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Determination of the migratory capacity of embryonic cortical cells lacking the transcription factor Pax-6

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SUMMARY

The cerebral cortex forms by the orderly migration and subsequent differentiation of neuronal precursors generated in the proliferative ventricular zone. We studied the role of the transcription factor Pax-6, which is expressed in the ventricular zone, in cortical development. Embryos homozygous for a mutation of *Pax-6* (Small eye; *Sey)* had abnormalities suggesting defective migration of late-born cortical precursors. When late-born *Sey*/*Sey* precursors were transplanted into wild-type embryonic rat cortex, they showed similar integrative, migrational and differentiative abilities to those of transplanted wild-type mouse precursors. These results suggest that postmitotic cortical cells do not need *Pax-6* to acquire the capacity to migrate and differentiate, but that *Pax-6* generates a cortical environment that permits later-born precursors to express their full developmental potential.

Key words: bromodeoxyuridine, cerebral cortex, neuronal migration, *Pax-6*, Small eye mice, transplantation

INTRODUCTION

The cerebral cortex is crucial for perceptual and higher cognitive processes. Although its formation is completed postnatally under the influence of environmental stimuli, much of its architecture is assembled prenatally. This involves the orderly migration of cells from the proliferative ventricular zone, at the inner surface of the telencephalon, through the overlying intermediate zone to the cortical plate (Angevine and Sidman, 1961; Rakic, 1974, 1988). Postmitotic neurones migrate along radial glia, and possibly other substrates, in the order in which they are born (Rakic, 1988; O'Rourke et al., 1995). As they arrive in the cortical plate they assume progressively more superficial positions and so create the layers of the cortex in an inside-first, outside-last sequence. The laminar identity of deep layer neurones is determined by cues in the ventricular zone, just prior to final mitotic division (McConnell and Kaznowski, 1991). The laminar identity of superficial layer neurones is probably determined by restriction of the developmental potential of progenitor cells (Frantz and McConnell, 1996). The *reelin* gene, which encodes an extracellular matrix protein secreted by Cajal-Retzius neurons in cortical layer 1, plays a role in the ordering of cortical layers (D'Arcangelo et al., 1995).

The transcription factor Pax-6 is crucial for CNS development. It contains two DNA-binding motifs, a paired domain and a paired-like homeodomain. It is widely expressed in the developing eyes, nasal structures, spinal cord and brain, including the telencephalon, from embryonic day 8.5 (E8.5; Walther and Gruss, 1991; Stoykova and Gruss, 1994; Grindley et al., 1995, 1997; Mastick et al., 1997). Mutations in the mouse *Pax-6* gene produce the Small eye phenotype and two alleles (*Sey* and Sey^{Neu}) have similar abnormalities (Hill et al., 1991; Mastick et al., 1997). Homozygotes have severe defects of the eyes, face and CNS, including the cerebral cortex, and die at birth (Hogan et al., 1986; Schmahl et al., 1993; Quinn et al., 1996; Stoykova et al., 1996; Grindley et al., 1997; Warren and Price, 1997). At present the roles of transcription factors such as Pax-6 in regulating cortical laminar development remain unclear. Schmahl et al. (1993) suggested that a failure of migration of cortical precursors might explain the histological appearance of the Small eye cortex. We tested this hypothesis by following the migration of cortical precursors after labelling them with bromodeoxyuridine in Small eye mice with a point mutation of *Pax-6* (*Sey*, in which a stop codon is introduced between the paired box and homeobox; Hill et al., 1991). We then transplanted *Sey*/*Sey* cells into a normal cortical environment so that their fates could be studied into postnatal life and their developmental abilities could be assessed.

MATERIALS AND METHODS

Animals

Mice were from isolated laboratory colonies. Adult *Sey*/+ mice were distinguished by their abnormally small eyes. *Sey*/*Sey* mice die at birth and *Sey*/*Sey* embryos were derived from *Sey*/+×*Sey*/+ matings. Homozygotes were recognized by the absence of eyes and a shortened snout (Hogan et al., 1986; Hill et al., 1991). Wild-type embryos were derived from +/+ × +/+ and +/*Sey* × +/*Sey* matings. In +/*Sey* × +/*Sey* matings, wild-type embryos were recognized at the time of dissection

by inspection of eye size and the genotype was confirmed later by polymerase chain reaction and restriction enzyme analysis of tail DNA, as described in Quinn et al. (1996). Wild-type Long-Evans hooded rats were obtained from external suppliers. The day after mating, on which the vaginal plug was found, was deemed E1.

In situ hybridizations

Pregnant dams were deeply anaesthetized with urethane (0.3 ml of a 25% solution in normal saline i.p.) and embryos were removed on E13 or E16. Embryos were dissected in cold phosphate-buffered saline (PBS) and the heads were fixed overnight in 4% paraformaldehyde in PBS at 4°C. Fixed heads were embedded in wax, sectioned parasagittally at 7 μm and mounted on slides coated with 3-aminopropyltriethoxysilane. In situ hybridization using a digoxygenin-labelled riboprobe transcribed from a *Pax-6* cDNA clone was performed on sections at a range of lateromedial levels. Sections were coverslipped without counterstaining and examined by bright-field and Nomarski microscopy. Adjacent sections were stained with cresyl violet.

Immunohistochemistry for L1, RC2 and TuJ1

For L1 and RC2 immunohistochemistry, 10 μm parasagittal frozen sections fixed in methanol at –20°C for 5 minutes were incubated overnight (4°C) with rat anti-L1 (Boehringer Mannheim, clone 324; 1:100, v:v) and/or mouse anti-RC2 (a gift from P. Gressens, University of Louvain, Brussels, Belgium; 1:3, v:v). Anti-L1 was revealed with biotinylated rabbit anti-rat antiserum (DAKO; 1:100, v:v) followed either by avidin-biotin complex and diaminobenzidine or by avidin-fluorescein complex (all from Vector). Anti-RC2 was revealed with Texas red-conjugated goat anti-mouse antiserum (Vector; 1:50, v:v). For TuJ1 immunohistochemistry, 10 μm paraffin sections were incubated sequentially with mouse anti-β-tubulin class III neuron-specific isotype (TuJ1 clone, a gift from A. Frankfurter, University of Virginia, Charlottesville, VA, USA; 1:500, v:v; overnight at room temperature), biotinylated horse anti-mouse antiserum (1:200, v:v), avidin-biotin complex and diaminobenzidine (all from Vector).

BrdU labelling

Pregnant dams were given a single injection of bromodeoxyuridine (BrdU; 70 μg/g in sterile saline i.p.) on E13, E14 or E16. Mothers were deeply anaesthetized with urethane (0.3 ml of a 25% solution in normal saline i.p.) and embryos were removed 30 minutes later or on E19. Homozygous and wild-type embryos were identified as described above. After dissection, heads of E13-E16 embryos or brains of E19 embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature, embedded in wax, serially sectioned parasagittally at 10 μm, mounted on slides coated with poly-L-lysine and reacted immunohistochemically to reveal BrdU labelling using the peroxidase method described previously (Gillies and Price, 1993). The last few sections on each slide were counterstained with cresyl violet.

