

G1 phase regulation, area-specific cell-cycle control and cytoarchitectonics in the primate cortex

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Abstract

We have investigated the cell-cycle related mechanisms that lead to the emergence of the primate areas 17 and 18. These areas are characterized by striking differences in cytoarchitectonics and neuron number. We show *in vivo* that (i) area 17 precursors of supragranular neurons exhibit a shorter cell-cycle duration, a reduced G1 phase and a higher rate of cell-cycle re-entry than area 18 precursors (ii) area-specific levels of expression of Cyclin E (high in area 17, low in area 18) and p27^{Kip1} (low in area 17, high in area 18) (iii) *Ex vivo* up and down modulation of Cyclin E and p27^{Kip1} show that both regulators influence cell-cycle kinetics by modifying rates of cell-cycle progression and cell-cycle re-entry (iv) Modeling the areal differences in cell-cycle parameters suggests that they contribute to areal differences in numbers of precursors and neuron production.

Introduction

Primate corticogenesis shares key features with that in the rodent including peripheral control of arealization and early patterning of the germinal zone and cortical plate (Kennedy and Dehay, 1993; Rakic, 1974; Sestan et al., 2001). However, a number of recent studies have revealed the existence of primate-specific developmental features (Letinic et al., 2002; Meyer et al., 2000; Smart et al., 2002), indicating that the monkey is of special importance for understanding human cortical development.

Areal differences in cytoarchitectonics and neuron number are a general feature of the cortex that has been documented in rodents, carnivores and primate (Beaulieu and Colonnier, 1989; Garey et al., 1985; Rockel et al., 1980; Skoglund et al., 1996). In the primate, areas 17 and 18 of the visual cortex have strikingly different numbers of neurons in granular and supragranular layers (Rockel et al., 1980). We have used this model system in which the germinal zones of presumptive areas 17 and 18 can be unambiguously identified during early stages of corticogenesis to investigate the contribution of the cell-cycle regulation of cortical precursors in areal specification.

In the primate, there are regional differences in tritiated thymidine uptake by the precursors in the germinal zones producing areas 17 and 18 during the generation of supragranular layer neurons, suggesting possible regional variations of cell-cycle dynamics (Dehay et al., 1993). Patterning and growth are linked via cell-cycle control. Regional differences in the proliferative program can have far reaching consequences for histogenesis of cortical areas. Firstly, there is evidence of molecular coordination of cell-cycle components and factors promoting neuronal determination/differentiation process (Ohnuma and Harris, 2003; Ohnuma et al., 2001), suggesting that cell-cycle regulation could impact on cell fate in cortical precursors. Secondly, because the pyramidal phenotype is specified by signals acting on cycling precursors (McConnell and Kaznowski, 1991; Polleux et al., 2001), variations in rates of proliferation at a given stage of histogenesis will lead to variations in the number of a particular phenotype being produced and therefore suggests a mechanism which can effectively link areal and laminar fate.

The inter-relationship of areal and laminar specification requires that the specification of cells to a regional fate is coupled to the regulated expansion of the precursor pool so as to generate the appropriate numbers of neurons in each of the layers that make up the cytoarchitecturally distinct areas. The differences in tritiated-thymidine uptake in the germinal zone generating areas 17 and 18 could hypothetically reflect modulation of neuron production via the regulation of the size of the precursor pool (Dehay et al., 1993).

To investigate this issue, we have examined area-specific cell-cycle kinetics and its molecular correlate in precursors of areas 17 and 18. The coordinated regulation of two cardinal parameters of the cell cycle is essential to regulate the size of the precursor pool (Rakic, 1995): (i) the balance between re-entry or exit of the cell cycle and (ii) rate of cell-cycle progression.

These two parameters are controlled by cell-cycle regulators, cyclins, cyclin-dependent kinases (Cdk), and cdk inhibitors (Cki). Of prime importance is cyclin E which binds to and activates Cdk2, thereby promoting entry in S-phase in a rate-limiting manner (Resnitzky et al., 1994). Contrarily, the CKI p27kip1 is chiefly a negative regulator of the G1 to S-phase transition, that binds to Cyclin E:Cdk2 and inhibits its activity (Polyak et al., 1994; Sherr and Roberts, 1999). p27kip^{-/-} mice have enlarged and hypercellular brain structures as well as general hyperplasia reflecting impaired withdrawal from the cell-cycle in response to anti-mitogenic signals (Kiyokawa et al., 1996). Hence, Cyclin E and p27kip1 play antagonistic roles in the control of Cdk2 activity and promotion of S-phase entry and here we have explored the involvement of these genes in the regulation of the cell-cycle in the germinal zones of areas 17 and 18.

The present study demonstrates important differences both in the duration of the G1 phase of the cell-cycle and mode of division of precursors of areas 17 and 18. Can the observed shorter cell-cycle of area 17 precursors lead to increased neuron production despite their lower rate of cell-cycle exit? To investigate this issue we modeled the observed differences in cell-cycle parameters and this shows that areal differences in these parameters lead to significantly higher rates of neuron production in area 17 compared to area 18.

Ex vivo experimental up and down modulation of Cyclin E and p27^{kip1} show that these two regulators of cell-cycle progression -acting via the control of the G1/S transition- are involved in the observed areal variation in cell-cycle kinetics .

The present study suggests that G1 phase regulation could have far reaching consequences for cortical development in the building of distinct cortical areas. Interestingly, a number of neuronal determination and proliferation promoting signals exert their influence through G1 cell-cycle components (Baek et al., 2003; Kioussi et al., 2002; Oliver et al., 2003).

