

We have further characterized the mitogenic effect of TCM by determining its influence on (1) GF, and (2) LI of the population of cycling cells (i.e., the percentage of PCNA-positive cells that have incorporated BrdUrd after a pulse exposure) (Fig. 5). The LI indicates the proportions of precursors in S phase at the time of the BrdUrd pulse and therefore reflects the relative duration of the S phase (Ts) with respect to the total duration of the cell cycle. Because Ts is largely invariant, variations of LI reflect changes in the duration of the cell cycle (Tc) (Waechter and Jaensch, 1972; Schultze et al., 1974; Schmahl, 1983; Reznikov and van der Kooy, 1995) (Fig. 5).

We examined the effects of TCM on cultures in which high levels of proliferation and minimal levels of cell death lead to a doubling of cell density during the first 48 hr and sustained growth for 5 DIV (see above). Under these conditions, TCM caused a 17–36% increase in cell density, an 11–17% increase in GF, and a 12–28% increase in LI (Fig. 6*B–E*, Table 1). The magnitude of the increase in CD, GF, and LI was significantly augmented when TCM was concentrated 10 or 20 times with Centricon filters (Fig. 6, compare *H*, *I*, and *J* to *E*).

One possibility is that the mitogenic factor released by thalamic axons acts by increasing the responsiveness of precursor cells to exogenous mitogens provided by FCS present in the culture media. To test this hypothesis, we examined whether TCM can increase proliferation in the absence of FCS (Fig. 6*F*). This showed that TCM alone leads to a 42–60% increase in density, an 11–26% increase in GF, and a 24–26% increase in LI (Table 1).