The distributions of BrdU-labelled cells throughout the depth of the E19 telencephalic wall were analysed in parasagittal sections taken from embryos that had been injected on E13, E14 or E16. Four non-consecutive parasagittal sections approximately one third of the distance from the midline to the lateral edge of the brain were chosen from the dorsal neocortex of each embryo. The caudal portion of the neocortical ventricular zone was studied; the most anterior portion of the neocortex, which lies above the striatum and lacks an easily delineated ventricular zone, was not included in the quantification. For each section, camera lucida drawings were made at three different rostrocaudal levels in the caudal neocortex, indicated as positions a, b and c in Fig. 2A,C. At each level, 100 μm wide radial strips were divided into 50 μm deep bins (see Fig. 4A) and the position of each heavily and lightly labelled cell was assigned to a bin to generate histograms of average cell density against depth. In each location, the numbers of labelled cells above the ventricular and subventricular zones in +/+ embryos (i.e. above the broken lines in Fig. 4A,C,E) or above the ventricular and subventricular zones and heterotopic clusters in *Sey*/*Sey* embryos (i.e. above the broken lines in Fig. 4B,D,F) were expressed as percentages of the total number of labelled cells in the strip. Data from four sections from three animals in each experimental group were averaged to give the histograms in Fig. 6. Means were compared statistically using Student's *t*-test, or Welch's *t*-test in the cases where the difference between the two standard deviations was significant.

Transplants

In some transplants, Small eye and wild-type pregnant mice were injected with BrdU (70 μg/g in sterile saline i.p.) 1 hour before death. In other transplants, no BrdU was given. Mothers were killed on E16 by cervical dislocation, embryos were removed into chilled Hank's balanced salt solution (HBSS) on ice and their brains were dissected. The dissociation protocol followed that in Huettner and Baughman (1986). For each dissociation, 8-12 cerebral neocortices were separated from all surrounding tissue (by cutting as indicated by broken line in Fig. 2A,C) and placed in Earle's balanced salt solution (EBSS) containing 200 units of papain (Worthington LS03126) at 30-35°C and pH 7.4 for 45 minutes. The tissue was then triturated gently in EBSS containing 10 mg/ml trypsin inhibitor (Boehringer Mannheim 109-878). The dissociated cells were incubated in HBSS containing the red fluorescent marker PKH26 (Sigma) for 4 minutes and were then washed in HBSS to remove any dye that had not incorporated into the cells. Some cells were plated onto poly(lysine)-coated glass slides to check the PKH26 staining with fluorescence microscopy or BrdU labelling after immunocytochemical processing. The viabilities of the dissociated cells, assessed by trypan blue exclusion, were 90-95%. In experiments where BrdU was used, sections of cortex were reserved undissociated for histological processing and evaluation of labelling indices.

The transplantation protocol followed those used by Brustle et al. (1995) and Fishell (1995). E16 pregnant rats were anaesthetized with ketamine (60 mg/kg i.m.) and xylazine (6 mg/kg i.m.) and the uterine horns were exposed under sterile conditions. A single 2 μl injection of $0.3\text{-}2.0\times10^6$ dissociated cells was made into the telencephalic vesicle of each embryo. The abdomen was sutured and the rat recovered. Some rats were killed at E20 by cervical dislocation and the fetal heads were placed in 4% paraformaldehyde. Other rats gave birth and the young were deeply anaesthetised with sodium pentobarbitone (1 mg i.p.) and perfused with 4% paraformaldehyde on postnatal day 7 (P7).

Brains that contained BrdU-labelled cells were processed as described above. Approximately every tenth section was lightly counterstained with cresyl violet. Sections were viewed with bright-field and Normaski microscopy. 11 of these rat brains (aged E20 and P7) were sectioned serially at 50 μm on a freezing microtome and reacted with a rat monoclonal antibody to the neuronal mouse-specific antigen M6 (a gift from C. Lagenaur, University of Pittsburgh, Pittsburgh, PA, USA; 1:10, v:v, dilution; overnight at room temperature; Lagenaur et al., 1990). Binding of the primary was revealed with a fluorescein-conjugated anti-rat secondary antibody. All brains that contained PKH26-labelled cells were sectioned serially at 200 μm on a vibrotome, counterstained with bisbenzimide (5 μg/ml) and viewed with a fluorescence microscope.

All sections from brains that contained BrdU-labelled or PKH26-labelled cells were analysed for the presence of labelled cells in the neocortex. Each labelled cell was assigned to a cortical layer. For each brain, the percentage of cells in each layer was calculated. For each set of experimental conditions, average percentages in each layer were obtained by combining results from 6-8 transplants (to generate the histograms in Fig. 8). Injected cells were free to distribute themselves through the brain's ventricular system, and both *Sey*/*Sey* and +/+ cells integrated not only at cortical sites but also extracortically. Quantitative data on integration into extracortical areas are not presented here and the Results deal only with cortical integration.

RESULTS

In the mouse, most cortical neurones are born between E12 and E16 (Gillies and Price, 1993). During this time, telencephalic expression of *Pax-6* is restricted to the vast majority of cells in the ventricular zone of +/+ embryos (Fig. 1). Early in cortical neurogenesis, there is no expression in the overlying primordial plexiform layer, in which the cortical plate forms (Fig. 1A,B). Later, *Pax-6* expression extends into the subventricular zone but is still absent from the cortical plate (Fig. 1C,D).

Cortical morphology in Small eye mice

Fig. 2 shows sections through the cortex of E19 +/+ (Fig. 2A,B) and *Sey*/*Sey* (Fig. 2C,D) embryos. *Sey*/*Sey* cortices were smaller than +/+ cortices; the cortical plate was thinned, the subventricular zone was thickened and clusters of cells with similar density and appearance to cells in the subventricular zone intruded into the intermediate zone (Fig. 2D). By following the clusters through serial sections, it was clear that they were usually continuous with the subventricular zone. This is illustrated by photomicrographs of two sections from the same embryo: Fig. 2D shows clusters that were apparently separated from the subventricular zone but Fig. 2L shows that they were continuous at a different mediolateral level (arrows). The marginal zone was present in *Sey*/*Sey* embryos but its lower border was blurred (Fig. 2D), because of the absence of the thin band of densely packed cells at the top of the cortical plate that comprises recently arrived postmitotic cells in +/+ embryos (arrowheads in Fig. 2B). These observations suggested that precursor cells were accumulating in the ventricular zone, causing it to bulge into the intermediate zone, rather than migrating to the cortical plate.

L1 and TuJ1 expression

To investigate differentiation in the cortex of *Sey*/*Sey* embryos, we stained for molecules expressed specifically by developing neurones. The cell adhesion molecule L1 is localized principally on cortical axons but also on the cell bodies of relatively mature neurones (Godfraind et al., 1988; Fushiki and Schachner, 1986; Tuttle et al., 1995). In +/+ E19 embryos (Fig. 2E,F), there was intense L1 staining in the intermediate zone but no staining of the ventricular and subventricular zones, allowing the border between the intermediate and subventricular zones to be recognized (Godfraind et al., 1988; Fushiki and Schachner, 1986; Tuttle et al., 1995). There was moderate staining of the deep cortical plate and marginal zone and weak staining of the upper part of the cortical plate. In *Sey*/*Sey* E19 embryos (Fig. 2G,H), the ventricular and subventricular zones (including the overlying clusters) were devoid of L1 staining. There was strong staining in the intermediate zone around the clusters; staining in the cortical plate and marginal zone was similar to that in +/+ embryos. These findings further identify the thickened layer of densely packed cells and the overlying clusters in the deep half of the telencephalic wall of E19 *Sey*/*Sey* embryos as a hypertrophic equivalent of the ventricular and subventricular zones in wild types, and suggest that these regions do not contain axons.

