Pre- and Post-mitotic Events Contribute to the Progressive Acquisition of Area-specific Connectional Fate in the Neocortex

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The adult primary motor cortex (area 6) is characterized by a stronger projection to the spinal cord than the primary somatosensory cortex (area 3). Here we have explored the progressive and regressive phenomena that determine these areal differences in the number of corticospinal neurons (CSNs). CSNs were birthdated with [^{3}H]thymidine and subsequently retrogradely labeled from the spinal cord. The time window of CSN production is identical in both areas. The probability that a cohort of neuroblasts project to the spinal cord is indicated by the percentage of [^{3}H]thymidine-positive neurons that can be back-labeled from the spinal cord. In the neonate this *fate index* is significantly higher in area 6 compared with area 3, indicating that early regionalization of cell fate contributes to areal differences in CSN number. In neonatal *reeler* mice, an increase in CSN number was accompanied by an increased fate index, showing that the integrity of the post-mitotic environment is required for the specification of the appropriate number of neurons expressing a given connectional phenotype. Postnatal development in *reeler* and normal is characterized by an area-specific elimination of CSN axons, which reduces areal differences in CSN number. These results show a progressive acquisition of CSN fate in the neocortex and indicate that both early regionalization and late environmental signals contribute to determining areal differences of connectional phenotype.

Introduction

While there have been considerable advances in our understanding of the determination of the phenotype of cortical neurons, very little is known concerning the developmental mechanisms controlling areal differences in the numerical values of neuronal phenotypes. This is an important issue because the distinctive physiological roles of individual neocortical areas are thought to be largely determined by the patterns of their afferent and efferent connections (Gilbert, 1983; Douglas and Martin, 1991). In the adult sensorimotor cortex of rodents the number of corticospinal neurons (CSNs) is significantly higher in the primary motor cortex, area 6, compared with the primary somatosensory cortex, area 3 (Miller, 1987; Schreyer and Jones, 1988a,b; Oudega *et al.*, 1994). In the present study we have used the areal differences of CSN number in these two areas in the normal and the *reeler* mutant as a model system for studying the developmental mechanisms that specify neuron number of a precisely defined projection phenotype.

Mechanisms of areal specification of CSN number have been at least partially explored in investigations of the developmental control of cortical differentiation. It is generally agreed that the timing of the specification events of different cortical traits are staggered throughout corticogenesis (Levitt *et al.*, 1993; Eagleson and Levitt, 1999). A number of studies have shown an early regionalization of the neuroepithelium in terms of molecular marker expression (Barbe and Levitt, 1991; Arimatsu *et al.*, 1992; Ferri and Levitt, 1993; Cohen-Tannoudji *et al.*, 1994; Barbe and Levitt, 1995; Tole and Patterson, 1995; Miyashita-Lin *et al.*, 1999; Bishop *et al.*, 2000). However, the notion that molecular phenotype is specified early in development has recently been refined by evidence that molecular fate of cortical neurons is determined progressively during proliferative and post-mitotic phases of neuron production (Arimatsu *et al.*, 1999a,b; Gitton *et al.*, 1999b). In contrast to molecular phenotypes, neuropeptide expression is thought to be specified relatively late (Parnavelas and Cavanagh, 1988; Obst and Wahle, 1995).

There is evidence that the specification of connectional phenotypes like the specification of the molecular phenotype occurs at different stages of corticogenesis (Innocenti, 1981; Schwartz *et al.*, 1991). Transplantation studies show that there is an early regionalization and that newly born neurons are committed to the CSN phenotype shortly after their final mitosis (McConnell, 1988; McConnell and Kaznowski, 1991; Ebrahimi-Gaillard *et al.*, 1994; Ebrahimi-Gaillard and Roger, 1996; Frantz and McConnell, 1996). These findings contrast with previous transplantation experiments showing that the CSN connectional phenotype continues to be specified late in development (Stanfield *et al.*, 1982; O'Leary and Stanfield, 1985, 1986; Stanfield and O'Leary, 1985). Elsewhere, it has been speculated that in general terms, pruning of exuberant connectivity constitutes a late specification event (Innocenti, 1995).

During cortical development many connections are lost, raising the possibility that the adult number and distribution of neurons displaying a given connectional phenotype results from a process of selective elimination of connections (Bates and Killackey, 1984; O'Leary and Stanfield, 1985; Schreyer and Jones, 1988a,b; O'Leary, 1992). This issue has been extensively studied in the corticospinal pathway where, compared with the adult, the immature brain has been shown to have a more widespread distribution of CSNs across the cortex (Bates and Killackey, 1984; O'Leary and Stanfield, 1985). However, in the immature brain the distribution of CSNs is non-uniform so that in the neonate the location of peak densities corresponds to the location of peak densities in the adult (Meissirel *et al.*, 1993; Oudega *et al.*, 1994). This could mean that regressive phenomena are not selective and in which case they would not contribute significantly to determining adult areal differences in CSNs. The alternative is that areal differences in CSN phenotype are established earlier in development as a result of regional differences in CSN number produced by the ventricular zone. This would imply that regions of the ventricular zone that are fated to produce CSNs show local variations in the expression of this fate.

At the present time we do not know the relative contributions of early regionalization and late elimination of connections to the determination of areal differences in CSN number. This is conceptually important because the relative timing of specification events determines which environmental factors can influence cortical development (Kennedy and Dehay, 1993; Levitt *et al.*,

1993). Because connectional phenotype plays such a crucial role in cortical physiology the timing of the specification of these traits is an important issue. One approach to monitoring the specification of connectional phenotype is to study the mitotic history of numerically and ontogenetically defined populations of neurons in the adult.

Such an approach is provided by pulse injections of tritiated thymidine ([^{3}H]thymidine) at regular intervals throughout the generation of CSNs (Polleux *et al.*, 1997a). Precursors that quit the cell cycle immediately after the pulse contain maximum grain counts, correspond to first generation (FG) neurons and can be accurately distinguished from precursors that return to the cell cycle and dilute out their radioactivity (Polleux *et al.*, 1998). CSNs that underwent their final mitosis immediately after the [^{3}H]thymidine pulse (i.e. FG neurons) are double-labeled (DL) and the ratio DL/CSN defines a *generation index* that accurately indicates the timetable of CSN production (Carter-Dawson and LaVail, 1979; Mustari *et al.*, 1979; Reznikov, 1990; Valverde *et al.*, 1995; Polleux *et al.*, 1997b). The fraction of DL with respect to FG (DL/FG) defines a *fate index* that reflects the probability that a neuron born on a given date becomes a CSN (Polleux *et al.*, 1998). In the present study, measurement of this fate index reveals that the increased number of CSNs produced in area 6 compared with area 3 is due to a higher proportion of precursors being specified to express the corticospinal phenotype.

It is known that signals at the level of the ventricular zone determine neuronal phenotype (McConnell, 1988; McConnell and Kaznowski, 1991; Ferri and Levitt, 1993; Cohen-Tannoudji *et al.*, 1994; Frantz and McConnell, 1996; Polleux *et al.*, 1998; Arimatsu *et al.*, 1999a,b; Miyashita-Lin *et al.*, 1999; Bishop *et al.*, 2000). The present results show that signals at the ventricular zone also contribute to specifying areal differences in CSN number. However, at this stage we could not exclude the possibility that the cortical post-mitotic environment might also contribute to areal differences in CSN number. To address this issue we have examined the CSN fate index in the perturbed cortical environment of the *reeler* mutant mouse (Caviness and Sidman, 1973; Caviness, 1976, 1982; Terashima *et al.*, 1983, 1985; Inoue *et al.*, 1991; Hoffart *et al.*, 1995). The *reeler* returns considerably higher fate indices compared with the normal and this increase in the fate index in the mutant correlates with a large increase in CSN number. This difference between normal and *reeler* suggests that the cortical environment does contribute to the specification of CSNs and that cell fate in normal development occurs in a two-step fashion in response to signals found in the ventricular zone and in the cortical plate (Arimatsu *et al.*, 1999a,b; Gitton *et al.*, 1999a).

Postnatal development is characterized by an important reduction in cortical connectivity (Cragg, 1975; Finlay *et al.*, 1987; Schreyer and Jones, 1988a,b; Oudega *et al.*, 1994), which could be expected to influence the final number of connections and which importantly could be influenced by evoked neural activity [reviewed in Katz and Shatz (Katz and Shatz, 1996)]. Monitoring postnatal changes in DL/FG provides an efficient and sensitive measure of axon loss. This shows that there is an important postnatal elimination of CSN projections which actually reduces the areal differences in CSN number found transiently in the neonate. The fact that the rate of loss differs in areas 3 and 6 and that it is greatly increased amongst the expanded CSN population of the *reeler* suggests that the protracted postnatal pruning of connections serves to adjust CSN number to physiological needs.

Materials and Methods

Experimental Animals

BALB/c mice (Iffa-Credo, Lyon, France) were maintained under a constant photoperiod of 12 h of light (08:00–20:00 h). The offspring of homozygous *reeler* mutant males and heterozygous females with BALB/c background carrying the Orleans allele of the *reeler* gene (rl^{Or}) were used. In this study, we used heterozygous littermates (+/rl^{Or}) as controls and refer to these animals as normal. Comparison between hetero- and homozygous animals were made within litters. The morning of the vaginal plug was designated E1.

Injections

Pregnant females received an intraperitoneal injection of [^{3}H]methyl thymidine (Amersham, UK, 5 μCi/g body wt, sp. act. 25 Ci/mmol) between 12:00 and 14:00 h on four different dates of embryonic development. The spinal cord was exposed by section of ligaments and the tectorial membrane between the first and the second cervical vertebrae in adults (2–4 months old) under ketamine/xylazine anesthesia (Ketalar 10 mg/kg, Rompun 4 mg/kg) and hypothermia in neonates. The corticospinal tracts, visualized under the dissection microscope, received a large pressure injection of Fluorogold (2.5%) (Schmued and Fallon, 1986) (0.4–0.8 μl in adults; 0.05–0.1 μl in neonates). The wounds were sutured and the animals returned to their cages for a survival period of 5 days in the adults and 2 days in the young pups.

Perfusion and Tissue Processing

After the survival period, all animals received an overdose of sodium pentobarbital and were perfused intracardially with 0.9% NaCl solution including a vasodilator (procaine 1 g/l) followed by a phosphate-buffered (PB; pH 7.4, 0.1 M) fixative solution of 4% paraformaldehyde. The brain was removed and post-fixed in the same solution for 1 week, rinsed in running tap water for 24 h and dehydrated in ethanol, cleared with toluene and embedded in paraffin. The brains were cut in the coronal plan (10 μm) and mounted on glass slides from an albumin–gelatine mixture. Sections were processed for autoradiography as described elsewhere (Polleux *et al.*, 1997a). Spinal cord were cryoprotected in 30% sucrose and sectioned horizontally at 40 μm using cryostat in order to check systematically the size and the position of Fluorogold injection sites (see Fig. 3).

The Fluorogold labeling was observed under epifluorescence (D type filter; Leitz, UV epifluorescence 325–455 nm). After analysis of the fluorescent and autoradiographic labeling, selected sections were counterstained with cresyl violet (0.1%, 10 min), dehydrated and coverslipped with Depex in order to observe with greater precision the autoradiographic labeling over individual cortical cells and to count and measure neuron profiles.

