Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain

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SUMMARY

Mutations in the gene for the transcription factor, Pax6, induce marked developmental abnormalities in the CNS and the eye, but the cellular mechanisms that underlie the phenotype are unknown. We have examined the adhesive properties of cells from the developing forebrain in Small eye, the Pax6 mutant mouse. We have found that the segregation normally observed in aggregates of cortical and striatal cells in an in vitro assay is lost in Small eye. This correlates with an alteration of in vivo expression of the homophilic adhesion molecule, R-cadherin. Moreover, the boundary between cortical and striatal regions of the telencephalon is dramatically altered in Small eye: radial glial fascicles do not form at the border, and the normal expression of R-cadherin and tenascin-C at the border is lost. These data suggest a link between the transcription factor, Pax6, R-cadherin expression, cellular adhesion and boundary formation between developing forebrain regions.

Key words: Small eye mutant mice, patterning, cortex, striatum, radial glia, RC2, tenascin-C

INTRODUCTION

The molecular mechanisms that generate regional and cellular diversity in the developing central nervous system are largely unknown. Transcription factors, signalling and adhesion molecules are all likely to be involved in these mechanisms, and the manner in which the pattern of expression of many of these genes anticipates prospective brain regions is consistent with such a role (for review: Krumlauf, 1994; McMahon 1992; Redies and Takeichi, 1996). How the different elements of these mechanisms might interact, however, is less well understood. The present study aims at elucidating a functional link between transcription factors and adhesion molecules co-expressed in a region-specific manner in the developing brain.

The analysis of genes, such as from the Hox, Emx, Otx, Dlx, Pax, POU (reviewed in Lumsden and Krumlauf, 1996; Mansouri et al., 1994; Rosenfeld, 1991; Rubenstein and Puelles, 1994), zinc-finger (reviewed in Beato, 1989) or Wnt and FGF gene families (reviewed in Nusse and Varmus, 1992; Baird, 1994), has confirmed their important role in the specification of developing brain regions. For example, the elimination of Hoxa-1 by homologous recombination results in a deletion of rhombomeres 5 and 4, while the disruption of the zinc-finger gene Krox-20 leads to a loss of the territory of rhombomeres 3 and 5 (reviewed in Lumsden and Krumlauf, 1996). Furthermore, the targeted inactivation of Hoxb-1 changes the identity of rhombomere 4 (Studer et al., 1996). After targeted mutation of the Emx2 gene, the dentate gyrus is entirely missing and the hippocampus and medial limbic cortex are also abnormal (Pellegrini et al., 1996; Yoshida et al., 1997), whereas the elimination of Otx1 affects severely the cortical morphogenesis and the cerebellar foliation and leads to a spontaneous epileptic behaviour (Acampora et al., 1996).

Mutation of the Pax6 gene generates a variety of CNS abnormalities in brain and eye development in various vertebrates including humans (reviewed in Tremblay and Gruss, 1994). Pax6 is a member of the evolutionary conserved Pax gene family of transcriptional regulators and its expression is mostly confined to the developing CNS and eye (Walther and Gruss, 1991). The naturally occurring Small eye mutation (Sey) is a point mutation within the Pax6 gene. It results in a protein that is truncated between the two DNA-binding domains (the paired domain and the paired type homeodomain) and is assumed to be non functional (Hill et al., 1991). Homozygous mice die soon after birth having failed to develop eyes or a nasal cavity (Hogan et al., 1986). They also have a malformed cerebral cortex (Schmähl et al., 1993). The cellular mechanisms underlying these malformations have not yet been thoroughly investigated, although failures in neural cell migration have been observed in the rat Small eye mutation (Matsuo et al., 1993). Recently we demonstrated severe patterning defects in the Small eye mutant forebrain in regions where Pax6 is specifically expressed (Stoykova et al., 1996). We found that the morphogenesis of the hypotalamo-telemencephalic transition zone was distorted, and that the specification of nuclei and structures in the ventral thalamus was abolished. In the wild-type telencephalon, Pax6 and Dlx1 are expressed in a complementary pattern in the cortex and the basal ganglia, and thereby delineate the boundary between the lateral ganglionic eminence (LGE) and the cortex (CTX). In the Small eye
mutant, the expression of Dlx1 is distorted at this and other expression borders (Stoykova et al., 1996). These observations prompted the suggestion that loss of Pax6 function leads to a distortion of forebrain boundaries.

In the present study, we have examined the behaviour of cells from the two forebrain regions that appear compromised (cortex and striatum). This follows our recent study, which showed that cells from these forebrain regions differ in their adhesive properties (Götz et al., 1996). In a short-term adhesion assay, cells from a given region prefer to adhere to other cells from the same region and to segregate from cells of the adjacent domain. This property is dependent on Ca\(^{2+}\)-dependent adhesion molecules, and two such homophilic molecules, R-cadherin (Matsunami and Takeichi, 1995) and Lewis X (Götz et al., 1996), are located exclusively in the cortex and thus may mediate the differential adhesion. Similar region-specific segregation has been observed for other telencephalic (Whitesides and LaMantia, 1995) and rhombencephalic regions (Wizenmann and Lumsden, 1997). These in vitro results have shown that cells of prospective brain regions differ in their adhesive properties and suggested that region-specific adhesion might be a fundamental aspect of pattern formation in the developing brain in vivo. This hypothesis predicts a link between the patterning instructed by transcription factors, the region-specific segregation in vitro and the delineation of adjacent brain regions in vivo. We tested this hypothesis by analysing the region-specific segregation and boundary formation between distinct regions in the Small eye mutant in comparison to the wild type.