Neurone-specific class III β-tubulin isotype TuJ1 is the earliest marker for postmitotic neurones (Lee et al., 1990; Ferreira and Caceres, 1992). In E19 +/+ embryos, TuJ1 is expressed by very few cells in the ventricular zone (the thin strip of unlabelled cells above the broken line in Fig. 2I) but by most cells in the subventricular zone and more superficial layers (Fig. 2I,J; Menzes and Luskin, 1994). In E19 *Sey*/*Sey* embryos, TuJ1 is expressed by many cells in the ventricular zone and by most cells in more superficial locations, including the subventricular zone and its associated clusters (Fig. 2K,L), indicating that cells in these regions of *Sey*/*Sey* embryos are in an early state of neuronal differentiation.

Radial glia

We used the glial-specific monoclonal radial cell (RC2) antibody (Misson et al., 1988; Gressens and Evrard, 1993) to show that radial glial processes are present in E16 *Sey*/*Sey* embryos (Fig. 3G; the radial glial processes were cut into short segments by sectioning). In the intermediate zone, the radial glial processes ran mainly in the spaces between the clusters overlying the subventricular zone and relatively few were in them (Fig. 3E,F).

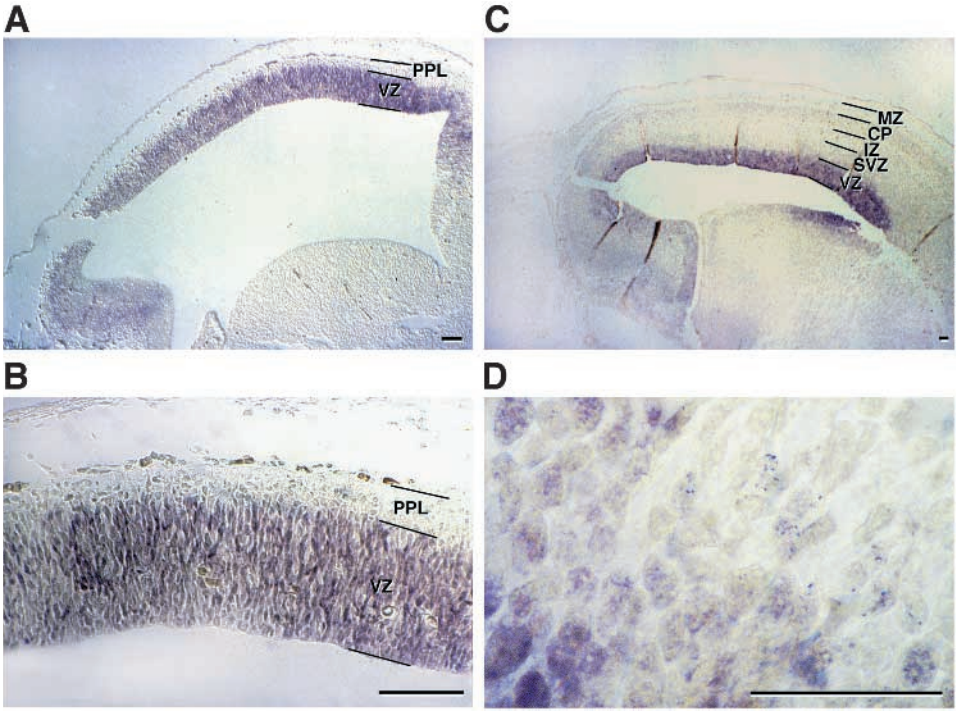

Fig. 1. In situ hybridizations for *Pax-6* expression on parasagittal sections of the telencephalon of E13 +/+ (A,B) and E16 +/+ (C,D) embryos (in D, ventricular zone is on the left, subventricular zone is on the right). CP, cortical plate; IZ, intermediate zone; MZ, marginal zone; PPL, primordial plexiform layer; SVZ, subventricular zone; VZ, ventricular zone. Scale bars, 50 μm.

Radial migration

BrdU given to E13 or E16 embryos 30 minutes before death labelled cells in similar positions in *Sey*/*Sey* and +/+ embryos (Fig. 3A-D), indicating that most precursor cells were generated at the same positions in the mutants and wild types. The band of labelled cells was of slightly less uniform density in the mutants. Figs 4 and 5 show the positions of cells labelled with BrdU on E13, E14 and E16 in +/+ and *Sey*/*Sey* embryos on E19. BrdU-labelled cells in these embryos were classified into two groups, heavily labelled cells (more than half of the nucleus covered by reaction product) and lightly labelled cells (less than half of the nucleus covered by reaction product). Previous work has shown that heavily labelled cells are those born on the day of injection (first generation), whereas the majority of lightly labelled cells are the product of subsequent progenitor cell divisions (second and perhaps third generation cells; Gillies and Price, 1993).

In both +/+ and *Sey*/*Sey* E19 embryos, most E13- and E14-born cells (heavily labelled) were in the superficial half to two thirds of the telencephalic wall, i.e. in the cortical plate (Figs 4A,B,D,E and 5A,B,E,F). In E19 +/+ embryos, most E16-born cells were still in the deep half of the telencephalic wall, i.e. in the ventricular, subventricular and intermediate zones (Figs 4C and 5I). In E19 *Sey*/*Sey* embryos, most E16-born cells were at similar relative depths but, in contrast to wild-types, these positions corresponded to the ventricular zone and subventricular zone, with its associated heterotopic clusters (Figs 4F and 5J). As shown in Fig. 6A,B, for +/+ and *Sey*/*Sey* embryos injected with BrdU on E13 and E16, we calculated the average percentages (from all E19 embryos studied) of labelled cells lying superficial to the border between the subventricular and intermediate zones (marked by broken lines in Fig. 4; cell clusters in the mutants were considered part of the subventricular zone). After BrdU labelling on E13, the proportions of heavily labelled cells superficial to this boundary were slightly lower in E19 *Sey*/*Sey* embryos (shaded bars in Fig. 6B) than in E19 +/+ embryos (shaded bars in Fig. 6A; significant only in anterior cortex). After BrdU labelling on E16, the proportions of heavily labelled cells superficial to this border were very much lower in E19 *Sey*/*Sey* embryos (shaded bars in Fig. 6D) than in E19 +/+ embryos (shaded bars in Fig. 6C; highly significant in all areas). These results suggest that many later-born cells accumulate in the subventricular zone of the mutants.