Results

We have investigated corticogenesis specifically in relationship to cytoarchitecture. We have addressed five main issues using *in vivo* and *ex vivo* approaches: (i) The identity of the precursor pool of supragranular layers of areas 17 and 18; (ii) The cell-cycle parameters of the precursors of the supragranular layers of areas 17 and 18; (iii) The differential expression in areas 17 and 18 of cell-cycle regulators; (iv) up and down modulation of the G1/S transition regulators on organotypic slices to confirm their role in modifying cell-cycle parameters; (v) modeling of the observed areal differences in cell-cycle parameters.

Germinal origin of supragranular layer neurons

The germinal zones of the monkey embryonic cortex are characterised by the emergence of a novel precursor pool: the OSVZ (outer subventricular zone) which is unique to the primate. The OSVZ individualises from the SVZ circa E62 (Smart et al., 2002). During the production of supragranular layers, the SVZ is subdivided into an inner SVZ (ISVZ) and an outer SVZ (OSVZ) as revealed by the cytological organisation of the precursors and the location of abventricular mitosis (**Fig 1**) (Smart et al., 2002).

So as to identify the pool of precursors that generate the supragranular layers we have carried out a series of H3-thymidine injections at different development stages coupled with variable survival times. A H3-thymidine pulse at E64 followed by a short survival time (3 hours) shows that during the production of infragranular layers (Rakic, 1974), 70% of labeled S-phase neuroblasts are located in the ventricular zone (VZ) (**Fig 2A,D**). In contrast, short survival periods (1 hour) following E78 and E89 pulses, reveal that 80-90% of labeled precursors are located in the OSVZ (**Fig 2B,D**), which has reached its maximum extent at these ages (Smart et al., 2002). Examination in the newborn cortex of the autoradiographic labeling following a H3-thymidine pulse at E78, shows large numbers of labeled neurons in layers 2/3 (**Fig 2C**). These results show that the OSVZ is the major site of production of neurons destined for the supragranular layers.

Characterization of cell-cycle kinetics of areas 17 and 18 *in vivo*

First we developed a stable *in vitro* assay system in which cycling precursors (corresponding to the growth fraction-GF) are reliably identified by PCNA expression and postmitotic neurons by MAP2 expression (Dehay et al., 2001; Lukaszewicz et al., 2002) (**Fig 3A**). At E78 compared to area 18, area 17 dissociated precursors show higher rates of proliferation, as indicated by a more rapid increase in cell density (**Fig 3B**). Using BrdU as an S phase marker, we further characterized the proliferation rates by computing the labelling index (LI) i.e. the percentage of PCNA-positive cells that have incorporated BrdUrd after an 8 hour exposure. The LI indicates the proportions of precursors in S phase at the time of the BrdUrd pulse and therefore reflects the relative duration of S phase (T_s) with respect to the total duration of the cell cycle (T_c). LI values did not differ between the two sets of precursors at E62 (Fig3C). However, at E78, area 17 precursors showed significantly higher LI values than area 18, indicating differences in cell-cycle kinetics (Fig 3D).

Second, we investigated the *in vivo* duration of the individual phases of the cell-cycle in precursors of the OSVZ in each area by combining S phase cumulative labeling technique (Nowakowski et al., 1989) and computation of the percentage of labeled mitosis (PLM)(Quastler and Sherman, 1959). The cumulative S phase labeling procedure makes it possible to calculate T_c , T_s and the combined duration of G1, G2 and M phases ($T_{g1+g2/m}$) (**Fig 3E**). The proportion of cells labeled by 3H-thymidine (LI) was monitored at two points within the estimated duration of T_c (Kornack and Rakic, 1998) . The LI values have been corrected with the GF values measured in the OSVZ of area 17 and area 18 at E78 by means of PCNA immunostaining (see methods). The cumulative 3H-thymidine labeling performed at E78 shows that the two sets of precursors have identical T_s (9 hours) and significant differences in the duration of $T_{g1+g2/m}$ (area 17: 27 hours; area 18: 37 hours) (**Fig 3F**).

Following a pulse injection of 3H-thymidine, the time interval required for 100% labeling of mitotic figures corresponds to the passage through G2 and M (PLM method) (**Fig 3G**). This returns identical values for Tg2+m of 8 hours for both area 17 and area 18 precursors (**Fig 3H**). Hence, Tc is 36 hours for area 17 precursors and 46 hours for area 18 precursors. This difference in cell cycle length is exclusively due to a 51 % difference in Tg1 (area 17: 19 hours; area 18: 29 hours). Computation of the percentage of mitotic figures in the OSVZ of presumptive area 17 and area 18 shows a significantly higher proportion of precursors in M phase in area 17, confirming a faster cell-cycle progression in this area (**fig 3I**).

Area-specific levels of expression of cell-cycle regulators.

Next, we have analysed the molecular correlate of the area-specific regulation of Tg1 in the OSVZ precursors. We focused our analysis on Cyclin E and p27^{Kip1}. *In vivo*, both p27^{Kip1} and Cyclin E are expressed by cortical precursors of the VZ and the OSVZ (**Fig 4A,B**). Whereas p27^{Kip1} is expressed at detectable levels in all precursors and postmitotic cells (Lukaszewicz et al., 2002; Mitsuhashi et al., 2001; Zindy et al., 1999; Zindy et al., 1997) (**Fig 4 A,B, supplemental data**), Cyclin E expression is restricted to the fraction of precursors that are in G1/S transition (Ekholm et al., 2001; Wimmel et al., 1994) (**Fig 4C**).

In a first approach, we investigated *Cyclin E* mRNA levels in area 17 and area 18 germinal zones at E82. Transcriptional activation of *Cyclin E* plays a significant role in the cell-cycle dependent regulation of cyclin E expression (Bartek and Lukas, 2001). Real-time PCR analysis shows increased expression of Cyclin E mRNA in area 17 germinal zones compared to area 18 (**Fig 4 D**).