The proliferative behavior of the cultures supplemented with

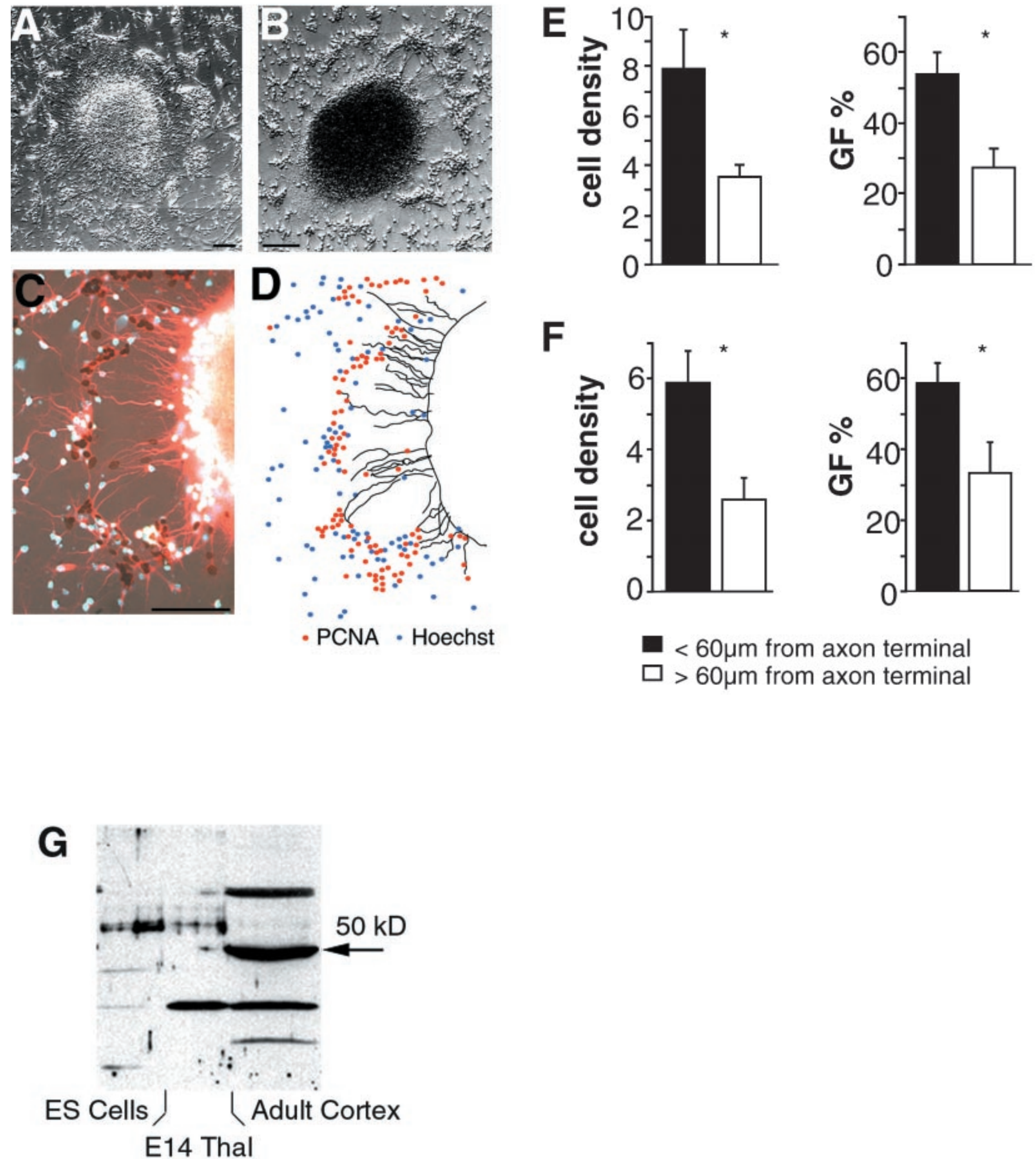


Figure 4. *In vitro* proliferation of E14 cortical precursors in the presence of E14 thalamic axons. *A*, *B*, Examples of thalamic explants (E14) cultured with E14-dissociated cortical precursors (GMEM + 10% FCS). Cell densities are higher in the proximity of neurite termination. *C*, Cortical precursors growing in the region of thalamic putative axons revealed by immunocytochemical identification of MAP2 (*red*) and PCNA (*brown*). *D*, Drawing of the explant shown in *C*. PCNA+ cells (*red dots*) and differentiated cells (*blue dots*). *E*, *F*, Cell density and proportion of undifferentiated cycling precursors in the vicinity of thalamic axon terminals (2 experiments). *G*, Western blot for GFAP detection in E14 dorsal thalamus, adult mouse cortex (positive control), and embryonic stem (*ES*) cells (negative control). The 50 kDa protein was detected with anti-GFAP antibody in the adult cortex but not in the embryonic thalamus. Statistical significance of the results: Mann–Whitney *U* test; $^{*}p < 0.05$. Scale bars, 100 μm.

TCM and no FCS (Fig. 6*F*) compared with cultures with FCS alone (Fig. 6*G*) shows that the magnitude of the mitogenic effect of TCM is similar to that of FCS, which is known to contain a number of growth-promoting factors.

The specificity of the mitogenic effect was investigated by determining its developmental time window (Fig. 7). This shows that the mitogenic effect of TCM is restricted to a narrow time period because E18 cortical precursors are no longer competent to respond to the thalamic mitogenic effect (Fig. 7*A–C*). At later developmental stages, thalamic neurons cease to influence neurogenesis because TCM obtained from E18 thalamus is unable to promote proliferation of E15 cortical precursors (Fig. 7*D–F*). This absence of a mitogenic effect of E18 thalamus is found with and without FCS (data not shown). The loss of response of E18 precursors to TCM is relatively selective because the mitogenic effect of FCS is conserved at this age (Fig. 7*G–I*). Altogether, these results indicate that the responsiveness of cortical cells to the thalamus-derived factor is selectively modulated during development.

We characterized the influence of TCM by estimating the duration of individual phases of the cell cycle by means of BrdUrd cumulative labeling (Nowakowski et al., 1989; Alexiades and Cepko, 1996) and the PLM technique (Quastler and Sherman, 1959). Here, we have improved the BrdUrd cumulative labeling method as a tool to measure the length of the cell cycle by computing the LI values within the population of cycling cells. In such a system, prolonged exposure to BrdUrd at least equal to Tc − Ts generates LI values of 100%. This cannot be the case when LI is computed with respect to the total population (cycling and quiescent cells). This makes it possible to accurately determine the Tc value by projecting the extrapolated 100% LI value on the *x*-axis.

BrdUrd cumulative labeling has been performed in E14 precursors. The results show significantly different slopes for control

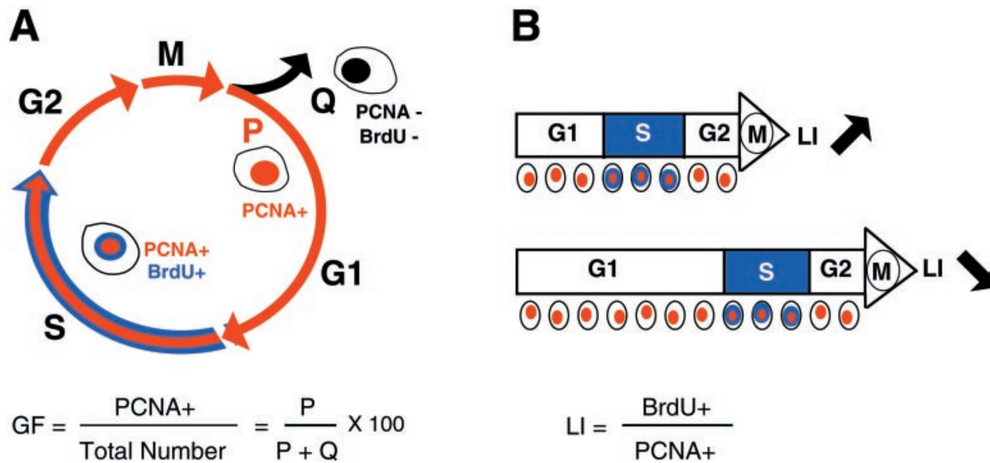


Figure 5. Calculation of cell-cycle parameters. *A*, Cell cycle. Early divisions are proliferative so that both daughter cells return into the cell cycle. Asymmetrical divisions, giving rise to a precursor (*P*) and a quiescent (*Q*) neuroblast, become progressively more frequent during corticogenesis (Takahashi et al., 1995). *B*, BrdUrd incorporation identifies the fraction of precursors (*PCNA+*) in S phase and determines the labeling index (*LI*). Because the Ts is largely invariant, variations of LI reflect changes in the Tc (Schmahl, 1983).

