



HAL
open science

The concerted modulation of proliferation and migration contributes to the specification of the cytoarchitecture and dimensions of cortical areas

Agnès Lukaszewicz, Véronique Cortay, Pascale Giroud, Michel Berland, Iain H. M. Smart, Henry Kennedy, Colette Dehay

► To cite this version:

Agnès Lukaszewicz, Véronique Cortay, Pascale Giroud, Michel Berland, Iain H. M. Smart, et al.. The concerted modulation of proliferation and migration contributes to the specification of the cytoarchitecture and dimensions of cortical areas. *Cerebral Cortex*, 2006, 16 Suppl 1, pp.i26-34. 10.1093/cercor/bhk011 . hal-00077632

HAL Id: hal-00077632

<https://hal.science/hal-00077632>

Submitted on 31 May 2006

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

The concerted modulation of proliferation and migration contributes to the specification of the cytoarchitecture and dimensions of cortical areas

Agnès Lukaszewicz^{1,2,6}, Véronique Cortay^{1,2}, Pascale Giroud^{1,2}, Michel Berland^{1,2,4}, Iain Smart⁵, Henry Kennedy^{1,2} and Colette Dehay^{1,2,3}

¹ INSERM, U371, Department of Stem Cell and Cortical Development, , Cerveau et Vision, 18 avenue du Doyen Lépine, 69500 Bron France

² Université Claude Bernard Lyon I, IFR19, 18 avenue du Doyen Lépine, 69500 Bron France

³ PrimaStem, 18 avenue du Doyen Lépine, 69500 Bron France

⁴ Hôpital Lyon Sud, Chemin du Grand Revoyet, 69495 Pierre-Bénite, France

⁵ Department of Anatomy and Physiology, School of life sciences, Old Medical School, The University, Dundee, Scotland DD1 4HN.

⁶ Present address: California Institute of Technology, Division of Biology, 216-76, 1200 East California Blvd, Pasadena, CA 91125

Correspondence should be addressed to Colette Dehay (dehay@lyon.inserm.fr)

Abbreviated title: Primate corticogenesis

7023 words

Text pages: 17

Figures: 4

Abstract: 111 words.

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

Abstract:

Regionalization of cell-cycle kinetics of cortical precursors has been described in non-human primates and rodents indicating a fate map of areal 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 two areas involve an orchestration of diverse phenomena including differential rates of proliferation, migration and tangential expansion.

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 is accompanied by a multiplication of the number of areas. Information processing in the cortex is increasingly understood in terms of co-operative 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 (Beaulieu and Colonnier, 1989; Garey *et al.*, 1985; Rockel *et al.*, 1980; Skoglund *et al.*, 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 *et al.*, 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 progenitors numbers necessary for the proper morphogenesis of the brain (Kuan *et al.*, 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 *et al.*, 1999; Kuan *et al.*, 1999).

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

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 specialisations 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 (Dehay *et al.*, 1989; Rakic, 1988).

A number of studies have shown that there is a regionalization of the proliferative behavior of precursors in the germinal zone of non-human primates and rodents (Dehay *et al.*, 1993; Polleux *et al.*, 1997). These studies have revealed a fate map of distinct proliferative programs in the germinal zones that are spatially and temporally co-ordinated to generate the layout of cortical areas. In rodents and primates mathematical modelling of results of birthdating experiments have shown that variation 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 (Lukaszewicz *et al.*, 2005; Polleux *et al.*, 1997).

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 neuronal production. This has made it possible to look at those cell cycle features which ensure the large increase in the number of supragranular layer pyramidal cells that distinguishes area 17 from area 18 (Rockel *et al.*, 1980). This shows that the higher rates of neuron production in 17 compared to 18 is due to area 17 precursors having a shorter G1 phase and higher rates of proliferative divisions than do area 18 precursors (Lukaszewicz *et al.*, 2005).