As the abundance of p27^{Kip1} -and also of cyclin E- is largely regulated by posttranslational mechanisms, in particular by proteolysis mediated by the ubiquitin-proteasome pathway

(Nakayama et al., 2001), we therefore investigated p27^{Kip1} and Cyclin E protein levels using confocal quantification of immunofluorescent labeling (Durand et al., 1997; Lukaszewicz et al., 2002; Tokumoto et al., 2002). Confocal quantification of immunofluorescent labeling against cyclin E over the full-width of the OSVZ in presumptive area 17 and 18 shows significantly higher levels of tissular expression in area 17 compared to area 18 (**Fig 4E**). p27^{Kip1} in the OSVZ shows a contrasting expression pattern (**Fig 4F**).

However, because expression of p27^{Kip1} is up regulated in post-mitotic cells (Lukaszewicz et al., 2002), areal differences in p27^{Kip1} expression at the tissue level could reflect regional variations in the proportions of precursors and postmitotic cells in the OSVZ rather than variations in cell-cycle regulatory gene activity within the cycling precursor pool. Further, Cyclin E tissular expression could also be influenced by areal differences in the proportion of precursors in late G1 and in G1/S transition (cf above). In order to pin down areal differences in the regulatory mechanisms that directly control cell-cycle progression, it is therefore necessary to monitor the levels of gene expression at the single cell level within the cycling precursor pool. Confocal microscopy quantification of the protein levels of expression of Cyclin E shows that individual precursors exhibit significantly higher levels of Cyclin E expression in area 17 compared to area 18 (**Fig 4G**).

So as to restrict p27^{Kip1} analysis to the cycling cells, we used dissociated cell cultures (see above) where PCNA expression makes it possible to reliably identify the cycling population of precursors (**Fig 4H,I, 3A**). 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 (**Fig 4J**).

The present results show that at both the population and single cell levels, there are contrasting levels of expression of p27^{Kip1} and Cyclin E in area 17 and area 18 precursors. These findings are in agreement with the higher rates of proliferation found *in vivo* in area 17.

Areal differences in frequency of cell-cycle re-entry. We now need to address the frequency of cell-cycle re-entry, which also influences rates of neuron production in the cerebral cortex (Rakic, 1973; Rakic, 1995). The G1 phase is divided into pre- and post restriction point (R) components and markers of the post restriction G1 phase can be used to identify daughter cells that are committed to cell-cycle re-entry (Ekholm et al., 2001; Zetterberg et al., 1995). Passage through R is a prerequisite for accumulation of Cyclin E during a two hour period prior to entry in S phase and Cyclin E expression is limited to this narrow time window. Most of the protein is rapidly down regulated within 1 to 2 hours after entry into S phase (Ekholm et al., 2001). Hence, Cyclin E expression constitutes a reliable marker of late G1 phase and therefore of precursor engagement in cell-cycle re-entry (**Fig 5A**). Analysis in the OSVZ shows that the proportion of precursors that are Cyclin E positive and therefore engaged in cell-cycle re-entry is significantly higher in area 17 compared to area 18 (**Fig 5B**).

Role of p27^{Kip1} and Cyclin E in modifying rates of cell-cycle progression and cell-cycle re-entry revealed by *ex vivo* upregulation and downregulation experiments. The above data suggest that differential expression levels of p27^{Kip1} and Cyclin E in areas 17 and 18 OSVZ could be responsible for the area-specific differences in cell cycle parameters of supragranular precursors. To directly assess p27^{Kip1} and Cyclin E gene function in areal differences in cell-cycle kinetics, we performed upregulation (GOF) and downmodulation (LOF) experiments by using LipofectamineTM 2000 mediated transfection on organotypic slices of E80 visual cortex spanning through presumptive areas 17 and 18. By using low levels of transfection we ensured that change in gene expression was induced in isolated cells that were maintained in an intact environment (**Fig 6A-C**). Specifically, we asked whether up or down modulation of these two cell-cycle regulators is able to alter the two cardinal parameters of cell-cycle kinetics that are area-specifically regulated: Tc and frequency of cell-cycle re-entry. Down regulation of Cyclin E and p27^{Kip1} was implemented on organotypic

slices of E80 visual cortex by means of RNAi technique. siRNA were colipofected with the EGFP expression plasmid, so as to monitor cell-cycle modifications of identified precursors (**Fig 6A-C**). Some lipofected precursors showed a radial glial-like morphology with extended radial process, suggesting that, as in the mouse, a fraction of cortical precursors in the primate could be radial glial cells (Malatesta et al., 2000; Noctor et al., 2001) (**Fig 6A-C**). We first checked that siRNA transfection resulted in a significant decrease of p27^{Kip1} and Cyclin E expression. Confocal microscopy quantification of the immunolabeling against p27^{Kip1} in p27-siRNA-EGFP colipofected cells performed after 3DIV, indicate a 33 % decrease ($p < 0.001$) in expression levels compared to EGFP singly lipofected cells. Using an identical procedure, we found that Cyclin E protein level expression was reduced by 28 % ($p < 0.001$) in Cyclin E-siRNA-EGFP colipofected cells compared to EGFP singly lipofected cells. Forced expression of p27^{Kip1} and Cyclin E was obtained via co-transfection of p27^{Kip1} and Cyclin E expression plasmids with the EGFP expression plasmid respectively. Transfection resulted in an expression increase of 55% for p27^{Kip1} and 100% for Cyclin E as revealed by confocal quantification of immunolabeling.

Analysis of the influence of p27^{Kip1} down-modulation in cortical OSVZ precursors shows a significant increase in the proportion of Ki67 positive cells within the germinal zone, indicating an increase in cell-cycle re-entry (see methods) (22.6% increase, $p < 0.001$ using X^2 test with Yate's correction, fig 6D) while p27^{Kip1} forced expression results in a significant decrease of cell-cycle re-entry (19.8% decrease, $p < 0.001$, **fig 6D**). In contrast, Cyclin E down modulation results in a decrease of cell-cycle re-entry as shown by lower percentages of Ki67 positive cells in the colipofected population compared to the EGFP singly lipofected cells (29.1% decrease, $p < 0.001$, **fig 6D**) whereas Cyclin E forced expression leads to a significant increase in the frequency of cell-cycle re-entry (14% of increase, $p < 0.001$, **fig 6D**).