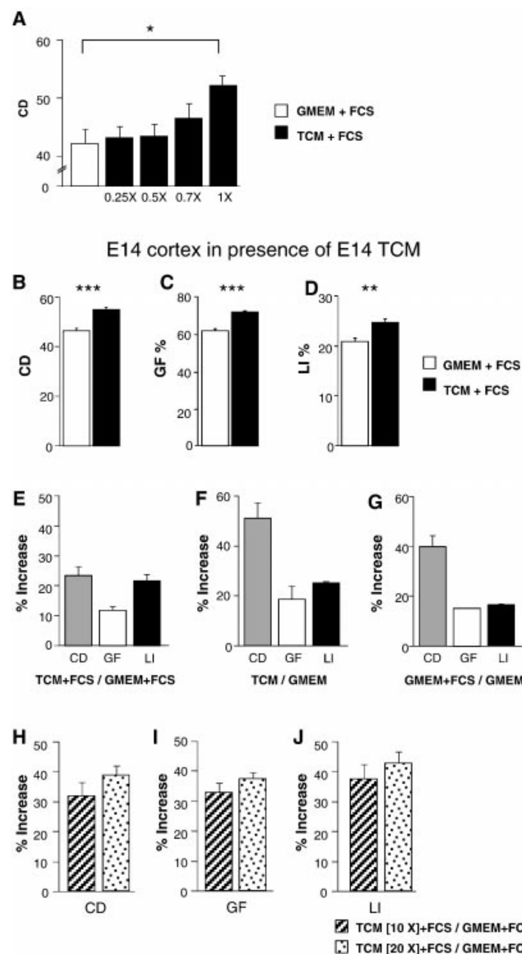


Figure 6. *A*, Dose–response effect of E14 TCM on cell density of E14 cortical precursors. *B–D*, Effect of TCM on cell density (*CD*), growth fraction (*GF*), and labeling index (*LI*), respectively, in E14 cultures. *E–G*, Mean percentage increase of CD, GF, and LI in cortical precursors grown in TCM (Table 1). *E*, TCM + 10% FCS compared with cortical precursors grown in GMEM + 10% FCS (values from 6 experiments). *F*, TCM alone, two experiments. *G*, FCS alone, two experiments. *H–J*, Mean percentage increase of CD, GF, and LI in cortical precursors grown in 10× and 20× concentrated TCM.

and TCM-treated precursors, indicating different cell-cycle times (Fig. 8*A*). Extrapolation to the 100% LI value shows that Tc is reduced in TCM cultures compared with controls, whereas the length of Ts (derived from the extrapolation of LI = 0 on the negative limb of the *x*-axis) is identical under both conditions (Fig. 8*A*). The data return a duration of S phase of the order of 3 hr, which is close to the 4 hr Ts value reported *in vivo* (Takahashi et al., 1995). Measurements of the length of the G_2/M phases by means of the PLM technique return identical values of 3.5 hr under both culture conditions (Fig. 8*B*). Subtraction of Ts + TG_2/M values from Tc returns a G_1 duration (TG_1) of 19 hr in the case of TCM-treated cultures, which indicates a reduction of 30%, compared with the TG_1 of control cultures (25 hr). Together, these results show that the thalamus-derived factor modulates Tc of cortical precursors by selectively shortening the G_1 duration.

Inhibition of TCM mitogenic effect by anti-bFGF

We have sought to investigate the identity of the thalamus-derived extracellular signal. A candidate molecule is bFGF, which is a particularly effective mitogen for cortical precursors (Ghosh and Greenberg, 1995; Cavanagh et al., 1997; Vaccarino et al., 1999) and is present *in vivo* in the E15 thalamus neurons (Lotto et al., 1997) and axons as shown in Figure 9*A*. We first characterized the influence of bFGF (10–50 ng/ml) on E14 precursors. bFGF was found to stimulate proliferation by significantly increasing the cell density, GF, and LI values (data not shown). We explored the potential role of bFGF by the addition of a neutralizing monoclonal antibody against bFGF (which recognizes bFGF but not acidic FGF) to cortical precursors cultured in TCM and to precursors grown in the vicinity of thalamic axons. In both cases, although this treatment did not totally abolish the mitogenic effect of TCM, it significantly decreased proliferation rates resulting in reduced GF values (Fig. 9*B*,*C*). This result suggests that bFGF partly mediates the mitogenic thalamic effect either by increasing the responsiveness of cortical precursors to another growth factor (Ciccolini and Svendsen, 1998) or by acting directly in cooperation with other factors.

Influence of TCM on cortical precursor differentiation

The present findings show that under the influence of TCM, a greater proportion of cortical precursors do not differentiate, but rather continue their progression in the cell cycle and exhibit a shorter G_1-phase duration. This raises several questions. For instance, are the cortical precursors treated by TCM prevented from differentiation? Does TCM selectively influence the proliferation of glial or neuronal lineages?

Table 1. Influence of thalamus-conditioned medium (TCM) and fetal calf serum (FCS) on cell-cycle parameters

	Cell density (% increase)	Growth fraction (% increase)	Labelling index (% increase)
TCM in the presence of FCS (TCM + FCS versus GMEM + FCS) six experiments	+19***	+17**	+18**
	+17***	+11**	+28**
	+36***	+7***	+25**
	+19**	+12***	+22*
	+28**	+12***	+12*
	+22***	+12***	+25***
TCM in the absence of FCS (TCM versus GMEM) two experiments	+42***	+11*	+24***
	+60***	+26***	+26**
FCS in the absence of TCM (GMEM + FCS versus GMEM) two experiments	+46***	+15*	+16 ns
	+34***	+15**	+17*

Statistical analysis of the differences: Mann–Whitney U test (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$).