Neuronal Profile Counts

All soma measurements were made using an interactive plotting system (Biocom), which accurately records the position and size of individual retrogradely labeled and unlabeled neurons as well as the number of autoradiographic silver grains per cell nucleus. Measures of the soma size were made on images from a CCD Cohu camera and projected on a Barco CD233 screen. The average cell profile diameter (d) was corrected using the equation described by Schüz and Palm (Schüz and Palm, 1989).

Concerning the autoradiographic labeling, the number of silver grains overlying individual nucleus profile has been corrected for different nuclear profile diameters using the Appleton formula (Appleton *et al.*, 1969) [see also Polleux *et al.* (Polleux *et al.*, 1997b)]. Autoradiographic background was estimated by counting the number of grains over neocortical tissue which does not present any nuclear profiles. This showed background levels to be inferior to one grain per 400 μm2, i.e. much less than one grain per mean nuclear surface (~250 μm2).

Counts of neuron number per unit area of cortical surface were corrected for split cell error (Abercrombie, 1946) using a cytoarchitectonic analysis based on categorization of cell body diameters (Clarke, 1992) as described earlier (Polleux *et al.*, 1998). Using this procedure, the maximal error attributable to profile splitting is <0.5% for neurons such as CSNs with soma diameters exceeding 16 μm. Statistical analysis of CSN counts was performed using a non-parametric Mann–Whitney

test. Finally, glial and endothelial cell profiles were identified using the morphological criteria described by Heumann and Leuba (Heumann and Leuba, 1983) and were excluded from the profile counts.

Estimation of Total Cortical Volume (V_c) and Injection Site Volume in Normal and Reeler Mice

The total cortical volume was estimated in three animals of each genotype and age (newborn and adult) from three different litters using Cavalieri's principle (Cavalieri, 1966). Briefly, the neocortical surface (from midline to pyriform cortex) was measured using an image analysis system (Biocom) on coronal paraffin sections (thickness 10 μm) at 300 μm intervals throughout the entire rostro-caudal extent. Then the following formula derived from the Cavalieri's principle was applied to estimate cortical volume (V_c):

$$V_c = A \times T \times S$$

where A is the mean cortical surface computed from all sections, T is section thickness and S is the numbers of sections.

The average volume of Fluorogold injection sites was also estimated using the same approach (Cavalieri, 1966) by measuring the surface occupied by the tracer on 40 μm thick horizontal sections of the spinal cord (spacing 120 μm) of four animals of each genotype and age group.

Exhaustive analysis of the relationship between the intensity of [^{3}H]thymidine labeling of cortical neurons and their radial height in the cortex shows that, provided the Appleton corrections (Appleton *et al.*, 1969) are applied, there is a halving principle of the radioactive signal with each round of mitosis (Polleux *et al.*, 1997a,b). This means that, following a single [^{3}H]thymidine pulse, each generation of labeled neurons can be unambiguously identified. Neurons that show >50% of the maximum labeling have exited the cell cycle at the first mitosis following the [^{3}H]thymidine pulse and are first generation neurons (FG neurons) (Rakic, 1973; Polleux *et al.*, 1997a,b). The timing of the [^{3}H]thymidine pulse that generates a cohort of FG neurons defines the birthdate of the FG neurons. Neurons that, following the pulse, complete two or more divisions before quitting the cycle have <50% maximum labeling and are subsequent generation neurons.

Statistical Analysis

Statistical analysis of the two ratios DL/CSN and DL/FG was performed using a chi-square analysis as described earlier (Polleux *et al.*, 1997b, 1998). Briefly, variability in proportions among all animals of a given genotype or among all values within a given area (intra-group variability) was systematically tested, and failed to show any statistical significance. Then inter-group variability (between normal and *reeler* animals or between areas 6 and 3) was analyzed on pooled values obtained from different animals (see text for numbers). Throughout this study, DL/CSN and DL/FG values were obtained by examining 300–600 neurons in three or four animals at each given time point.

Results

Technical Considerations

In the present study we have used [^{3}H]thymidine pulse injections to investigate the developmental processes that specify CSN number. This approach can be used to describe accurately the mitotic history of specific subsets of neurons (Polleux *et al.*, 1997b, 1998). There are numerous technical difficulties associated with the autoradiographic process and the quantitative use of [^{3}H]thymidine labeling which globally result in an underestimation of FG labeling (Polleux *et al.*, 1997a). However, because in the present study we are examining ratios of FG neurons, the underestimation of the autoradiographic signal can not be expected to influence our principal conclusions.

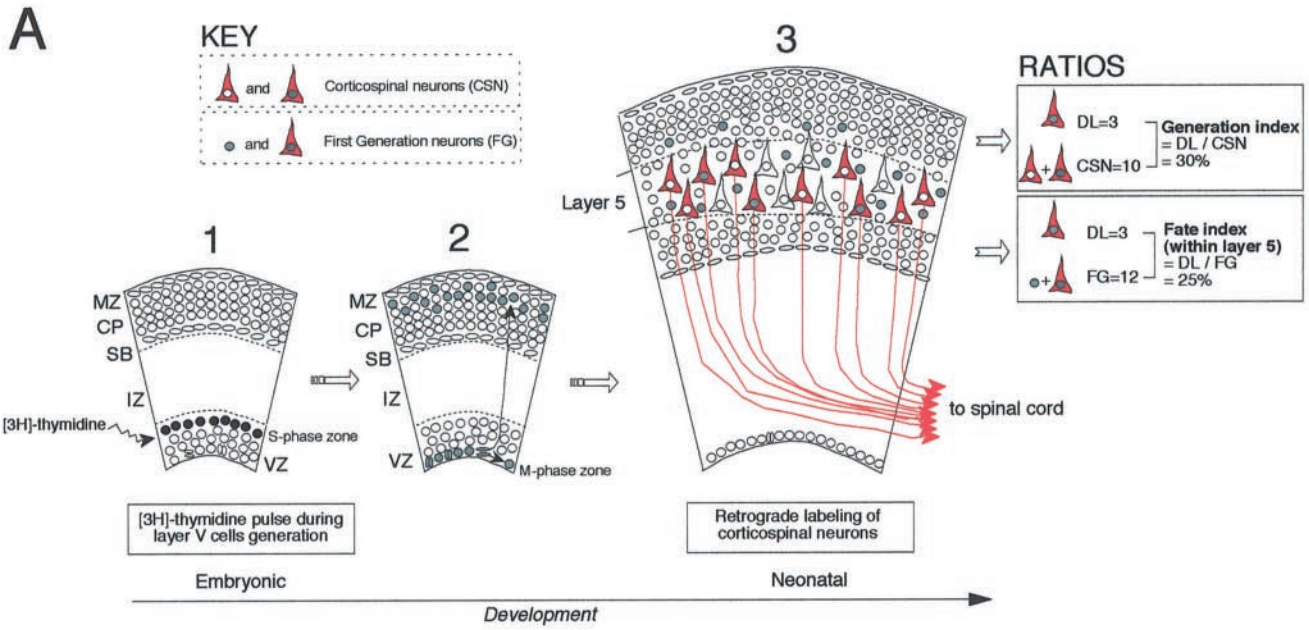
The percentage of FG neurons within a given population of adult neurons defines a *generation rate* which quantifies the rate of neuron production of that population (Carter-Dawson and LaVail, 1979; Mustari *et al.*, 1979; Reznikov, 1990; Vaccarino *et al.*, 1995; Valverde *et al.*, 1995; Polleux *et al.*, 1997b) (see Fig. 1*A*). The generation rate of the CSN population is determined by the number of CSNs that are FG neurons (i.e. double-labeled neurons – DL) and expresses this number as a percentage of the total CSN population. DL/CSN describes the relative rate at which the daughter cells of neuroblasts undergoing their final mitosis are incorporated into the CSN population. More precisely, changes in DL/CSN reflect the changes in labeling index (LI, i.e. percentages of [^{3}H]thymidine-labeled precursors) in the ventricular zone *and* in frequencies of differentiative divisions of the cohort of precursors that generate the CSN population (Schultze *et al.*, 1974; Schultze and Korr, 1981; Miller, 1988; Reznikov, 1990; Polleux *et al.*, 1997b). Given the constancy of S-phase, variation of the LI in the ventricular zone reflects changes in the duration of the cell cycle (Kaufmann, 1968; Waechter and Jaensch, 1972; Hoshino *et al.*, 1973; Schmahl, 1983). Because differences in numbers of precursors do not contribute to variations of DL/CSN, this means that variation of this ratio reflects changes in rates of neuron production due to modulation of cell-cycle parameters (cell-cycle duration and mode of division) (Polleux *et al.*, 1997b). Importantly, DL/CSN is independent of the size of the precursor pool.

The probability that a neuron born on a specific date forms and maintains a projection to the spinal cord is determined by the ratio of DL/FG neurons, which is defined as the fate index (Polleux *et al.*, 1998) (see Fig. 1*A*,*B*).

Areal and Laminar Boundaries in the Neonate

Cytochrome oxidase histochemistry in the post-natal day (PND) 6 neonate reveals a well defined primary sensorimotor cortex (Fig. 2*A*). At this age the barrel field has clearly defined borders and individual barrels are present. The present study critically requires that the counts of birthdated neurons are accurately performed in areas 3 and 6. This requires a method of positive identification of areas 3 and 6 in the neonates and adults in 10 μm thick paraffin sections (see Materials and Methods). The location of areal boundaries on such sections is achieved by tracing out the profiles of all neurons encountered in a 250 μm wide stripe of cortex using the image analysis system (see Methods). This procedure reveals the laminar organization of the cortex and shows that areas 3 and 6 in the neonate can be reliably distinguished as shown in Figure 2*B*,*C*. While some indication of areal identity can be obtained from observation of pooled neuronal profiles (far left panel, Fig. 2*B*,*C*), areal and laminar localization is further enhanced by allocating profiles to one of six categories according to soma size (right-hand side panels in Fig. 2*B*,*C*). This shows that in the neonate, area 3 has a well developed layer 4, which contrasts with a very thin layer 4 in area 6 (compare panels 2 in Fig. 2*B*,*C*) (Morin and Beaulieu, 1994). The large pyramidal neurons of layer 5 are more numerous in area 6 than in area 3 (compare section 5 in Fig. 2*B*,*C*). This procedure enabled us to make our analysis of labeling in the neonate in identified areas which validates the inter-areal comparisons.