In a second step, we investigated the influence of p27^{Kip1} and Cyclin E down and up regulation on the rate of cell-cycle progression. LI analysis in colipofected dissociated

precursors reveals that both p27^{Kip1} forced expression and Cyclin E down-modulation results in significantly decreased LI values, suggesting a lengthening of Tc whereas p27^{Kip1} down-modulation and Cyclin E forced expression lead to an increase in LI values, suggesting a shorter Tc, possibly via a reduced G1 phase (Lukaszewicz et al., 2002; Lukaszewicz et al., 2001) (**Fig. 6E**). These results indicate that the p27^{Kip1} and Cyclin E expression levels are at least partly responsible for regulating Tc in areas 17 and 18 primate cortical precursors.

By implementing low levels of transfection in E80 occipital cortical precursors, we have been able to show that p27^{Kip1} and Cyclin E expression levels contribute to variations of cell-cycle parameters that are observed *in vivo* in supragranular precursors of area 17 and 18. We now need to address the significance of these results with respect to early areal specification. This requires implementing a large scale population changes in cell-cycle regulators to examine whether for instance area 17 precursors can be induced to show cell-cycle parameters comparable to that of area 18 precursors. This approach is possible using adenoviral mediated-gene transfer that allows a nearly 100% rate of infection (Craig et al., 1997; Lukaszewicz et al., 2001). Human p27^{Kip1} was overexpressed in area 17 precursors in E80 dissociated cultures, leading to an increase of 42% in p27^{Kip1} level of expression. Compared with area 18 precursors, precursors of area 17 overexpressing p27^{Kip1} exhibit lower LI values (**Fig 6G**), pointing to a lengthening of Tc within the cycling population. Area 17 dissociated cell cultures overexpressing p27^{Kip1} also show diminished values of the fraction of PCNA positive cells (GF) with respect to the total population (**Fig 6H**), indicating an increase in the fraction of precursors withdrawing from the cell-cycle and therefore corresponding to a decrease in cell-cycle re-entry. As a consequence of the decrease in both LI and GF values, area 17 precursors overexpressing p27^{Kip1} are characterized by lower rates of proliferation as indicated by decreased cell density (CD) (**Fig 6I**). Therefore, the present results show that p27^{Kip1} ectopic expression in area 17 precursors induces altered cell-cycle characteristics. Under these conditions, area 17 precursors show lower rates of proliferation than area 18

precursors, indicating the involvement of p27^{Kip1} in regulating area-specific parameters of cortical proliferation.

These results provide evidence that p27^{Kip1} and Cyclin E expression levels play a key role in regulating both cell-cycle re-entry and Tc. The present experimental up and down modulation of Cyclin E and p27^{Kip1} show that, like *in vivo* in area 17 and area 18 of the primate (see above) and as demonstrated *in vitro* in mouse cortical precursors (Lukaszewicz et al., 2002), both parameters are regulated in a coordinated fashion: short Tg1 is associated with a higher frequency of cell-cycle re-entry whereas long Tg1 is correlated with lower frequencies of cell-cycle re-entry.

Modeling of cell-cycle parameters

So as to understand how regulation of Tc and frequency of cell-cycle re-entry can influence both the dimensions of the precursor pool and rates of neuron production, we have implemented modeling of cell-cycle parameters using a compartmental mathematical model of cortical neurogenesis (Polleux et al., 1997).

Using the cell-cycle parameters from the present report, we simulated corticogenesis spanning E70 to E85 to explore if experimentally observed areal differences in frequency of cell-cycle re-entry and Tc can account for both the increase in neuron production and the amplification of precursor pool of area 17 compared to area 18 (Smart et al., 2002). At E70, precursor pools are identical in both areas. Between E70 and E85 there is an amplification of the precursor pool and an upsurge in neuron production which are more pronounced in area 17 compared to area 18 (Smart et al., 2002). To obtain the correct approximation of the number of cycles in the 15 day period we used Tc values of 36 hours (area 17) and 46 hours (area 18) (cf results). We implemented differences in the cell-cycle re-entry of areas 17 and 18 obtained from Cyclin E LI values after correcting for areal differences in Tc.

Implementing a shorter Tc in model area 17 precursors while maintaining an identical frequency of cell-cycle re-entry in both sets of precursors, leads to only a modest increase in

the rate of neuron production in area 17 relative to area 18. Further, under these conditions there is a failure to amplify the precursor pool in area 17 relative to area 18 (**Fig 7A**). Modeling shows that increased frequency of cell-cycle re-entry does in fact lead to increases and not decreases in rates of neuron production. This can be seen by implementing the calculated 20% higher rate of cell-cycle re-entry in model area 17 precursors while maintaining identical T_c values in both sets of precursors. This leads to a relatively larger amplification of the precursor pool of area 17 compared to area 18 as well as to an increase in the relative rate of neuron production in area 17. However, the 75% increase in rate of production of neurons in area 17 (**Fig 7B**) is inferior to the experimentally observed values that correspond to a 2 to 2.5 fold increase in supragranular neurons number in area 17 compared to area 18 (Rockel et al., 1980)(Dehay and Kennedy, unpublished observations). It turns out that it is necessary to combine both the higher rates of cell-cycle re-entry and the shorter T_c values as observed in the present study in model area 17 precursors in order to obtain the increase the rates of neuron production that are comparable to those observed in area 17 (**Fig 7C**).