These cell-cycle related areal features have to be considered in the context of the neurogenic gradients that occur during corticogenesis (Bayer and Altman, 1991; Polleux *et al.*, 1997; Smart and Smart, 1982). 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 two areas involve an orchestration of diverse phenomena including differential rates of migration (Kennedy *et al.*, 1996), proliferation (Dehay *et al.*, 1993; Lukaszewicz *et al.*, 2005) and tangential expansion (Smart *et al.*, 2002).

Material and Methods :

In vivo studies:

Two fetuses from timed-pregnant monkeys (E64 and E78) received approximately 10-20 $\mu\text{Ci/g.b.w.}$ (ip or im) of H3-thymidine in saline (specific activity: 40/60 $\mu\text{Ci/m.mole}$) and were replaced in the uterus according to previously described protocols (Dehay *et al.*, 1993; Lukaszewicz *et al.*, 2005; Smart *et al.*, 2002). Pregnant monkeys were returned to their cages and medicated for two days with an analgesic (Visceralgine im). A muscular relaxant (Salbumol im) was given twice daily and the fetuses were delivered by caesarian section after 7 (E64->E71) and 14 days (E78 ->E92) of survival. Following caesarian section, anaesthetized fetuses were perfused through the heart with 4% paraformaldehyde. Histological observations of the embryonic occipital pole were made on paraffin sections (see below) from time pregnant fetuses aged E46, E55, E65, E72, E78, E88 and E94 from a previous study (Smart *et al.*, 2002) that were delivered by caesarian section and perfused as indicated above. In situ immunolabelling of p27kip1 and Cyclin E was performed on frozen

parasagittal sections from a E80 fetus (Lukaszewicz *et al.*, 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 *et al.*, 2002) and E75 (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 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 *et al.*, 2002).

Brains were embedded in paraffin wax and 5 or 10 μm thick parasagittal sections were cut. Sections were processed for autoradiography (K2 autoradiographic emulsion from Ilford) to give maximum counts of 40 grains per differentiated cell. Proportions of labelled cells in the different anatomical compartments were computed. Using a X50 or X63 oil objective, labelled neurons were observed on a video screen and positions charted by a high precision computer controlled plotting system (Mercator, ExploraNova technology)

Dissociated cell culture: An E80 fetal brain was removed under sterile conditions in iced Hank's Balanced Salt Solution containing 10 mM Hepes. The cerebral hemispheres were detached and the neopallium 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%, three minutes at 37°C) followed by mechanical dissociation and were suspended in GMEM+10%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 two-step procedure. Briefly, sections were incubated overnight at 4°C with primary antibodies p27^{Kip1} (Santa Cruz, SC-528, 1:200) and Cyclin E (Santa Cruz, SC-481; 1:200). Sections were further incubated for three hours at RT with goat anti-rabbit-Cy3 (Interchim, 1:400 in DAKO-diluent). Sections were counterstained with Syto16.

In vitro PCNA/p27 double-immunolabeling: After PCNA immunochemistry (described in

Lukaszewicz *et al.*, 2002), coverslips were processed for p27^{Kip1} immunocytochemistry. Briefly, primary antibody incubation (rabbit anti-p27^{Kip1} from Santa-Cruz Biotechnology, SC-528) was incubated at 1:20 in Dako diluent at RT for 1 hour. After three rinses, goat anti-rabbit Cy2 (1:200 in Dako diluent; Jackson ImmunoResearch) was incubated for 1 hour at RT. Coverslips were mounted with 0.1% n-propylgallate (P3130; Sigma) in a 1 M phosphate buffer and glycerol (1:1) to prevent fading on fluorescent illumination (Lukaszewicz *et al.*, 2002).