**MATERIALS AND METHODS**

**Animals**

The allele of the Small eye mouse used in this work is Sey, a spontaneous mutation originally described by Roberts (1967) and Hogan et al. (1986) on a C57BL/6J X DBA/2J background. Heterozygous Sey parents were crossed to obtain homozygous, heterozygous and control wild-type embryos. The day of vaginal plug was considered as embryonic day (E) 0.5. In some experiments, we included heterozygous animals from the small eye allele Sey\(^{3766}\) (Jackson Laboratories, Theiler et al., 1978), which has a large chromosomal deletion including the Pax6 region.

**Short-term aggregation assay**

Experiments were performed as previously described in Götz et al. (1996). The lateral and medial ganglionic eminence (for simplification in the text referred to as ‘striatum’) and neocortical anlage (‘cortex’) were isolated in Dulbecco’s modified medium (DMEM) containing 10 mM Hepes, and dissociated in Ca/Mg-free HBSS containing 1 mM EDTA (dissociation buffer). After several washes in chemically defined medium (Bottenstein and Sato, 1979), cells were labelled with the fluorescent cell tracker dyes (Molecular Probes, staining solution and several washes, cells of each fraction were mixed in equal numbers (200 µl of 1x10^6 cells/ml) in 24-well plates and incubated at 37°C in 5% CO\(_2\) on a rotary shaker (70-80 revs/minute) incubator for 60 minutes. Aggregates were then fixed in 3.7% formalin and mounted as described previously (Götz et al., 1996).

Analysis was performed after fixation overnight and aggregates were classified according to the following criteria as described in Götz et al. (1996):

- as ‘pure’ when only 1-3 cells in an aggregate exhibit a different fluorescence.
- as ‘clustered’ when cells of different fluorescence are arranged in separate clusters within the same aggregate (Fig. 1E-G). Four and more cells of the same fluorescence next to each other were regarded as a ‘cluster’.
- as ‘mixed’ when none of the above criteria was appropriate and cells with different fluorescence were intermingled (see Fig. 1A-D).

The ‘pure’ and ‘clustered’ aggregates were considered as ‘segregated’, and their frequency as a percentage of all aggregates was designated the segregation ratio. Experiments with high proportion of dead cells, weak aggregation, incomplete or equivocal fluorescent labelling were excluded from the analysis. Data were collected from a total of 9 different experiments and the aggregates were analysed double-blind.

**Brdu labeling and immunohistochemistry**

In order to label cells on their day of birth, time-mated heterozygous Sey mice were injected intraperitoneally with 14 mg bromodeoxyuridine (Brdu, Sigma) per 100 g body weight (Miller and Nowakowski, 1988). Animals were killed after 1 hour by cervical dislocation, the brain was removed in ice-cold phosphate-buffered saline (PBS), pH 7.4, embedded in Tissue Tek and immediately frozen on dry ice. Frontal or sagittal cryostat sections were cut at 10 µm thickness. For Brdu detection, sections were fixed for 10 minutes in 4% paraformaldehyde in PBS (pH 7.5) and then processed as described previously (Götz and Bolz, 1992; Brdu antisem, IgG1, 1:10, Bioscience, Switzerland). The monoclonal antibody RC2 (IgM; kindly provided by P. LePrince) was used to detect radial glial cells at a 1:60 dilution after fixation in 2% paraformaldehyde in PBS, pH 7.5 for 30 minutes. The monoclonal antibody directed against β-tubulin III (IgG2b; Sigma) was used at 1:100 after fixation in methanol at ~20°C for 5 minutes. Secondary rhodamine- or fluorescein-coupled antibodies were used at 1:50 (for further description see Götz and Bolz, 1992). For detection of the Lewis X antigen, we used the monoclonal antibody RB11.2 (IgM; Götz et al., 1996) and the avidin-biotin reaction (Vektor). After washing, slides were mounted in the glycerol-based mounting medium Mowiol (Aldrich). Serial sections of WT and Sey/Sey brains were stained simultaneously to compare the staining intensity. At least two different WT and mutant brains were analysed at each stage (E13.5-E18.5), except for Lewis X, which was examined at E14.5.

**In situ hybridisation**

Sectioning, in situ hybridisation, washing and emulsion autoradiography were performed as previously described (Stoykova and Gruss, 1994). 35S-labelled sense and anti-sense RNA probes were synthesised in the presence of two radioactive nucleotides according to the suppliers instructions (Promega) from linearised plasmid templates as described for Pax6 in Walther and Gruss (1991), En1/2 in Simeone et al. (1992), Otx1/2 in Simeone et al. (1993), Prox 1 in Oliver et al. (1993), R-cadherin in Matsunami and Takeichi (1995), N-cadherin in Redies and Takeichi (1993) and tenascin-C in Götz et al. (1997). Two independent in situ analyses were performed for each stage on serial sagittal and transverse sections from WT and Sey/Sey littermate embryos with all the probes mentioned above (see also Stoykova et al., 1996). Signals were compared at corresponding levels in WT and
Sey/Sey brains processed in the same in situ hybridisation experiment. The terminology for the prosomeric subdivision is according to Puelles and Rubenstein (1993) and the rat brain atlases of Paxinos et al. (1991) and Altman and Bayer (1995) were used for the regional identification.