Discussion

Primate-specific features of cortical neurogenesis. The SVZ undergoes an evolutionary expansion and is a major germinal zone in the monkey where it is established at the onset of neuron production at E55 (Smart et al., 2002). The OSVZ from E72 to the end of neuron production at E100 (Rakic, 1974), constitutes the major site of neuron production, both in terms of size and numbers of mitotically active cells (present work). This contrasts with the SVZ in mouse which is not apparent before E13, and although it increases in volume up to the end of corticogenesis it fails to acquire the importance observed in the monkey (Kostovic and Rakic, 1990; Smart et al., 2002; Smart and McSherry, 1982).

The present study showing that the OSVZ is the major site of the supragranular layer neuron production in the monkey agrees with findings showing common specific gene expression in both the subventricular zone and the supragranular neurons in rodents (Nieto et al., 2004; Tarabykin et al., 2001; Zimmer et al., 2004) as well as recent studies showing neurogenic production in the rodent SVZ (Haubensak et al., 2004; Noctor et al., 2004).

Tc of cortical precursors is considerably extended in primates (Kornack and Rakic, 1998). It has been suggested that the prolonged Tc in monkey cortical precursors is an adaptive feature related to the evolutionary expansion of neocortex in primates (Rakic, 1995). Because environmental signals are known to determine cortical precursor fate (McConnell and Kaznowski, 1991; Polleux et al., 2001) during the cell-cycle as well as regulate rates of proliferation (Dehay et al., 2001), the prolonged primate Tc may serve to ensure a fine adjustment of the rates of production of phenotypically defined neurons.

Contribution of cell-cycle regulation and frequency of cell-cycle re-entry in early cortical patterning. The results show that germinal zones of presumptive areas 17 and 18 are characterized by differences in control of the cell-cycle, indicating early mechanisms of areal specification. The *in vivo* quantification of cell-cycle kinetics demonstrates for the first time the existence of areal differences in Tc via an area-specific control of Tg1. Kornack and Rakic reported combined variations in Ts and Tg1 phase duration in the VZ during corticogenesis of the rhesus monkey (Kornack and Rakic, 1998). This contrasts with the mouse where Tg1 is considered to be the only regulated phase during corticogenesis (Caviness et al., 2003). Our measurements of Tc in the OSVZ fail to show areal variations in Ts and demonstrate that Tg1 variation accounts for the areal differences in Tc. Interestingly, this area-specific decrease in Tg1 occurs specifically during the amplification of the supragranular precursors pool (Smart et al., 2002). There is evidence pointing to G1 phase of the cell-cycle being a window of increased sensitivity to differentiation signals (Mummery et al., 1987). It is thus tempting to

speculate that shortening of G1 phase might shield area 17 cortical precursors from signals that induce cell-cycle exit and differentiation.

The present *in vivo* observations provide the first evidence for an area-specific regulation of the frequency of cell-cycle re-entry during corticogenesis. Together, with the observations of the G1 phase duration, the present results show an area-specific regulation of Tg1 associated with regulation of the balance between cell-cycle exit and cell-cycle re-entry.

Conclusion and perspectives.

The areal differences in levels of expression of p27^{Kip1} and Cyclin E combined with the single cell and tissue level up and downmodulation experiments converge to indicate that levels of expression of these two G1/S transition regulators could contribute to area-specific differences in cell-cycle kinetics. Mathematical modeling of areal differences in cell-cycle parameters shows that alone they could account for the differences in neuron number which characterize these two areas. These results point to the essential role of cell-cycle regulation in the early regional patterning of the cortex, at the level of the germinal zones (Piao et al., 2004; Rakic, 1988; Rakic, 2004).

The present results showing an area-specific cross-coordination of G1 phase duration and frequency of cell-cycle re-entry provide additional evidence for the link between G1 phase duration and frequency of cell-cycle re-entry that are regulated in a concerted fashion in mouse cortical progenitors (Lukaszewicz et al., 2002; Lukaszewicz et al., 2001).

It remains to be determined whether these areal differences in cell-cycle regulation are intrinsic to the developing germinal zones where they could constitute a read-out of regionalized gene expression (Grove and Fukuchi-Shimogori, 2003; Monuki and Walsh, 2001; O'Leary and Nakagawa, 2002; Rakic, 1988). Areal cell-cycle differences could also be the consequence of environmental factors acting on a protomap (Job and Tan, 2003; Kennedy and Dehay, 1993; Rakic, 1988; Sur and Leamey, 2001). Thalamic afferents which have been

shown to shorten Tg1 and increase cell-cycle re-entry (Dehay et al., 2001) could therefore fulfill this role in area 17.

What ever the upstream control of area-specific corticogenesis is, a central problem that remains is how the proliferative gradient in the germinal zone generating areas 17 and 18 is transcribed in to a stepwise function at the 17/18 border in terms of differences in numbers of neurons (Kennedy and Dehay, 1993; Kennedy and Dehay, 2001). The morphogenetic events that are involved in the formation of the sharp border will involve differential rates of migration (Kennedy et al., 1996), proliferation (Dehay et al., 1993)(this report) and tangential expansion (Smart et al., 2002). While we do not know the molecular mechanisms integrating these diverse phenomena, they form part of an intriguing challenge in cortical development since at no time during corticogenesis are sharply bordered patterns of genes observed in the germinal zones. Even genes differentially expressed in the early cortical plate show graded patterns, although at later stages of development many acquire an expression pattern with abrupt changes that correlate with borders between areas (Rubenstein et al., 1999; Sestan et al., 2001) as pointed out in Nagakawa and O'Leary review (O'Leary and Nakagawa, 2002).

Experimental procedures

In vivo studies: Cynomolgus monkey fetuses of known gestational dates received approximately 10-20 $\mu\text{Ci/g.b.w.}$ (ip or im) or 2mCi (intra-amnios) 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). Following caesarian section, anaesthetized fetuses were perfused through the heart with 4% paraformaldehyde. All experiments were performed in compliance with the national and European laws as well as with institutional guidelines concerning animal experimentation.