Further evidence for this hypothesis came from analysis of the distributions of lightly labelled cells on E19 after injections of BrdU on E13 and E14. After injections on E13, most of these cells lay superficial to the peak of the distribution of heavily labelled cells in +/+ embryos (Fig. 5A,C), in agreement with the known deep-to-superficial sequence of laminar formation (Wood et al., 1992; Gillies and Price, 1993; Price et al., 1997). In *Sey*/*Sey* embryos, the opposite was the case (Fig. 5B,D), and there were fewer lightly labelled cells above the border between the intermediate and subventricular zones than in +/+ embryos (Fig. 6A,B). These data show that, in the mutants, most lightly labelled cells failed to overtake the heavily labelled cells, even though the period between an E13 injection and E19 was sufficient for them to do so in +/+ embryos.

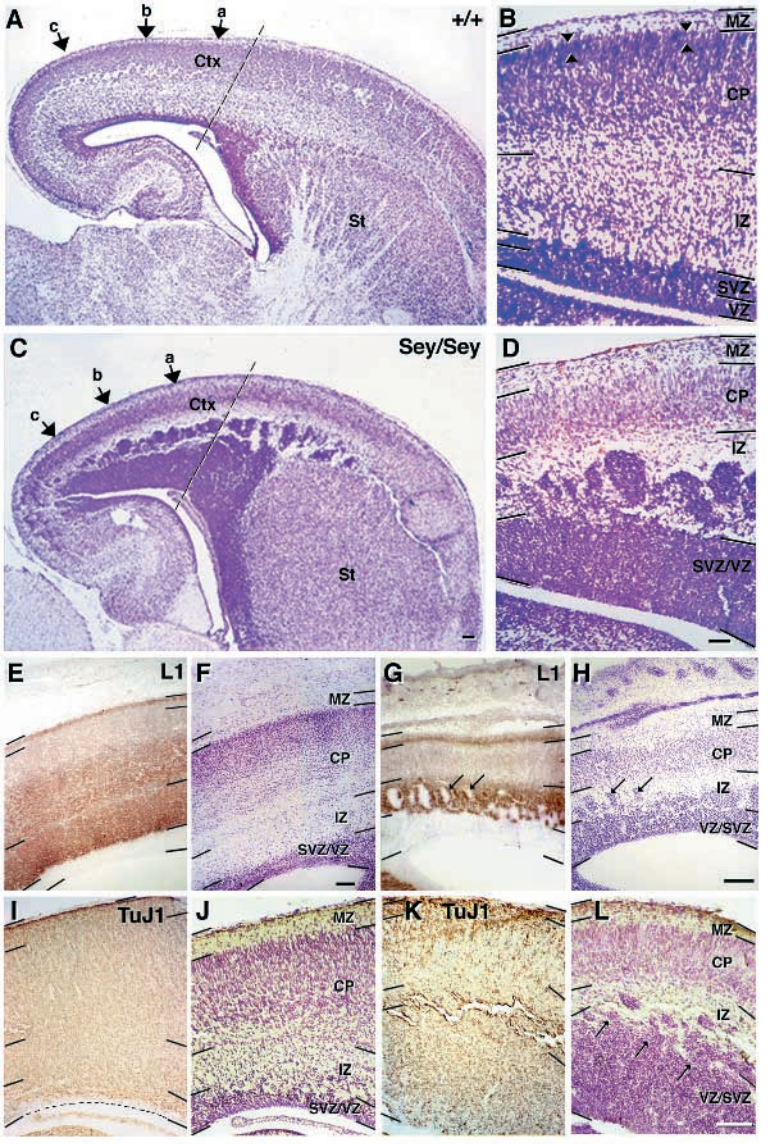

Fig. 2. Parasagittal sections of +/+ and *Sey*/*Sey* E19 embryos. (A,B) +/+ and (C,D) *Sey*/*Sey* cortices stained with cresyl violet; photomicrographs in B and D are from around position b in A and C. (A,C) Material caudal to the positions marked by the broken lines was used in transplant experiments. In the BrdU-labelling experiments, analysis of cell migration was done at positions a, b and c. (E,G) L1 expression in +/+ (E) and *Sey*/*Sey* (G) embryos; (F,H) corresponding adjacent counterstained sections. (I,K) TuJ1 expression in (I) +/+ and (K) *Sey*/*Sey* embryos; (J,L) corresponding adjacent counterstained sections. K and L show slightly more lateral and hence thicker sections than G and H. Arrowheads in B mark the dense band of newly arrived cortical precursors. Arrows in G and H mark the same clusters. Results in D and L are from the same animal; arrows in L show clusters in D merging with the subventricular zone. Tissue above the marginal zone in E-H comprises the meninges, skull and skin. Broken line in I marks the ventricular edge. St, striatum; Ctx, cortex; other abbreviations as in Fig. 1. Scale bar in C (also applies to A), 100 μm; all others, 50 μm.

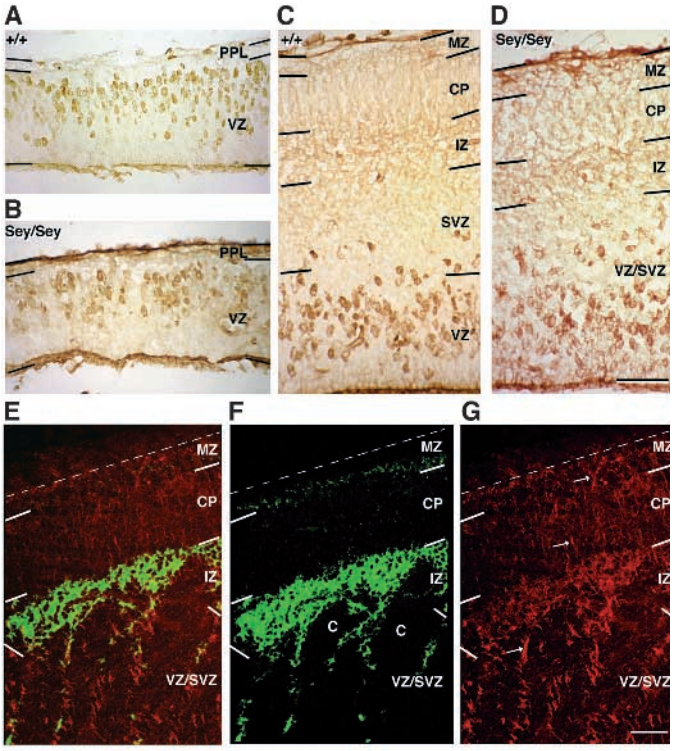

Fig. 3. Parasagittal sections of +/+ and *Sey*/*Sey* telencephalon from (A) an E13 +/+ embryo, (B) an E13 *Sey*/*Sey* embryo, (C) an E16 +/+ embryo and (D) an E16 *Sey*/*Sey* embryo, all stained for BrdU given 30 minutes before death. (E-G) Parasagittal section of an E16 *Sey*/*Sey* embryo showing staining for both anti-L1 and anti-RC2 (E), anti-L1 (clusters are surrounded by L1 label) (F) and anti-RC2 (arrows mark sectioned radial glial fibres) (G). C, clusters; broken lines, upper edge of marginal zone; other abbreviations as in Fig. 1. Scale bars, 50 μm.