RESULTS

Segregation of cortical and striatal cells Small eye mutant mice

First, we examined whether the segregation of cortical and striatal cells is altered in the Small eye mutant using the previously described short-term aggregation assay (Götz et al., 1996). Cortical and striatal cells (see Methods) from wild-type (WT) and Sey/Sey littermates were isolated, labelled with different fluorescent dyes and allowed to aggregate for an hour on a rotary shaker. Aggregates were classified as either ‘pure’, ‘clustered’ or ‘mixed’ as before (Götz et al., 1996) and the segregation ratio was calculated as described in the Methods.

When cortical and striatal cells from E13.5 WT mice were mixed, 71% of all aggregates (n=249) were segregated, i.e. clustered or pure (Figs 1A-D, 2B). These results are in good agreement with our previous data from rat and chick telencephalon (Götz et al., 1996). In contrast, however, when cortical and striatal cells from Sey/Sey littermates were mixed, only 37% of all aggregates (n=861) were segregated (Fig. 2B) and most aggregates were mixed (see example in Fig. 1E-G). This is a significant reduction (χ² test, P=0.01) compared to the segregation ratio of WT cortical and striatal cells. A comparably low segregation ratio was observed when cortical and striatal cells from heterozygous Small eye (Sey/WT) or Dickies' Small eye (SeyD ey/WT) were mixed (Sey/WT: 43% segregated aggregates, n=150; heterozygous animals with severe eye defects were selected. SeyD ey/WT: 47%, n=164).

The low percentage of segregated aggregates formed by cortical and striatal cells from Sey/Sey mutants is not significantly different from the negative control when two samples of WT striatal cells were differently labelled and mixed with each other (37%, n=484; Fig. 2B). These results suggest that the cortical/striatal segregation is attenuated in Sey/Sey mutant mice.

Next, we analysed the developmental profile of the adhesive behaviour to ask whether segregation might appear at later stages in the Sey/Sey mutants. Corticostriatal aggregates from WT and Sey/Sey littermates ranging from E12.5-E15.5 were assessed. Throughout this developmental window, the majority of aggregates was mixed with a segregation ratio of 38% (n=1823). In contrast, WT cells displayed the developmental profile previously described (Götz et al., 1996) – between E12.5 and E14.5, most WT cortico-striatal aggregates were segregated (70%, n=418) but, by E15.5, most aggregates were mixed and only 33% were segregated (n=103; Fig. 2A). As depicted in Fig. 2A, the segregation ratio of E15.5 Sey/Sey cortical and striatal cells remained as low as at earlier stages (37%, n=444). Taken together, throughout the developmental stages analysed (E12.5-15.5) cells from Sey/Sey cortex and striatum predominantly mix in contrast to the developmentally regulated region-specific adhesion in the WT animals.

The failure of cortico-striatal segregation observed in the Sey/Sey mutant implies that cells have altered adhesive prop-

Fig. 1. Fluorescence micrographs of aggregates of wild-type (WT) and Sey/Sey cortical and striatal cells labeled with different fluorescent dyes isolated at embryonic day 13.5. (D,H) The phase contrast of the respective aggregates, photographed in green (C,G), red (B,F) and green+red fluorescence (A,E). Green fluorescent cells appear yellow in the double filter (A,E). To illustrate the distinct labeling of cells arrows and arrowheads in A,D and E,H indicate examples of cells that are labeled either by red (arrowheads in B,F) or green (arrows in C,G) fluorescent dyes. (A-D) Example of an aggregate formed by green fluorescent Sey/Sey cortical and red fluorescent Sey/Sey striatal cells. This aggregate was classified as mixed. (E-H) Example of an aggregate formed by red fluorescent WT cortical and green fluorescent WT striatal cells. This aggregate was classified as segregated. Scale bar: 25 μm.
Since Pax6 is expressed by cortical but not striatal cells (Walther and Gruss, 1991; Stoykova and Gruss, 1994), we postulated that the adhesive properties of just the cortical cells might have been altered. To test this, we mixed WT and Sey/Sey cells from the same region. When E13.5 Sey/Sey and WT striatal cells were mixed, they failed to segregate (segregation ratio 32%, n=412; Fig. 2B). Similar results were observed with E15.5 cells (segregation ratio 30%, n=56). In contrast, when Sey/Sey and WT cortical cells were mixed, a significant proportion of the aggregates segregated. This was true of both E13.5 and E15.5 cells (segregation ratio E13.5 = 50%, n=514, Fig. 2B: E15.5 = 54%, n=433; χ² test, P=0.01). Interestingly, the segregation ratio of WT and Sey/Sey cortical cells is intermediate between the negative control (37%) and the high segregation ratio of WT cortical and striatal cells (72%; Fig. 2B). These results indicate that the adhesive properties of the Sey/Sey cortical cells are changed so that they now segregate from WT cortical cells, but that the striatal cells of the Sey/Sey brain still mix with WT striatal cells.