Observations in fetuses aged E64, E78 were made prior to the appearance of a clear cytoarchitectonic border of striate cortex, transient expression of acetylcholine esterase (Kostovic and Rakic, 1984) was used to infer the area17/18 boundary (Smart et al., 2002)-

Brains used for H3-thymidine studies were embedded in paraffin wax and 5 or 10 μm thick parasagittal sections were cut. An E80 fetus was transcardially perfused with saline (0,9%) followed by 4% paraformaldehyde in 0.1 M phosphate buffer and an increasing sucrose gradient (10 to 20%) for cryoprotection. 60 microns thick sections were cut on a freezing microtome and were used for p27^{Kip1} and Cyclin E immunocytochemistry.

In vivo cell-cycle kinetics measurements:

3H-thymidine cumulative labeling (Nowakowski et al., 1989) makes it possible to derive the duration of S and G1+G2/M phases. Two cumulative 3H-thymidine labeling experiments were performed at E78, during the generation of supragranular neurons. 3H-thymidine was administered intra-amniotically via ultrasound guided injection using a 22G gauge. In one experiment, cumulative labeling was performed during 14 hrs by means of 8 injections (each injection consisted of a constant dose of 2mCi) every 120 minutes. The successive injections spaced 2 hours apart (Kornack and Rakic, 1998) cumulatively label precursors that enter S phase during the 14 hour period. In the second experiment, two injections of 3H-thymidine were made (2mCi) with the same time interval (120 minutes), corresponding to a 3hrs period exposure to 3H-thymidine.

One hour following the last injection, the foetuses were removed by caesarian section, lethally anaesthetized with sodium pentobarbital and transcardially perfused with a mixture of paraformaldehyde (PFA)(4%) in PB.

The labeling index (LI) values were determined as the proportion of 3H-thymidine positive cells (i.e. cells that were in S phase during the 3H-thymidine exposure) with respect to the OSVZ precursor population. In order to get accurate measurements, we have corrected those LI values so as to take into account the value of the growth fraction (GF). GF in the OSVZ of area 17 and area 18 was measured on 40 micron thick frozen sections from a -20°C ethanol fixed E78 embryonic brain, using PCNA as a marker of cycling cells. Computing percentage of PCNA positive cells with the respect of the total number of precursors using confocal

microscopy examination reveals GF values of 88.9% and 90.2% for area 17 and area 18 respectively. As in the Kornack and Rakic 1998 study, we assumed that 3H-thymidine accumulation to saturation in the monkey OSVZ is linear, as demonstrated in rodents (Takahashi et al., 1995). In this context it is appropriate to estimate Tc and Ts from a linear slope best-fit to data from two survival times (3 hrs and 14 hrs).

PLM labeling (Quastler and Sherman, 1959) was used to determine G2/M duration. Two fetuses aged E78 received a pulse injection of 3H-Thymidine (1.5mCi) and were perfused respectively 2 and 7 hours following the pulse.

Statistical analysis: For the PLM experiments statistical significance was tested by means of a F test applied to the ascending slope of the curve. For 3H-thymidine cumulative labelling, statistical significance was tested by means of a generalized linear model using a binomial family (equivalent to a logistic regression) in which the covariates were time and visual area (McCullagh and Nelder, 1989). The statistical difference between areas was tested by means of a X2 test. All tests were performed in the R statistical computing environment (R Development Core Team, 2004). While only two points in time were sampled, 4 measurements were made at each time point and for both conditions. This makes it possible to model the data. In fact, there is not a single overlap in the sample distributions.. Logistic regression shows that the visual area of origin generates a significant different temporal evolution in the proliferation rate (X2: 41.128; $p=1.223 \times 10^{-10}$).

Dissociated Cell culture: Fetal brains at E62, E78 and E 80 were removed under sterile conditions in iced Hank's Balanced Salt Solution containing 10 mM Hepes. 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 were seeded and cultured as described elsewhere (Dehay et al., 2001)

Organotypic slice culture: 200 micron thick slices were cut through the occipital lobe (E80) spanning area 17 and area 18 with a Leica vibratome in 4°C HBSS. Slices were maintained in GMEM+10%FCS on polylysine-laminin coated transwells (Falcon Cell Culture Inserts, 1.0 micron pore size, 6 well format).

In vitro Immunocytochemistry: - PCNA/BrdU double-labeling: Following fixation (ethanol 70%, -20°C) immunostaining was performed as described elsewhere (Dehay et al., 2001). Cells in S-phase at the time of the exposure were positively stained for BrdU. Cycling precursors were identified by means of PCNA labeling. Nuclei were counterstained with Hoechst.

- GFP/Ki67 double-labelling: Following PFA fixation, slices and dissociated cells were processed as follows. Briefly, coverslips were rinsed three times in TBS + triton 0.5% . Following incubation in Normal Goat Serum (1:5 for 20 minutes) and the anti-Ki67 (mouse monoclonal Ab, clone NCL-Ki67-MM1, NovoCastra; 1:100) and anti-GFP (Alexa Fluor 488 conjugate, Molecular Probes; 1:1000) were incubated in TBS overnight at 4°C. After three rinses, goat anti-mouse Cy3 (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).

- GFP/BrdU double labelling: Slices and dissociated cells were fixed with PFA. BrdU immunostaining was performed as described elsewhere (Dehay et al., 2001). In addition, anti-GFP (Alexa Fluor 488 conjugate, Molecular Probes; 1:1000) was incubated in TBS overnight at 4°C. Coverslips were mounted as described above.

- p27^{Kip1} immunocytochemistry: After PCNA immunochemistry, coverslips were processed for p27^{Kip1} immunochemistry. 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 as described above.

