Roles of *Pax-6* in murine diencephalic development

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**SUMMARY**

*Pax-6* is one of the earliest regulatory genes to be expressed in the diencephalon. We tested whether normal Pax-6 protein is required for early diencephalic development by examining morphology, precursor proliferation and patterns of regulatory gene expression in the embryonic diencephalon of Small-eye mice (*Pax-6* mutants). In Small-eye mice, diencephalic morphology was abnormal at all the embryonic ages studied (days 10.5, 12.5 and 14.5). Regional differences in diencephalic cell density were lost, the diencephalon/mesencephalon boundary was unclear and the third ventricle was enlarged. We estimated diencephalic proliferative rates after labelling with bromodeoxyuridine and found that they were abnormally low in mutants aged embryonic day 10.5. In older mutants, the diencephalon contained fewer cells than normal.

In wild-type E14.5 diencephalon, *Pax-6*, *Dlx-2* and *Wnt-3* are expressed in discrete regions along the rostrocaudal and dorsoventral axes. In situ hybridizations for these genes in E14.5 Small-eye mice revealed discrete zones of diencephalic expression that had similar relative positions to those in wild-type mice. Some differences of detail in their expression were seen: *Pax-6* had an expanded rostral domain of expression and an abnormally indistinct caudal boundary; *Dlx-2* had a diffuse, rather than a sharp, caudal boundary of expression; the normally high dorsal midline expression of *Wnt-3* was lost. We conclude that normal expression of *Pax-6* is required for the correct regulation of diencephalic precursor proliferation. *Pax-6* may also control some aspects of diencephalic differentiation, but its mutation in Small-eye mice does not preclude the development of a degree of diencephalic regionalization resembling that in normal mice.

Key words: cell proliferation, diencephalon, *Dlx-2*, *Pax-6*, Small-eye mouse, *Wnt-3*

**INTRODUCTION**

Recent studies of the temporally and spatially restricted patterns of expression of regulatory genes in the murine forebrain (prosencephalon), together with earlier anatomical descriptions of this complex structure, have generated a framework for understanding the morphological development of the prosencephalon (the Prosomeric Model: Puelles and Rubenstein, 1993; Rubenstein et al., 1994). The early prosencephalon (primary prosencephalon) divides into the secondary prosencephalon and the diencephalon, the subject of this study. The diencephalon is subdivided into three prosomeres (p1-p3) identified by morphological constrictions in the tissue and the expression domains of regulatory genes including *Pax-6*, *Dlx-2* and *Wnt-3* (Bulfone et al., 1993; Puelles and Rubenstein, 1993; Stoykova and Gruss, 1994). The pretectum forms in p1, the dorsal thalamus in p2 and the ventral thalamus in p3.

As yet the molecular mechanisms that establish the regionalization and specification of the forebrain are unclear. We examined the role of *Pax-6* in the development of the diencephalon. *Pax-6*, independently isolated by homology to *gooseberry-distal* (Walther and Gruss, 1991) and from positional cloning at the aniridia locus (Ton et al., 1991), encodes two DNA-binding motifs, a paired domain (Bopp et al., 1986; Treisman et al., 1991) and a paired-like homeodomain (Frigerio et al., 1986). Its mRNA is first detected in alar regions of the murine diencephalon on embryonic day 8.5 (E8.5), as well as in more rostral and caudal regions of the nervous system (Walther and Gruss, 1991; Puelles and Rubenstein, 1993). Its expression becomes progressively more restricted during embryogenesis (Stoykova et al., 1996). *Pax-6* is therefore a prime candidate for a regulator of diencephalic development, regionalization and differentiation (Mansouri et al., 1994; Stoykova and Gruss, 1994).

*Dlx-2* is a homeobox-containing gene homologous to *Drosophila distal-less* (Porteus et al., 1991; Price et al., 1991; Robinson et al., 1991; Bulfone et al., 1993). It is first detected on E9.5 in two domains in the forebrain, one caudal the other rostral. The caudal domain of *Dlx-2* expression overlaps with *Pax-6* expression in the rostral diencephalon, and so acts as a marker of this region. It forms a caudal boundary at the junction between ventral and dorsal thalamus. This caudal domain of *Dlx-2* expression extends rostrally, outside the diencephalon, as far as the suprachiasmatic area. The second, rostral domain of *Dlx-2* expression overlaps with *Pax-6* expression in the ventral telencephalon. *Wnt-3* encodes a secreted peptide and is first expressed on E9.0 in the dorsal thalamus and also further caudally in the mesencephalon and rhombencephalon, with a rostral expression boundary where the dorsal thalamus meets the ventral thalamus (Salinas and
Nusse, 1992). Its expression is highest dorsally and decreases ventrally (Bulfone et al., 1993) and overlaps with Pax-6 expression in the dorsal thalamus and along the dorsal midline as far as the diencephalon/mesencephalon border.