**Differentiation of WT and Sey/Sey cortex are comparable during the stages analysed in the adhesion assay**

Next we asked whether the adhesive differences of Sey/Sey cortical cells might be caused by alterations in the differentiation of WT and mutant cortical cells. We used BrdU-pulse labelling to detect dividing precursor cells and an antibody directed against β-tubulin III to detect differentiating neurons. During normal cortical development, precursor cells are located in the ventricular zone (VZ) and generate postmitotic neurons that migrate towards the pial surface and form the cortical plate (CP). Accordingly, in sections through the cortex from both WT and Sey/Sey littermates at E13.5, β-tubulin-stained cells were observed in the cortical plate, whereas BrdU-
pulse-labelled precursor cells were located in the ventricular zone (Fig. 3A-D). We could detect only small differences in the ventricular zone of the Sey/Sey cortex as compared to the wild-type: in the mutant VZ, more BrdU-labelled cells seem to be located closer to the ventricular surface. This observation merits further investigation, which is beyond the scope of this study. In the context of this study, the significant point is that, at the time when the adhesive behaviour of the mutant cortical cells is significantly altered, there are no obvious differences in the differentiation state of WT and Sey/Sey cortices.

Colocalized and specific expression domains of R-cadherin and Pax6 in developing mouse forebrain

The intriguing similarity between the expression of R-cadherin and Pax6 at early developmental stages (Gänzler and Redies, 1995; Matsunami and Takeichi, 1995) and the altered adhesive properties of the cortical cells in the Small eye brain prompted us to examine the effect of the Pax6 mutation on the expression of the homophilic adhesion molecule R-cadherin. We first analysed the expression of the two genes on serial sagittal and cross sections in WT brain by in situ hybridisation analysis.

At E12.5 (preplate stage), R-cadherin and Pax6 were both strongly expressed in the proliferative neuroepithelium of neocortex, hippocampus, subiculum, amygdala and anterior rhinencephalon and in a portion of the lateralmost striatal neuroepithelium (Fig. 4A,B). Both genes are expressed in the ventricular zone of the lateral cortex, but expression of each ceases abruptly at the boundary between the cortex and the lateral ganglionic eminence (LGE). Thus the expression...
of the two genes helps delineate precisely this boundary between embryonic cortex and striatum. They help delineate another boundary in the preoptic area. Both genes are expressed in the VZ of the posterior preoptic area (POP), while R-cadherin alone is also expressed in the mantle zone of this region (Fig. 4K,L). The transcripts of both genes are absent from the adjacent region of the anterior preoptic area (POA), thus outlining a second expression boundary (POA/POP).

In addition, there are regions where one of these genes is expressed and the other not. The R-cadherin expression in the mantle zone of the POA/POP. The identity of these different nuclei was confirmed at E18.5 when their morphology is clearer and the gene expression pattern is still clear (Figs 7A,B,F,G, 8).

At early cortical plate stage (E14.5), the pattern of expression seen earlier is maintained (Fig. 5). Consistent with the reported data (Matsunami and Takeichi, 1995), R-cadherin is faintly expressed in medial cortical regions (data not shown). The Pax6 expression in the cortical ventricular and subventricular (SVZ) zones remains. In addition, R-cadherin expression is now detected throughout the cortical thickness including the cortical plate (Fig. 5C-E).

We also examined the expression of the Pax6 and R-cadherin in the developing diencephalon and hypothalamus. At E12.5 there is an extensive overlap of Pax6 and R-cadherin expression in the ventricular and mantle zone of the ventral thalamus, especially in its caudal part (Fig. 6; see also Matsunami and Takeichi, 1995). By E14.5, however, distinct structures in the mantle zone of the ventral thalamus are clearly delineated by virtue of their R-cadherin or Pax6 expression (Fig. 6C,D). We reported previously that the optoeminential zone expresses Pax6 (Stoykova et al., 1996). This is a forebrain domain, including the caudal ganglionic eminence (CGE), eminentia thalami (EMT), supraoptic paraventricular area (SPV), anterior hypothalamus (AH), posterior preoptic area (POP: see Puelles and Rubenstein, 1993). This optoeminential zone also expresses R-cadherin and is therefore another area of co-expression of these two genes (Fig. 6). In contrast, several hypothalamic regions were labelled by R-cadherin only; i.e. suprachiasmatic area (SCH), hypothalamic cell cord (HCC), the tuberal hypothalamus (TU) and the retrochiasmatic domain (RCH). In the pretectum (PT), Pax6 and R-cadherin transcripts were detected within its posterior region and in the
R-cadherin and Pax6 transcripts colocalize in the cortical and olfactory proliferating neuroepithelium, in the optoeminenctal zone, in the pretectum and along two forebrain boundary zones (CTX/LGE, POP/POA). In addition, there are a number of regions where only one or the other of these two genes are expressed.