Coverslips were scanned at regular intervals with a grid corresponding to a field of 0.128 mm². 100 to 150 fields were observed per coverslip. A minimum of two coverslips was observed for each condition. Fluorescent labeling was analysed using a Leica confocal microscope, using a X40 objective (see below for details)

Confocal measurements of Cyclin E and p27^{Kip1} expression levels: We have quantified protein expression levels by means of confocal microscopy analysis (Durand *et al.*, 1997; Lukaszewicz *et al.*, 2002; Tokumoto *et al.*, 2002). Confocal examination of the fluorescent labelling was carried out on a LEICA TCS SP equipped with an Argon-krypton laser. Analysis of p27^{Kip1} and Cyclin E expression was performed using an x40 (40 micron brain sections) or an x63 (dissociated cells) oil objective. p27^{Kip1} 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^{Kip1} 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^{Kip1} or Cyclin E labelling with respect to the total number of cells (indicated either by the intensity of Syto16 fluorescence or by cell counts), as described in Lukaszewicz *et al.* (2005). Several fields of view were analyzed so as to span the entire thickness of the 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 labelling); >110 (high intensity labeling). The 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 (Fig1). The expression level of p27^{Kip1} was further analyzed at the single cell level in the cycling dissociated precursors, as identified by PCNA expression (Lukaszewicz *et al.*, 2002) (**Fig1D**). 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 presumptive areas 17 and 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 3H-thymidine at early and mid-corticogenesis followed by survival time of 7 to 14 days respectively. This allows to visualise the migration of neurons that were in their final rounds of mitosis at the moment of injection. At mid-corticogenesis (E78), the 14 days 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 *et al.*, 2005). These results show that the area 17 germinal zone with high rates of neuron production generate neuroblasts which show faster migratory rates compared to 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 H3-thymidine labeling in the OSVZ, the principal germinal zone in the monkey and the major site of supragranular neuron production (Lukaszewicz *et al.*, 2005; Smart *et al.*, 2002). We computed the areal density of H3-thymidine positive cells per unit area of the OSVZ along the full extent of the occipital pole, encompassing presumptive areas 17 and 18. We have used AChE labelling to determine the position of areal borders (data not shown) (Dehay *et al.*, 1993)(Dehay and Kennedy, unpublished). 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 3-4 millimeters gradient (**Figure 1A**). This contrasts with the hair-line 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, CKI p27^{Kip1} a negative regulator of the cell-cycle and of G1 phase progression (Polyak *et al.*, 1994; Sherr and Roberts, 1999) - and

Cyclin E -which is rate-limiting for progression from G1 to S phase (Resnitzky *et al.*, 1994; Wimmel *et al.*, 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 *et al.*, 2005).

Confocal quantification of immunolabelling against CyclinE and p27^{kip1} over several millimeters of the OSVZ of the occipital lobe going through the 17/18 presumptive border shows two opposite gradients of expression. Whereas area 17 precursors have reduced levels of p27^{kip1} and increased levels of cyclin E expression, area 18 precursors exhibit high levels of p27^{kip1} with low levels of cyclin E. This points to a gradient of relative facilitation of the progression through G1 phase in area 17 compared to area 18 precursors (**Fig 1B,C**), in agreement with the higher rates of proliferation observed in vivo (**Fig 1A**, Lukaszewicz *et al.*, 2005).

Because expression of p27^{kip1} is usually up-regulated in post-mitotic cells (Lukaszewicz *et al.*, 2002), the analysis performed on the overall population does not make it possible to determine whether the regional differences in the level of p27^{kip1} 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 *et al.*, 2005). So as to restrict p27^{kip1} 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^{kip1} restricted to the PCNA-positive precursors revealed significantly lower levels of p27^{kip1} expression in area 17 precursors compared to area 18 precursors. Comparison of the levels of p27^{kip1} 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 re-entry (Ekholm *et al.*, 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 division) 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 millimeters 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^{Kip1} and Cyclin E between area 17 and area 18 precursors. Because the CKI p27^{Kip1} 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 to 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 3-4mm from the 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 3H-thymidine labeling following intermediate survival periods.