Single and double-labeled neurons can be readily identified in the 10 μm thick sections by making simultaneous observations under fluorescent- and bright field illumination (Fig. 3*C*). In the present experiments, the exposure time for the autoradiographic procedure was chosen in order to standardize maximum grain counts at ~80 per profile (Fig. 3*C*). Elsewhere we have shown that neurons with >50% maximum labeling correspond to the first generation (FG) neurons leaving the cell cycle only one division following the [^{3}H]thymidine pulse (Polleux *et al.*, 1997a,b). Thus this double labeling method allows the identification of CSNs that have undergone their final division on the date


A
KEY
and
Corticospinal neurons (CSN)
and
First Generation neurons (FG)
1
2
3
MZ
CP
SB
IZ
VZ
[3H]-thymidine
S-phase zone
M-phase zone
Layer 5
to spinal cord
RATIOS
DL=3
CSN=10
Generation index
= DL / CSN
= 30%
DL=3
FG=12
Fate index
(within layer 5)
= DL / FG
= 25%
[3H]-thymidine pulse during
layer V cells generation
Retrograde labeling of
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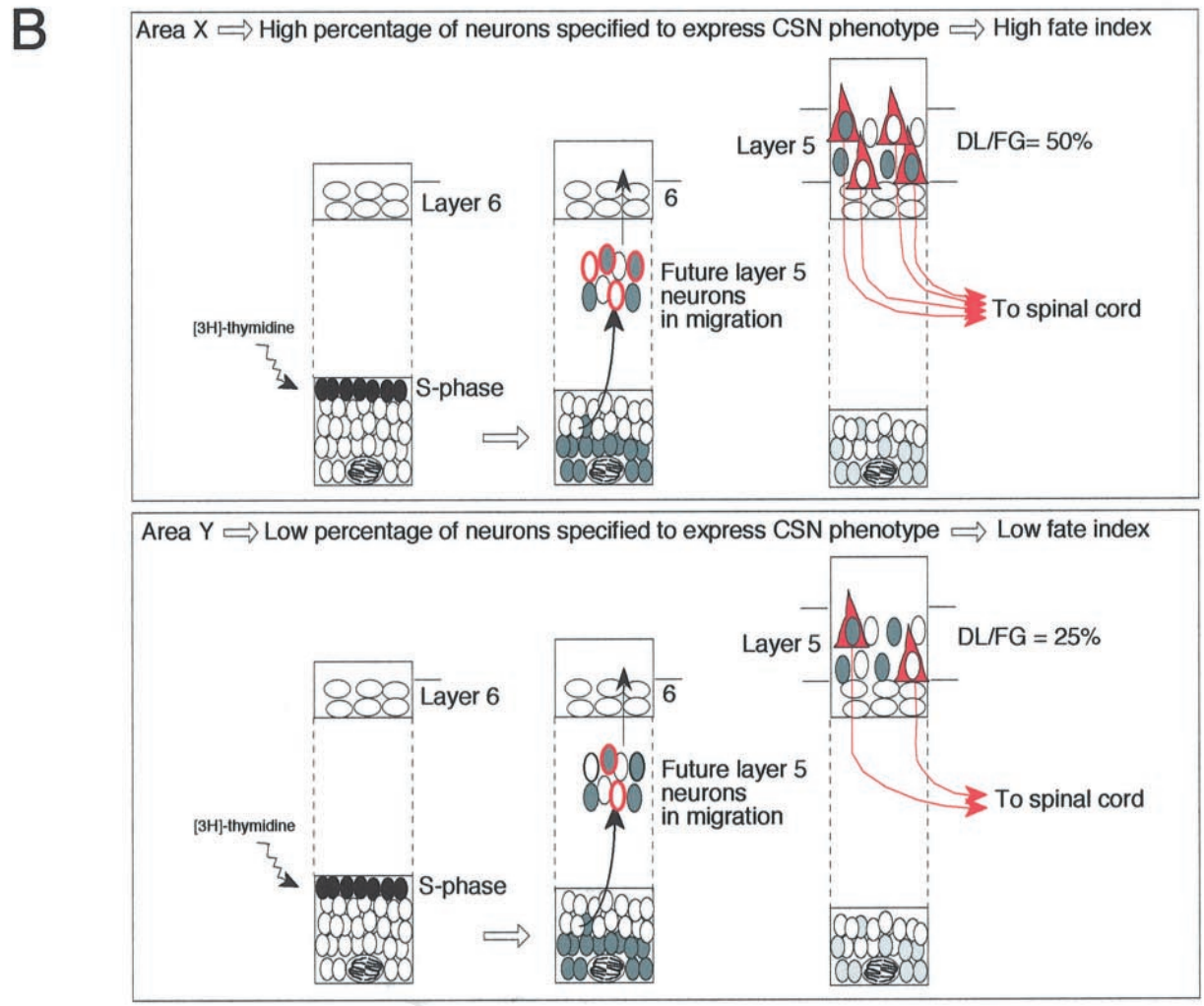
B
Area X ⇨ High percentage of neurons specified to express CSN phenotype ⇨ High fate index
Layer 6
6
Layer 5
DL/FG= 50%
Future layer 5
neurons
in migration
To spinal cord
[3H]-thymidine
S-phase
Area Y ⇨ Low percentage of neurons specified to express CSN phenotype ⇨ Low fate index
Layer 6
6
Layer 5
DL/FG = 25%
Future layer 5
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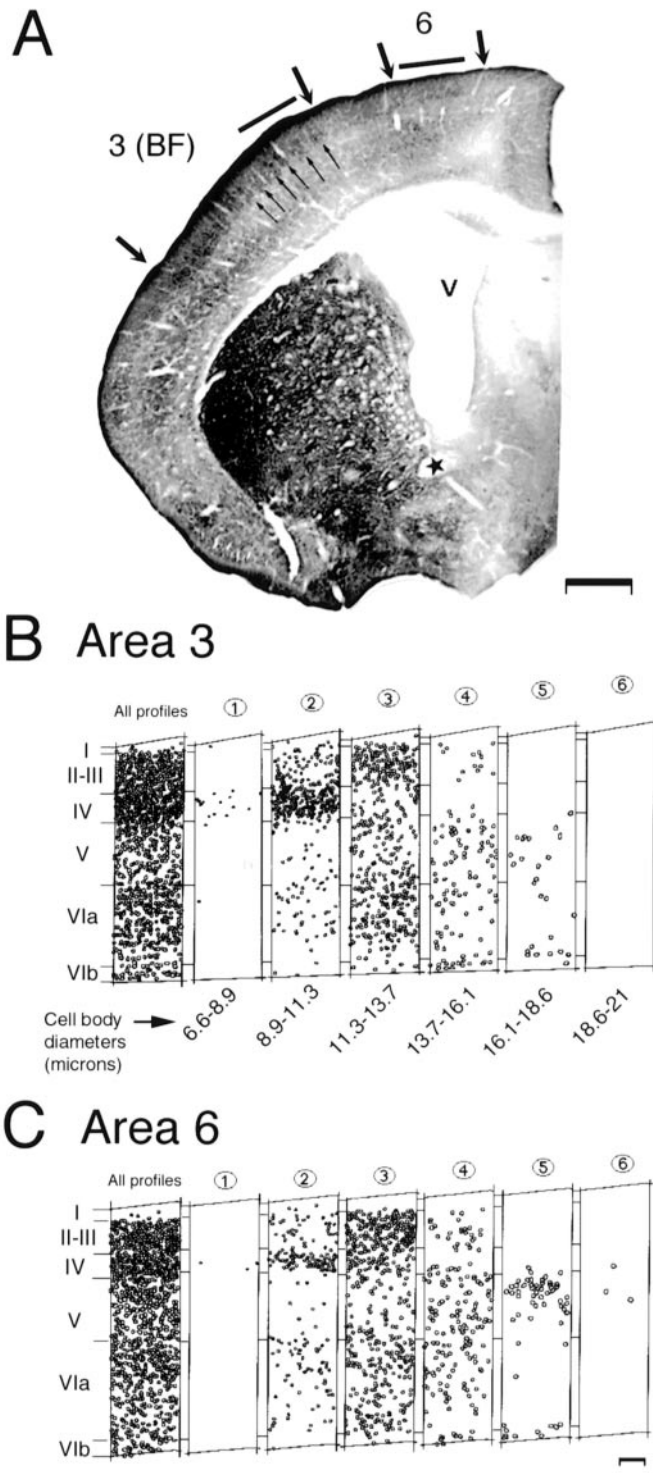

of the [^{3}H]thymidine pulse (Polleux *et al.*, 1998) (double-labeled neurons DL, see Fig. 3*C*).

Early Areal Differences of CSN Density

Injections performed at PND4 reveal a CSN distribution stretching from the medial limit of the cingulate cortex across to the pyriform cortex (Fig. 4*A*). High labeled cell densities as well as the immature widespread distribution are maintained at PND6 (Fig. 4*B*). Following injection at PND8, however, CSNs in the cingulate cortex and in the lateral part of area 3 are less numerous than at PND6, and in the adult these regions are devoid of CSNs (Fig. 4*C*,*D*). These observations suggest that the peak of corticospinal projection occurs around PND6, in line with earlier studies in rat (Bates and Killackey, 1984; O'Leary and Stanfield, 1985; Joosten *et al.*, 1987; Schreyer and Jones, 1988a,b; Joosten and van Eden, 1989; Oudega *et al.*, 1994) and mouse (Uematsu *et al.*, 1996; Gianino *et al.*, 1999). Because PND6 injections are performed after the completion of target invasion and prior to noticeable elimination of connections, they are expected to label optimally the CSN population.

Our quantitative analysis shows that in normal mouse cortex at PND6, the areal density of CSNs in area 6 is nearly twice that in area 3 (Fig. 5*A*,*B*,*E*; $P < 0.001$, Mann–Whitney test). During late postnatal development there is a reduction of areal density that is non-significantly different between area 6 (–41%) compared with area 3 (–27%) (Fig. 5*E*). These results show that there are significant areal differences in CSN number in the neonate. The postnatal decrease in the CSN density in normal and *reeler* is greater in area 6 than in area 3 (Fig. 5, see also Fig. 7). This suggests that there is an area-specific decrease in CSN density. However, it needs to be pointed out that this decrease reduces areal differences and does not create them.

The differences of CSN density between area 6 and 3 are conserved in the *reeler* mouse newborn ($P < 0.001$, Mann–Whitney test) and adult cortex ($P < 0.05$, Mann–Whitney test) (Fig. 5*C*–*E*). Importantly, the density of CSNs in *reeler* newborn is largely increased compared with normal both in area 6 (40% increase; $P < 0.001$, Mann–Whitney test) and in area 3 (52% increase; $P < 0.001$, Mann–Whitney test).

The measurement of total cortical volume in normal newborn (mean ± SD: 18.56 ± 0.41 mm^3; $n = 3$) and *reeler* newborn

Figure 2. Morphological features of frontal and parietal areas in the early postnatal mouse cortex allows accurate determination of areal boundaries. (*A*) Low-power microphotograph showing the location of the barrel field (or primary somatosensory cortex area 3) and motor cortex (area 6) on a coronal section of a PND6 mouse brain processed for cytochrome oxidase. (*B–C*) Analysis of the cytoarchitecture of parietal area 3 (*B*) and frontal area 6 (*C*) at PND6. Cell bodies stained with cresyl violet were drawn (all profiles left panel). The following six panels show the radial distribution of neurons according to their cell body apparent diameter (see Materials and Methods for details). Scale bars: (*A*) 500 μm, (*B*) 100 μm. Abbreviations: v: lateral ventricle, BF barrel field.