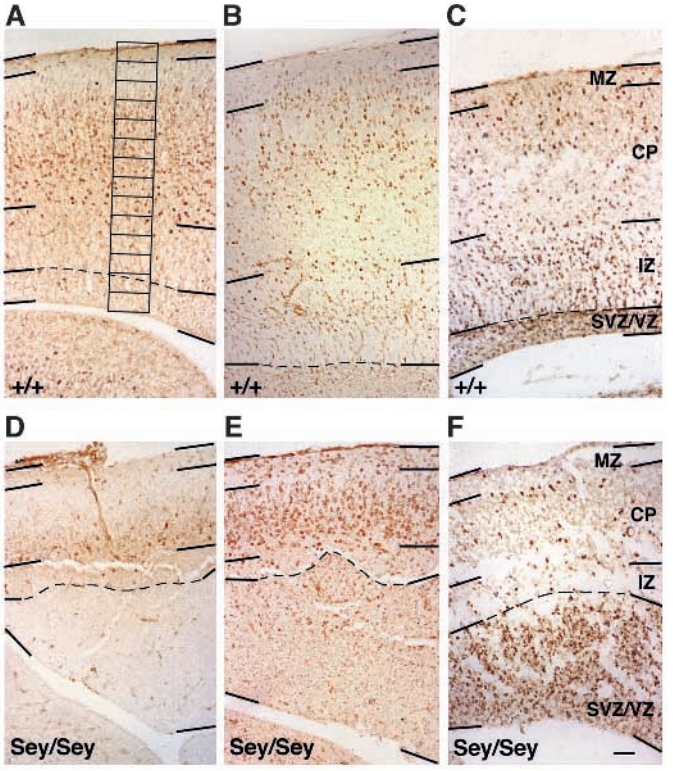

Fig. 4. Analysis of patterns of BrdU labelling in the E19 cortex (all examples are at the central position b in Fig. 2A,C) of +/+ (A,B,C) and *Sey*/*Sey* (D,E,F) embryos after injections of BrdU on E13 (A,D), E14 (B,E) and E16 (C,F). In the photomicrographs, broken lines indicate the upper edge of the subventricular zone (in +/+ embryos: A,B,C) or the heterotopic clusters (in *Sey*/*Sey* embryos: D,E,F). This upper edge was identified by reference to adjacent cresyl violet-stained sections. The ladder in A illustrates the method used to generate data for the histograms in Fig. 5. Abbreviations as in previous Figs. Scale bars, 50 μm.

ficient to allow this in wild types and the absolute distance that these cells would have had to cover was less than in the wild types. Results in E19 embryos injected with BrdU on E14 appeared similar (Fig. 5E-H): whereas most lightly labelled cells were intermingled with the heavily labelled cells in the wild types (Fig. 5E,G), most remained deep to the majority of the heavily labelled cells in the mutants (Fig. 5F,H). In summary, our results suggest that, in *Sey*/*Sey* embryos, many later-generated cortical precursors fail to migrate and form an enlarged subventricular zone. Although the absolute distances travelled by many E16-born cells from their sites of proliferation to give the distributions shown in Fig. 5I,J are similar for wild-type and mutant cells, this is most likely explained by displacement within the enlarging subventricular zone.

Transplants

Based on our results in +/+ and *Sey*/*Sey* embryos, we formulated the hypothesis that *Pax-6* gives precursors born late in cortical neurogenesis the capacity to migrate to appropriate cortical locations. To test this, we compared the ability of E16 *Sey*/*Sey* and +/+ cortical cells to migrate by dissociating them and injecting them into the ventricles of normal E16 rat brains. The donor cells were labelled either with BrdU injected on E16, shortly before transplantation, or with the fluorescent dye PKH26, during the dissociation protocol. Over the days following injection, many of the injected cells integrated into the overlying ventricular zone and migrated. Fig. 7A,B shows examples of BrdU-labelled and PKH26-labelled *Sey*/*Sey* cells in layers 2 and 3 of the cortex of the P7 rat. The vast majority of BrdU-labelled *Sey*/*Sey* and +/+ cells in the rat cortex were classified as heavily labelled. This suggested either that no further division of donor cells occurred after transplantation or that, if further divisions did occur, the later-generated cells did not integrate into the cortex. Fig. 8A,B shows the data combined from all transplants (Fig. 8A: data from BrdU-labelled transplants; Fig. 8B: data from PKH26-labelled transplants). Layers 2 and 3 contained the largest proportions of integrated BrdU-labelled and PKH26-labelled cells, irrespective of whether these cells were *Sey*/*Sey* or +/+. There was a slight difference in the distributions of cells depending on whether BrdU-labelled cells (only cells proliferating at the time of transplant) or PKH26-labelled cells (all transplanted cells) were studied. Where BrdU was the label (Fig. 8A), layers 2 and 3 contained the majority of the integrated *Sey*/*Sey* or +/+ cells. Where PKH26 was the label (Fig. 8B), 42-44% of integrated *Sey*/*Sey* or +/+ cells were in layers 2 and 3. This difference might have been because the total population of mouse

cells integrating into the rat brain (i.e. PKH26-labelled) included at least a few cells that did not undergo their final mitosis on E16.

In fact, by comparing the total numbers of PKH26- and BrdU-labelled cells in the cortex we obtained evidence that most of the cells that integrated were born on E16 in both wild types and mutants. The numbers of incorporated cells varied from several hundred to several thousand (as found by Brustle et al., 1995, using similar methods). The most important determinant of the number of integrated cells was