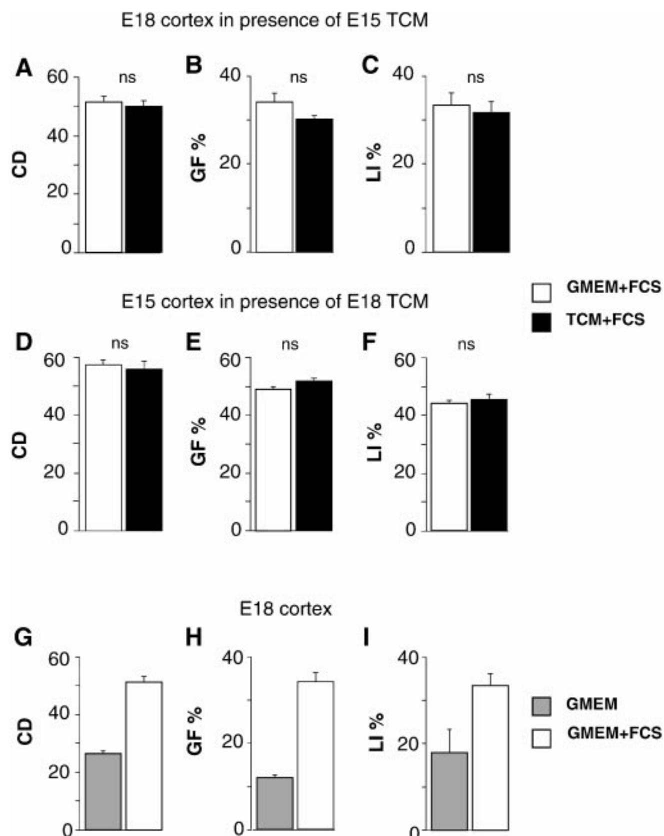


Figure 7. *A–C*, CD, GF, and LI in E18 cortical precursors grown in GMEM + 10% FCS and in TCM prepared from E15 thalamus (1 of 2 experiments). *D–F*, CD, GF, and LI in E15 cortical precursors grown in GMEM + 10% FCS and in TCM prepared from E18 thalamus (1 of 2 experiments). *G-I*, CD, GF, and LI in E18 cortical precursors grown in GMEM and in GMEM + 10% FCS, showing that FCS exerts a strong mitogenic effect on the E18 cortical precursors.

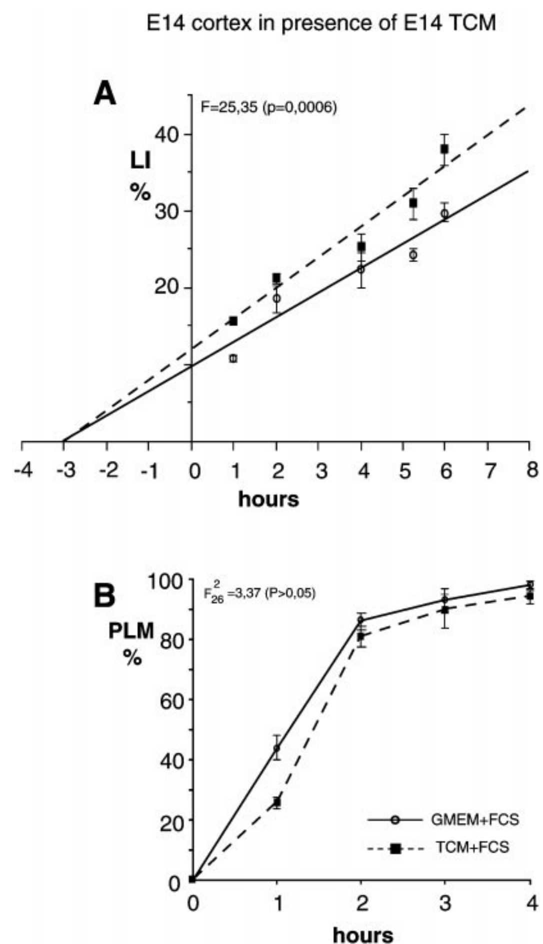
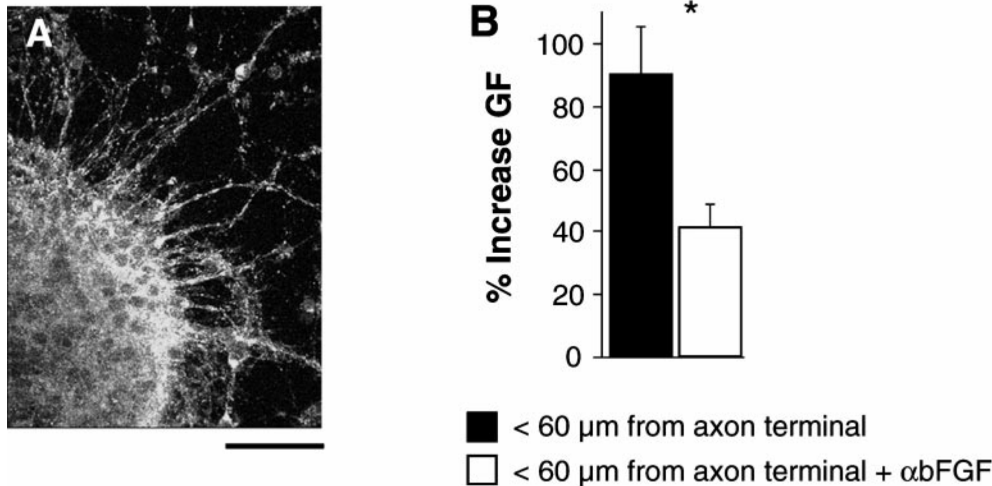