Figure 1. (*A*) Schematic representation of the paradigm used in the present study. Panels 1–3 represent successive developmental stages: (1) Single injections of [^{3}H]thymidine during cortical neurogenesis label precursors of layer 5 in the ventricular zone. (2) As shown previously (see text), progenitors that undergo only one division after the pulse give rise to a cohort of post-mitotic neurons characterized by a maximum autoradiographic labeling (gray dots — first generation, FG) which migrate to the cortical plate. (3) Among differentiating layer 5 neurons, a subpopulation projects to the spinal cord and is back-labeled by an injection of a retrograde fluorescent tracer (red — CSNs). [^{3}H]Thymidine injections spanning the generation of layer 5 followed by back-labeling from the spinal cord are used to compute two ratios. The *generation index* (DL/CSN) reflects the proportion of CSNs born on the day of [^{3}H]thymidine injection. This ratio is used to determine the timetable of layer 5 formation. The fate index (DL/FG) reflects the probability for a neuron born on a given day to project to the spinal cord. (*B*) Significance of areal differences in the fate index. Neuronal phenotype is thought to be specified at the level of the ventricular zone (Caviness, 1982; Jensen and Killackey, 1984; McConnell, 1988; Barbe and Levitt, 1991; McConnell and Kaznowski, 1991; Arimatsu *et al.*, 1992; Ferri and Levitt, 1993; Barbe and Levitt, 1995; Frantz and McConnell, 1996; Polleux *et al.*, 1998), during cell cycle progression (McConnell and Kaznowski, 1991; Arimatsu *et al.*, 1999a,b). Thus a putative areal difference in the proportion of neurons specified to project to the spinal cord (red circle, migrating neurons) will be transcribed into a difference in the DL/FG ratio (compare DL/FG in areas X and Y). Abbreviations: CP,cortical plate; IZ, intermediate zone; MZ, marginal zone; SB, subplate.

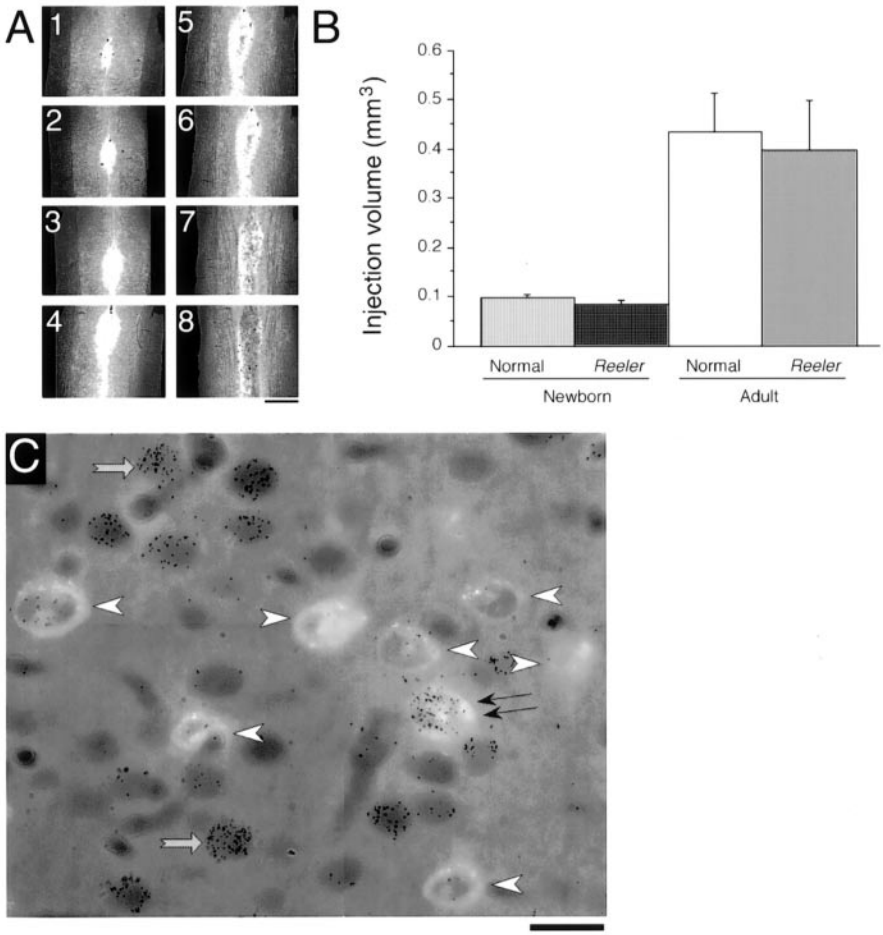

Figure 3. (*A*) Photomicrographs of a representative series of horizontal sections (spacing 120 μm) of cervical spinal cord [from dorsal (1) to ventral (8) level of section] illustrating a Fluorogold injection site in an adult normal mouse. (*B*) Histogram showing the mean Fluorogold injection volumes computed using the Cavalieri's formula (see Materials and Methods) in four animals of each genotype (normal and *reeler*) and each age group (newborn and adult). No statistical difference is detected between normal and *reeler* at each ages (see Results for details). (*C*) Photomicrograph illustrating Fluorogold and [^{3}H]thymidine single- and double-labeled neurons. Combining fluorescence and bright field illuminations reveals [^{3}H]thymidine (first generation, FG) single-labeled neurons (shaded arrows), Fluorogold single-labeled neurons (arrowheads) and double-labeled thymidine–Fluorogold neurons (double arrow). Scale bars: (*A*) 250 μm, (*C*) 20 μm.

(18.20 ± 2.27; n = 3) revealed no statistically significant difference according to an ANOVA analysis (PLSD Fisher test, P = 0.883). Therefore, the observed differences in CSN densities between normal and *reeler* cortex are not the reflection of differences of total cortical volume that would artefactually affect the estimation of cell packing density.

Finally, in order to rule out possible differences in the size and position of Fluorogold injections, we systematically checked the spinal cord injection sites and estimated the volume occupied by the tracer using Cavalieri's principle (Cavalieri, 1966) applied to serial sections (see Materials and Methods; Fig. 3*A*). We found no statistical differences in the average injection volumes between normal and *reeler* in the newborn (n = 8, P > 0.27, Mann–Whitney test; Fig. 3*B*) or in the adult injections (n = 8, P > 0.50, Mann–Whitney test; Fig. 3*B*).

Areal Timetable of CSN Generation — Normal Cortex

Since postnatal regressive phenomena are not responsible for establishing adult CSN areal differences in density an alternative possibility is that areal differences are the consequence of CSNs being generated over a longer time period in area 6 than they are in area 3. To investigate this issue we have examined the incorporation of FG neurons (i.e. neurons that underwent their final round of mitosis shortly after the [^{3}H]thymidine pulse) into the CSN population.

Successive injections of [^{3}H]thymidine reveal the inside-first outside-last histogenic gradient of cortical layers (Fig. 6*A*). Quantification of the percentage of DL neurons with respect to the population of CSNs defines the generation rate (Carter-Dawson and LaVail, 1979; Mustari *et al.*, 1979; Reznikov, 1990; Valverde *et al.*, 1995; Polleux *et al.*, 1997b) (Fig. 6*B*). Although DL/CSN is expected to be immune to developmental regressive events (since cell death or axon elimination will influence identically both terms of the fraction), this index needs to be defined in the neonate, since a transient population of CSNs generated at either early or late stages of development can not be excluded. FG neurons returned from the E13.5 pulse are concentrated in layer 6b and a few are found at the bottom of layer 5. At this stage in both areas ~3% of the CSN population are FG neurons, indicating that generation of the CSN population is initiated simultaneously in both areas. By E14.5, there is an increase in the generation rate of CSNs and this rate of increase is greater in area 3 than in area 6. Hence, while the overall numbers of CSNs are lower in area 3 compared with area 6, pulse injections of [^{3}H]thymidine at E14.5 double-labeled larger numbers of CSNs in 3 compared with 6. At E15.5 CSN production in area 6 is greatly

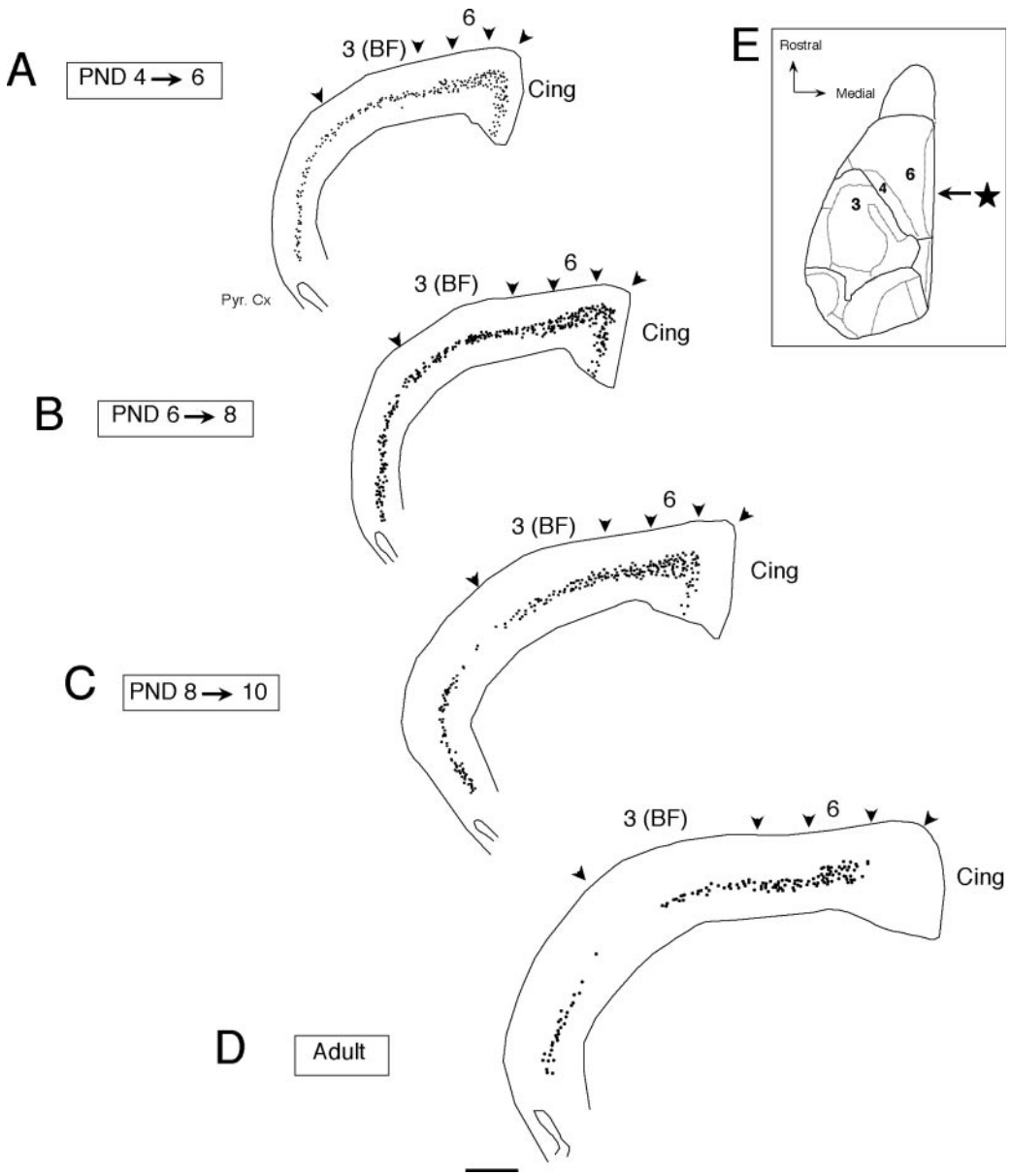

Figure 4. Developmental changes of the distribution of CSNs in mouse fronto-parietal cortex. The date of tracer injection and perfusion are respectively indicated on the left-hand side of each plot. The relative level of section is the same as in Figure 2 and is indicated in the inset on a dorsal view of the mouse cortical areas map adapted from Caviness (Caviness, 1975). Scale bars: 500 μm. Abbreviations: Cing, cingulate cortex; BF, barrel field; Pyr. Cx, pyriform cortex.

increased compared with area 3. This illustrates the use of the generation index to describe the timetable of CSN production.