We used a mouse with a mutation of Pax-6 (Small-eye) to study the role of this gene in the development of the diencephalon. There are several alleles of Small eye: the one used here was the original spontaneous mutation designated Sey, in which a point mutation in the Pax-6 locus results in a dysfunctional Pax-6 protein (Hill et al., 1991). We tested the hypothesis that disruption of Pax-6 disrupts embryonic diencephalic precursor proliferation, anatomy (morphological constrictions in the tissue, cell numbers and densities) and expression of regulatory genes (Dlx-2, Wnt-3 and Pax-6 itself).

**MATERIALS AND METHODS**

Embryonic development was assumed to have begun at midnight of the night of mating. Sey/Sey mice die at birth (Hogan et al., 1986), and Sey/Sey embryos (easily distinguished by the absence of eyes and a shortened snout) were obtained from Sey/+ × Sey/+ matings (heterozygotes were readily distinguished by their smaller than normal eyes; Hill et al., 1991). Age-matched controls were +/+ littersmates from these matings and +/+ embryos from +/+ × +/+ matings.

**Precursor proliferation**

Mothers were injected with bromodeoxyuridine (BrdU: 70 μg/g in sterile saline i.p.) on E10.5, E12.5 and E14.5 and were killed by cervical dislocation after 30 minutes. Fetuses were fixed in 4% paraformaldehyde. They were sectioned at 10 μm (in the planes shown in Results) and reacted to reveal BrdU as described by Gillies and Price (1993). Sections were lightly counterstained with cresyl violet. To estimate proliferative rates, average labelling indices (LIs) in the proliferative zone of the diencephalon were obtained at each age (LI: labelled cells as a proportion of total cells; Takahashi et al., 1993). At E10.5, the proliferative zone was the entire thickness of the wall of the diencephalon, at E12.5 and E14.5 it was restricted to its medial aspect (see Results for further details of counts). In addition, total numbers of BrdU-labelled cells in E12.5 wild-type and Sey/Sey diencephalons were counted in serial sections.

**Cell death**

Sections of E10.5, E12.5 and E14.5 wild-type and Sey/Sey embryos were stained with bisbenzimide (5 μg/ml for 10 minutes) to selectively stain the cell nuclei. Apoptotic cells were identified by their densely condensed nuclei, as first described by Kerr et al. (1972). For each of four wild-type and four Sey/Sey embryos at each age, the proportion of cells that were apoptotic was measured in 30 randomly selected 0.5×0.5 mm areas through the diencephalon, to give average rates for apoptosis in +/+ and Sey/Sey E10.5, E12.5 or E14.5 embryos.

**Anatomical study**

E14.5 embryos were removed from anaesthetized mothers (0.3 ml urethane in sterile saline, i.p.) by Cesarean section. The brains were removed in phosphate-buffered saline (PBS) at 4°C, and fixed in 4% paraformaldehyde for 2-3 hours at room temperature. The brains were washed briefly in PBS, dehydrated in alcohol, placed in chloroform overnight and embedded in wax. 10 μm parasagittal serial sections were cut and Nissl-stained.

Diencephalic regions were identified using several criteria, including the presence of strips of low cell density, tissue constrictions and domains of gene expression in both normal and mutant mice (see Results). We analysed cell densities in the mesencephalon and in regions of the diencephalon, namely the pretectum, dorsal thalamus and ventral thalamus, and measured the volumes of dorsal thalamus and ventral thalamus (the diencephalic regions whose borders could be delineated in both +/+ and Sey/Sey mice, see Results). To estimate densities, 4-5 sections taken at equally spaced mediolateral positions from each of three brains were selected. For each section, a 10×10 square graticule was placed over the mesencephalon, pretectum, dorsal thalamus and ventral thalamus in +/+ and Sey/Sey brains. All cells with a visible nucleus covered by the grid were counted and the average cell density (per mm 3 ) was calculated for each diencephalic region. To estimate volumes, ten sections taken at equally spaced mediolateral positions through the diencephalic regions in each of three brains were selected. The area of each region in each section was measured using NIH IMAGE 1.58 VDM software. For each brain, volumes were obtained by multiplying each area by the thickness of tissue between sections and summing the values. An estimate of total cell number in each region was obtained by multiplying its volume by its corresponding average cell density.