Figure 8. Analysis of cell-cycle kinetics in E14 cultures, showing that TCM reduces cell-cycle duration via the G_1 phase. *A*, BrdUrd cumulative labeling indicates the duration of the cell cycle (Tc; 31.5 hr), S phase (Ts; 3 hr), and G_1 + G_2 + M phases (28.2 hr) in E14 control cultures (*GMEM+FCS*). TCM + FCS gives a 6 hr reduction of Tc; Ts remains constant. LI values are ± SEM. F test statistical analysis indicates that the two slopes are different. *B*, Percentage of labeled mitoses (*PLM*) indicates the duration of the G_2 + M phases. In the presence and absence of TCM, G_2 + M duration was 3.5 hr, indicating that TCM regulates the duration of Tc via the G_1 phase (see Results). Values are ± SEM. Statistical analysis with an F test shows that the two slopes are identical.

To examine whether TCM prevents differentiation, we have investigated the influence of TCM on the proportions of MAP2- and GFAP-positive cells in dissociated cultures maintained for 5 DIV. Both GFAP- and MAP2-positive cell numbers are found to increase with time in control cultures (Fig. 10*A*,*B*). In TCM-treated cultures, the GFAP-positive cell number increase is bigger than in control cultures (Fig. 10*A*). We found that all GFAP-positive cells are also PCNA positive (see Fig. 1*F*). Although the GF substantially increases in TCM-treated cultures, the GFAP/PCNA ratio remains unchanged compared with control cultures and reaches 13% at 5 DIV (Fig. 10*C*). This indicates that the



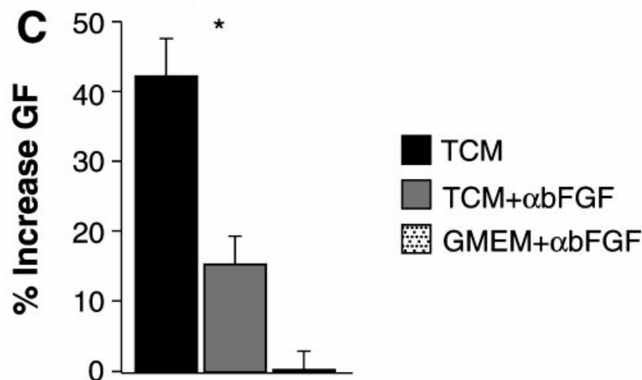


Figure 9. *A*, Immunostaining of an E15 thalamic explant showing bFGF localization in cell bodies as well as in outgrowing neurites. Scale bar, 50 μm. *B*, Effect of treatment with an anti-bFGF antibody (60 ng/ml) on GF in E15 cortical precursors grown in the vicinity of E15 thalamic axons (2 experiments). *C*, Effect of treatment with an anti-bFGF antibody (60 ng/ml) on GF in E15 cortical precursors grown in GMEM and TCM (2 experiments). The antibody directed against bFGF significantly reduces the increase in GF induced by TCM but does not modify the GF value in the control culture.

fraction of precursors following the glial fate is not altered in 5 DIV TCM-treated cultures and the increase of glial cells in TCM-treated cultures compared with control (Fig. 10*A*) is directly attributable to the increase in the GF. TCM-treated cultures are characterized by lower MAP2-positive cell numbers (Fig. 10*B*), in agreement with the fact that TCM treatment results in a higher GF and, conversely, a reduced postmitotic cell proportion compared with normal.

To determine whether more neurons are produced under the influence of TCM, we computed the proportion of MAP2-positive cells in a 7 DIV culture; the cells were exposed to TCM for the first 2 DIV and left to survive for an additional 5 DIV. This shows that TCM-exposed cultures are characterized by a higher proportion of MAP2-positive cells (Fig. 10*D*) and that precursors engage in neuronal differentiation subsequent to the TCM mitogenic effect.

Together, these findings show that the thalamic mitogenic effect (1) influences the proliferation of both glial and neuronal precursors and (2) does not preclude the action of signals that induce differentiation in these two lineages.

Cortical neuroblast proliferation in organotypic culture

To assess the relevance of the above findings to the *in vivo* situation, we have sought to determine whether embryonic thalamic axons exert a mitogenic effect in the intact cortex. Proliferation parameters of cortical precursors were examined in organotypic cultures with intact thalamocortical innervation. E15 cortical hemispheres devoid of subcortical innervation and cortical hemispheres attached to the thalamus were cultured for 24 hr and exposed to BrdUrd for 2 hr. BrdUrd immunolabeling was then examined on thin sections (Fig. 11*A*,*B*). The results show an increased number of BrdUrd-positive cells in the ventricular zone of the cortex, which was innervated by thalamic afferents, compared with the isolated cortex.

