

# The Concerted Modulation of Proliferation and Migration Contributes to the Specification of the Cytoarchitecture and Dimensions of Cortical Areas

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**Regionalization of cell cycle kinetics of cortical precursors has been described in nonhuman primates and rodents indicating a fate map of areas as distinct proliferative programs in the germinal zones of the neocortex. It remains to be understood how proliferative gradients during corticogenesis are transcribed into a stepwise function to form adult areal borders. Here we have used the monkey areas 17 and 18, which show striking cytoarchitectonic differences, as a model system for studying how developmental events establish areal boundaries in the adult. We present data indicating that the events that are involved in the formation of a sharp border separating 2 areas involve an orchestration of diverse phenomena including differential rates of proliferation, migration, and tangential expansion.**

**Keywords:** cortical plate, neocortex, primate, subventricular zone

## Introduction

The cerebral cortex is composed of multiple cortical areas that are characterized by distinct functional roles in sensory perception, language, and motor control. During mammalian evolution, the expansion of the cerebral cortex was accompanied by a multiplication of the number of areas. Information processing in the cortex is increasingly understood in terms of cooperative interactions between areas in turn linked to the unique connectivity and cytoarchitectonics of each area. Cortical cytoarchitecture is largely determined by variation of the number and density of neurons in individual layers.

There are a number of studies showing that the adult cortex shows very exact numbers of neurons in the layers that make up a cortical area (Rockel and others 1980; Garey and others 1985; Beaulieu and Colonnier 1989; Skoglund and others 1996). There are theoretical reasons for believing that neuron number is a cardinal feature in the cortical algorithm (Douglas and Martin 2004). Early theories of cortical development postulated that adult patterns of connectivity and neuron number were the consequence of regressive phenomena including cell death and axon elimination (Finlay and Slattery 1983; O'Leary 1989). In some ways, it was assumed that elimination (of neurons and connections) was a simple solution to the complex problem of establishing precise patterns of connections. In recent years, on the one hand, the precision of connectivity has been refined to mean exact numbers of neurons and connections (Scannell and others 2000), and increasingly progressive rather than regressive phenomena have been seen to dominate the development of the cortex (Goodman and Shatz 1993; Kennedy and Dehay 1993). However, recent evidence has shown that cell death plays a determinant role in the adjustment of the progenitor

numbers necessary for the proper morphogenesis of the brain (Kuan and others 2000). A series of influential studies using mutant mice have underlined the need for the precise coordination of proliferation and differential apoptosis during early neurogenesis for the proper regulation of cortical dimensions (Haydar and others 1999; Kuan and others 1999).

There is growing evidence for the importance of the patterning of the germinal zones in cortical areal development. The graded expression of transcriptional regulators (noticeably *Emx2* and *Pax6*) and of other genes (Eph kinase receptors, cell adhesion molecules, and nuclear receptors) among the proliferating cells in the germinal zones is considered to lay down the initial blueprint of cortex arealization (Donoghue and Rakic 1999; Nakagawa and others 1999; Bishop and others 2000, 2002; Mallamaci and others 2000). Although there is evidence for the role of these transcription factors in the control of regional identity, cell type specification, proliferation, and differentiation (Mallamaci and others 2000; Rubenstein 2000; Heins and others 2001; Estivill-Torrus and others 2002; Galli and others 2002; Bishop and others 2003), how these graded patterns of regulatory molecules translate into the genesis of individual areas is a major issue that remains to be elucidated (Monuki and Walsh 2001; Ragsdale and Grove 2001; Bishop and others 2002; O'Leary and Nakagawa 2002).

Beside the above-reviewed evidence for a genetic control of patterning intrinsic to the neocortex, another major extrinsic source of patterning is provided by the thalamocortical axons that convey modality-specific information from the sensory periphery to the neocortex and impose functional specializations on primary sensory areas. The thalamocortical projections originating from the principal sensory nuclei of the dorsal thalamus are characterized by area specificity throughout development and have been shown to exert a major influence on both areal size and cortical specification during the early stages of corticogenesis (Rakic 1988; Dehay and others 1989).

A number of studies have shown that there is a regionalization of the proliferative behavior of precursors in the germinal zone of nonhuman primates and rodents (Dehay and others 1993; Polleux and others 1997). These studies have revealed a fate map of distinct proliferative programs in the germinal zones that are spatially and temporally coordinated to generate the layout of cortical areas. In rodents and primates, mathematical modeling of results of birthdating experiments has shown that variations in rates of proliferation are correlated to the neuron numbers of cortical layers and that adjacent areas showing different neuron number are generated by distinct differences

in the cell cycle parameters during corticogenesis (Polleux and others 1997; Lukaszewicz and others 2005).

Because of the striking and quantifiable cytoarchitectural differences between the adjacent areas 17 and 18 of the monkey, associated with the highly developed and distinctive germinal zones, the embryonic monkey visual cortex offers the unique opportunity to relate events in the germinal zone to the final outcome in terms of neuron production. This has made it possible to look at those cell cycle features that ensure the large increase in the number of supragranular layer pyramidal cells that distinguishes area 17 from area 18 (Rockel and others 1980). This shows that the higher rates of neuron production in 17 compared with 18 are due to area 17 precursors having a shorter G1 phase and higher rates of proliferative divisions than do area 18 precursors (Lukaszewicz and others 2005).