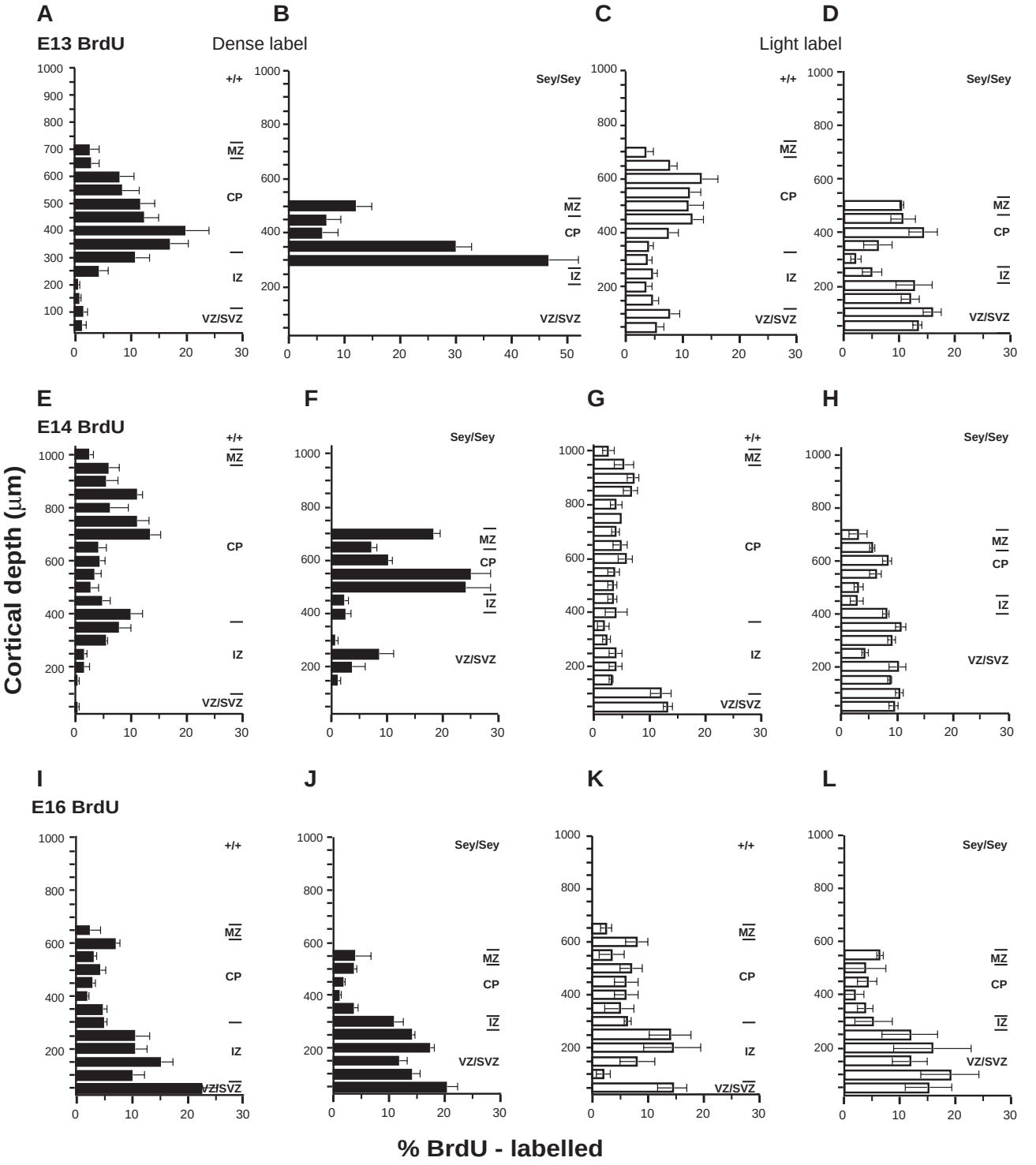

Fig 5. Histograms plot the average densities (± s.e.m.) of BrdU-labelled cells in +/+ E19 embryos (A,C,E,G,I,K) and *Sey*/*Sey* E19 embryos (B,D,F,H,J,L) against depth. Percentages of heavily labelled cells (graphs on left; filled bars) and lightly labelled cells (graphs on right; open bars) in each 50×100 μm bin (illustrated in Fig. 4) are plotted against depth. Each histogram shows representative data from one animal (n=4 sections per animal). Abbreviations as in previous Figs.

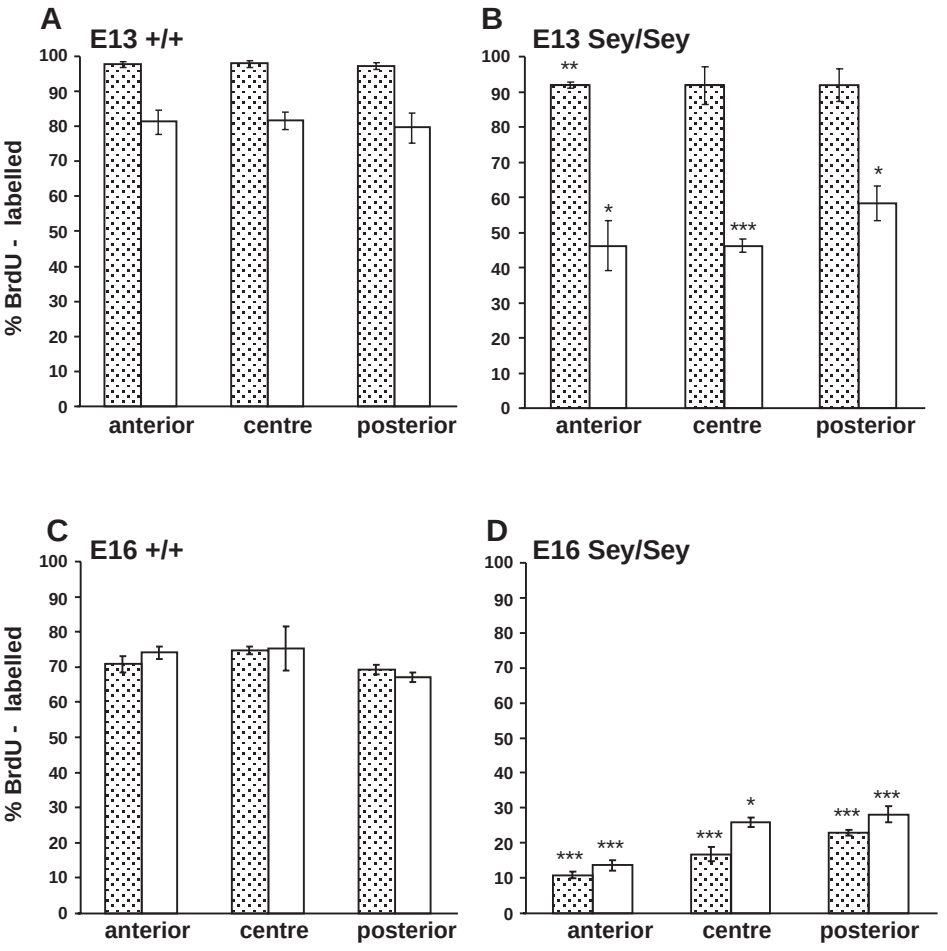

Fig. 6. The average proportions (± s.e.m.) of BrdU-labelled cells above the superficial border of the subventricular zone (in +/+ embryos; broken lines in Fig. 4A,C,E) or the heterotopic clusters (in *Sey*/*Sey* embryos; broken lines in Fig. 4B,D,F) at E19 after injections at (A,B) E13 or (C,D) E16. Filled bars are for heavily labelled cells, open bars are for lightly labelled cells. Data are from the three regions marked a (anterior), b (central) and c (posterior) in Fig. 2A,C. Values from +/+ embryos are compared with corresponding values in *Sey*/*Sey* embryos and significant differences are marked by asterisks (Student's *t*-test): * $P<0.05$; ** $P<0.01$; *** $P<0.005$.

the total number of cells injected (correlation coefficient = 0.84; data from transplants of *Sey*/*Sey* and +/+ cells did not differ significantly and were combined for this analysis). We standardised the numbers of BrdU-labelled cells and the numbers of PKH26-labelled cells in the rat cortices for comparison by expressing the number of cells in the cortex of each rat as a percentage of the number of cells injected, and found that the average percentage for BrdU-labelled cells was about a third that for PKH26-labelled cells. We found that about one third of ventricular zone cells (all of which divide during a 14 hour period in E16 mice; Waechter and Jaensch, 1972) were labelled by a 1 hour pulse of BrdU in both *Sey*/*Sey* and +/+ E16 cortices. Thus, had we labelled all ventricular zone cells prior to transplantation with a longer pulse of BrdU, we would have found numbers of incorporated BrdU-labelled cells similar to those of incorporated PKH26-labelled cells. These values agree with the conclusion that the majority of the integrated wild-type and mutant cells were born around the time of transplantation (in agreement with previous findings; Barbe, 1996).

