Modulation of the cell cycle contributes to the parcellation of the primate visual cortex

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AN as-yet unresolved issue in developmental neurobiology is whether the discrete areas that form the mammalian cortex emerge from a uniform cortical plate or whether they are already specified in the germinal zone[1,2]. A feature of the primate striate cortex is that the number of neurons per unit area is twice that of anywhere else in the cerebral cortex[3]. Here we take advantage of this unique structural feature to investigate whether the extra striate cortical cells are due to increased neuron production during neurogenesis. We labelled precursors undergoing terminal cell division with ^{3}H-thymidine and allowed them to migrate to the cortical plate. Cell counts revealed that their rate of production in the germinal zone of striate cortex is higher than in that giving rise to extrastriate cortex. Also, we used ^{3}H-thymidine pulse injections to investigate cell cycle dynamics and found that this phase of increased production of striate cortical cells is associated with changes in the parameters of the cell cycle. These results show that cortical area identity is at least partially determined at the level of the ventricular zone.

A total of eight ^{3}H-thymidine pulse injections have been carried out on fetal monkeys. Fetuses received an intraperitoneal injection of ^{3}H-thymidine at known gestational ages and were returned to the uterus for a survival period before being processed for autoradiographic histological observation. The position and density of labelled cells were determined in either the cortical plate or the underlying germinal zone (Fig. 1*a*). Using frequent sampling, we were able to employ a statistical test (ANOVA) to show that differences between striate and extrastriate cortex were not a result of spurious variations in labelling density in each area; we found significant differences in labelling for all ages except in the E64 case (see Table 1). Experimental cases fell into two groups (I and II in Table 1) according to the length of the survival period.

In the first group (5 cases), a long survival period of 14–84 days allowed neurons to migrate out to the cortical plate as well as a variable degree of cortical differentiation. In this group the numbers of labelled cells per unit area of cortical plate were estimated in striate and extrastriate cortex. In all cases, regardless of the age of the fetus at the moment of injection, striate cortex was found to have significantly more labelled cells per unit area than adjacent extrastriate cortex. Quantitative analysis of the results showed that there were developmental changes in the percentage increase of labelled cells in striate cortex compared with extrastriate cortex (Table 1).

Differences in labelling of striate and extrastriate cortex are illustrated in Fig. 1*b*, which shows cortical labelling in a neonate that had received a ^{3}H-thymidine pulse injection on embryonic day 81 (E81). Different intensities of labelling broadly correspond to successive generations of cells, because there is roughly a halving of labelling intensity at each mitosis[4]. The radial location of successive generations of cells can be determined from the positions of cells with maximum, 50% of maximum and 25% of maximum labelling. Besides the increased rate of cell production in striate cortex (as indicated by the higher densities of labelled neurons in this area), Fig. 1*b* illustrates two common

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features seen at all ages. First, the fact that heavily labelled cells tend to be at deeper locations than more lightly labelled cells confirms the inside-first, outside-last sequence of cortical neuron production[5]. Second, labelled cells in extrastriate cortex are slightly nearer the surface in the supragranular layers than those in striate cortex, confirming that cell production in striate cortex lags behind that in extrastriate cortex[6].

The ages for ^{3}H-thymidine injection were chosen to span the period of neuron production of infra- and supragranular layers[7]. The developmental age of the fetus at the time of injection strongly influenced the increase in labelling of striate cortex (Fig. 2). The youngest fetus at the time of injection was E66; this showed an increase in labelling of striate cortex of 7%. Increases were larger in older fetuses, reaching a maximum of 150% in the fetus injected on day E78. At later stages of cortical neuron production, the percentage increase in labelled striate neurons fell and the two fetuses injected on E81 gave values of 70 and 100%. These results show that increased rates of striate cortex neuron production are much lower at early stages of neurogenesis when infragranular layers are being generated. Increased striate cortex neuron production peaks at about E78 when superficial layers are being generated[7].