These cell cycle-related areal features have to be considered in the context of the neurogenic gradients that occur during corticogenesis (Smart IHM and Smart M 1982; Bayer and Altman 1991; Polleux and others 1997). A central question that remains is how proliferative gradients during corticogenesis are transcribed into a stepwise function at the adult areal borders? Here, we have used the monkey area 17-18 border as a model system for studying how events in the germinal zone establish areal boundaries in the adult. There is reason to believe that the developmental events that are involved in the formation of a sharp border separating 2 areas involve an orchestration of diverse phenomena including differential rates of migration (Kennedy and others 1996), proliferation (Dehay and others 1993; Lukaszewicz and others 2005), and tangential expansion (Smart and others 2002).

## Materials and Methods

### *In Vivo Studies*

Two fetuses from timed-pregnant monkeys at embryonic (E) day 64 and E78 (E64 and E78) received approximately 10–20  $\mu\text{Ci}$ /grams body weight (intraperitoneally or intramuscularly [i.m.]) of  $^3\text{H}$ -thymidine in saline (specific activity: 40/60  $\mu\text{Ci}/\text{mmol}$ ) and were replaced in the uterus according to previously described protocols (Dehay and others 1993; Smart and others 2002; Lukaszewicz and others 2005). Pregnant monkeys were returned to their cages and medicated for 2 days with an analgesic (Visceralgine i.m.). A muscular relaxant (Salbumol i.m.) was given twice daily, and the fetuses were delivered by caesarian section after 7 (E64  $\rightarrow$  E71) and 14 days (E78  $\rightarrow$  E92) of survival. Following caesarian section, anesthetized fetuses were perfused through the heart with 4% paraformaldehyde. Histological observations of the embryonic occipital pole were made on paraffin sections (see below) from timed-pregnant fetuses aged E46, E55, E65, E72, E78, E88, and E94 from a previous study (Smart and others 2002) that were delivered by caesarian section and perfused as indicated above. In situ immunolabeling of p27<sup>Kip1</sup> and cyclin E was performed on frozen parasagittal sections from an E80 fetus (Lukaszewicz and others 2005). All experiments were performed in compliance with the national and European laws as well as with institutional guidelines concerning animal experimentation.

Observations in fetuses younger than E72 were made prior to the appearance of a clear cytoarchitectonic border of striate cortex. Acetylcholine esterase (AChE) is transiently expressed by extrastriate cortex (Kostovic and Rakic 1984). Despite the fact that the area 17-18 border emerges on a gradient of morphological and proliferative features, it can be clearly distinguished as early as E86 (Smart and others 2002) and E75 (C. Dehay, I. H. M. Smart, H. Kennedy, unpublished results) using AChE histochemistry. We have taken advantage of this to approximate the location of the borders of striate cortex in younger fetuses. Because striate cortex is a large cortical area that has a tight spatial relationship to the paleocortex, it is possible to extrapolate its position from E75 to younger fetuses. The brain used to detect AChE was cut on a freezing

microtome in the parasagittal section and processed according to the protocols described elsewhere (Smart and others 2002).

Brains were embedded in paraffin wax, and 5- or 10- $\mu\text{m}$ -thick parasagittal sections were cut. Sections were processed for autoradiography (K2 autoradiographic emulsion from Ilford Imaging, Mobberley, UK) to give maximum counts of 40 grains per differentiated cell. Proportions of labeled cells in the different anatomical compartments were computed. Using a  $\times 50$  or  $\times 63$  oil objective, labeled neurons were observed on a video screen and positions charted by a high-precision, computer-controlled plotting system (Mercator, ExploraNova Technology, La Rochelle, France).

### *Dissociated Cell Culture*

An E80 fetal brain was removed under sterile conditions in iced Hank's balanced salt solution containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The cerebral hemispheres were detached, and the neopallium was isolated. Area 17 cells were obtained from the most caudal part of the occipital lobe and presumptive area 18 cells from more rostral location, on the posterior bank of the presumptive lunate sulcus. Cells underwent enzymatic dissociation (trypsin 0.2%, 3 min at 37 °C) followed by mechanical dissociation and were suspended in Glasgow minimum essential medium (GMEM) + 10% fetal calf serum (FCS). Cells were seeded at a density of  $4 \cdot 10^5$  cells per 14-mm diameter polylysine-laminin-coated glass coverslip and cultured in 1 mL of GMEM + 10% FCS.

### *Immunocytochemistry*

In situ immunolabeling was performed according to a 2-step procedure. Briefly, sections were incubated overnight at 4 °C with primary antibodies p27<sup>Kip1</sup> (Santa Cruz Biotechnology [Santa Cruz, CA], SC-528, 1:200) and cyclin E (Santa Cruz Biotechnology, SC-481, 1:200). Sections were further incubated for 3 h at room temperature (RT) with goat anti-rabbit Cy3 (Interchim [Montluçon, France], 1:400 in Dako diluent). Sections were counterstained with Syto16.

### *In Vitro PCNA/p27 Double Immunolabeling*

After proliferating cell nuclear antigen (PCNA) immunocytochemistry (described in Lukaszewicz and others 2002), coverslips were processed for p27<sup>Kip1</sup> immunocytochemistry. Briefly, primary antibody (rabbit anti-p27<sup>Kip1</sup> from Santa Cruz Biotechnology, SC-528) was incubated at 1:20 in Dako diluent at RT for 1 h. After 3 rinses, goat anti-rabbit Cy2 (1:200 in Dako diluent; Jackson ImmunoResearch, Baltimore, MD) was incubated for 1 h at RT. Coverslips were mounted with 0.1% *n*-propylgallate (P3130; Sigma St. Louis, MO) in 1 M phosphate buffer and glycerol (1:1) to prevent fading on fluorescent illumination (Lukaszewicz and others 2002).