Evidence for efficient integration of E16-born cells, at rates that were similar for wild-type and mutant cells, came from analysis of the numbers of BrdU cells in the ventricles of rats. As shown in Fig. 7C, the ventricles of rats that had been injected with mouse cells contained large numbers of unincorporated donor cells, but hardly any of these cells were BrdU-labelled. Of the entire population of BrdU-labelled wild-type or mutant cells identified in the cortex and ventricle, hardly any (only about 1%) were in the ventricles. The vast majority of both types were in the cortex. This suggested that most of the transplanted wild-type and mutant mouse cells that underwent final mitosis on E16 integrated into the rat cortex and migrated to layers 2 and 3 (the layers that they would have generated in the donor; Gillies and Price, 1993), whereas relatively few of the other transplanted cells integrated at all, and ruled against the possibility that the integrated mutant cells were a subpopulation of E16-born cells.

In all the transplants, labelled donor cells were scattered in the tangential direction over very wide areas of the cortex. They were often found in both hemispheres, and within each hemisphere they covered areas of cortex with surface areas of up to about 10mm^2. The vast majority of PKH26-labelled *Sey*/*Sey* or +/+ cells in the cortex had neuronal morphologies; examples are shown in Fig. 7B. Many had the appearance of pyramidal cells, some of stellate cells. A few cells had the appearance of astrocytes. As described before by Brustle et al. (1995), the M6 antibody (specific for mouse neurones) stained numerous processes in the cortex of rats transplanted with *Sey*/*Sey* or +/+ cells (data not shown). This antibody was not used routinely as it did not stain cell bodies well, and so it was of limited value for quantification, although it served to confirm that both *Sey*/*Sey* and +/+ cells in the rat cortex dif-

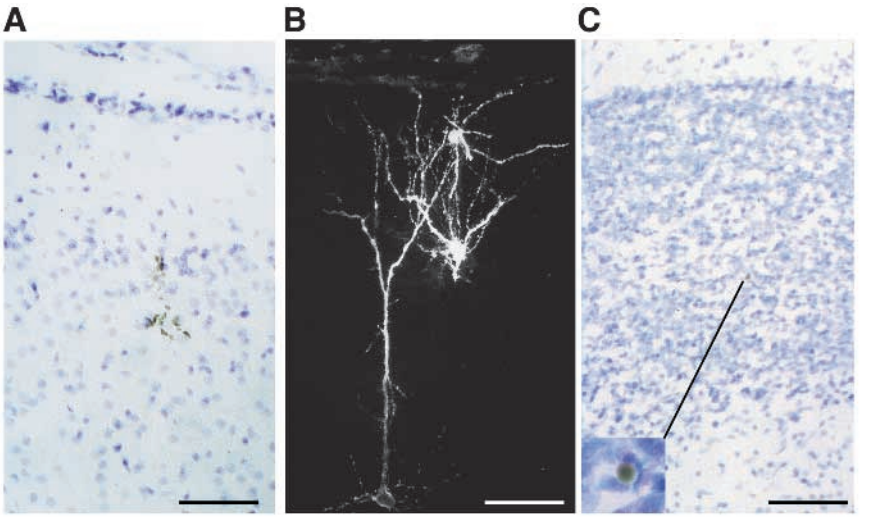

Fig. 7. Sections through the cortex of P7 rats that had received transplants of E16 *Sey*/*Sey* mouse cells on E16. Transplants of +/+ cells gave results that were qualitatively indistinguishable from those shown here; quantitative comparisons are in Fig. 6. (A) A small cluster of nuclei of E16-born mouse cells labelled with BrdU (brown stain) in the superficial layers (cresyl violet counterstain). (B) PKH26-labelled mouse cells in the superficial layers. (C) Transplanted cells, from a mouse injected with BrdU on E16, that did not integrate and were still in the lateral ventricle. Only one BrdU-labelled cell is seen (inset) in the midst of many hundreds of unlabelled cells (cresyl violet counterstained). Scale bars: A,C, 100 μm; B, 50 μm.

ferentiated into neurones, as would be expected for their date of birth.

Although our analysis of P7 host rats had revealed no difference in the migrational capacity of E16 *Sey*/*Sey* and +/+ cells, we checked for a possible delay in migration of the *Sey*/*Sey* cells by studying some transplants of PKH26-labelled cells on E20. This age was closer to that at which E16-born cells were studied in the *Sey*/*Sey* embryos. The data are shown in Fig. 8C. The distributions of *Sey*/*Sey* and +/+ cells were not significantly different (χ-squared test). We conclude that E16-born *Sey*/*Sey* cells themselves have the capacity to migrate and differentiate in a fashion indistinguishable from E16-born +/+ cells if placed in a normal environment.

DISCUSSION

Pax-6 is expressed by most cells in the ventricular zone during cortical neurogenesis (Walther and Gruss, 1991; Stoykova and Gruss, 1994) and mutations of *Pax-6* cause severe abnormalities of neocortical development (Schmahl et al., 1993). Our analysis of Small eye mice suggested that a major abnormal-