**R-cadherin expression in the Small eye mutant**

Given that a number of developing brain regions co-express R-cadherin and Pax6, we wanted to know what happened to R-cadherin expression in Sey mice. We discovered that R-cadherin expression was considerably reduced in areas that in the WT showed co-expression. In E12.5 Sey/Sey mice, the prominent R-cadherin expression that abutted the two boundary zones, LGE/CTX and POA/POP, was almost completely abolished (Fig. 43/N, L/P). R-cadherin expression in the cortical anlage was severely reduced (Fig. 4C/G), and even the very low level of R-cadherin expression in the WT hippocampal anlage was decreased (Fig. 4D/H). In the structures of the basal telencephalon where R-cadherin and Pax6 were not colocalized, however, the expression level of R-cadherin was not reduced.

At E14.5, the reduction of R-cadherin expression in the Sey/Sey cortex was pronounced through the width of the telencephalic wall including the VZ where co-expression had been detected in the WT (compare in Fig. 5E/L, D/I, C/H). However, expression was retained in the MZ where co-expression had not been observed. The strongly labelled cells in the MZ are almost certainly Cajal-Retzius cells given their location, horizontal orientation and their expression of the reelin gene (not shown), a specific marker for these cells (D’Arcangelo et al., 1995; Ogawa et al., 1995). The R-cadherin expression in the Sey/Sey basal ganglia was not substantially changed compared with WT, this not being an area of co-expression (Fig. 5B/G).

Interestingly, the vesicle-like structure that is formed in the rostralmost region of the Small eye telencephalon (Schmahl et al., 1993) was moderately labelled by the R-cadherin (Fig. 5G, arrowhead) and reelin probes (not shown).
The decrease in the R-cadherin expression persisted until E18.5. The enlarged VZ/SVZ in the mutant brain showed R-cadherin expression only slightly above background (Fig. 7/E), whereas the signal appeared unchanged in the MZ as at earlier stages (compare in Fig. 8L/D,B). An intermediate level of R-cadherin expression was detected in the superficial regions of the rostromedial cortex and the vesicle-like structure (see above) (Figs 5G, 8L). The strongly R-cadherin labelled cortical layer, seen in this region in the WT brain, was not detectable in corresponding sections from the mutant brain (Fig. 8D,H,L).

In the diencephalon also, R-cadherin expression was substantially decreased in those forebrain regions where it colocalized with Pax6. The expression of R-cadherin decreased almost to the background level in the ventral thalamus (prosomere 3) in Sey/Sey (Fig. 6E,F). In contrast, no substantial changes in the level of R-cadherin expression were detected in the presumptive hypothalamic cell cord and retrochiasmatic area where the two genes are not co-expressed.

To check for the specificity of the observed reduction in R-cadherin expression in the mutant brain, we examined the expression of the closely related adhesion molecule N-cadherin (Redies and Takeichi, 1993) and Lewis X (Götz et al., 1996). In sagittal and cross sections from E14.5 WT and mutant brains no changes in the level of N-cadherin expression could be detected in the forebrain (Fig. 9), as well as in the diencephalon, mesencephalon and myelencephalon (data not shown). Likewise, no obvious changes were observed in Lewis X immunoreactivity (at E14.5, data not shown) and in any of the genes (Emx2, Otx1, Otx2, Prox1) whose expression is restricted to the developing cortex (data not shown; Stoykova et al., 1996). These results strengthen the suggestion that the reduced expression of R-cadherin in the Small eye forebrain regions is a specific consequence of the Pax6 loss of function condition in the mutant brain.

Disturbances at the cortico-striatal boundary in the Small eye brain

We next analysed the cortico-striatal boundary in the WT and Sey/Sey mutant. Using the RC2-antiserum as a specific marker for radial glial cells (Misson et al., 1988), we found that radial glial fascicles develop along the cortico-striatal transition zone in the wild-type brain and are most prominent around E16.5 (Fig. 10A, arrows) as previously described (Edwards et al., 1990). In Sey/Sey littersmates, RC2-immunoreactive cells are present but fascicles could not be detected at the cortico-striatal boundary (Fig. 10B).

We also examined the expression of another marker of the cortico-striatal boundary, the extracellular matrix molecule tenascin-C (TN-C). As shown previously in the rat (Götz et al., 1997), cells at this boundary and the cortical VZ cells show a high level of TN-C expression. In the Sey/Sey mutant, this expression is abolished (Fig. 10E,F, G/H). Interestingly, no alterations in expression could be detected in the VZ of the medial (hippocampal) telencephalic wall (Fig. 10F/H) or in other regions (mesencephalon, cerebellum) of the mutant brain (data not shown).

Taken together, these observations support the notion that the cortico-striatal boundary in the Small eye brain is compromised.

DISCUSSION

Pax6 function and cortico-striatal adhesive behaviour

In this study, we have detected altered cellular adhesion and expression of R-cadherin in the developing forebrain of the Pax6 mutant Small eye. These changes are restricted to the Pax6 expression domains, which fits with the idea that they are caused by the loss of Pax6 function. These results confirm a
link between patterning of the developing brain and adhesive properties of cells in embryonic brain regions. A similar link has been suggested for patterning by engrailed and Wnt-1 genes and the adhesion molecule E-cadherin (Shimamura and Takeichi, 1992; Shimamura et al., 1994).