In a second series of experiments, E15 cortical hemispheres devoid of subcortical innervation and cortical hemispheres attached to the thalamus were cultured 8 hr after dissection. BrdUrd was added to the culture medium for 4 hr before microdissection and dissociation of the embryonic telencephalic wall. Dissociated cells were then plated on polylysine–laminin-coated coverslips and fixed 8 hr after plating. Because labeled and unla-

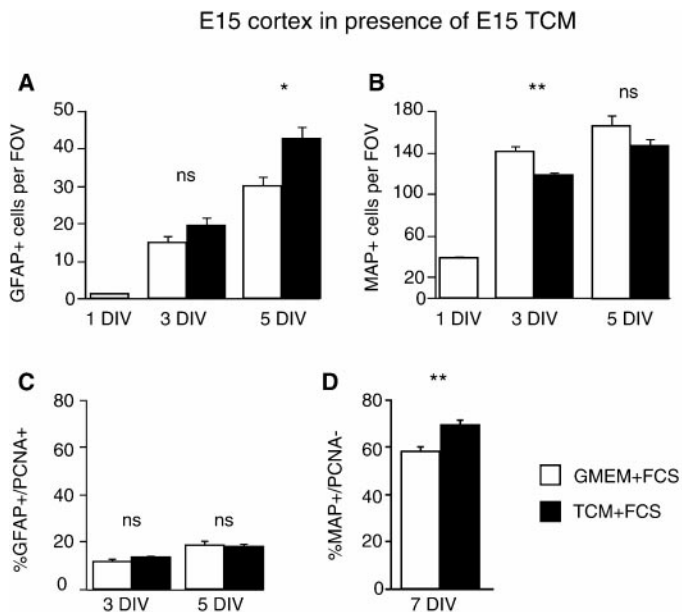


Figure 10. Quantitative analysis of glial and neuronal populations in E15 control cultures (*GMEM+FCS*) and in TCM + FCS-treated cultures. *A*, Number of GFAP+ cells per FOV. *B*, Number of MAP2+ cells per FOV. *C*, Proportions of GFAP+ cells with respect to the GF. *D*, Percentage of MAP2+ cells with respect to the postmitotic population in a 7 DIV culture. Values are ± SEM.

beled precursors will have identical rates of proliferation subsequent to the pulse, the labeling indices can be examined after the time period that is required to allow precursors to attach to the coverslips (Fig. 11*C*).

The results show that both GF and LI values are significantly increased in the case of the thalamocortical organotypic culture, compared with the isolated hemispheres (Fig. 11*C*). These experiments performed in organotypic cultures show that in the cytoarchitecturally intact system, thalamic afferents stimulate the proliferation of cortical precursors. Therefore, the mitogenic effect of thalamic axons on dissociated cell cultures is not an artifact of cell dissociation, but instead reflects a developmental role of thalamic innervation in the cytoarchitecturally intact system.

DISCUSSION

Before discussing the significance of these results for neurogenesis and corticogenesis, we need to address the relevance of the present findings to *in vivo* development. Thalamic afferents *in vivo* are in a position to exert a mitogenic effect on cortical precursors. Axonal tracing experiments at E15 show that ingrowing thalamocortical axons lie within 80 μm of the ventricular zone (Erzurumlu and Jhaveri, 1992; Polleux et al., 1996). One possibility is that thalamic fibers signal over these distances to the cortical precursors in the germinal zones or via their close contacts with radial glia (Godement et al., 1987; Erzurumlu and Jhaveri, 1992; Kageyama and Robertson, 1993). Furthermore, thalamic axons can directly contact those precursors located in the intermediate zone (Smart, 1973; Shoukimas and Hinds, 1978; Valverde et al., 1995).

We have directly addressed the issue of a mitogenic influence of thalamic afferents *in vivo* with organotypic cultures. The increased proliferation observed in the hemispheres that are attached to the thalamus cannot be the result of monoaminergic innervation from the brainstem, which is excluded in this preparation, nor of cholinergic innervation from the basal forebrain, which is not formed until later in development (Dinopoulos et al., 1989; Kiss and Patel, 1992).

The specificity of the effect has been addressed by examining its developmental timetable. The mitogenic effect is observed when thalamic fibers enter the lateral wall of the E15 telencephalic vesicle (Bicknese et al., 1994; Polleux et al., 1996). TCM derived from E18 thalamus fails to show a mitogenic effect, and this corresponds to a stage when thalamic axons are growing into the cortical plate (Bicknese et al., 1994). The competence of cortical precursors to respond to the thalamic mitogenic effect is temporally restricted because E18 precursors fail to respond to TCM. Hence, the short period during which a mitogenic effect can be demonstrated is restricted to the developmental time window when cortical afferents are in close proximity to cortical precursors.

Candidate molecule for the mitogenic effect

Numerous extrinsic factors have been shown to modulate cortical neuroblast proliferation (Cameron et al., 1998). These include widely expressed growth factors [bFGF, EGF, IGF, TGF-β, and pituitary adenylate cyclase-activating polypeptide (PACAP)], neurotrophic factors (NT3, NT4, BDNF, and NGF), and neurotransmitters (GABA, glutamate, VIP, and monoamines). Factors that have been reported to promote neuroblast proliferation include NGF (Cattaneo and McKay, 1990), bFGF (Ghosh and Greenberg, 1995; Cavanagh et al., 1997), EGF (Burrows et al., 1997), IGF (Nielsen and Gammeltoft, 1990; Ye et al., 1996), and VIP (Gressens et al., 1998), whereas 5-hydroxytryptamine (5-HT) (Lavdas et al., 1997), norepinephrine (Ghiani et al., 1999), glutamate and GABA (LoTurco et al., 1995), and PACAP (Lu and DiCicco-Bloom, 1997; DiCicco-Bloom et al., 1998) have been shown to inhibit proliferation and/or to elicit cell-cycle withdrawal.

Although the signaling molecule that is delivered to the embryonic cortex by thalamic axons and acts as a positive regulator of neurogenesis has not been identified, this study suggests that bFGF is involved. The thalamus-derived signal can act directly or via the induction of secondary signals from resident cell populations. bFGF is one putative signal that embryonic thalamic axons could deliver to the embryonic cortex. bFGF immunoreactivity is present in embryonic thalamic neurons (Lotto et al., 1997) and axons (present results), in agreement with the demonstration that bFGF can be anterogradely transported (von Bartheld et al., 1996). Although bFGF is a potent mitogen for cortical precursors (Ghosh and Greenberg, 1995; Vaccarino et al., 1999), it may also exert its effects through the induction of responsiveness to other factors (Ciccolini and Svendsen, 1998).