The second short-survival group (3 cases; Table 1) enabled us to examine ^{3}H-thymidine labelling in the germinal zone lining the ventricles and to determine whether the higher cell production in striate cortex compared to the same area of adjacent cortex was due to changes in cell cycle parameters. The percentage of tritiated cells relative to the total population of germinal cells is referred to as the labelling index and reflects the proportion of precursor cells in S phase (the phase of DNA synthesis

TABLE 1 Experimental cases and statistical analysis of labelling differences of striate and extrastriate cortex

Group I Age at ^{3}H-thymidine injection	Age at perfusion	Differences of labelling density in cortex	
E66	E121	$F=8.28$	$P=0.0048$ $n=4,418$
E72	E94	$F=26.75$	$P=0.0001$ $n=2,458$
E78	E92	$F=40.39$	$P=0.0001$ $n=12,919$
E81*	E110	$F=105.82$	$P=0.0001$ $n=2,505$
E81	E165	Unpaired Student t-test	$P=0.0001$ $n=5,448$
Group II Age at ^{3}H-thymidine injection and perfusion	Labelling index (%)	Differences of labelling index in the germinal zone	
E64	A17, 20.15 A18, 20.38	$F=1.21$	$P=0.3029$ $n=13,223$
E78	A17, 29.65 A18, 19.88	$F=11.38$	$P=0.0097$ $n=19,006$
E94	A17, 6.97 A18, 5.41	$F=14.76$	$P=0.0049$ $n=14,220$

n, Total number of counted neurons; E represents the embryonic indicated by the number following.
* Two E81 animals also mentioned in Fig. 2.

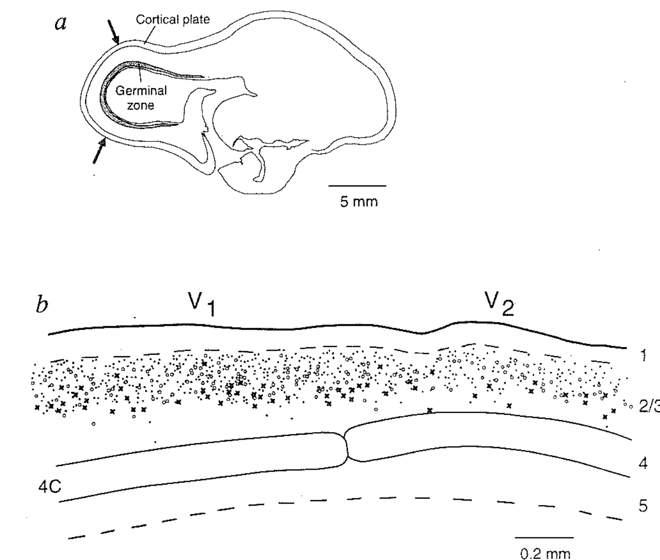

FIG. 1 *a*, E94 fetal brain showing the location of the cortical plate and germinal zones of presumptive striate cortex as revealed by acetylcholine esterase histochemistry. Arrows indicate the borders of the striate cortex. *b*, Distribution of the ^{3}H-thymidine labelling in the visual cortex of a newborn monkey (E165) after injection at E81. Small dots represent neurons containing 3–9 grains, open circles represent those containing 10–20 grains and crosses those containing >20 grains. Labelled neurons in striate cortex are nearer the surface than in extrastriate cortex, and are nearly double the number of those in extrastriate cortex. V_1, striate cortex; V_2, extrastriate cortex; 4C, layer 4C of striate cortex; 1, 2, 3, 4 and 5 indicate cortical layers.