In the short-term aggregation assay, the segregation of cortical and striatal cells observed in WT animals during most of neurogenesis is significantly reduced in Sey/Sey mutant cells. The low degree of segregation appears to be due to an alteration of the properties of the cortical, but not the striatal cells since Sey/Sey and WT striatal cells mix, but Sey/Sey and WT cortical cells segregate. This is a satisfying result since it is the cortical not the striatal cells that express Pax6. These

**Fig. 8.** Comparison of R-cadherin and Pax6 expression in the developing olfactory bulb and olfactory cortex in the wild-type and Small eye brain at stage E 18.5. Adjacent sections at two more medial levels (A,B; C,D) of sectioning than those in Fig. 6 from the wild-type brain were hybridised with the Pax6 and with the R-cadherin (B,D) antisense probes, as indicated. In A,B note the colocalization of the Pax6 and of the R-cadherin signal in the olfactory bulb VZ/SVZ and in the presumptive internal granular layer (thin arrows), while the presumptive periglomerular- and mitral cell layer (arrowheads in A and B) are specifically labelled either by Pax6 or R-cadherin probe. E,F and G,H are corresponding bright-/dark-field pictures at higher magnification of areas, shown in C and D respectively, illustrating the overlapping expression of the two genes in the cortical VZ/SVZ also at these more medial levels. (H) Note the strong hybridisation signal with the R-cadherin probe in the MZ and a layer of cells that appear to be below the subplate (arrowheads in H); I,K and J,L are bright-/dark-field pictures of adjacent sections from the Sey/Sey brain at approximately corresponding level to that of the wild-type brain, hybridised with the probe for Pax6 and R-cadherin. (L) Note the severely decreased expression of R-cadherin in the pathologically enlarged VZ/SVZ in the mutant cortex (compare L/D) in contrast to the preserved expression of the gene in the MZ, the superficial layer of the olfactory cortex and the vesicle-like structure (arrows in K,L). However, the R-cadherin-positive layer of cells seen in the wild-type brain is not detectable in the mutant olfactory cortex (compare D, H/I). CP, cortical plate; CTX, cortex; MZ, marginal zone; SE, septum; VZ/SVZ, ventricular/subventricular zone. Scale bars: in A (B-D) 50 μm; in E (F-H) 100 μm; in I (J-L) 50 μm.

**Fig. 9.** Unchanged level of N-cadherin expression in Small eye forebrain at stage E14.5. Sagittal (A,B) and cross (C,D) sections from wild-type (A,C) and Sey/Sey (B,D) brain were hybridized with a N-cadherin probe. The level of the sections in A and B are comparable to A and F respectively in Fig. 5. Hi, hippocampus; LGE, lateral ganglionic eminence; NCX, neocortex; SE, septum. Scale bar, 300 μm.
results accord well with the expression data, which showed a marked reduction in the expression of R-cadherin in the cortical but not striatal regions of the Sey/Sey telencephalon throughout development. Other adhesion molecules, N-cadherin and Lewis X, were not affected by Pax6 mutation. Since we already know that the segregation of cortical and striatal cells depends on a Ca\(^{2+}\)-dependent mechanism (Götz et al., 1996), it seems likely that one component of this mechanism is R-cadherin.

The observation that the expression of N-cadherin and Lewis X, as well as Emx2, Otx1/2 and Prox1 (also in Stoykova et al., 1996) were not significantly changed in the mutant brain argues against an overall delay in differentiation of the Sey/Sey cortex. This conclusion is further supported by the analysis of differentiating neurons and BrdU-pulse-labelled precursor cells, which showed no gross differences between mutant and wild-type cortex. Nevertheless, we found that the BrdU-labelled cells were more dispersed through the ventricular zone of the Sey/Sey cortex (see Fig. 3), so we cannot exclude subtle aberrations in the cell cycle kinetics of Sey/Sey precursor cells and further experiments are needed to clarify this issue. Since the failure of the cortico-striatal segregation in the mutant brain cannot be explained by differentiation defects, our results suggest a specific (direct or indirect) link between Pax6, the decrease in R-cadherin expression and the region-specific segregation in the telencephalon.

**Pax6 function and cortico-striatal boundary formation**

Our experiments also show that the cortico-striatal boundary is distorted in the Sey/Sey mutant brain. RC2-immunostaining revealed that radial glia, whose fasciculated processes normally outline the cortico-striatal border at E16.5, are absent in the Sey/Sey mice. These morphological disturbances were preceded by changes in gene expression in this boundary region. In WT animals, R-cadherin is particularly strongly expressed in the Pax6-expressing, cortical VZ cells that abut the LGE/CTX boundary. The two genes also extensively colocalize in the region of POP at the expression border POP/POA. In Sey/Sey mice, R-cadherin expression at both of these boundaries was markedly down regulated as early as E12.5. Similarly, the normally high level of TN-C expression seen at E14.5 in the neuroepithelial cells at the LGE/CTX border was abolished in the Sey/Sey mutant. These results indicate that the cortico-striatal boundary is severely compromised in the Sey/Sey mutant. Moreover, the absence of cortico-striatal segregation in vitro and the absence of radial glia fasciculation at this boundary in the mutant brain in vivo raises the possibility that these phenomena are linked. It has been suggested in the developing hindbrain (see e.g. Lumsden and Krumlauf, 1996) that selective adhesion between distinct regions might be a prerequisite for boundary formation. This is at least consistent with