Mitogenic effect on the dynamics of the cell cycle

The influence of the thalamus-derived factor exclusively on the duration of the G_1 phase of the cell cycle is in agreement with a number of studies showing that, in most eukaryotic cell types, cell-cycle duration is regulated mainly via G_1 (Pardee, 1989) and that Ts and G_2/M-phase duration are highly conserved during neurogenesis (Kaufmann, 1968; Waechter and Jaensch, 1972; Schultze et al., 1974; Schmahl, 1983; Reznikov and van der Kooy, 1995; Miyama et al., 1997).

The fact that TCM influences the commitment of cortical precursors through cell-cycle progression suggests that it contains factors that inhibit growth arrest by promoting G_1/S transition in

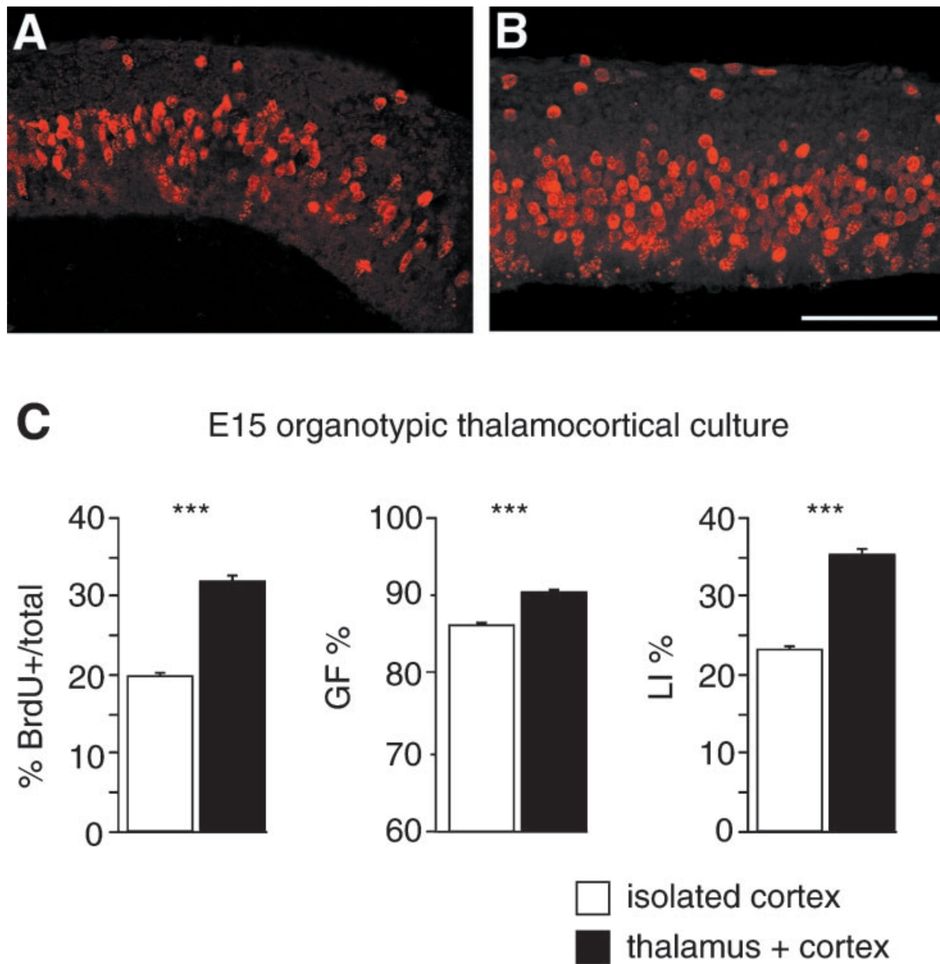


Figure 11. Proliferation parameters in E15 organotypic cultures. *A*, BrdUrd+ cells in a section of an organotypic culture of cortex innervated by thalamus. *B*, BrdUrd+ cells in a section of an organotypic culture of isolated cortex. *C*, Growth fraction (*GF*) and labeling index (*LI*) are increased in organotypic thalamocortical coculture compared with isolated cortex culture.

cortical precursors. Accordingly, the thalamus-derived factors may upregulate the expression of positive cell-cycle regulators such as D cyclins or downregulate the expression of cell-cycle inhibitors such as cyclin-dependent kinase inhibitors (Sherr and Roberts, 1999). Consistent with a major role in positive regulation of G_1 progression, the D-type cyclins are required for S-phase entry, and their overexpression accelerates G_1 and reduces dependency on exogenous growth factors (Baldin et al., 1993; Quelle et al., 1993; Lukas et al., 1994).

The present results concerning the effect of the thalamus-derived mitogenic factor on G_1/S transition agree with observations in invertebrates. Work in the *Drosophila* shows that innervation from the optic nerve facilitates the G_1/S transition of central precursors (Selleck et al., 1992; Huang and Kunes, 1998).