At E71 there are complex patterns of labelling with significant differences between area 17 and area 18 (**Figure 3A**). The uneven distribution of labelled 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 **Figure 2**) has few labelled cells while immediately above and below this layer there is an accumulation of labelled neuroblasts. In area 17, a substantial fraction of labelled 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 to area 18. This could mean that migrating cells reach their final destination earlier in area 17 compared to area 18. Alternatively it could simply reflect the rostro-caudal 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 rostro-caudal gradient of increased mitosis is accompanied by a rostro-caudal 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 (**Figure 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 to area 18, 3H-thymidine pulse injection was made at E78 and observations were carried out at E92 (**Figure 3B**). This shows that at E92 the proportion of labelled cells at the top of the cortical plate with respect to the number of labelled neurons in the full thickness of the cortical plate in presumptive area 17 continues to be higher compared to 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 (**Figure 2**, Smart *et al.*, 2002). Initially (E46-E72) the rostrocaudal decrease in thickness affects all the layers of the wall including germinal and supragerminal compartments (**Figure 2**). The greater thickness of the cortical plate in area 18 compared to 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 to area 17. From E72 to E94 the germinal compartment of area 17 is deeper in area 17 compared to area 18 (**Figure 2**). These rostro-caudal 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 (**Figure 4**).

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 labelled 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 whilst an inverse gradient is observed for the positive regulator Cyclin E.

The role of p27^{kip1} in down-regulating CDK activity, thereby slowing down cell-cycle progression is well documented in a large number of biological systems (Chellappan *et al.*, 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 *et al.*, 2005; Lukaszewicz *et al.*, 2002; Lukaszewicz *et al.*, 2001). Taken together, these data show that the increased p27^{kip1} expression levels and the decreased levels of CyclinE are likely to contribute to the lengthened duration of the G1 phase that characterizes area 18 precursors.

The opposite gradients of CyclinE and p27kip1, resulting in short G1 duration in area 17 are paralleled with a high in area 17-low in area 18 gradient in the percentages of cells that expressed high levels of Cyclin E and that correspond to the fraction of precursors engaged in proliferative divisions. 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 duration neurogenic divisions (Calegari *et al.*, 2005; Dehay *et al.*, 2001; Lukaszewicz *et al.*, 2002).

Recently, an alternative function of the CKI p27kip¹ has been discovered, independent from its cell-cycle function. In addition to being a proliferation inhibitor, p27kip1 has also been shown to regulate cytoskeletal organization and migration through the modulation of the Rho signalling pathway, depending on its subcellular localization (Besson *et al.*, 2004a; Besson *et al.*, 2004b). Therefore, p27kip1 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 *et al.*, 1990)(Dehay and Kennedy, unpublished). These findings need to be considered along with the reduction in size of area 17 following 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 *et al.*, 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 cortex that was originally destined to become area 17 takes on an alternative phenotype that corresponds more closely to extrastriate cortex (Dehay *et al.*, 1996b; Dehay *et al.*, 1996a; Dehay *et al.*, 1989; Rakic, 1988; Rakic *et al.*, 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 rostro-caudal 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 A 17/18 border following early enucleation does not differ from that found in the normal adult suggests that the thalamic afferents directly influences the gradient itself by modulating rates of proliferation (see below, Dehay *et al.*, 2001) and perhaps migration (Edgar and Price, 2001). The *in vitro* work of Edgar and Price (2001) showed that co-culture 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 (eg Lein *et al.*, 2000) and neurotransmitters (Ding and Casagrande, 1998; Montero, 1990) that may influence the migration of cortical precursors. BDNF which is expressed in the developing LGN (Lein *et al.*, 2000), has been shown to be anterogradely transported in axons (Altar *et al.*, 1997) and to stimulate migration of dissociated cortical neurons (Behar *et al.*, 1997). Its high affinity receptor TrkB is present on postmitotic migrating cortical neurons (Behar *et al.*, 1997). Glutamate which is present in geniculocortical axons (Ding and Casagrande, 1998; Montero, 1990) can also influence migration of embryonic neurons. The activity of N-methyl-D-aspartate (NMDA) receptors has been shown to regulate migration of cerebellar neurons (Komuro and Rakic, 1993) and cortical precursors (Behar *et al.*, 1999).