![Figure 10](image-url). Immunohistochemical and in situ hybridisation analysis of the border region between cortex and lateral ganglionic eminence in wild-type and Sey/Sey forebrain. (A-D) Fluorescent micrographs of RC2-labeled radial glial cells in cross sections from WT (A,B) and Sey/Sey (C,D) brains at embryonic day 16.5. B and D are higher magnifications of the boundary zone shown in A and C, respectively. Note the prominent radial glial fascicle between cortex (CTX) and lateral ganglionic eminence (LGE) in WT (indicated by arrowheads), which is missing in Sey/Sey mutant brains. Scale bar: 125 μm (A,C); 75 μm (B,D). (E-H) Dark-field micrographs of adjacent cross sections from E14.5 wild-type (E,G) and Sey/Sey (F,H) brains, hybridised with antisense in situ probe for tenascin-C. Note that, in the mutant brain, the expression of the tenascin-C is abolished in the ventricular zone of the dorsolateral telencephalic wall and at the border CTX/LGE, that is strongly labelled in sections at a corresponding level from the wild-type brain (arrowheads in E,G). CTX, cortex; EMT, eminentia thalami; Hi, hippocampus; LGE, lateral ganglionic eminence; LV, lateral ventricle; SE, septum; vz, ventricular zone. Scale bar, 200 μm.
our present results which show that the cortico-striatal segregation precedes the morphological specification of the boundary.

The defects observed at the cortico-striatal boundary in Sey/Sey mice are particularly intriguing because the formation of this boundary is one of the earliest regionalisation events within the forebrain VZ. The border neuroepithelium between the cortical and basal ganglia primordia forms a wedge that generates the neurons of the endopiriform nucleus of the basal telencephalon (Bayer and Altman, 1991). This subdivision is accompanied by the confinement of many transcription factors and regulatory molecules to either the cortical or to the striatal compartment (reviewed in Rubenstein and Puelles, 1994). Interestingly, the cortico-striatal expression boundary of many of those genes as well as the radial glial fascicle described above (Edwards et al., 1990) do not coincide with the morphological cortico-striatal sulcus (also Puelles and Rubenstein, 1993; Gänzler and Redies, 1995). Genes expressed within the cortical neuroepithelium such as AP-2.2 (Chazaud et al., 1996; Fig. 3), Prox-1 (Oliver et al., 1993; our unpublished data), Tbr-1 (Bulfone et al., 1995) extend over this border region, whereas genes that are selectively expressed in the striatal anlage – Dlx1/Dlx2 (Bulfone et al., 1993; Price et al., 1991; Stoykova et al., 1996), Mash1 (Guillemot and Joyner, 1993; our own unpublished data) and Six3 (Oliver et al., 1993; our observations) exclude the cortico-striatal wedge from their lateralmost expression domains. It is therefore reasonable to accept this peculiar region of the telencephalic neuroepithelium as a cortical rather than a striatal primordium, a suggestion initially made by Bayer and Altman (1991). If and how the molecular specialisations of the neuroepithelium at the cortico-striatal wedge contribute to the generation of particular neuronal descendants, located in structures of the basal telencephalon (e.g. endopiriform nucleus, piriform cortex; see Bayer and Altman, 1991), and to the formation of specialised radial glial fascicles (De Carlos et al., 1996) remains to be determined.

The alterations that we observed at the forebrain boundaries in Sey/Sey mice may also explain our previous results in which heterotopic expression of Dlx1 was observed in the mutant cortex and POP (Stoykova et al., 1996), precisely at the boundary areas that our current data show are patterned by Pax6. The extension of Dlx1 expression into cortical regions could be explained either by a transcriptional up-regulation of the Dlx1 gene in the cortical cells due to loss of Pax6 function, or by a failure in the mutant of the border to restrict cellular migration of Dlx1-expressing cells into the cortical domain. A restriction of migration has been reported at this boundary in wild-type mice (Fishell et al., 1993). In light of our results showing altered adhesive properties of Sey/Sey mutant cells, we tend to favour the latter explanation.