By E16 the production of CSNs is terminated in both areas. These results show negligible differences in the profiles of CSN generation rate calculated in the adult and neonate, confirming that regressive phenomena do not influence the generation index (Polleux *et al.*, 1998). This means that the adult values obtained with eight different time points (E12.5, E13.5, E14, E14.5, E15.5, E16, E16.5, E17.5) provide a high temporal resolution of the timetable of CSN production (Polleux *et al.*, 1998). The important finding is that both the onset and duration of CSN generation is identical in the two areas.

This result provides further evidence that the timing of the final mitosis is an important constraint in the determination of cortical neuron phenotype (Caviness and Sidman, 1973; Caviness, 1982; McConnell, 1988; McConnell and Kaznowski, 1991; Arimatsu *et al.*, 1992, 1994; Frantz and McConnell, 1996; Polleux *et al.*, 1998). However, a single [^{3}H]thymidine pulse leads to a relatively wide vertical spread of FG neurons across several cortical layers (Fig. 6*A*). At later stages of corticogenesis occasional FG neurons take up a deep position and we have investigated whether those late generated neurons that occupy layer 5b can form projections to the spinal cord albeit with a reduced frequency. We examined large numbers of FG neurons resulting from a [^{3}H]thymidine pulse on E16 and which occupy layer 5b in the neonate. This showed that there was 0% double-labeling in both areas (n = 163 FG neurons, 27 sections). Together these results show that timing of the final mitosis is critically important for the specification of cortical neuron phenotype.

Areal Differences in Fate Index — Normal Cortex

The percentage of FG neurons that are retrogradely labeled from the spinal cord (fate index — see Fig. 1) indicates the probability

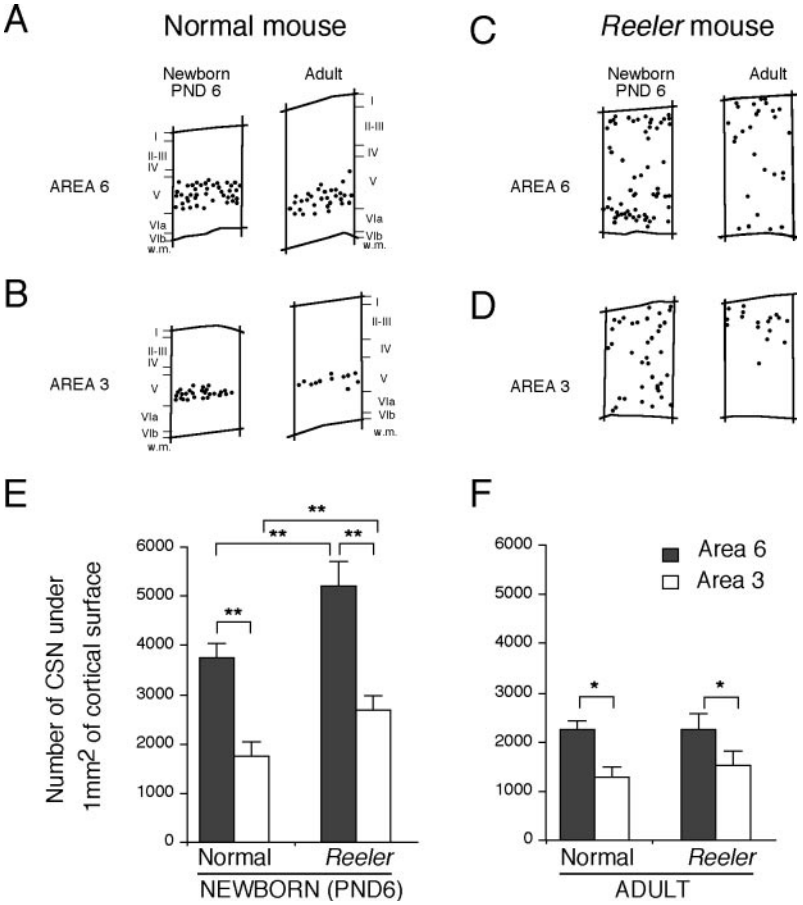

Figure 5. Early differences of CSN areal density in areas 6 and 3. (*A*, *B*) Higher magnification plot of the CSN distributions in normal newborn (PND 6; left panel) and (*C*, *D*) *reeler* in areas 6 and 3. (*E*, *F*) Quantitative analysis of the areal density of CSNs in neonate (PND 6; *E*) and adult (*F*) areas 6 and 3. Error bars indicate standard error to the mean. $*P < 0.05$, $**P < 0.01$, according to a Mann–Whitney non-parametric test.

of a neuroblast of a given birthdate acquiring the CSN phenotype. In Figure 6*C* the fate index for each time point is calculated from a minimum of 12 sections taken from two or three different pups (two pups from one litter injected at E13.5 and E16 and three pups taken randomly from two different litters injected at E14.5). At E13.5 both areas return identical fate indices (χ^2 = 0.41; d.f. = 1, $P > 0.50$). At E14.5 there is a substantial increase in the fate indices in both areas and importantly the rate of increase in fate index is substantially greater in area 6 compared with area 3 (χ^2 = 8.23; d.f. = 1, $P < 0.01$). This result indicates that at E14.5 the probability that a neuroblast undergoing its final round of mitosis acquires the CSN phenotype is significantly greater in area 6 compared with area 3 (Fig. 6*C*). These areal differences in DL/FG are not likely to be the expression of a mitogenic gradient because elsewhere we have shown that the areal differences in cell-cycle characteristics are not part of a continuum but are area-restricted (Polleux *et al.*, 1997a,b).

DL/FG is sensitive to late elimination of corticospinal axons by pruning (Bates and Killackey, 1984; O'Leary and Stanfield, 1985; Joosten *et al.*, 1987; O'Brien *et al.*, 1987; Joosten and van Eden, 1989; O'Leary and Stanfield, 1989; Oudega *et al.*, 1994). Postnatal decrease in DL/FG values reflect the extent of axon elimination, making it possible to examine whether selective elimination of CSN axons contributes to the adult differences in CSN number. In fact the postnatal decrease in DL/FG is higher (51%) in area 6 than in area 3 (10%) showing that this regressive factor, although area specific, actually reduces the initial areal differences in CSN number observed in the adult (Fig. 7*B*).

Influence of the Cortical Environment on CSN Fate — DL/FG in Reeler Cortex

So far the results show that there is a regionalization of cell fate that contributes to the specification of areal differences in CSN number. Does the commitment to a CSN fate take place entirely in the ventricular zone during the final mitosis? If not, does the cortical environment also contribute to the determination of CSN phenotype? Because the *reeler* shows a highly disorganized cortex (Caviness and Sidman, 1973; Caviness, 1977; Caviness and Rakic, 1978; Terashima *et al.*, 1983, 1985; Inoue *et al.*, 1991), the difference in cell fate index between normal and *reeler* would indicate that the cortical environment does contribute to cell-fate decisions. Note that because layering is absent in the *reeler* mutant the counts of DL/FG used to compare normal and *reeler* neonate are based on FG neurons throughout the full thickness of the cortex. This shows that the nearly 40–50% increase in CSN number in *reeler* compared with normal neonates (Fig. 6) is accompanied by a 55–58% increase in DL/FG ratio both in area 6 (χ^2 = 7.21; d.f. = 1, $P < 0.01$; Fig. 7*A*) and in area 3 (χ^2 = 8.43; d.f. = 1, $P < 0.01$; Fig. 7*A*).

The massive increase in the CSN number and fate index in the *reeler* mutant provides a challenge to the postnatal pruning of CSN axons that occurs during normal development. In the *reeler*, the postnatal decrease in DL/FG is area-specific as in the