The large expansion of the SVZ in monkey is also found in human (Zecevic *et al.*, 2005) and is possibly linked to the increased importance of the supragranular layers in primates compared to non-primates. The large expansion of the cerebral cortex in primates and the increase in the numbers of neocortical areas leads to a massive increase in the numbers of cortico-cortical 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 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 (Baptista *et al.*, 1990; Gong and Shipley, 1995; Kollros, 1953, 1982; Selleck *et al.*, 1992; Selleck and Steller, 1991; Williams and Herrup, 1988), 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 *et al.*, 2001; Lukaszewicz *et al.*, 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 (Dehay *et al.*, 1996b; Dehay *et al.*, 1996a; Rakic *et al.*, 1991; Suner and Rakic, 1996).

Acknowledgements: We are indebted to Christelle Merrouche and Delphine Autran who provided technical help. Financial support was provided by the EU grant Concorde (QLG3-2000-00158), Nets and Representations (QLG3 1999-01064), Ministère de la Recherche; ACI 2003 Neurosciences Integratives et computationnelles grant No 03668

Figure Legends

Figure 1. In vivo regional differences in the control of proliferation

A. Number of 3H-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 insert); 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 p27kip1 (**C**) as measured by confocal microscopy quantification in the OSVZ at E80 :Statistical significance assessed with a Mann-Whitney U test. **D:** Level of p27kip1 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, percent of Cyclin E positive cells in the OSVZ at E80; Statistical significance assessed with a Mann-Whitney U test.

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).

Abbreviations, CP cortical plate, IFL inner fiber layer, ISVZ inner subventricular zone, MZ marginal zone, OFL outer fiber layer, OSVZ outer subventricular zone, SP subplate, SVZ subventricular zone, VZ ventricular zone.

Figure 3. Differential rates of migration. A: 3H-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: 3H-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).

Abbreviations see Figure 2.

Figure 4 Parasagittal serial sections at equivalent levels in a E65 and E72 brains.

Despite the fact that the occipital lobe is characterised by higher rates of neuron production the neuroepithelium is narrower than at more anterior locations.

References

- Altar CA, Cai N, Bliven T, Juhasz M, Conner JM, Acheson AL, Lindsay RM, Wiegand SJ (1997) Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* 389: 856-860.
- Baptista CA, Gershon TR, Macagno ER (1990) Peripheral organs control central neurogenesis in the leech. *Nature* 346: 855-858.
- Bayer SA, Altman J (1991) Neocortical development. New York: Raven Press.
- Beaulieu C, Colonnier M (1989) The number of neurons in individual laminae of areas 3B, 4B and 6a alpha of the cat cerebral cortex: a comparison with major visual areas. *J Comp Neurol* 279: 228-234.
- Behar TN, Dugich-Djorjevic MM, Li YX, Ma W, Somogyi R, Wen X, Brown E, Scott C, McKay RDG, Barker JL (1997) Neurotrophins stimulate chemotaxis of embryonic cortical neurons. *Eur J Neurosci* 9: 2561-2570.