**Method of cell counting**

At various stages throughout this study, we counted cellular profiles in sectioned material. For clarity, we refer to these as ‘cell’ counts rather than ‘profile’ counts (Coggeshall and Lekan, 1996). We were interested in making comparisons, i.e. in the ratios of counts or densities between wild-type and Sey/Sey embryos, rather than absolute cell counts in any one type of embryo. As discussed more fully by Saper (1996), comparisons based on uncorrected profile counts are accurate provided that the structures being counted do not vary in size and shape between the two conditions, since systematic biases in estimations of population size should cancel. Where we have made comparisons, we did not find any such variations between +/+ and Sey/Sey embryos. We counted 400 randomly selected cell diameters from the diencephalon of each of 3 +/+ and 3 Sey/Sey embryos of each age and found no significant differences. Therefore, corrections were not applied to the profile counts.

**In situ hybridizations**

E14.5 mouse brains were dissected and fixed overnight in 4% paraformaldehyde at 4°C. Whole-mount in situ hybridizations were performed as described by Conlon and Rossant (1992). The Dlx-2 antisense riboprobe was transcribed from a 1.7 kb fragment derived from the 3’ untranslated region of the Dlx-2 cDNA clone (a gift from J. L. R. Rubenstein). The Wnt-3 antisense riboprobe was transcribed from a 0.6 kb fragment derived from a noncoding and nonconserved region of the Wnt-3 cDNA clone (a gift from R. Nusse). The Pax-6 antisense riboprobe was transcribed from a 1.7 kb fragment derived from the Pax-6 cDNA clone (a gift from R. Hill). Sense probes were generated from these plasmids for controls. Stained embryos were cleared in glycerol and photographed. Some embryos were embedded in wax as described above, and sectioned at 10 μm.

**RESULTS**

Early defect in cell proliferation

Fig. 1A,D shows the planes in which the E10.5 +/+ and Sey/Sey forebrains were cut, giving coronal sections. The exact planes of section varied very little between embryos of either type. This was confirmed by identifying the relative locations of major head structures (such as the telencephalic vesicles, the optic stalk, Rathke’s pouch, and the trigeminal ganglia), and following the changes in shape of the diencephalon and its transition to the mesencephalon through serial sections in both +/+ and Sey/Sey brains. To study the alar diencephalon, we took 6-9 equally spaced sections between lines a and b in Fig. 1A,D from each of four +/+ and four Sey/Sey mice. These sections
passed through regions that express Pax-6 at this age (see Fig. 5A), and entered the mesencephalon caudally (at least in wild-types, see below). To study the basal diencephalon and more caudal mesencephalon, we took 4 equally spaced sections between lines c and d in Fig. 1A,D from each of four +/+ and four Sey/Sey mice. These sections, which looked like the drawing in the inset of Fig. 1A in both types of embryo, cut through regions that do not express Pax-6 (Fig. 5A). Fig. 1B,E shows coronal sections through the alar diencephalon of +/+ and Sey/Sey embryos at low magnification. In the mutants, the ventricular space broadened abnormally towards the caudal end of the diencephalon (Fig. 1E). Thus, even at this early stage the gross morphology of the Sey/Sey diencephalon was abnormal.

For sections through the alar diencephalon, LI was measured on one side of each brain, in bins that were 75 μm wide (in the rostrocaudal direction) and ran through its entire wall. For each section, this gave a rostrocaudal profile of LIs through the diencephalon. Measurements began immediately caudal to the optic stalk (at the positions marked by the black lines in Fig.