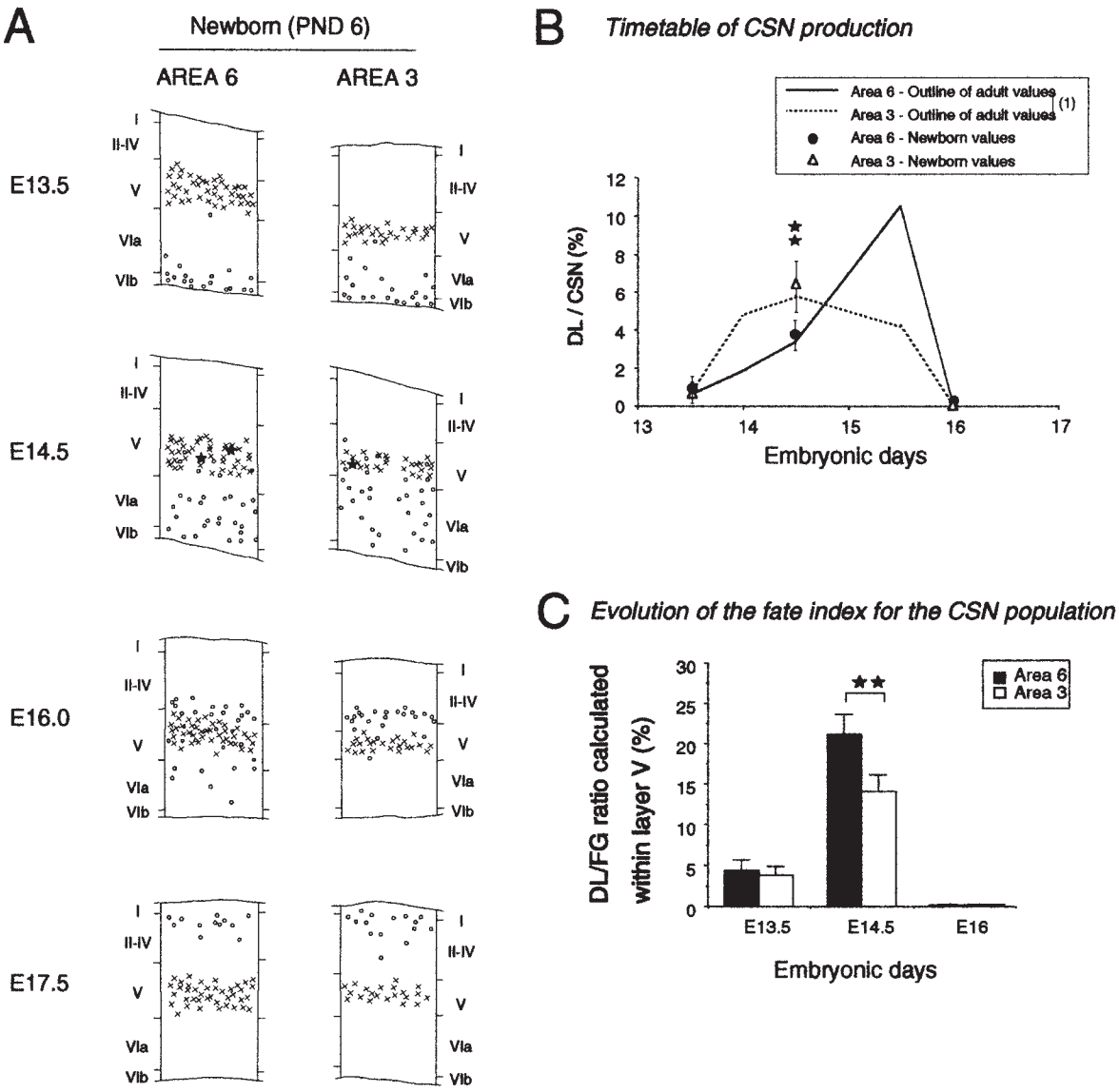

Figure 6. Timetable of CSN production and areal differences in specification of CSN phenotype. (*A*) In the neonate, [^{3}H]thymidine pulses on E13.5, 14.5, 16.0 and 17.5 reveal cohorts of neurons that undergo their final mitosis before, during and after the generation of CSNs. [^{3}H]Thymidine-labeled (first generation) neurons are represented by circles and Fluorogold-positive CSNs by crosses. First-generation CSNs (i.e. double-labeled neurons — see text) are represented by stars. (*B*) Developmental profile of the percentage of double-labeled neurons calculated among the population of CSNs (DL/CSN) in areas 6 and 3. [(1)]Outline of DL/CSN adult values adapted from Polleux *et al.* (Polleux *et al.*, 1998). (*C*) In neonate, the percentage of double-labeled CSNs calculated among the population of first-generation (FG) neurons (DL/FG) located in the sub-layer containing the CSNs (layer 5b). $^{**}P < 0.01$, according to a χ^2 analysis.

normal, but is considerably higher than in the normal (–22% in *reeler* area 3 versus –10% in normal area 3 and –84% in *reeler* area 6 versus –51% in normal area 6; Fig. 7*B*,C), suggesting that axon elimination is a developmental adjustment that serves to counterbalance excess generation of CSNs.

Discussion

Significance of Indices with Respect to Cell-cycle Parameters

DL/CSN and DL/FG provide complementary information on the mitotic history of the population of CSN precursors. Variations of the generation index (DL/CSN ratio) mainly reflect variations of the dynamics of cell-cycle parameters underlying the production of a given phenotype (Polleux *et al.*, 1997b, 1998). In the present study, we have restricted the use of DL/CSN to estimate quantitatively the timetable of CSN production. In contrast, changes in DL/FG are not related to changes in the kinetics of proliferation. At early stages of postnatal development, prior to the phase of axon elimination, DL/FG reflects the fraction of neuroblasts born on a given date that acquires a CSN phenotype and can be thought of as a probability factor governing cell fate (Polleux *et al.*, 1998). During the period of peak production of layer 5b, DL/FG is ~30% higher in area 6 compared with area 3.

Cell Fate and Axon Elimination — Normal Cortex

The present results show that during normal development there are important areal differences in cell fate that correlate with differences in CSN number. This indicates that regionalization of cell fate contributes to determining the inter-areal differences in CSN phenotype in the adult. McConnell and Kaznowski challenged laminar identity by performing heterochronic grafts

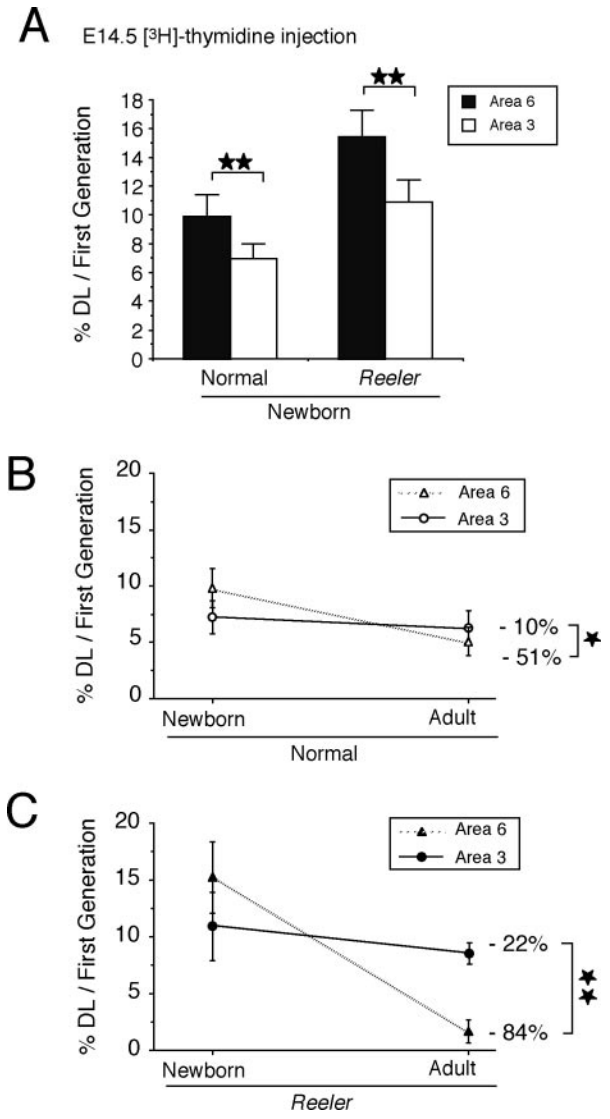

Figure 7. Postnatal changes in DL/FG in areas 6 and 3 of normal and *reeler* cortex. (*A*) Percentage of double-labeled CSNs calculated among the population of FG neurons computed throughout the full thickness of the cortex in normal neonates (PND 6) and adults. (*B*, *C*) Percentage of double-labeled CSNs calculated among the population of FG neurons computed throughout the full thickness of the cortex in normal (*B*) and *reeler* (*C*) newborn (PND 6) and adult mice. Error bars indicate standard error to the mean. $^{*}P < 0.05$ and $^{**}P < 0.01$, according to a χ^2 analysis.

(McConnell and Kaznowski 1991). Neuroblasts which were destined to migrate to layer 5 continued to do so when grafted into an older host which is generating supragranular layers. This work demonstrated that signals encountered in the ventricular zone during S-phase play a role in cell-fate determination, suggesting that an important aspect of cell fate is determined at the final mitosis.

DL/FG is exquisitely sensitive to loss of CSN axons and so during postnatal development can be used to monitor refinement of the CSN pathway. During normal postnatal development we found that the decrease in DL/FG was significantly different in the two somatomotor areas — whereas there is a 51% reduction in DL/FG in area 6 there was only a 10% reduction in area 3. These findings show that although the magnitude of axon elimination is area-specific, this phenomenon does not contribute to creating the areal difference in CSN number. Previous studies have shown regional elimination of axon collaterals (O'Leary *et al.*, 1981; O'Leary and Stanfield, 1985; O'Leary and Terashima, 1988) and here we show that it can be area-specific. Because the rate of axon elimination is different in neighboring areas 3 and 6, and because it reduces the areal differences, it would seem that axon elimination could serve to finely tune numbers of projection neurons within a given cortical area.

Cell Fate and Axon Elimination — Reeler Cortex

The *reeler* mutant provides an alternative experimental paradigm to the heterochronic transplants used by McConnell for challenging cell fate during corticogenesis. The possibility that the environment of the cortex contributes to the specification of phenotype is directly addressed by birthdating experiments in the *reeler* (Caviness and Sidman, 1973; Caviness, 1982; Polleux *et al.*, 1998). These experiments show that in the absence of cortical lamination, the timing of the final mitosis continues to determine the CSN phenotype as stringently as in the normal cortex (Polleux *et al.*, 1998). The importance of the timing of the final mitosis, however, has to be reconciled with the fact that the radial distribution of FG neurons in the normal cortex from a pulse at E14.5 is considerably wider than layer 5b, suggesting either that the ventricular zone at a given moment is generating a range of phenotypes destined for different layers (Price and Thurlow, 1988; Fishell *et al.*, 1990; Walsh and Cepko, 1992; Luskin *et al.*, 1993; Kornack and Rakic, 1995) or that FG neurons that do not terminate in layer 5b are inhibited from forming a projection to the spinal cord. This last possibility would mean that the cortical environment contributes to specifying the CSN phenotype.

If the cortical environment contributes to specifying CSN fate then one would predict that CSN number should be modified in the perturbed environment of the *reeler* mutant. This in fact is the case, the increase in CSNs is accompanied by an increase in DL/FG, indicating that the increase in CSNs in the mutant is the result of a fate change and is not due to some other mechanisms such as an increased survival or reduced elimination. Although the contribution of the cortical environment to the specification of cell fate is clearly established by the present results in the *reeler*, we do not know if the cortex exerts its influence post-migratory, or whether it signals down to the ventricular zone and influences cell fate during mitosis. Such a possibility would be in line with studies suggesting that post-migratory cortical neurons exert a feedback control on neurogenesis as has been suggested in the retina (Reh, 1987; McConnell and Kaznowski, 1991; Gillies and Price, 1993; Polleux *et al.*, 1998).

If the normal cortical environment outside of layer 5b inhibits formation of projections to the spinal cord (consistent with the fact that FG neurons resulting from a pulse injection of [^{3}H]thymidine at E14.5 have a wider radial distribution than the CSN population) then the removal of this inhibition in the non-laminated cortex found in *reeler* could lead to the observed increase of DL/FG values found in both areas of the mutant. In normal cortex, the present finding that the extremely rare late FG neurons that come to occupy layer 5b fail to project to the spinal cord, even at a very low frequencies, shows that whereas the timing of the final mitosis is a necessary factor governing cell fate, laminar location is not a sufficient one. This result shows that birthdates discriminate between subclasses of layer V neurons.

The fact that the final cellular environment can contribute to the specification of neuronal connectivity is supported by recent results showing that in the developing spinal cord, the appropriate specification of the phenotype of late-generated motor neurons pool depends on the interaction they perform

while migrating through previously generated motor neurons (Sockanathan and Jessell, 1998). Our results in the *reeler* are compatible with the fact that early-born neurons can regulate the expression of the connectional phenotype of later-generated neurons through cell–cell interactions.

Theories of Areal Specification and Numerical Control of Connectional Phenotype

The present study sets out to explore if the connectional phenotype of cortical neurons is specified according to similar mechanisms as other phenotypic traits such as the neurotransmitter or the laminar phenotype. Recently, it has been shown that in the developing spinal cord, the connectivity of motor neurons is specified early at the level of dividing progenitors by the expression of specific subsets of transcription factors by inductive cues that restrict the competence of precursors to produce a limited set of neuronal phenotypes [reviewed by Edlund and Jessell (Edlund and Jessell, 1999)]. However, it remains to be determined if the developmental mechanisms governing the acquisition of neuronal phenotype in the spinal cord can be applied to higher order, laminated structures of the forebrain such as the cerebral cortex.