- Behar TN, Scott CA, Greene CL, Wen X, Smith SV, Maric D, Liu QY, Colton CA, Barker JL (1999) Glutamate acting at NMDA receptors stimulates embryonic cortical neuronal migration. *J Neurosci* 19: 4449-4461.
- Besson A, Assoian RK, Roberts JM (2004a) Regulation of the cytoskeleton: an oncogenic function for CDK inhibitors? *Nat Rev Cancer* 4: 948-955.
- Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM (2004b) p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev* 18: 862-876.
- Bishop KM, Garel S, Nakagawa Y, Rubenstein JL, O'Leary DD (2003) Emx1 and Emx2 cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical efferents, and thalamocortical pathfinding. *J Comp Neurol* 457: 345-360.
- Bishop KM, Goudreau G, O'Leary DD (2000) Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. *Science* 288: 344-349.
- Bishop KM, Rubenstein JL, O'Leary DD (2002) Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. *J Neurosci* 22: 7627-7638.
- Calegari F, Haubensak W, Haffner C, Huttner WB (2005) Selective lengthening of the cell cycle in the neurogenic subpopulation of neural progenitor cells during mouse brain development. *J Neurosci* 25: 6533-6538.
- Chellappan SP, Giordano A, Fisher PB (1998) Role of cyclin-dependent kinases and their inhibitors in cellular differentiation and development. *Curr Top Microbiol Immunol* 227: 57-103.
- Dehay C, Giroud P, Berland M, Killackey H, Kennedy H (1996b) Phenotypic characterisation of respecified visual cortex subsequent to prenatal enucleation in the monkey: development of acetylcholinesterase and cytochrome oxidase patterns. *J Comp Neurol* 376: 386-402.
- Dehay C, Giroud P, Berland M, Killackey HP, Kennedy H (1996a) The contribution of thalamic input to the specification of cytoarchitectonic cortical fields in the primate: Effects of bilateral enucleation in the foetal monkey on the boundaries and dimensions of striate and extrastriate cortex. *J Comp Neurol* 367: 70-89.
- Dehay C, Giroud P, Berland M, Smart I, Kennedy H (1993) Modulation of the cell cycle contributes to the parcellation of the primate visual cortex. *Nature* 366: 464-466.
- Dehay C, Horsburgh G, Berland M, Killackey H, Kennedy H (1989) Maturation and connectivity of the visual cortex in monkey is altered by prenatal removal of retinal input. *Nature* 337: 265-267.

- Dehay C, Savatier P, Cortay V, Kennedy H (2001) Cell-cycle kinetics of neocortical precursors are influenced by embryonic thalamic axons. *J Neurosci* 21: 201-214.
- Ding Y, Casagrande VA (1998) Synaptic and neurochemical characterization of parallel pathways to the cytochrome oxidase blobs of primate visual cortex. *J Comp Neurol* 391: 429-443.
- Donoghue MJ, Rakic P (1999) Molecular evidence for the early specification of presumptive functional domains in the embryonic primate cerebral cortex. *J Neurosci* 19: 5967-5979.
- Douglas RJ, Martin KA (2004) Neuronal circuits of the neocortex. *Annu Rev Neurosci* 27: 419-451.
- Durand B, Gao FB, Raff M (1997) Accumulation of the cyclin-dependent kinase inhibitor p27/kip1 and the timing of oligodendrocyte differentiation. *EMBO J* 16: 306-317.
- Edgar JM, Price DJ (2001) Radial migration in the cerebral cortex is enhanced by signals from thalamus. *Eur J Neurosci* 13: 1745-1754.
- Ekholm SV, Zickert P, Reed SI, Zetterberg A (2001) Accumulation of cyclin E is not a prerequisite for passage through the restriction point. *Mol Cell Biol* 21: 3256-3265.
- Estivill-Torres G, Pearson H, van Heyningen V, Price DJ, Rashbass P (2002) Pax6 is required to regulate the cell cycle and the rate of progression from symmetrical to asymmetrical division in mammalian cortical progenitors. *Development* 129: 455-466.
- Finlay BL, Slattery M (1983) Local differences in amount of early cell death in neocortex predict adult local specializations. *Science* 219: 1349-1351.
- Galli R, Fiocco R, De Filippis L, Muzio L, Gritti A, Mercurio S, Broccoli V, Pellegrini M, Mallamaci A, Vescovi AL (2002) Emx2 regulates the proliferation of stem cells of the adult mammalian central nervous system. *Development* 129: 1633-1644.
- Garey LJ, Winkelmann E, Brauer K (1985) Golgi and Nissl studies of the visual cortex of the bottlenose dolphin. *J Comp Neurol* 240: 305-321.
- Gong QH, Shipley MT (1995) Evidence that pioneer olfactory axons regulate telencephalon cell cycle kinetics to induce the formation of the olfactory bulb. *Neuron* 14: 91-101.
- Goodman CS, Shatz CJ (1993) Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell* 72 Suppl: 77-98.
- Haydar TF, Kuan CY, Flavell RA, Rakic P (1999) The role of cell death in regulating the size and shape of the mammalian forebrain. *Cereb Cortex* 9: 621-626.