Coverslips were scanned at regular intervals with a grid corresponding to a field of 0.128 mm<sup>2</sup>. A total of 100–150 fields were observed per coverslip. A minimum of 2 coverslips were observed for each condition. Fluorescent labeling was analyzed using a Leica confocal microscope, using a  $\times 40$  objective (see below for details)

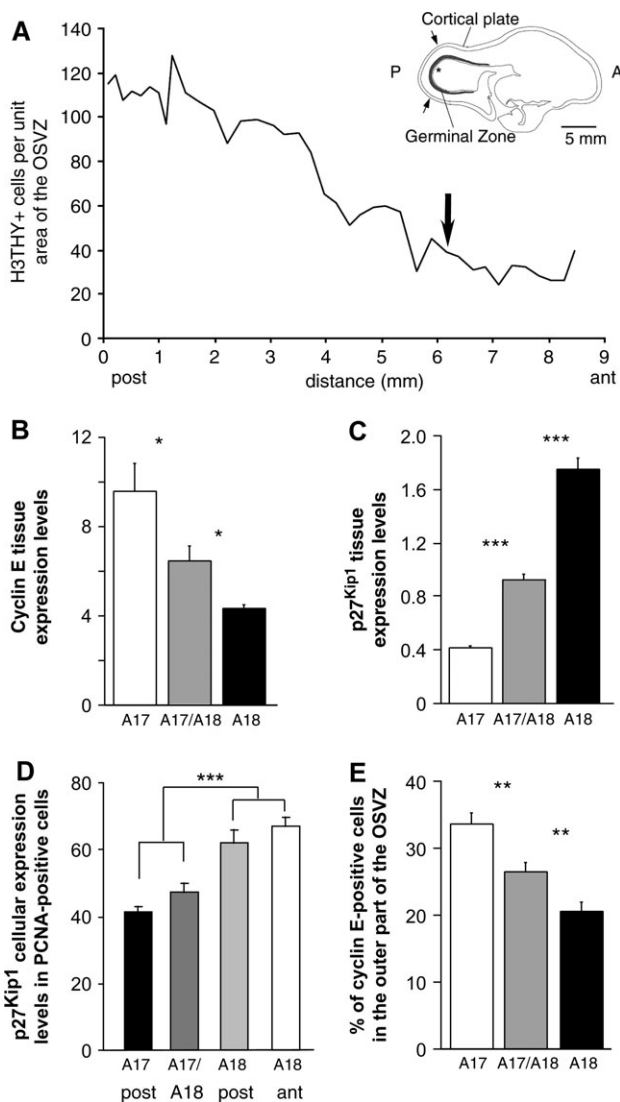
### *Confocal Measurements of Cyclin E and p27<sup>Kip1</sup> Expression Levels*

We have quantified protein expression levels by means of confocal microscopy analysis (Durand and others 1997; Lukaszewicz and others 2002; Tokumoto and others 2002). Confocal examination of the fluorescent labeling was carried out on a Leica TCS SP equipped with an Argon-krypton laser. Analysis of p27<sup>Kip1</sup> and cyclin E expression was performed using a  $\times 40$  (40- $\mu\text{m}$  brain sections) or a  $\times 63$  (dissociated cells) oil objective. p27<sup>Kip1</sup> is expressed during all phases of the cell cycle, whereas cyclin E expression is restricted to those precursors in G1 and G1/S transition phases. p27<sup>Kip1</sup> and cyclin E expression levels were analyzed at the tissue level. Quantitative analysis of Cy2 and Cy3 fluorescence was performed using Leica software (TCS NT). The tissue expression levels in the germinal zone were determined by calculating the intensity of fluorescence (arbitrary units) of p27<sup>Kip1</sup> or cyclin E labeling with respect to the total number of cells (indicated either by the intensity of Syto16 fluorescence or by cell counts), as described in Lukaszewicz and others (2005). Several fields of view were analyzed so as to span the entire thickness of the outer subventricular zone (OSVZ), in area 17 and area 18. Levels of cyclin E immunofluorescence intensity have been measured for each cell expressing detectable levels

of cyclin E. Four categories of labeling intensity have been defined: <50 (negative), 50–80 (low-intensity labeling), 80–110 (intermediate-intensity labeling), and >110 (high-intensity labeling). The labeling index (LI) of cyclin E-positive cells is determined by the percentage of cells showing detectable immunofluorescent intensity (>50) with respect to the total population of precursors (Fig. 1). The expression level of p27<sup>Kip1</sup> was further analyzed at the single-cell level in the cycling dissociated precursors, as identified by PCNA expression (Fig. 1D) (Lukaszewicz and others 2005). Statistical significance between area 17 and area 18 values was assessed with a Mann-Whitney *U*-test.

## Results

In a first step, using both *in vivo* and *in vitro* analysis, we characterized the neurogenic gradient spanning through pre-



**Figure 1.** *In vivo* regional differences in the control of proliferation. (A) Number of <sup>3</sup>H-thymidine-positive precursors per unit of surface area in the OSVZ of an E92 fetus after a short survival. First bin corresponds to the occipital pole (see star in inset); arrows indicate presumptive area 17–18 borders. (B, C) Going from area 17 to area 18, changing level of tissular expression of cyclin E (B) and p27<sup>Kip1</sup> (C) as measured by confocal microscopy quantification in the OSVZ at E80: statistical significance assessed with a Mann-Whitney *U*-test. (D) Level of p27<sup>Kip1</sup> expression in dissociated precursors of the germinal zones isolated from area 17, area 18, and intermediate positions at E80: statistical significance assessed with a Mann-Whitney *U*-test. (E) Going from area 17 to area 18, percentage of cyclin E-positive cells in the OSVZ at E80: statistical significance assessed with a Mann-Whitney *U*-test.