There are two long-standing views of cortical development that are relevant to understanding area-specific connectional fate. Protomap theory views the neuroepithelium as being regionalized prior to the arrival of the thalamic afferents (Rakic, 1988). Protocortex theory views the early formed cortex as having a wide developmental potential (O'Leary, 1989). Two recent studies using *in vitro* approaches were able to show that while there is early regionalization of molecular markers expression prior to arrival of thalamic afferents, environmental signals continue to contribute to the acquisition of the molecular phenotype in the post-mitotic population (Arimatsu *et al.*, 1999a,b; Gitton *et al.*, 1999b). In both studies late environmental signals were found to influence the extent that the early specified molecular phenotype is expressed. The studies of Arimatsu *et al.* and Gitton *et al.* (Arimatsu *et al.*, 1999a,b; Gitton *et al.*, 1999b) are conceptually important on several accounts. Firstly, they reconcile protomap and protocortex theories and provide the conceptual basis required by the fact that there is extensive mixing of cortical precursors (Walsh and Cepko, 1992; Fishell *et al.*, 1993; O'Rourke *et al.*, 1995; Tan *et al.*, 1995) and an early regionalization of the cortex at least with regard to the expression of molecular markers (Barbe and Levitt, 1991; Arimatsu *et al.*, 1992; Cohen-Tannoudji *et al.*, 1994; Miyashita-Lin *et al.*, 1999; Gitton *et al.*, 1999a; Bishop *et al.*, 2000). The present results provide *in vivo* evidence for a similar progressive specification of a cardinal feature of neuronal identity, i.e. the efferent connectivity. The areal differences in CSN cell fate at E14.5 bare witness to early regionalization prior to the arrival of thalamic afferents (E14.5). This early regionalization of cell fate can be challenged by the perturbed cortical environment of the *reeler*, which the present study shows, leads to a radical increase in CSN fate index.

The progressive acquisition of CSN phenotype reconciles apparently contradictory findings in the literature. Fetal heterotopic grafting experiments of somatosensory cortex originally were thought to show that regional locus in the host determined the connectional CSN phenotype (O'Leary and Stanfield, 1989). However, quantification of results showed that heterotopically transplanted neurons retain some of the connectional features corresponding to their site of origin (Ebrahimi-Gaillard *et al.*, 1994; Ebrahimi-Gaillard and Roger, 1996). One could expect that the environmental signals in the heterotopic graft would lead to reduced levels of acquisition of the phenotype so that while it remains true that site of origin determines the connectional phenotype, signals in the final location will modulate the expression of this phenotype.

The large excess of CSNs in the neonatal *reeler* allows us to investigate the control mechanism underlying the postnatal elimination of axons from the corticospinal pathway. The postnatal decrease of DL/FG is greatly increased in *reeler* compared with normal and the decrease is area-specific, being significantly larger in area 6 compared with area 3. One consequence of this is that the final numbers of CSNs in adult *reeler* and normal mice are actually very similar (Fig. 5*E*). These findings suggest that the elimination of axons during postnatal development reflects an adaptive process that allows the numerical value of CSN in different areas of the cortex to be adjusted to the physiological needs of the developing organism. We do not exclude that cell death can also play a role in the acquisition of area-specific number of CSNs. In fact, the role of cell death has been already well documented in the control of cell number elsewhere in the nervous system (Williams and Herrup, 1988; Finlay and Pallas, 1989; Finlay, 1992). However, our results in the normal and in the *reeler* show that the final numbers of CSNs are specified by an early two-step cell-fate determination as well as by late elimination of corticospinal axons during postnatal development.

Conclusion

Our results underscore that laminar and areal specification are interrelated (Eagleson *et al.*, 1997; Polleux *et al.*, 1997a,b). They provide *in vivo* evidence of a progressive specification of connectional phenotype due to signals originating from the ventricular zone, cortical plate and maturing cortex. In this way they suggest that connectional phenotype is determined by early regionalization of the neuroepithelium as well as environmental signals in the post-migratory cortex (Jensen and Killackey, 1984; McConnell, 1988; Rakic, 1988; O'Leary, 1989; O'Leary and Stanfield, 1989; Barbe and Levitt, 1991; McConnell and Kaznowski, 1991; Arimatsu *et al.*, 1992, 1999a,b; Ferri and Levitt, 1993; Gillies and Price, 1993; Gitton *et al.*, 1999a,b). While the mitotic history of CSNs shows that adult values of the number of neurons expressing a given connectional phenotype are achieved by multiple events occurring at the level of the ventricular zone, the cortical plate and in the maturing cortex, there are a number of key events that remain unclear. For example, it is not known whether the component of cell-fate control that is cortical in origin is pre-migratory or post-migratory [as in the studies of Arimatsu *et al.* and Gitton *et al.* (Arimatsu *et al.*, 1999a,b; Gitton *et al.*, 1999b)] or both.

Notes

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References

Abercrombie M (1946) Estimation of nuclear population from microtome sections. Anat Rec 94:239–247.

Appleton TC, Pelc SR, Tarbit MH (1969) Formation and loss of DNA in intestinal epithelium. J Cell Sci 5:45–55.

Arimatsu Y, Miyamoto M, Nihonmatsu I, Hirata K, Uratani Y, Hatanaka Y, Takiguchi-Hayashi K (1992) Early regional specification for a

molecular neuronal phenotype in the rat neocortex. Proc Natl Acad Sci USA 89:8879–8883.

Arimatsu Y, Nihonmatsu I, Hirata K, Takiguchi-Hayashi K (1994) Cogeneration of neurons with a unique molecular phenotype in layer-V and layer-VI of widespread lateral neocortical areas in the rat. J Neurosci 14:2020–2031.

Arimatsu Y, Ishida M, Sato M, Kojima M (1999a) Corticocortical associative neurons expressing latexin: specific cortical connectivity formed *in vivo* and *in vitro*. Cereb Cortex 9:569–576.

Arimatsu Y, Ishida M, Takiguchi-Hayashi K, Uratani Y (1999b) Cerebral cortical specification by early potential restriction of progenitor cells and later phenotype control of postmitotic neurons. Development 126: 629–638.

Barbe MF, Levitt P (1991) The early commitment of fetal neurons to the limbic cortex. J Neurosci 11:519–533.

Barbe MF, Levitt P (1995) Age-dependent specification of the corticocortical connections of cerebral grafts. J Neurosci 15:1819–1834.

Bates CA, Killackey HP (1984) The emergence of a discretely distributed pattern of corticospinal projection neurons. Dev Brain Res 13: 265–273.

Bishop KM, Goudreau G, O'Leary DD (2000) Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science 288:344–349.

Carter-Dawson LD, LaVail MM (1979) Rods and cones in the mouse retina: II. Autoradiographic analysis of cell generation using tritiated thymidine. J Comp Neurol 188:263–272.

Cavalieri B (1966) Geometria degli indivisibili. Turin: Unione Tipographico-Editrice.

Caviness VS (1975) Architectonic map of the neocortex of the normal mouse. J Comp Neurol 164:247–264.

Caviness VS (1976) Patterns of cell and fiber distribution in the neocortex of the *reeler* mutant mouse. J Comp Neurol 170:435–448.

Caviness VS (1977) *Reeler* mutant mouse: a genetic experiment in developing mammalian cortex. Soc Neurosci Symp 2:27–46.

Caviness VS (1982) Neocortical histogenesis in the normal and *reeler* mice: a developmental study based upon [^{3}H]-thymidine autoradiography. Dev Brain Res 4:293–302.

Caviness VS, Rakic P (1978) Mechanisms of cortical development: a view from mutations in mice. Annu Rev Neurosci 1:297–326.

Caviness VS, Sidman RL (1973) Time of origin of corresponding cell classes in the cerebral cortex of normal and *reeler* mice: an autoradiographic analysis. J Comp Neurol 148:141–152.

Clarke PGH (1992) How inaccurate is the Abercrombie correction factor for cell counts? Trends Neurosci 15:211–212.

Cohen-Tannoudji M, Babinet C, Wassef M (1994) Early determination of a mouse somatosensory cortex marker. Nature 368:460–463.

Cragg BG (1975) The development of synapses in the visual system of the cat. J Comp Neurol 160:147–166.

Douglas RJ, Martin KAC (1991) A functional microcircuit for cat visual-cortex. J Physiol Lond 440:735–769.

Eagleson KL, Levitt P (1999) Complex signaling responsible for molecular regionalization of the cerebral cortex. Cereb Cortex 9:562–568.

Eagleson KL, Lillien L, Chan AV, Levitt P (1997) Mechanisms specifying area fate in cortex include cell-cycle-dependent decisions and the capacity of progenitors to express phenotype memory. Development 124:1623–1630.

Ebrahimi-Gaillard A, Roger M (1996) Development of spinal-cord projections from neocortical transplants heterotopically placed in the neocortex of newborn hosts is highly dependent on the embryonic locus of origin of the graft. J Comp Neurol 365:129–140.

Ebrahimi-Gaillard A, Guitet J, Garnier C, Roger M (1994) Topographic distribution of efferent fibers originating from homotopic or heterotopic transplants: heterotopically transplanted neurons retain some of the developmental characteristics corresponding to their site of origin. Dev Brain Res 77:271–283.

Edlund T, Jessell TM (1999) Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell 96:211–224.

Ferri RT, Levitt P (1993) Cerebral cortical progenitors are fated to produce region-specific neuronal populations. Cereb Cortex 3:187–198.

Finlay BL (1992) Cell death and the creation of regional differences in neuronal numbers. J Neurobiol 23:1159–1171.

Finlay BL, Pallas SL (1989) Control of cell number in the developing mammalian visual system. Prog Neurobiol 32:207–234.

Finlay BL, Wikler KC, Sengelaub DR (1987) Regressive events in brain development and scenarios for vertebrate brain evolution. Brain Behav Evol 30:102–117.

Fishell G, Rossant J, Van der Kooy D (1990) Neuronal lineages in chimeric mouse forebrain are segregated between compartments and in the rostrocaudal and radial planes. Dev Biol 141:70–83.

Fishell G, Mason CA, Hatten MY (1993) Dispersion of neuronal progenitors within the germinal zones of the forebrain. Nature 362:636–638.

Frantz GD, McConnell SK (1996) Restriction of late cerebral cortical progenitors to an upper-layer fate. Neuron 17:55–61.

Gianino S, Stein SA, Li H, Lu X, Biesiada E, Ulas J, Xu XM (1999) Postnatal growth of corticospinal axons in the spinal cord of developing mice. Brain Res Dev Brain Res 112:189–204.

Gilbert CD (1983) Microcircuitry of the visual cortex. Annu Rev Neurosci 6:217–247.

Gillies K, Price DJ (1993) The fates of cells in the developing cerebral-cortex of normal and methylazoxymethanol acetate-lesioned mice. Eur J Neurosci 5:73–84.