- MAP2 immunocytochemistry was performed as described elsewhere (Dehay et al., 2001).

Coverslips were examined using oil objective (x50 or x100) under UV light to detect FITC (filter 450-490 nm) and Hoechst (filter 355-425 nm). 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. All experiments were done at least in duplicate.

Fluorescent labeling on organotypic slices was analysed using a Leica confocal microscope, using a X40 objective (see below for details)

In situ immunolabeling: 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.

Anti-H3 immunocytochemistry was used to visualize mitotic figures in the OSVZ at E78. On frozen sections from PFA fixed tissue. Briefly Rabbit anti-H3 (Upstate, 07-145) was incubated at 4°C overnight in TBS-T, followed by incubation of goat anti-rabbit Cy2 1/1000 in TBST at RT for one hr.

Real-time PCR: Real-time PCR was performed on E82 microdissected germinal zones of area 17 and area 18 using the light Cycler Fast Start DNA Master SYBR Green I kit and a LightCycler (Roche). Primers for detection of CyclinE and β -actin were as follows:

- cyclin E : cDNA length, 133 bp; annealing temperature, 64°C; 5' primer (5' to 3'), AGC ACT TCA GGG GCG TCG C; 3' primer (5' to 3'), CTG GGG AGA GGA GAA GCC C.

- β -actin : cDNA length, 101 bp; annealing temperature, 60°C; 5' primer (5' to 3'), GCG TGA TGG TGG GCA TGG; 3' primer (5' to 3'), GAT GCC GTG CAC GAT GGG.

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 labeling 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. 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 labeling with respect to the total number of cells (indicated either by the intensity of Syto16 fluorescence or by cell counts) (**Fig 5**). 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 labeling); >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 (**Fig 5**). Confocal quantitative analysis of p27^{Kip1} immunofluorescence in PCNA-positive cell nuclei was performed on dissociated cell cultures. Statistical significance between area 17 and area 18 values was assessed with a Mann-Whitney U test.

Plasmids constructs: Full-length cDNAs encoding mouse p27^{Kip1} (Toyoshima and Hunter, 1994) and mouse Cyclin E (Dulic et al., 1992) were inserted into the unique BstXI site of the pHPCAG expression plasmid (Niwa et al., 1998) to generate pHPCAG-mp27 and pHPCAG-cyle, respectively.

siRNA duplex: The siRNA were purchased from Ambion and the sequences were as follows GGAAUAAGGAAGCGACCUGTT (sens) and CAGGCGCUUCCUUAUUCCTG (antisens) pre-designed siRNA p27, and GGAAAAGACAUAUCUUAAGGTT (sens) and CCUUAAGUAUGUCUUUUCCTT (antisens) for silencerTM validated siRNA cyclin E1. The *Silencer Validated* siRNAs from Ambion are single siRNA duplexes that have been verified experimentally to reduce the expression of their individual target genes. Each siRNA has been shown and is guaranteed to reduce target gene expression by at least 70% forty-eight hours post transfection via electroporation in human Hela cells. The extent of mRNA knockdown elicited was compared to cells transfected with a nonsense control siRNA: the Silencer Negative Control #1 from Ambion (Ref 4611).

Lipofection: Constructs were lipofected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions with minor modifications. An EGFP reporter plasmid was colipofected to mark transfected cells. Colipofection efficiency has been estimated to 85 to 100% (Ohnuma et al., 2002). Briefly, transfection was carried out 24 hours following dissociated cells seeding or organotypic slice culture. After one day of incubation, the medium was changed. Slices and dissociated cultures were fixed and processed for cell-cycle parameters 48 and 96 hours following lipofection. All experiments were done in triplicate.

Adenoviral infection: Dissociated neuroblasts from a E80 brain were plated as described above. 15 hours later a dose of 5 moi. Adp27, coding for the human form of p27^{Kip1} (hp27^{Kip1}) (Craig et al., 1997) was added to the culture medium. The proliferation rates were assayed 48 hours after infection. Control cultures were infected with Adnul (an empty virus) (Craig et al., 1997).

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Figure Legends

Figure 1: Compartmentation of the primate germinal zones

A 5 micron thick Nissl stained parasagittal sections cutting through area 17 and area 18 germinal zones at E64. **B** 5 micron thick Nissl stained parasagittal sections cutting through area 17 and area 18 germinal zones at E78. **C** H3 immunolabelling showing the location of mitosis in area 17 germinal zones at E78. Abventricular mitosis are observed in the ISVZ and OSVZ. Adjacent microphotograph shows fluorescent nuclei staining with Propidium Iodide. **D** H3 immunolabelling showing the location of mitosis in area 18 germinal zones at E78. Adjacent microphotograph shows fluorescent nuclei staining with Propidium Iodide. **E** 5 micron thick eosin-haematoxyllin stained section of the germinal zones of area 17 at E64. Mitotic figures are observed in the ISVZ and OSVZ as indicated on the chart. **F** High power microphotograph showing abventricular mitosis from the insert in panel E.

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, VZ ventricular zone, WM white matter. Scale bars: A to E : 100 microns, F : 10 microns.

Figure 2: Germinal origin of cortical neurons.

A Area 17, E64 H3-thymidine pulse, three-hour survival. **B** Area 17, E78 H3-thymidine pulse, one-hour survival. **C** Area 17, E78 H3-thymidine pulse, 87-day survival. **D** Distribution of labeled precursors in OSVZ and VZ at E64, E78 and E89. Abbreviations as in Fig 1. Scale bars: 100 microns.

Figure 3: Cell-cycle kinetics of area 17 and 18 precursors.