sumptive area 17 and area 18 at the time of supragranular layer neuron production in the embryonic monkey. Areal differences in rates of neuron production might be expected to lead to local crowding of the migratory lanes to the cortical plate. To examine if this is the case, we have made pulse injections of <sup>3</sup>H-thymidine at early and midcorticogenesis followed by survival time of 7–14 days, respectively. This will allow us to visualize the migration of neurons that were in their final round of mitosis at the moment of injection. At midcorticogenesis (E78), the 14-day survival time allows a migration period of 12.5 days for area 17 precursors and 12.1 days for area 18 precursors, given the cell cycle duration of monkey cortical precursors (Lukaszewicz and others 2005). These results show that the area 17 germinal zone with high rates of neuron production generates neuroblasts that show faster migratory rates compared with the neuroblasts exiting the area 18 germinal zone. The higher rates of neuron production and migration should lead to a thicker cortical plate in area 17. Unexpectedly, we found that during corticogenesis the cortical plate of area 17 is thinner and not thicker than the cortical plate of area 18. This last result suggests that there is a tangential expansion of area 17 that accommodates the increased rate of production from this area.

### *Proliferative Gradients in the Primate OSVZ, Spanning through Presumptive Area 17 and Area 18*

So as to determine how proliferation changes with respect to the 17–18 border, we examined <sup>3</sup>H-thymidine labeling in the OSVZ, the principal germinal zone in the monkey and the major site of supragranular neuron production (Smart and others 2002; Lukaszewicz and others 2005). We computed the areal density of <sup>3</sup>H-thymidine-positive cells per unit area of the OSVZ along the full extent of the occipital pole, encompassing presumptive areas 17 and 18. We used AChE labeling to determine the position of areal borders (data not shown) (Dehay and others 1993; C. Dehay and H. Kennedy, unpublished data). This shows that there is not an abrupt change in proliferation at the 17–18 border but rather that the highest proliferation rate is found on the pole of the cortex and progressively decreases to minimal levels at the 17–18 border, over a 3- to 4-mm gradient (Fig. 1A). This contrasts with the hairline 17–18 border found in the adult.

Area 17 and area 18 OSVZ precursors differ in the level of expression of key regulators of cell cycle control. More specifically, cyclin-dependent kinase inhibitor (CKI) p27<sup>Kip1</sup>, a negative regulator of the cell cycle and of G1 phase progression (Polyak and others 1994; Sherr and Roberts 1999)—and cyclin E—which is rate limiting for progression from G1 to S phase (Resnitzky and others 1994; Wimmel and others 1994)—have been shown to be expressed in contrasting patterns in area 17 and area 18 OSVZ progenitors, contributing to areal variations in rates of cell cycle progression (Lukaszewicz and others 2005).

Confocal quantification of immunolabeling against cyclin E and p27<sup>Kip1</sup> over several millimeters of the OSVZ of the occipital lobe going through the 17–18 presumptive border shows 2 opposite gradients of expression. Whereas area 17 precursors have reduced levels of p27<sup>Kip1</sup> and increased levels of cyclin E expression, area 18 precursors exhibit high levels of p27<sup>Kip1</sup> with low levels of cyclin E. This points to a proliferation gradient reflecting relative facilitation of the progression through G1 phase in area 17 compared with area 18

precursors (Fig. 1B,C), in agreement with the higher rates of proliferation observed in vivo (Fig. 1A, Lukaszewicz and others 2005).

Because expression of p27<sup>Kip1</sup> is usually upregulated in postmitotic cells (Lukaszewicz and others 2002), the analysis performed on the overall population does not make it possible to determine whether the regional differences in the level of p27<sup>Kip1</sup> expression are the consequences of changes in the proportions of precursors and postmitotic cells in the OSVZ or whether they truly reflect variations in cell cycle regulatory gene activity within the cycling precursor pool. When maintained in dissociated culture, area 17 precursors show higher rates of proliferation than area 18 precursors, therefore providing a reliable in vitro test (Lukaszewicz and others 2005). So as to restrict p27<sup>Kip1</sup> quantification to the cycling cells, we used dissociated cell cultures where PCNA expression makes it possible to reliably identify the cycling population of precursors. Quantification of fluorescent immunoreactivity against p27<sup>Kip1</sup> restricted to the PCNA-positive precursors revealed significantly lower levels of p27<sup>Kip1</sup> expression in area 17 precursors compared with area 18 precursors. Comparison of the levels of p27<sup>Kip1</sup> expression in precursors from intermediate locations between posterior area 17 and anterior area 18, thereby covering the 17-18 transition regions, shows intermediate levels of expression (Fig. 1D), suggesting a progressive lengthening of the G1 phase in a transition zone going from presumptive area 17 to area 18.

Cyclin E is a reliable marker of daughter cells committed to cell cycle reentry (Ekholm and others 2001), and the percentage of cyclin E-positive cells indicates frequency of precursors in G1/S transition. In proliferative divisions (also referred to as symmetrical divisions), both daughter cells return to the cell cycle and will therefore express high levels of cyclin E. This contrasts with differentiative (or asymmetrical) divisions where one daughter cell will cease to express cyclin E and quit the cell cycle. Therefore, variations in the percentage of cyclin E LI values reflect variations in the mode of division. High rates of proliferative divisions in a precursor pool are associated with an increase in the percentage of cells exhibiting high levels of cyclin E expression. Analysis of cyclin E LI over a stretch of 4 mm spanning through area 17 and area 18 OSVZ reveals that there is a gradual decrease in the percentage of cells engaged in proliferative divisions going from presumptive area 17 to presumptive area 18 (Fig. 1E).