In the mouse telencephalon, thalamus afferents exert a mitogenic influence in midcorticogenesis. Because of the spatial proximity of the thalamic axons to the ventricular and subventricular zones, the mitogenic effect would be expected to differentially affect subventricular and ventricular precursors (Haydar et al., 2000). Cell-cycle progression is controlled by multiple (intrinsic and extrinsic) inhibitory and excitatory regulators (see above). The present result suggests that thalamic afferents exert a control over corticogenesis by modulating rates of proliferation and thereby offsetting the lengthening of the cell cycle in late corticogenesis (Schmahl 1983; Takahashi et al., 1995). Such a control mechanism could serve to adjust final cortical cell numbers with respect to the sensory periphery (Kennedy and Dehay, 1997).

Afferent control of morphogenesis in the CNS

A number of studies in invertebrates suggest that peripheral axons regulate morphogenesis of target structures. Work on leech genitalia shows that peripheral organs can regulate central neurogenesis (Baptista et al., 1990). In the visual system of the crustacea *Daphnia magna*, lesions of growing optic axons reduce the numbers of target neurons (Macagno, 1979). In *Drosophila*, optic axons promote the proliferation of target precursor neurons (Selleck et al., 1992). In vertebrate CNS development, only a few reports have examined the role of afferent axons in the regulation of CNS proliferation. Eye removal during early frog development results in lower mitotic rates in the regions of the tectum that are innervated by the optic axons (Kollros, 1953, 1982). In the olfactory system, afferent axons influence the proliferation and differentiation of target progenitors in the telencephalon (Gong and Shipley, 1995).

A number of studies in rodent and primate provide indirect evidence in favor of an afferent control of corticogenesis. Regionalization of cell-cycle kinetics in the ventricular zone plays a determinant role in the generation of cytoarchitecturally distinct neocortical areas characterized by different numbers of neurons per unit area of cortical surface (Dehay et al., 1993; Polleux et al., 1997), and increased rates of proliferation are characteristic of precursors of areas containing high numbers of neurons (Dehay et al., 1993; Polleux et al., 1997, 1998). Note that the increase in proliferation rates that are characteristic of A17 precursors in the

primate visual cortex is observed at a stage when thalamic axons are in close proximity to the germinal zones (Dehay et al., 1993). Significantly, depletion of geniculocortical axons leads to a drastic reduction of neuron number and of surface area of the target area (Rakic, 1988; Dehay et al., 1989, 1996).

Thalamic influence on cortical proliferation is likely to be one of many extracellular factors regulating cortical neurogenesis. A number of potential sources of neurotransmitters can influence precursor proliferation in the ventricular zone (LaMantia, 1995). Monoaminergic afferents originating from the brainstem and midbrain (Moore et al., 1978) are among the first axons to innervate the embryonic telencephalic wall shortly after the onset of neuron production (Wallace and Lauder, 1983) and are likely to influence cortical proliferation because monoaminergic receptors are expressed by neuroepithelial cells of the ventricular zone (Johnson and Heinemann, 1995). A recent study (Lavdas et al., 1997) has provided evidence that 5-HT promotes the differentiation of glutamatergic neurons, without affecting precursor proliferation. Precursors of the cortical ventricular zone express adrenergic receptors (Lidow and Rakic, 1995; Wang and Lidow, 1997), and norepinephrine triggers cell-cycle arrest in oligodendrocytes (Ghiani et al., 1999). GABAergic cells and processes (as well as other neurotransmitter-containing processes) are located in close proximity to the germinal zones (Lauder et al., 1986; Parnavelas and Cavanagh, 1988). Glutamate and GABA influence DNA synthesis of cortical precursors in the rat ventricular and subventricular zones (LoTurco et al., 1995, Haydar et al., 2000). There is evidence suggesting that the cortical plate exerts an inhibitory feedback influence on proliferation in the ventricular zone (DiCicco-Bloom et al., 1998; Polleux et al., 1998) that could be relayed by descending corticofugal axons (Kim et al., 1991; Miller et al., 1993; McConnell et al., 1994; Meyer et al., 1998).

The present results could appear at odds with the recent findings reported by Miyashita-Lin et al. (1999) on Gbx2 mutants and by Nakagawa et al. (1999) on Mash mutants characterized by an impairment of thalamocortical projections throughout development and in which neocortical region-specific gene expression is reported to develop normally. Although these studies convincingly show that regionalized gene expression in the mutants followed a normal developmental process, they did not address their areal cytoarchitecture and regionalized differences in neuron number, which would require stereological examination of the newborn cortex (Skoglund et al., 1996). More recently, Bishop et al. (2000) and Mallamaci et al. (2000) provided further evidence for a genetic determination of areal identity in the neocortex. Together, these findings underlie the notion that specification of cortical area identity results from an interplay between intrinsic and extrinsic factors.

Conclusion

The differential distribution of thalamocortical projections, acting in combination with other neurotransmitter-containing afferent systems, could provide a tight spatiotemporal regulation of proliferation rates in the ventricular zone. Precursors of the six layers of the neocortex exit the cell cycle according to a precisely defined spatiotemporal pattern (Angevine and Sidman, 1961; Smart and Smart, 1982; Bayer and Altman, 1991; Takahashi et al., 1999). The modulation of cell-cycle withdrawal will have far-reaching consequences because the environmental signals encountered during the final round of mitosis play a major role in determining neuronal fate (McConnell and Kaznowski, 1991; Gotz and Bolz, 1994; Bohner et al., 1997; Eagleson et al., 1997). Therefore, the interplay between positive and negative regulators of cortical proliferation, by regulating the timing of cell-cycle exit, will contribute to the control of neuron number, cell fate, and ultimately areal and laminar specification.

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