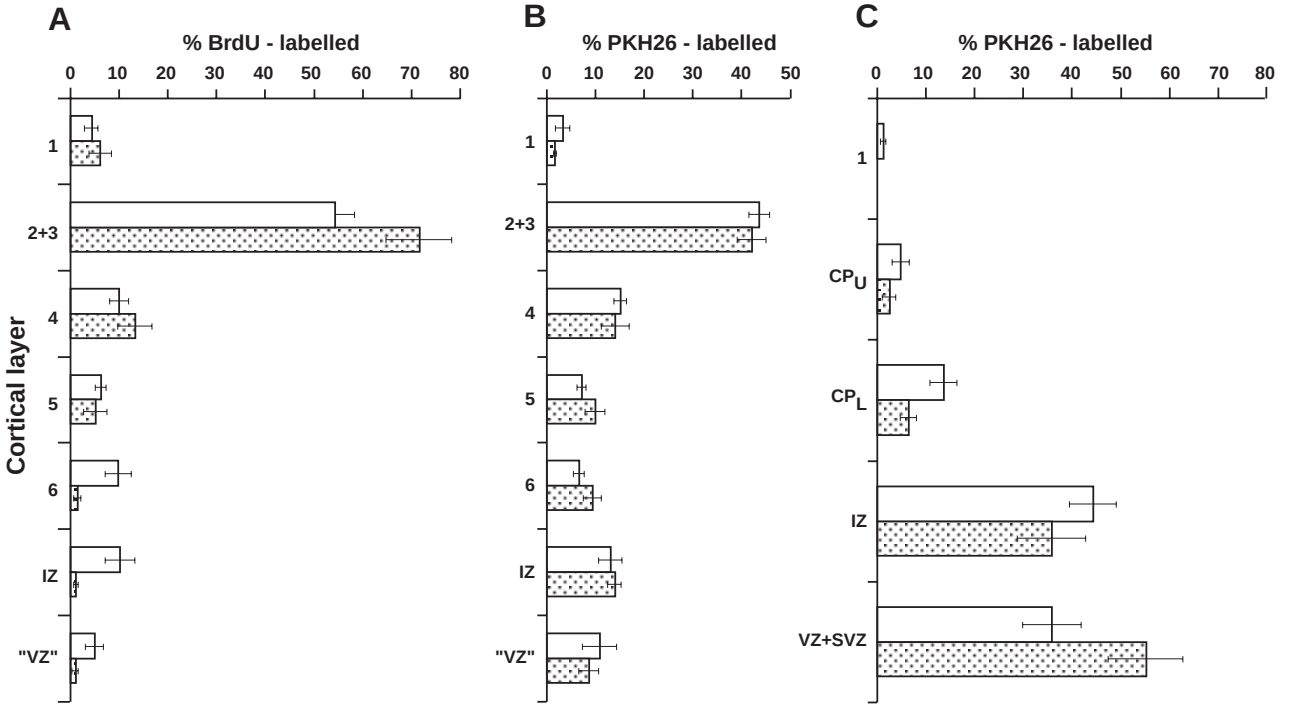

Fig. 8. Histograms showing the average percentages (± s.e.m.) in each layer of (A) BrdU-labelled or (B,C) PKH26-labelled *Sey*/*Sey* cells (shaded bars) or +/+ cells (open bars) in P7 rats (A,B) or E20 rats (C) (for all three sets of *Sey*/*Sey* transplants, n=6; for all three sets of +/+ transplants, n=8). We could not distinguish layers 2 and 3 at P7, nor layers in the cortical plate at E20 (which is split into upper (CP_U) and lower (CP_L) halves). 'VZ' indicates the thin remnant of the proliferative neuroepithelium at P7.

ity is the accumulation specifically of later-born cortical precursor cells on the deep side of the telencephalic wall. Despite their apparent inability to exit from the subventricular zone, the later-born cells in *Sey*/*Sey* embryos do express the neurone-specific marker, TuJ1. Mutant cortical precursors born early in neurogenesis migrate into the cortical plate and adopt laminar positions that are very similar to the positions adopted by wild-type cells. Results from several previous studies and some of our observations indicate, however, that there may be other defects in the early *Sey*/*Sey* cortex, including an abnormally thin telencephalic wall comprising reduced numbers of postmitotic neurones (Schmahl et al., 1993; Mastick et al., 1997; Grindley et al., 1997; Figs 4D and 5). The relationship between such early defects and the migratory abnormalities that appear later remains to be explored.

These observations suggested that later-born *Sey*/*Sey* cells are unable to migrate normally, but we could not test this hypothesis further by studying *Sey*/*Sey* embryos. Firstly, Small eye homozygotes die at birth and, since corticogenesis is not complete until after birth in normal mice, this restricted our ability to compare migration in normal and mutant mice. Secondly, *Pax-6* is expressed widely by a range of cells, including many whose cell bodies or processes surround and/or interact with cortical precursors, such as cortical glia and diencephalic afferents (Stoykova and Gruss, 1994; Stoykova et al., 1996; Warren and Price, 1997), preventing distinction of primary and secondary defects in the mutants. To overcome these limitations, we transplanted *Sey*/*Sey* cells into normal rat brains, as described by Brustle et al. (1995), and kept them alive into postnatal life.

When wild-type mouse cells were transplanted into rat brains, they behaved in a fashion compatible with the results of previous transplants between individuals of a single species. Frantz and McConnell (1996) used heterochronic transplants to show that later-born cortical cells have a restricted developmental potential such that they adopt positions in the superficial layers even when placed into a younger cortical environment. In the rat, deep cortical layers are born on E16 (Bayer and Altman, 1991; Frantz et al., 1994) and so the transplantation of E16-born mouse cells, that form superficial layers 2 and 3 (Gillies and Price, 1993), is the equivalent of a heterochronic transplant. In agreement with findings of Frantz and McConnell (1996), we found that most E16-born mouse cells migrated into the superficial layers of the rat cortex, implying that they migrated to positions appropriate to their own birthdate and were not influenced by their new postmitotic neighbours.

In other respects, the results of our transplants of wild-type mouse cells were in good agreement with the findings of others. They indicate that cortical cells undergoing their final mitosis at the time of tranplantation integrate very efficiently into the host brain, whereas non-dividing cells do not (Barbe, 1996). They also show that transplanted E16 mouse cortical cells are able to adopt appropriate phenotypes, mainly neuronal, in the host brain (Brustle et al., 1995; Fishell, 1995).

Sey/*Sey* E16-born cortical cells integrated into the host cortex with the same high efficiency as E16-born wild-type cells, migrated to similar laminar positions and differentiated in indistinguishable ways. This indicates that *Pax-6* is not required for E16-born cells to acquire the intrinsic ability to migrate and differentiate. Our observations on *Sey*/*Sey* embryos suggested that many late-born cortical precursors were not migrating normally but were accumulating in, and hence enlarging, the subventricular zone. These precursors did express a neurone-specific marker, indicating that they were able to develop early components of a neuronal phenotype even in the mutant brains. Clearly, the finding that mutant precursor cells born late in neurogenesis have the intrinsic ability to migrate normally has to be reconciled with the apparent inability of many of these cells to migrate normally in the mutant. The most parsimonious conclusion, which would encompass all of our data, is that late-born cells in the mutants have a non-autonomous defect of migration that results from an abnormality in the environment of the newborn cells.

What is the primary cause of the cortical defect in *Sey*/*Sey* embryos? There are two sets of possibilities: the primary cause may be either intracortical or extracortical. Given that *Pax-6* expression is mainly in the ventricular zone, several possible intracortical causes of the mutant phenotype are worthy of consideration. It is possible that the mechanisms that normally permit late-born cells to exit the ventricular and subventricular zones are defective or blocked in the mutants. Our results show that radial glia are present in E16 *Sey*/*Sey* embryos, although few of them are in the heterotopic clusters. It is not yet clear whether this is a primary cause of the clustered cell's failure to migrate or whether radial glia are simply displaced by an accumulation of cells that do not migrate for some other reason. There is a possiblity that the numerous extracortical defects present in the *Sey*/*Sey* embryo contribute to the cortical phenotype. For example, diencephalic afferents grow to the cortex about midway through cortical neurogenesis in the mouse (Ferrer et al., 1992; Lotto and Price, 1995) and normal diencephalic afferent innervation has been suggested to influence the survival and migration of cells in the cerebral cortex (Price and Lotto, 1996). In Small eye embryos, the diencephalon is reduced in size, is not differentiated to a normal extent (Stoykova et al., 1996; Warren and Price, 1997), and its innervation of the cortex is very weak (Schmahl et al., 1993). However, it would be most surprising if extracortical abnormalities alone induce all the cortical defects in the mutants since this would imply that cortical *Pax-6* expression is redundant. At other sites where *Pax-6* is strongly expressed, such as the eye, its mutation causes cell-autonomous defects (Quinn et al., 1996).

In conclusion, we suggest that *Sey*/*Sey* cortical cells have an intrinsic ability to migrate and differentiate. Whereas most early born *Sey*/*Sey* cortical cells migrate normally in the mutant embryo, it appears that most late-born cortical *Sey*/*Sey* cells do not, most likely due to a defect in the mutant environment. These findings indicate that newborn cortical precursors do not require normal *Pax-6* expression to acquire the ability to migrate and differentiate appropriately, but that normal *Pax-6* expression is required to generate a cortical environment that permits later-born cells to express fully their developmental potential.

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