The present results show that there are contrasting and graded levels of expression of p27<sup>Kip1</sup> and cyclin E between area 17 and area 18 precursors. Because the CKI p27<sup>Kip1</sup> acts as a negative regulator of the cell cycle and the level of cyclin E expression is rate limiting for progression from G1 to S phase, areal variations in these molecules point to differences in control of cell cycle progression. The present findings show that there is a gradient of proliferation going from area 17 to area 18.

#### ***Areal Differences in Rates of Neuron Migration***

Compared with area 18, area 17 has nearly twice the number of neurons in a radial unit of cortex. During development, there is a gradient of neurogenesis, and peak levels of neuron production in area 17 are located at 3–4 mm from the area 17-18 border. This raises the question of how a neurogenetic gradient is converted to a step function at the 17-18 border. One possibility is that the high rate of proliferation in area 17 is

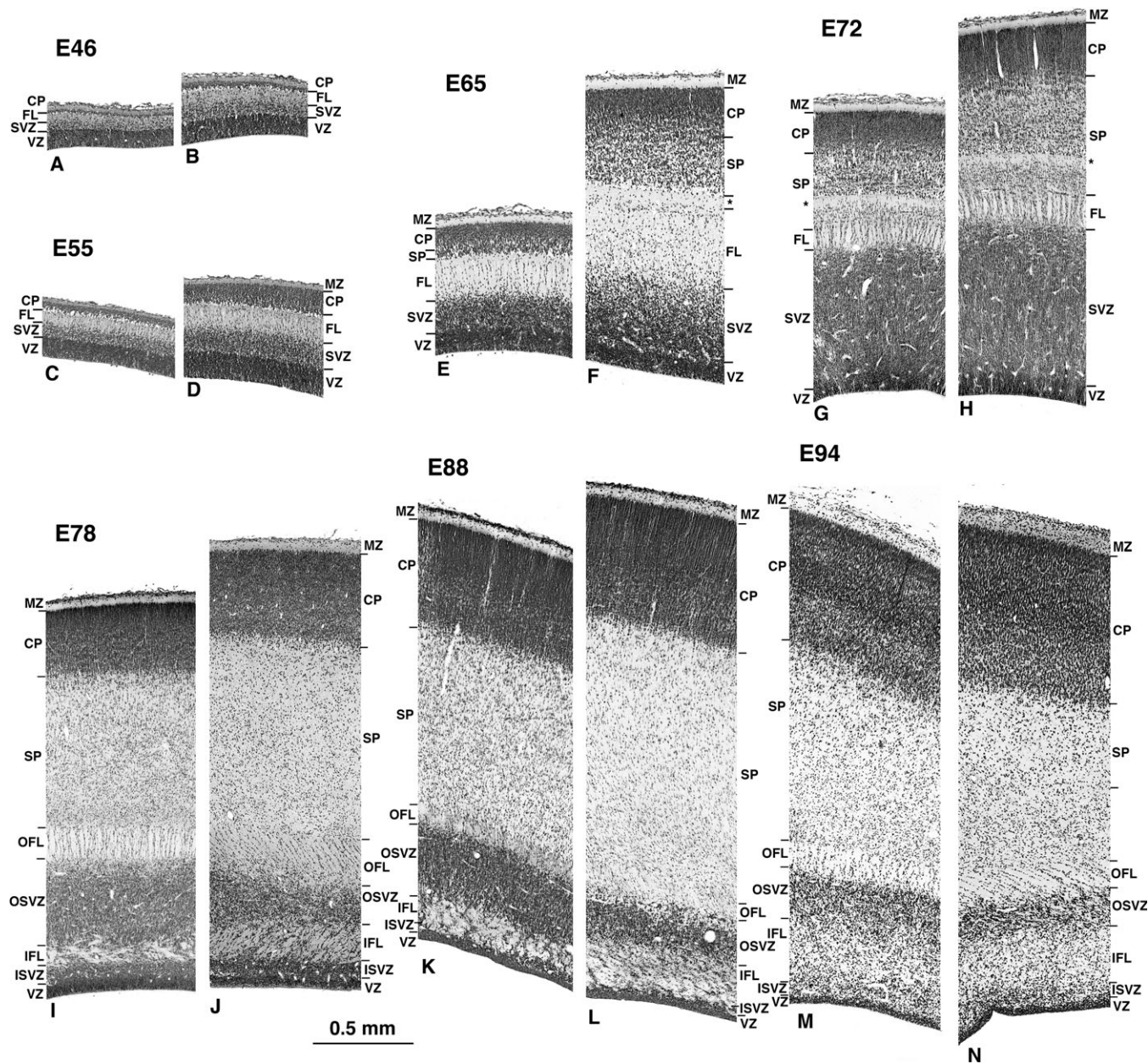
accompanied by faster rates of migration and tangential expansion. To explore migration rates, we have examined <sup>3</sup>H-thymidine labeling following intermediate survival periods.