Fig. 1. (A,D) Drawing of parasagittal views of an E10.5 +/+ (A) and an E10.5 Sey/Sey (D) forebrain to show the planes of section in which cellular proliferation was measured (broken lines). Sections analyzed in G were taken between lines a and b (through alar diencephalon) and appeared as in B and E. Sections between lines c and d appeared as in the inset of A. mes, mesencephalon; di, diencephalon; t, telencephalon; bp, basal plate. Scale bar, 100 μm. (B,E) Sections through a +/+ (B) and a Sey/Sey (E) embryo. LIs were measured starting at the short line just caudal to the optic recess (or), and continued through the diencephalon (d) and into the mesencephalon (m) of +/+ embryos (border marked with arrow in B) or presumptive mesencephalon (m) of Sey/Sey embryos (likely position of the border marked with asterisk in E). Scale bar, 500 μm. (C,F) Higher magnification views of the diencephalic wall of +/+ (C) and Sey/Sey (F) embryos taken from regions marked by white lines in B and E; arrow in C marks the apical surface. Scale bar, 50 μm. (G) Average LIs in a series of 75 μm wide bins running rostrocaudally through the diencephalon (± s.e.m.). Data are from four +/+ (broken line) and four Sey/Sey (continuous line) brains. All data points shown are n=4. CM indicates data from caudal mesencephalic regions between lines c and d in A and D. The extent of the diencephalon is underlined for each type of embryo; the right-hand end of the line for the Sey/Sey brains is broken to indicate our uncertainty about the exact position of the transition to the mesencephalon in the mutants.
and continued to the caudal extreme of each section. In +/+ embryos, the most caudal measurements were in the mesencephalon (recognized by the lateral bulging of the neural wall and a progressive transition to the appearance in the inset of Fig. 1A in more caudal sections). In Sey/Sey mice, the broadening of the ventricular space of the caudal diencephalon made recognition of the transition to the mesencephalon less obvious, and further evidence described below indicated that other markers of the diencephalon/mesencephalon border were also lacking. Therefore, only the likely position of this border is marked (with an asterisk) in Fig. 1E.

Averaged results (± s.e.m.s) for LIs through these alar regions are shown in Fig. 1G. In +/+ embryos, LIs were higher in the diencephalon than in the mesencephalon, and diencephalic LIs were about 150% greater than those in Sey/Sey embryos. By contrast, in Sey/Sey embryos LIs were very similar throughout the diencephalon and presumptive rostral mesencephalon. In the latter region they were no different to values in +/+ mesencephalon. To check this conclusion regarding the lack of differences between LIs in +/+ and Sey/Sey mesencephalon, we measured LIs in more caudal regions where we were confident that we were well within the mesencephalon in both normal and mutant embryos (between lines c and d in Fig. 1A,D). We found no significant difference between the LI for normal and mutant embryos (values are plotted on the right of Fig. 1G). Finally, our measurements of LIs in the basal plate of the diencephalon were 47±4 (s.e.m.) % in +/+ embryos and 60±7 % in Sey/Sey embryos, and this difference was not significant (Student’s t-test). Therefore, we conclude that in Sey/Sey mice LIs were abnormal in specifically those regions where Pax-6 is normally expressed.

Fig. 1C,F shows sections of the diencephalic wall at higher magnification. In addition to the reduction in the LI, the pattern of BrdU labelling was altered in the mutants. BrdU-labelled cells were present at the apical surface of the neuroepithelium of E10.5 +/+ embryos (arrow in Fig. 1C), and at the apical surface of the mesencephalon of E10.5 Sey/Sey embryos. There were very few BrdU-labelled cells at the apical surface in the alar diencephalon of Sey/Sey embryos (Fig. 1F). From a close inspection of serial sections, it was clear that this reduction of apical labelling was much greater than the overall reduction in LIs. In +/+ embryos, the apical labelled cells often appeared to be undergoing M-phase, i.e. the BrdU was fragmented rather than presenting a smooth nuclear profile as in the overlying neuroepithelium. It is known that, during neurogenesis, S-phase occurs in nuclei deep to the apical surface and they then move to undergo M-phase at the apical surface (Takahashi et al., 1993). Taken together, these observations indicate that the rate of cell division throughout the diencephalon of E10.5 Sey/Sey embryos is abnormally low.

We then measured the LI in the proliferating regions of the diencephalon in parasagittal sections of older embryos, aged E12.5 and E14.5. The average LIs from four +/+ and four Sey/Sey embryos at each age were obtained by measuring LIs in 30-40 serial sections from E12.5 embryos and 4-5 serial sections from E14.5 embryos. The proliferating regions of E14.5 embryos were very small; at this age, diencephalic neurogenesis is almost complete. There were no significant differences in the average LIs between different regions (ventral thalamus, dorsal thalamus and pretectum) of either +/+ or Sey/Sey embryos, nor between +/+ and Sey/Sey embryos, at either age. Average LIs ranged from 20.8% to 28.8% at E12.5 and from 10.8% to 12.5% at E14.5.