- Heins N, Cremisi F, Malatesta P, Gangemi RM, Corte G, Price J, Goudreau G, Gruss P, Gotz M (2001) *Emx2* promotes symmetric cell divisions and a multipotential fate in precursors from the cerebral cortex. *Mol Cell Neurosci* 18: 485-502.
- Kageyama GH, Gallivan ME, Gallardo KA, Robertson RT (1990) Relationships between patterns of acetylcholinesterase activity and geniculocortical terminal fields in developing and mature rat visual cortex. *Brain Res Dev Brain Res* 53: 139-144.
- Kennedy H, Dehay C (1993) Cortical specification of mice and men. *Cerebral cortex* 3: 27-35.
- Kennedy H, Dehay C, Ziegel G, Smart I, Giroud P, Berland M (1996) Birthdating experiments in embryonic monkey reveals distinct patterns of cell proliferation and migration in presumptive visual areas 17 and 18. *Soc Neurosci Abst* 22: 525.
- Kollros JJ (1953) The development of the optic lobes in the frog: I The effects of unilateral enucleation in embryonic stages. *J Exp Zool* 123: 153-187.
- Kollros JJ (1982) Peripheral control of midbrain mitotic activity in the frog. *J Comp Neurol* 205: 171-178.
- Komuro H, Rakic P (1993) Modulation of neuronal migration by NMDA receptors. *Science* 260: 95-97.
- Kostovic I, Rakic P (1984) Development of prestriate visual projections in the monkey and human fetal cerebrum revealed by transient cholinesterase staining. *J Neurosci* 4: 25-42.
- Kuan CY, Roth KA, Flavell RA, Rakic P (2000) Mechanisms of programmed cell death in the developing brain. *Trends Neurosci* 23: 291-297.
- Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* 22: 667-676.
- Lein ES, Hohn A, Shatz CJ (2000) Dynamic regulation of BDNF and NT-3 expression during visual system development. *J Comp Neurol* 420: 1-18.
- Lukaszewicz A, Savatier P, Cortay V, Giroud P, Huissoud C, Berland M, Kennedy H, Dehay C (2005) G1 phase regulation, area-specific cell cycle control, and cytoarchitectonics in the primate cortex. *Neuron* 47: 353-364.
- Lukaszewicz A, Savatier P, Cortay V, Kennedy H, Dehay C (2002) Contrasting effects of bFGF and NT3 on cell cycle kinetics of mouse cortical stem cells. *J Neurosci* 22: 6610-6622.