At E71, there are complex patterns of labeling with significant differences between area 17 and area 18 (Fig. 3A). The uneven distribution of labeled neuroblasts below the cortical plate suggests that there are compartments where migration is hindered and others where it is more rapid. For instance, the clear layer in the posterior pole of area 17 (marked by a star in Fig. 2) has few labeled cells, whereas immediately above and below this layer there is an accumulation of labeled neuroblasts. In area 17, a substantial fraction of labeled cells lie at the top of the cortical plate where cells have completed their migration. The density of labeled neurons, which have completed their migration, is higher in area 17 compared with area 18. This could mean that migrating cells reach their final destination earlier in area 17 compared with area 18. Alternatively it could simply reflect the rostrocaudal mitotic gradient. To distinguish between these possibilities, we have compared the proportions of labeled cells in the top compartment of the cortical plate in presumptive areas 17 and 18 with respect to the number of labeled neurons in the full thickness of the cortical plate. This gives the following ratios—for injection on E64: posterior area 17, 41.4%; anterior area 17, 33.1%; area 18, 9%. These results suggest that the rostrocaudal gradient of increased mitosis is accompanied by a rostrocaudal gradient of increased rates of migration.

At E71, the neuroepithelium of presumptive area 17 is thinner than that of more anterior regions generating area 18 (Fig. 3). Hence, the increased proportion of neuroblasts that have completed their migration in area 17 at this stage could simply reflect a shorter migratory distance in presumptive area 17. To see if similar results are obtained at later stages when the migratory path is longer in area 17 compared with area 18, <sup>3</sup>H-thymidine pulse injection was made at E78 and observations were carried out at E92 (Fig. 3B). This shows that at E92 the proportion of labeled cells at the top of the cortical plate with respect to the number of labeled neurons in the full thickness of the cortical plate in presumptive area 17 continues to be higher compared with the proportion in presumptive area 18. The ratios are 85.2% in area 17 and 61.4% in area 18.

#### ***Gross Histological Changes of the Telencephalic Wall***

Inspection of parasagittal transects of the occipital lobe from E46 to E94 shows that the total depth of the wall of the cerebral hemisphere diminishes progressively when traced rostrocaudally (Fig. 2, Smart and others 2002). Initially (E46–E72), the rostrocaudal decrease in thickness affects all the layers of the wall including germinal and supragerminal compartments (Fig. 2). The greater thickness of the cortical plate in area 18 compared with area 17 is most pronounced at the very onset of corticogenesis but is still apparent at E94. This contrasts with the germinal compartment. From E46, there is an increase in the depth of the germinal compartments, and this is more pronounced up to E72 in area 18 compared with area 17. From E72 to E94, the germinal compartment of area 17 is deeper in area 17 compared with area 18 (Fig. 2). These rostrocaudal differences in the thickness of the cerebral wall are not due to the plane of section as can be seen from examining stacked sections throughout the full medial-lateral extent of the brain (Fig. 4).



**Figure 2.** Parasagittal sections of the occipital lobe at different stages of development (E46–E94) showing transects of area 17 (left panels) and area 18 (right panels). CP, cortical plate; FL, fiber layer; IFL, inner fiber layer; ISVZ, inner subventricular zone; MZ, marginal zone; OFL, outer fiber layer; SP, subplate; SVZ, subventricular zone; VZ, ventricular zone.