Gitton Y, Cohen-Tannoudji M, Wassef M (1999a) Specification of somatosensory area identity in cortical explants. J Neurosci 19:4889–4898.

Gitton Y, Cohen-Tannoudji M, Wassef M (1999b) Role of thalamic axons in the expression of H-2Z1, a mouse somatosensory cortex-specific marker. Cereb Cortex 9:611–620.

Heumann D, Leuba G (1983) Neuronal death in the development and aging of the cerebral cortex of the mouse. Neuropathol Appl Neurobiol 9:297–311.

Hoffart RM, Johnston JG, Krushel LA, van der Kooy D (1995) The mouse mutation *reeler* causes increased adhesion within a subpopulation of early postmitotic cortical neurons. J Neurosci 15:4838–4850.

Hoshino K, Matsuzawa T, Murakami U (1973) Characteristics of the cell cycle of matrix cells in the mouse embryo during histogenesis of telencephalon. Exp Cell Res 77:89–94.

Innocenti GM (1995) Exuberant development of connections, and its possible permissive role in cortical evolution. Trends Neurosci 18: 397–402.

Innocenti GM (1981) Growth and reshaping of axons in the establishment of visual callosal connections. Science 212:824–827.

Inoue K, Terashima T, Inoue I (1991) The intracortical position of pyramidal tract neurons in the motor cortex of the *reeler* changes from postnatal day 10 to adulthood. Dev Brain Res 62:146–150.

Jensen KF, Killackey HP (1984) Subcortical projections from ectopic neocortical neurons. Proc Natl Acad Sci USA 81:964–968.

Joosten EA, van Eden CG (1989) An anterograde tracer study on the development of corticospinal projections from the medial prefrontal cortex in the rat. Brain Res Dev Brain Res 45:313–319.

Joosten EAJ, Gribnau AAM, Dederen PJWC (1987) An anterograde tracer study of the developing corticospinal tract in the rat: three components. Dev Brain Res 36:121–130.

Katz LC, Shatz CJ (1996) Synaptic activity and the construction of cortical circuits. Science 274:1133–1138.

Kaufmann SL (1968) Lengthening of the generation cycle during embryonic differentiation of the mouse neural tube. Exp Cell Res 49: 420–424.

Kennedy H, Dehay C (1993) Cortical specification of mice and men. Cereb Cortex 3:27–35.

Kornack DR, Rakic P (1995) Radial and horizontal deployment of clonally related cells in the primate neocortex: relationship to distinct mitotic lineages. Neuron 15:311–321.

Levitt P, Ferri RT, Barbe MF (1993) Progressive acquisition of cortical phenotypes as a mechanism for specifying the developing cerebral cortex. Perspect Dev Neurobiol 1:65–74.

Luskin MB, Parnavelas JG, Barfield JA (1993) Neurons, astrocytes, and oligodendrocytes of the rat cerebral-cortex originate from separate progenitor cells: an ultrastructural analysis of clonally related cells. J Neurosci 13:1730–1750.

McConnell SK (1988) Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. J Neurosci 8:945–974.

McConnell SK, Kaznowski CE (1991) Cell-cycle dependence of laminar determination in developing neocortex. Science 254:282–285.

Meissirel C, Dehay C, Kennedy H (1993) Transient cortical pathways in the pyramidal tract of the neonatal ferret. J Comp Neurol 338: 193–213.

Miller MW (1987) The origin of corticospinal projection neurons in rat. Exp Brain Res 67:339–351.

Miller MW (1988) Development of projection and local circuit neurons in

neocortex. In: Cerebral cortex (Peters A and Jones EG, eds), pp. 133–175. New York: Plenum.
Miyashita-Lin EM, Hevner R, Wassarman KM, Martinez S, Rubenstein JL (1999) Early neocortical regionalization in the absence of thalamic innervation. Science 285:906–909.
Morin F, Beaulieu C (1994) Equivalent cell density in three areas of neonatal rat cerebral cortex. Neurosci Lett 176:85–88.
Mustari MJ, Lund RD, Graubard K (1979) Histogenesis of the superior colliculus of the albino rat: a tritiated thymidine study. Brain Res 164:39–52.
O'Brien TF, Steindler DA, Cooper NGF (1987) Abnormal glial and glycoconjugate dispositions in the somatosensory cortical barrel field of the early postnatal *reeler* mutant mouse. Dev Brain Res 32:309–317.
O'Leary DDM (1992) Development of connectional diversity and specificity in the mammalian brain by the pruning of collateral projections. Curr Opin Neurobiol 2:70–77.
O'Leary DDM (1989) Do cortical areas emerge from a protocortex? Trends Neurosci 12:400–406.
O'Leary DDM, Stanfield BB (1985) Occipital cortical neurons with transient pyramidal tract axons extend and maintain collaterals to subcortical but not intracortical targets. Brain Res 336:326–333.
O'Leary DDM, Stanfield BB (1986) A transient pyramidal tract projection from the visual cortex in the hamster and its removal by selective collateral elimination. Dev Brain Res 27:87–99.
O'Leary DDM, Stanfield BB (1989) Selective elimination of axons extended by developing cortical neurons is dependent on regional locale: experiments utilizing fetal cortical transplants. J Neurosci 9:2230–2246.
O'Leary DDM, Terashima T (1988) Cortical axons branch to multiple subcortical targets by interstitial axon budding: implications for target recognition and 'waiting periods'. Neuron 1:901–910.
O'Leary DDM, Stanfield BB, Cowan WM (1981) Evidence that the early postnatal restriction of the cells of origin of the callosal projection is due to the elimination of axonal collaterals rather than to the death of neurons. Dev Brain Res 1:607–617.
O'Rourke NA, Sullivan DP, Kaznowski CE, Jacobs AA, McConnell SK (1995) Tangential migration of neurons in the developing cerebral cortex. Development 121:2165–2176.
Obst K, Wahle P (1995) Areal differences of NPY mRNA-expressing neurons are established in the late postnatal rat visual cortex *in vivo*, but not in organotypic cultures. Eur J Neurosci 7:2139–2158.
Oudega M, Varon S, Hagg T (1994) Distribution of corticospinal motor neurons in the postnatal rat: quantitative evidence for massive collateral elimination and modest cell death. J Comp Neurol 347: 115–126.
Parnavelas JG, Cavanagh ME (1988) Transient expression of neurotransmitters in the developing neocortex. Trends Neurosci 11:92–93.
Polleux F, Dehay C, Kennedy H (1997a) The timetable of laminar neurogenesis contributes to the specification of cortical areas in mouse isocortex. J Comp Neurol 385:95–116.
Polleux F, Dehay C, Moraillon B, Kennedy H (1997b) Regulation of neuroblast cell-cycle kinetics plays a crucial role in the generation of unique features of neocortical areas. J Neurosci 17:7763–7783.
Polleux F, Dehay C, Kennedy H (1998) Neurogenesis and commitment of corticospinal neurons in *reeler*. J Neurosci 18:9910–9923.
Price J, Thurlow T (1988) Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. Development 104:473–482.
Rakic P (1973) Kinetics of proliferation and latency between final division and onset of differentiation of cerebellar stellate and basket neurons. J Comp Neurol 147:523–546.
Rakic P (1988) Specification of cerebral cortical areas. Science 241:170–176.
Reh TA (1987) Cell-specific regulation of neuronal production in the larval frog retina. J Neurosci 7:3317–3324.
Reznikov K (1990) Cell proliferation and cytogenesis in the mouse hippocampus. Adv Anat Embryol Cell Biol 122:1–83.
Schmahl W (1983) Developmental gradient of cell cycle in the telencephalic roof of the fetal NMRI-mouse. Anat Embryol 167: 355–364.
Schmued LC, Fallon JH (1986) Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. Brain Res 377: 147–154.
Schreyer DJ, Jones EG (1988a) Axon elimination in the developing corticospinal tract of the rat. Dev Brain Res 38:103–119.
Schreyer DJ, Jones EG (1988b) Topographic sequence of outgrowth of corticospinal axons in the rat: a study using retrograde axonal labeling with fast blue. Dev Brain Res 38:89–101.
Schultze B, Korr H (1981) Cell kinetic studies of different cell types in the developing and adult brain of the rat and the mouse: a review. Cell Tissue Kinet 14:309–325.
Schultze B, Nowak B, Maurer W (1974) Cycle times of the neural epithelial cells of various types of neuron in the rat. An autoradiographic study. J Comp Neurol 158:207–218.
Schüz A, Palm G (1989) Density of neurons and synapses in the cerebral cortex of the mouse. J Comp Neurol 286:442–455.
Schwartz ML, Rakic P, Goldman-Rakic PS (1991) Early phenotype expression of cortical-neurons — evidence that a subclass of migrating neurons have callosal axons. Proc Natl Acad Sci USA 88:1354–1358.
Sockanathan S, Jessell TM (1998) Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell 94: 503–514.
Stanfield BB, O'Leary DDM (1985) Fetal occipital cortical neurones transplanted to the rostral cortex can extend and maintain a pyramidal tract axon. Nature 313:135–137.
Stanfield BB, O'Leary DDM, Fricks C (1982) Selective collateral elimination in early postnatal development restricts cortical distribution of rat pyramidal tracts neurons. Nature 298:371–373.
Tan S-S, Faulkner-Jones B, Breen SJ, Walsh M, Bertram JF, Reese BE (1995) Cell dispersion patterns in different cortical regions studied with an X-inactivated transgenic marker. Development 121:1029–1039.
Terashima T, Inoue K, Inoue Y, Mikoshiba K, Tsukuda Y (1983) Distribution and morphology of corticospinal tract neurons in *reeler* mouse cortex by the retrograde HRP method. J Comp Neurol 218: 314–326.
Terashima T, Inoue K, Inoue Y, Mikoshiba K, Tsukuda Y (1985) Distribution and morphology of callosal commisural neurons within the motor cortex of normal and *reeler* mice. J Comp Neurol 232:83–98.
Tole S, Patterson PH (1995) Regionalization of the developing forebrain: a comparison of Forse-1, Dlx-2, and Bf-1. J Neurosci 15:970–980.
Uematsu J, Ono K, Yamano T, Shimada M (1996) Development of corticospinal tract fibers and their plasticity I: quantitative analysis of the developing corticospinal tract in mice. Brain Dev 18:29–34.
Vaccarino FM, Schwartz ML, Hartigan D, Leckman JF (1995) Basic fibroblast growth-factor increases the number of excitatory neurons containing glutamate in the cerebral-cortex. Cereb Cortex 5:64–78.
Valverde F, Lopez-Mascaraque L, Santacana M, De Carlos JA (1995) Persistence of early-generated neurons in the rodent subplate — assessment of cell-death in neocortex during the early postnatal-period. J Neurosci 15:5014–5024.
Waechter RV, Jaensch B (1972) Generation time of the matrix cells during embryonic brain development: an autoradiographic study in rats. Brain Res 46:235–250.
Walsh C, Cepko CL (1992) Widespread dispersion of neuronal clones across functional regions of the cerebral cortex. Science 255: 434–440.
Williams RW, Herrup K (1988) The control of neuron number. Annu Rev Neurosci 11:423–454.