**Pax6-regulated expression of R-cadherin and the forebrain phenotypes of the Small eye mutant**

Several aspects of brain morphology are disrupted in Sey/Sey animals: the cortical VZ and SVZ are enlarged (Schmahl et al., 1993; Stoykova et al., 1996); cortical lamination is aberrant at late embryonic stages (Schmahl et al., 1993); the nuclei of the ventral thalamus and the hypothalamo-telencephalic transition zone exhibit abortive growth and differentiation (Stoykova et al., 1996; Glaser et al., 1994) and the olfactory bulb is missing (Hogan et al., 1986). We now know that, in addition, R-cadherin expression is disturbed in regions where Pax6 and R-cadherin expression overlap, and where Sey/Sey brain is morphologically abnormal. It is not clear how many of the morphological abnormalities are linked to the disturbance of R-cadherin expression, but the failure of layer formation in the mutant cortex is one candidate. This hypothesis is supported not only by those cells whose R-cadherin expression is disrupted, but also by those whose expression is unaffected. The cells of the VZ and the cortical plate lose R-cadherin expression in the Sey/Sey cortex, but notably the cells of the marginal zone do not. The MZ is populated by the earliest generated neurons in the cortex (Bayer and Altman, 1991). It has been suggested that these early generated neurons move directly into the telencephalic wall (Smart and Sturrock, 1979; Valverde and Santacana, 1994), whereas the later generated neurons migrate along radial glia processes before settling in their final location (Bayer, 1986; DeCarlos et al., 1996; Rakic, 1971). In Sey/Sey animals, the marginal zone cells, including the Cajal-Retzius neurons, seem to achieve their normal position, in contrast to the later neurons that are dependent on radial glial guidance. It seems likely therefore that the radial glial migration is specifically affected in Sey/Sey mice because the migratory neurons and/or the radial glial cells have lost their expression of R-cadherin.

A similar explanation probably accounts for the abnormal olfactory bulb. Sey/Sey mice fail to form olfactory placode and olfactory bulb. Instead, a strange vesicle-like structure begins to form at the rostral-most telencephalic wall at about E14.5 in mice that carry either the Sey (Stoykova et al., 1996) or Sey/+ (Schmahl et al., 1993) allele. The cells in this structure express high levels of R-cadherin, and moderate levels of reelin as observed in the mitral cells of the WT olfactory bulb. Therefore this vesicle probably corresponds to the olfactory bulb, and the cells that accumulate in it correspond to mitral cells. Furthermore, they have characteristics (early birthdate and reelin expression) of cortical MZ cells (D’Arcangelo et al., 1995). Like the cortical MZ cells they also express R-cadherin but not Pax6. So, as in the cortex, the earliest generated neuronal cells express R-cadherin independent of Pax6, they are born normally, and invade the abnormal olfactory vesicle. The latter-generated granule and periglomerular cells of the olfactory bulb are born in the rostral-most telencephalic VZ and SVZ (reviewed in O’Rourke, 1996). Like the cortical plate cells, these cells normally express Pax6 and R-cadherin, but fail to express R-cadherin in the Sey/Sey condition, and there is a corresponding failure of these cells to invade the abnormal olfactory vesicle. So, in both cortex and olfactory bulb there is a correlation between Pax6-dependant R-cadherin expression and migration.

In the diencephalon also, R-cadherin expression is reduced in Sey/Sey mice only in the regions where it normally colocailizes with Pax6. As previously observed (Gänzler and Redies, 1995; Matsunami and Takeichi, 1995), Pax6 and R-cadherin expression overlap extensively in the neuroepithelium and in the mantle zone of the ventral thalamus (VT) at E12.5. Later in development, however, the R-cadherin transcripts appear mainly within the presumptive zona incerta and ventral lateral geniculate body in a fashion parallel to the appearance of Dlx1, whereas Pax6 expression is confined mostly to a subpart of the lateral geniculate body, the reticular nucleus and endopenduncular nucleus (Stoykova et al., 1996). In addition, R-cadherin
and Pax6 are coexpressed in the neuroepithelium of the optoeminenzial zone, a region that can be clearly delineated by its expression of Pax6 (Stoykova et al., 1996). Both the VT and the optoeminenzial zone are severely distorted in the Sey/Sey mutant brain, as demonstrated by a paucity of tissue within the optoeminenzial zone, and the abortive growth, the lateral displacement and failure to differentiate of the VT (Stoykova et al., 1996). In these two forebrain regions, the normally high level of R-cadherin expression is strongly reduced in the Sey/Sey mutant. Thus as elsewhere, R-cadherin expression is restricted to regions where it is not normally co-expressed with Pax6. As in the telencephalon, the failure of histogenesis correlates with loss of R-cadherin expression in the Sey/Sey mutant. This alteration of adhesive properties in the region of prosome 3 and 4 may also underlie the recently described failure of axon guidance in the Sey/Sey diencephalon only after entering prosome 4 and 3 (Mastick et al., 1997).

In conclusion, this study suggests a link between Pax6, R-cadherin and adhesive properties in the developing forebrain. The selective adhesion of embryonic cortical and striatal cells is disrupted in the Pax6-mutant, and there are morphological abnormalities at the cortico-striatal boundary, particularly in the radial glial cells. Meanwhile, there is a loss of expression of R-cadherin in areas in which this gene is normally co-expressed with Pax6, which shows that Pax6 regulates R-cadherin expression, directly or indirectly. The simplest model consistent with these data would be that the Small Eye mutation leads to a disruption of the Ca2+-dependent adhesive mechanisms involving R-cadherin, and this leads to the morphological disruption observed in the mutant. Nonetheless, our current data only show a correlation between these phenomena. In particular, a direct link involving R-cadherin has not been proven. Whatever the role of R-cadherin, we have demonstrated here a direct link between region-specific adhesion properties and patterning driven by the homeobox transcription factor, Pax6.

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