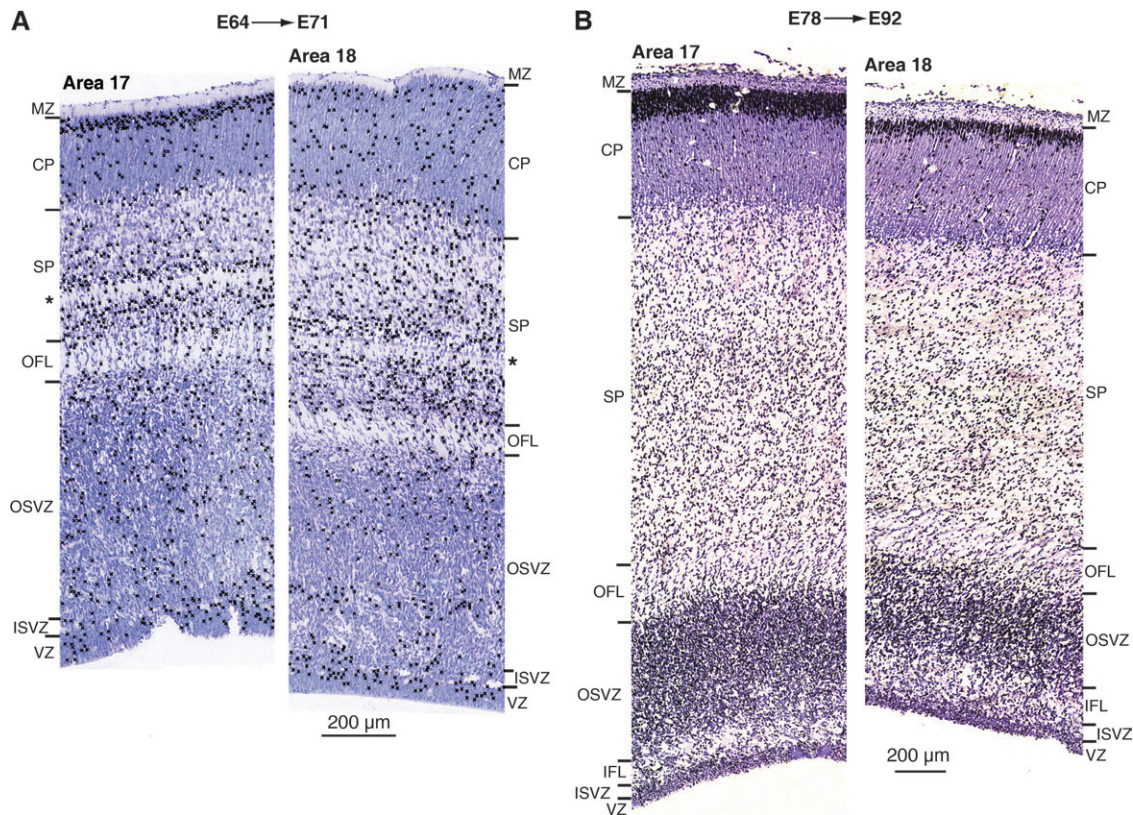
## Discussion

### *The Area 17-18 Border Is Set on a Proliferative and Migratory Gradient*

The *in vivo* analysis of proliferation based on the distribution of S phase-labeled neuroblasts in the germinal zones shows a gradual decrease in proliferation going from posterior area 17 to area 18 during the production of supragranular neurons in the OSVZ. We also found that positive and negative cell cycle regulatory molecules were expressed in opposite discrete gradients in the OSVZ of the embryonic occipital lobe. Levels of the inhibitor  $p27^{kip1}$  expression are lowest in area 17 precursors and increase progressively as one moves rostrally

to area 18, whereas an inverse gradient is observed for the positive regulator cyclin E.

The role of  $p27^{kip1}$  in downregulating cyclin-dependent kinase activity, thereby slowing down cell cycle progression, is well documented in a large number of biological systems (Chellappan and others 1998). Moreover, we have shown elsewhere that ectopic expression of  $p27^{kip1}$  in area 17 precursors induces a lengthening of cell cycle duration, indicating that  $p27^{kip1}$  expression level is rate limiting for progression through G1 and entry into S phase in cortical precursors (Lukaszewicz and others 2001, 2002, 2005). Taken together, these data show that the increased  $p27^{kip1}$  expression levels and the decreased levels of cyclin E are likely to



**Figure 3.** Differential rates of migration. (A)  $^3\text{H}$ -thymidine pulse injection at E64 and perfusion at E71 shows complex patterns of migration. Comparison of presumptive area 17 and area 18 suggests different rates of migration (see text). (B)  $^3\text{H}$ -thymidine pulse injection at E78 and perfusion at E92 shows complex patterns of migration. Comparison of presumptive area 17 and area 18 confirms different rates of migration (see text). For abbreviations, see Figure 2.

contribute to the lengthened duration of the G1 phase that characterizes area 18 precursors.

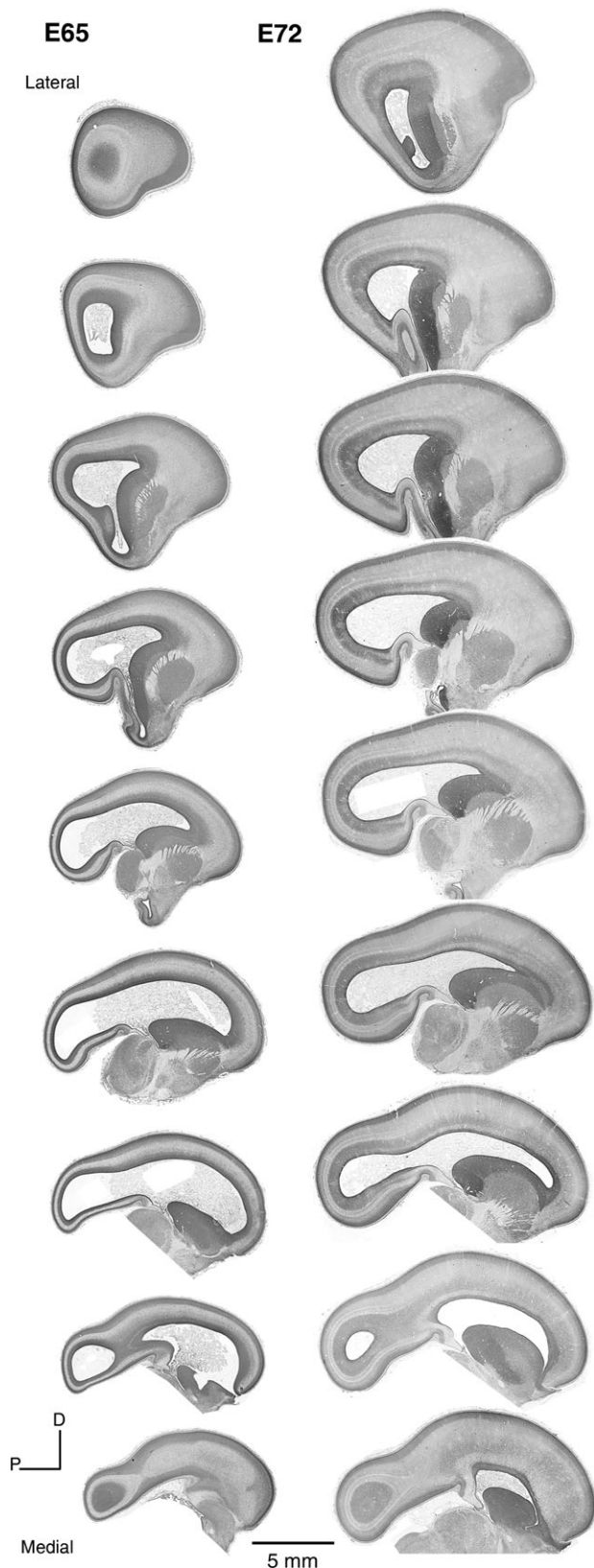
The opposite gradients of cyclin E and  $p27^{\text{kip1}}$  lead to a gradient of decreasing G1 duration as one goes from area 18 to area 17. In parallel going from area 18 to area 17 there is a low to high gradient in percentages of cells expressing high levels of cyclin E and corresponding to the fraction of precursors engaged in proliferative division. Interestingly, this points to the concerted regulation of G1 phase duration and mode of division that occurs in cortical precursors where short G1 phase duration characterize proliferative divisions and long G1 phase duration neurogenic divisions (Dehay and others 2001; Lukaszewicz and others 2002; Calegari and others 2005).