A Microphotograph of E78 area 17 dissociated precursors. MAP2 (red) and PCNA (brown) immunolabeling at 4 DIV. Scale bar: 20 microns. **B,C,D** *in vitro* values **B** cell density (CD) values at E78. **C**. LI values at E62 after 3DIV **D** LI values at E78 after 3DIV. Values \pm SEM. Statistical significance: t test. **E** Cartoon illustrating principles of ^3H -Thy cumulative labeling. This technique is based on a continuous ^3H -Thymidine exposure that leads to the incorporation of ^3H -Thymidine by successive cohorts of cycling cells progressing through S phase. The projection of the LI = 100% on the x -axis gives T_c - T_s . T_s is given by the projection of LI = 0 on the x -axis. **F** ^3H -Thy cumulative labeling at E78 *in vivo*. Logistic regression combined with a X2 analysis shows the two slopes are significantly different. **G** Cartoon showing principles of PLM (Percentage of labeled mitotic figures). This procedure, based on a brief exposure of cells to ^3H -Thymidine, measures the time required for cells in S phase to enter M phase and therefore returns $T_{g2/m}$. **H** PLM values at E78. Statistical analysis (F test) shows the slopes are identical. **I** Percentage of mitoses in area 17 and area 18 OSVZ at E78. Values are \pm SEM.

Figure 4: Tissular and cellular levels of expression of Cyclin E and p27^{Kip1}.

A Cyclin E, p27^{Kip1}, immunolabelling and Syto16 counterstaining in area 17 germinal zones at E80. **B** Cyclin E, p27^{Kip1}, immunolabelling and Syto 16 counterstaining in area 18 germinal zones at E80. **C** upper panel: high magnification of Cyclin E positive precursors in the OSVZ, taken from A; lower panel: high magnification of OSVZ precursors counterstained with Syto16, showing that only a fraction of precursors express high levels of Cyclin E. **D** Real Time PCR analysis of Cyclin E mRNA levels at E82. **E** Tissular Cyclin E expression levels (confocal quantification of Cyclin E fluorescent immunolabeling corrected for cell density in the OSVZ). **F** Tissular p27^{Kip1} expression in area 17 and area 18 OSVZ (confocal quantification of p27^{Kip1} fluorescent immunolabeling corrected for cell density in the full-width of the germinal zone). **G** Proportions of cells with high, medium and low Cyclin E

levels in area 17 and area 18 OSVZ. **H** Confocal image of PCNA nuclear immunostaining on dissociated precursors from area 17 after 2 DIV. **I** Confocal image of p27^{Kip1} immunolabeling (same field of view as H). **J** p27^{Kip1} cellular expression levels in PCNA positive cells after 2 DIV. p27^{Kip1} levels of expression are significantly lower in area 17 precursors than in area 18 precursors. Scale bars A, B: 100 microns ; C:25 microns ; H, I: 50 microns. Abbreviations see Figure 1.

Figure 5: Frequency of cell-cycle re-entry.

A Cartoon showing how Cyclin E informs on frequency of cell-cycle re-entry. The G1 phase is divided into early and late G1 separated by the restriction point (R). The restriction point corresponds to the time point where a cell is irreversibly committed to a new cycle. Molecules expressed after the restriction point distinguish between daughter cells that re-enter the cycle from those that exit the cycle. Cyclin E expression characterizes late G1 and is therefore only expressed by daughter cells that re-enter the cell cycle. Hence, changes in proportions of precursors that express Cyclin E reflect variations in proportions of cells re-entering the cell-cycle. **B** *In vivo* percentage of Cyclin E-positive precursors in area 17 and area 18 OSVZ with respect to the total population of precursors. Values are \pm SEM. Statistical analysis with Mann-Whitney U test; *** $p < 0.0005$.

Figure 6: Analysis of cell-cycle parameters in p27^{Kip1} and Cyclin E loss and gain of function experiments.

A Drawing showing a lipofected precursor in an organotypic slice. **B** Microphotograph showing the GFP lipofected precursor from A in the OSVZ. **C** Microphotograph of a Ki67 positive, GFP lipofected precursor. **D** Histograms showing the percentage variation of cell-cycle re-entry (fraction of Ki67 positive cells in the germinal zones) value on organotypic slices. Control values correspond to precursors colipofected with control (pHPCAG) and

EGFP plasmid or precursors colipofected with a nonsense control siRNA and EGFP plasmid

E Histograms showing LI values on dissociated cultures. Control values correspond to precursors colipofected with control (pHPCAG) and EGFP plasmid. Statistical significance with a X^2 test. **F** LI variations in area 17 and area 18 OSVZ precursors infected with Adnul. Results showing variations of LI (**G**), GF (**H**) and CD (cell density, **I**) in area 17 precursors overexpressing p27^{Kip1} via adenoviral infection compared to area 18 precursors infected by empty virus. Scale bars B: 50 microns ; C: 10 microns. Abbreviations see Figure 2.

Figure 7: Mathematical modeling showing the evolution of the size of the precursor pool and total number of postmitotic neurons produced during the 15 day simulation period.

Initial number of precursors (P) is identical in each case and arbitrary fixed at 100 (see text). Simulation is using in vivo Tc values obtained in this study (A17: 36 hours, A18: 46 hours) **A** Here, the frequency of cell-cycle re-entry is made identical for areas 17 and 18. The simulation shows that cell-cycle duration differences alone generate only a 14% difference in the number of neurons produced (N). **B** Here, Tc is fixed at 46 hours in both sets of precursors. Frequency of cell-cycle re-entry is set 20% higher in area 17 compared to area 18 (calculated from data of Figure 6, and corrected for variations in Tc). The simulation shows that cell-cycle re-entry difference alone generates a 75% difference in the number of neurons produced. **C** This simulation combines the experimentally observed a 22% difference in cell-cycle duration (36 vs 46 hours) and the observed 20% difference in cell-cycle re-entry (after correction for Tc). This generates a 151 % difference in the number of neurons produced.

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