METHODS. Cynomolgus monkeys with controlled dates of impregnation were used to provide 7 fetuses of known gestational age (Table 1). Surgery on pregnant monkeys and fetuses was performed as described elsewhere[24]. Fetuses were injected (i.p.) with ~10–20 μCi per g body weight of ^{3}H-thymidine in saline (specific activity, 40–60 μCi mmol^{-1}) and replaced in the uterus. In the long-survival group (14–84 days; group I in Table 1) pregnant monkeys were returned to their cages and medicated for two days with an analgesic (Visceralgine, injected i.m.). A muscular relaxant (Duvadilan, also given i.m.) was given twice daily for two weeks and fetuses were either delivered by caesarian section or left until birth (E165). During short survival (E64, 1 h; E78, 7 h; E94, 1.5 h), the pregnant monkey was maintained under anaesthesia. Fetuses were perfused through the heart with 4% paraformaldehyde. In fetuses aged E64, E78, E92 and E94, cells were counted before the appearance of a clear cytoarchitectonic border of striate cortex. Acetylcholine esterase is transiently expressed by extrastriate cortex[26] so we used an E94 fetus to determine the borders of presumptive striate cortex in the E64, E78 and E94 fetuses (see panel *a*). Striate cortex is a large cortical area close to paleocortex, so its position can be extrapolated from E94 to the E64 and E78 fetus. The brain used to detect acetylcholine esterase was cut on a freezing microtome and processed according to ref. 26. Brains used for ^{3}H-thymidine studies were embedded in paraffin wax and 5- or 10-μm-thick sections were cut. In the long-survival group, sections were processed for autoradiography to give a maximum of 40 grains per differentiated cell. Three intensities of labelling were distinguished: heavy (20–40 grains), moderate (10–19 grains) and light (3–9 grains). ^{3}H-thymidine-labelled cells were counted in 10- or 5-μm-thick sections counterstained with cresyl violet. This was not possible in the short-survival group because of the small size of the neuroblasts. Sections for quantitative analysis cut the dorsal cerebral operculum perpendicularly. Observations were made with a time-50 oil objective. Labelled cells were monitored on a video screen and their positions charted by a high-precision plotting system (Biocom, running on HISTO software). Because of local fluctuations in the density of labelled cells in the cortical plate and, to a lesser degree, in the germinal zone, we counted large numbers of labelled cells so as to determine reliably the differences between striate and extrastriate cortex. In the cortical plate, cells were counted in each animal (except the E81 fetus) in 2–7 sections, and in each section in 10–26 100-μm segments. In the germinal zone, cells were counted in 2 sections per animal and in 4–8 300-μm wide segments. In the E81 fetus, cells were counted continuously over a large area of striate and extrastriate cortex. Significance levels were determined with a 2-ways ANOVA test, except in the case of E81, for which a Student t-test was used. The ratio of labelled cells in equivalent areas of the cortical plate of striate and extrastriate cortex was expressed as a per cent (Fig. 2).

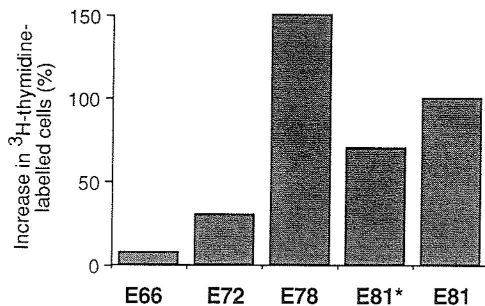

FIG. 2 Histograms showing per cent increase of labelled cells in striate cortex compared with extrastriate cortex after injection at different fetal ages.

when the labelled nucleotide is incorporated into the nucleus)[8-10]. After short survival times there was intense labelling in the germinal zones lining the ventricles, and there were more labelled cells in the germinal zone underlying the striate cortex than in that underlying extrastriate cortex (Fig. 3). Although the packing density of precursors in the germinal zone underlying striate cortex was higher than that underlying extrastriate cortex, counts of labelled and unlabelled cells showed that at later stages of corticogenesis the labelling index in the germinal zone underlying striate cortex was significantly higher than that of extrastriate cortex (Table 1). Injection at E78 gave a labelling index in the germinal zone underlying striate cortex that was 49% higher than in the germinal zone underlying extrastriate cortex. Injection at E94 led to a steep drop in the labelling index for both areas, indicating that neurogenesis was slower than in the younger fetus. At this age, the labelling index in the germinal zone of striate cortex was 29% higher than it was in the germinal zone of extrastriate cortex. By contrast, the labelling index at E64 did not differ significantly between the germinal zones of presumptive striate and extrastriate cortex.