Recently, an alternative function of the CKI  $p27^{\text{kip1}}$  has been discovered, independent from its cell cycle function. In addition to being a proliferation inhibitor,  $p27^{\text{kip1}}$  has also been shown to regulate cytoskeletal organization and migration through the modulation of the Rho signaling pathway, depending on its subcellular localization (Besson, Assoian, and Roberts 2004; Besson, Gurian-West, and others 2004). Therefore,  $p27^{\text{kip1}}$  could contribute to the coordination of cytoskeletal changes that occur during the different phases of the cell cycle and the regulation of cell migration.

Despite the fact that the area 17-18 border emerges on a gradient of morphological and proliferative features, it can be clearly distinguished as early as E75 by AChE activity in embryonic thalamic axons (Kageyama and others 1990; Dehay and Kennedy, unpublished data). These findings need to be considered along with the reduction in size of area 17 follow-

ing early bilateral enucleation. The consequence of bilateral removal of the retina during a critical developmental window is to reduce the number of thalamic afferents that in turn leads to a reduction in the dimensions of area 17. Despite the enormous reduction of the dimensions of area 17, the overall dimensions of the neocortex remains unaltered (Dehay and others 1996a). This last result means that a primary consequence of the decrease in the numbers of thalamic afferents is a shift in the location of the area 17 border so that the cortex that was originally destined to become area 17 takes on an alternative phenotype that corresponds more closely to extrastriate cortex (Rakic 1988; Dehay and others 1989, 1996a, 1996b; Rakic and others 1991). These findings suggest that in normal development, the final setting of the 17-18 border is in response to multiple factors that determine the position of the border along a rostrocaudal gradient of proliferation and migration. Given the important role of apoptosis in early neurogenesis, one could hypothesize that differential rates of apoptosis may also contribute in the setting up of a sharp areal boundary.

The fact that the abrupt differences in the numbers of neurons on either side of the area 17-18 border following early enucleation do not differ from those found in the normal adult suggests that the thalamic afferents directly influence the gradient itself by modulating rates of proliferation (see below, Dehay and others 2001) and perhaps migration (Edgar and Price 2001). The *in vitro* work of Edgar and Price (2001) showed that coculture of cortical slices with thalamic explants enhances the directed migration of E16 mouse cortical precursors. The developing thalamus could be the source of trophic factors



**Figure 4.** Parasagittal serial sections at equivalent levels in an E65 and E72 brains. Despite the fact that the occipital lobe is characterized by higher rates of neuron production, the neuroepithelium is narrower than at more anterior locations.

(e.g., Lein and others 2000) and neurotransmitters (Montero 1990; Ding and Casagrande 1998) that may influence the migration of cortical precursors. Brain-derived neurotrophic factor, which is expressed in the developing lateral geniculate nucleus (Lein and others 2000), has been shown to be anterogradely transported in axons (Altar and others 1997) and to stimulate migration of dissociated cortical neurons (Behar and others 1997). Its high-affinity receptor neurotrophic tyrosin kinase receptor of BDNF is present on postmitotic migrating cortical neurons (Behar and others 1997). Glutamate that is present in geniculocortical axons (Montero 1990; Ding and Casagrande 1998) can also influence migration of embryonic neurons. The activity of *N*-methyl-D-aspartate receptors has been shown to regulate migration of cerebellar neurons (Komuro and Rakic 1993) and cortical precursors (Behar and others 1999).

The large expansion of the subventricular zone in the monkey is also found in humans (Zecevic and others 2005) and is possibly linked to the increased importance of the supragranular layers in primates compared with nonprimates. The large expansion of the cerebral cortex in primates and the increase in the numbers of neocortical areas lead to a massive increase in the numbers of corticocortical connections that is accommodated by the larger supragranular layer compartment. Because thalamic axons specifically target appropriate areas, they are in a position to directly regulate numbers of supragranular layer neurons by amplifying the OSVZ precursor pool.

### Conclusion

The increased rates of proliferation and migration of area 17 precursors are accompanied by a greater rate of tangential expansion of the occipital lobe. This suggests that areal dimensions are determined by the rates of neuron production and migration. The regional rates of proliferation can be modulated by the extrinsic factors to the cortex. In agreement with results in lower vertebrates and invertebrates suggesting that afferents in development determine neuron number via a modulation of neurogenesis (Kollros 1953, 1982; Williams and Herrup 1988; Baptista and others 1990; Dehay and others 2001; Selleck and Steller 1991; Selleck and others 1992; Gong and Shipley 1995), it has been shown that thalamic afferents release a mitogenic factor that shortens G1 and increases the frequency of proliferative divisions. Therefore, embryonic thalamic axons could contribute to establishing the cell cycle parameters that distinguish the precursors pool of areas 17 and 18 (Dehay and others 2001; Lukaszewicz and others 2005). It is conceivable therefore that dimensions of area 17 are regulated by the sensory periphery via thalamic afferents. This would be in line with results from removal of the retina during development that leads to reductions in the numbers of surviving lateral geniculate neurons and a drastic decrease in the dimensions of area 17 (Rakic and others 1991; Dehay and others 1996a, 1996b; Suner and Rakic 1996).

### Notes

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