- Lukaszewicz A, Savatier P, Cortay V, Seth P, Kennedy H, Dehay C (2001) G1 phase regulation and differentiation of mouse cortical precursors. In: Stem and progenitor cells: Biology and applications. Cold Spring Harbor, New York. pp 50.
- Mallamaci A, Muzio L, Chan CH, Parnavelas J, Boncinelli E (2000) Area identity shifts in the early cerebral cortex of *Emx2*^{-/-} mutant mice. *Nat Neurosci* 3: 679-686.
- Montero VM (1990) Quantitative immunogold analysis reveals high glutamate levels in synaptic terminals of retino-geniculate, cortico-geniculate, and geniculo-cortical axons in the cat. *Vis Neurosci* 4: 437-443.
- Monuki ES, Walsh CA (2001) Mechanisms of cerebral cortical patterning in mice and humans. *Nat Neurosci* 4 Suppl: 1199-1206.
- Nakagawa Y, Johnson JE, O'Leary DD (1999) Graded and areal expression patterns of regulatory genes and cadherins in embryonic neocortex independent of thalamocortical input. *J Neurosci* 19: 10877-10885.
- O'Leary DD, Nakagawa Y (2002) Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. *Curr Opin Neurobiol* 12: 14-25.
- O'Leary DDM (1989) Do cortical areas emerge from a protocortex? *Trends Neurosci* 12: 400-406.
- Polleux F, Dehay C, Morailon B, Kennedy H (1997) Regulation of neuroblast cell-cycle kinetics plays a crucial role in the generation of unique features of neocortical areas. *J Neurosci* 17: 7763-7783.
- Polyak K, Kato J, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A (1994) P27kip1, a cyclin-Cdk inhibitor, links transforming growth factor- β and contact inhibition to cell cycle arrest. *Genes Dev* 8: 9-22.
- Ragsdale CW, Grove EA (2001) Patterning the mammalian cerebral cortex. *Curr Opin Neurobiol* 11: 50-58.
- Rakic P (1988) Specification of cerebral cortical areas. *Science* 241: 170-176.
- Rakic P, Suner I, Williams RW (1991) A novel cytoarchitectonic area induced experimentally within the primate visual cortex. *Proc Natl Acad Sci Usa* 88: 2083-2087.
- Resnitzky D, Gossen M, Bujard H, Reed SI (1994) Acceleration of the G1/S phase transition by expression of cyclin D1 and E with an inducible system. *Mol Cell Biol* 14: 1669-1697.
- Rockel AJ, Hiorns RW, Powell TPS (1980) The basic uniformity in structure of the neocortex. *Brain* 103: 221-244.

- Rubenstein JL (2000) Intrinsic and extrinsic control of cortical development. *Novartis Found Symp* 228: 67-75; discussion 75-82, 109-113.
- Scannell JW, Grant S, Payne BR, Baddeley R (2000) On variability in the density of corticocortical and thalamocortical connections. *Philos Trans R Soc Lond B Biol Sci* 355: 21-35.
- Selleck SB, Gonzalez C, Glover DM, White K (1992) Regulation of the G1-S transition in postembryonic neuronal precursors by axon ingrowth. *Nature* 355: 253-255.
- Selleck SB, Steller H (1991) The influence of retinal innervation on neurogenesis in the 1st optic ganglion of drosophila. *Neuron* 6: 83-99.
- Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 13: 1501-1512.
- Skoglund TS, Pasher R, Berthold CH (1996) Heterogeneity in the columnar number of neurons in different neocortical areas in the rat. *Neurosci Lett* 208: 97-100.
- Smart IHM, Dehay C, Giroud P, Berland M, Kennedy H (2002) Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cereb Cortex* 12: 37-53.
- Smart IHM, Smart M (1982) Growth patterns in the lateral wall of the mouse telencephalon: I. Autoradiographic studies of the histogenesis of the isocortex and adjacent areas. *J Anat* 134: 273-298.
- Suner I, Rakic P (1996) Numerical relationship between neurons in the lateral geniculate nucleus and primary visual cortex in macaque monkeys. *Vis Neurosci* 13: 585-590.
- Tokumoto YM, Apperly JA, Gao FB, Raff MC (2002) Posttranscriptional regulation of p18 and p27 Cdk inhibitor proteins and the timing of oligodendrocyte differentiation. *Dev Biol* 245: 224-234.
- Williams RW, Herrup K (1988) The control of neuron number. *Ann Rev Neurosci* 11: 423-454.
- Wimmel A, Lucibello FC, Sewing A, Adolph S, Muller R (1994) Inducible acceleration of G1 progression through tetracycline-regulated expression of human cyclin E. *Oncogene* 9: 995-997.
- Zecevic N, Chen Y, Filipovic R (2005) Contributions of cortical subventricular zone to the development of the human cerebral cortex. *J Comp Neurol* 491: 109-122.