The higher labelling index in the germinal zone giving rise to striate cortex indicates an increased proportion of cells in S phase, due either to a shortened cycle time or a maintained cycle duration with a proportionally longer S phase[8-10]. In the later stages of neurogenesis, duration of S phase is considered to remain fairly constant and variations in the cell cycle time to be due mainly to changes in the length of the G1 phase[11-14]. Hence under such conditions, when the cell cycle is short, S phase takes up a relatively larger part of the cycle and the labelling index is high. The higher rate of cell production characteristic of the germinal zone underlying striate cortex might thus be due to shorter cell-cycle times as well as a higher density of precursors. Differential cell death changes the number of supragranular neurons in different areas in rodents[15], but this is unlikely to be a factor affecting our results as our fetuses were examined before the principal phase of cell death in the cortex[16].

We have shown that the germinal zone giving rise to striate cortex has a higher rate of cell production than the germinal zone giving rise to the adjacent neocortex. Variations in the labelling index correspond to regional differences in the kinetics of the cell cycle. These differences in neurogenic activity of the germinal zone emerge during the later stages of corticogenesis when the main phase of excess striate neuron production occurs. The specialization of the primate striate area germinal zone ensures that this area has higher numbers of supragranular-layer neurons than other areas[3].

Our findings are relevant to theories of the specification of cortex. In rodents, thalamic fibres are important for specifying cortical areas at late stages of development[17,18], suggesting that the initially formed cortex is a uniform structure[1], but this view needs to be reconciled with recent findings. Molecular markers have revealed a remarkable degree of early regional specialization of phylogenetic subdivisions of the rodent cerebral cortex[19,20]. Gross subdivisions of the neocortex also show early specialization[21]. More importantly, early specialization of the immature rodent neocortex distinguishes different areas before the arrival of thalamic afferents[22], indicating that areal identity may be at least partially specified before neuroblasts arrive in the cortical plate[2]. A degree of heterogeneity of the germinal layers giving rise to the neocortex has been found[23]. We have shown that regional variation of neurogenesis in the germinal zone gives rise to a discrete neocortical area. As thalamic input influences the specification of striate cortex in the monkey[2,24], it may regulate neurogenesis in the germinal zone[25]. □

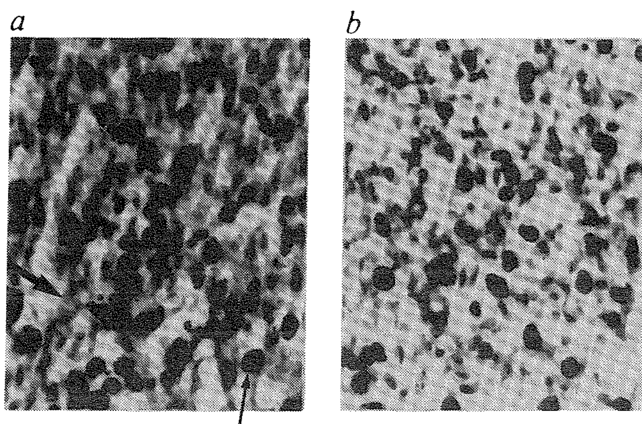

FIG. 3 High-power microphotograph illustrating cell density and ³H-thymidine labelling in the germinal zone of E78 fetus. *a*, Striate cortex; *b*, extrastriate cortex. Thin arrow, ³H-thymidine-labelled cell; thick arrow, non-labelled cell. Scale bar, 50 μm.

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