We then counted the total number of BrdU-labelled cells in the dorsal and ventral thalamus in serial sections through four +/+ and four Sey/Sey embryos aged E12.5. Only the dorsal and ventral thalamus were considered since their limits are clear in the mutant (we believe pretectum exists in the mutants, for...
reasons given below, but its posterior border with the mesencephalon is not clear morphologically. The average number of BrdU-labelled cells in the dorsal and ventral thalamus was 16,667±3,636 (s.e.m.) in +/+ embryos and 7,629±1,859 in Sey/Sey embryos. This was a significant reduction (Student’s t-test, P<0.008).

The proportions of apoptotic cells were very low throughout the diencephalon at all three ages studied in both +/+ and Sey/Sey embryos. The values ranged from 0.016% to 0.067%, with no significant differences between wild-type and mutant embryos at any age. We conclude that the loss of Pax-6 function in Sey/Sey embryos results in an early (E10.5) reduction in the rate of diencephalic precursor proliferation that, by E12.5, reduces the size of the proliferative pool in this region.

Gross morphological defects in older Sey/Sey diencephalon

We investigated the extent to which the diencephalon is morphologically abnormal in E12.5 and particularly E14.5 Sey/Sey mice, when diencephalic neurogenesis is almost complete. As has been described before (Bulfone et al., 1993; Puelles and Rubenstein, 1993), several transverse strips of low cell density run approximately dorsoventrally in the diencephalon of +/+ E12.5 (Fig. 2A) and +/+ E14.5 embryos (Fig. 2B). The most caudal strip (seen in Fig. 2A) coincides with the border between the diencephalon (its pretectal area or alar p1) and the mesencephalon. A second, more rostral strip lies at the border of the pretectum and the dorsal thalamus (alar p2; seen in Fig. 2A,B). The two most rostral strips (seen in Fig. 2B) converge on the base of the choroid plexus and delineate the ventral thalamus (alar p3). Associated with the strips are morphological constrictions (e.g. between the pretectum and the dorsal thalamus in Fig. 2B), and these can be useful markers of regional borders. However, as the neuroepithelium increases in size, these constrictions can move out of register with the strips of low cell density (Northcutt and Butler, 1993). In recent studies, the strips, which are in some cases the sites of developing axonal tracts, have been shown to be the true markers of prosomeric borders (Bulfone et al., 1993).

Parasagittal sections of E12.5 and E14.5 Sey/Sey embryos revealed a striking abnormality of the rostral diencephalon. Rostral to the choroid plexus, where the ventral thalamus and emt normally lie, the dorsal surface of the diencephalon, which normally has a smooth convexity (Fig. 2B), plunges...
**Fig. 4.** (A-E) *Dlx-2* and (F,G) *Wnt-3* expression in E14.5 embryos. (A) +/+ control showing normal *Dlx-2* expression in the ventral thalamus (vt), and more rostral structures (including the posterior entopeduncular area, hypothalamic cell cord, suprachiasmatic area (sch), tuberal hypothalamus (tu), and the medial ganglionic eminence (mge) and its surrounding tissues). Arrowhead marks the vt/dorsal thalamus (dt) border. emt, eminentia thalami; rch, retrochiasmatic area; ma, mammillary area. (B) Detail of A, showing the sharp caudal boundary of *Dlx-2* expression at the zli. (C) *Dlx-2* expression in Sey/Sey embryo. Arrowhead marks the position of the dt/vt border. (D) Detail of C: strong ectopic *Dlx-2* expression in the emt is separated by a strip of *Dlx-2*-negative tissue from a domain of weak expression in vt, with a blurred caudal border. The appearance of the staining in vt results from the superimposition of very weak, low contrast but specific label from different depths. (E) *Dlx-2* expression in tu of a Sey/Sey embryo. A sharp caudal boundary of expression is maintained. Arrow indicates *Dlx-2* mRNA transcripts detected in rch. (F,G) *Wnt-3* expression in E14.5 embryos. (F) Medial view of +/+ control: note the strong *Wnt-3* expression in cells of the dorsal midline. Arrow marks the position of the zli and the rostral boundary of *Wnt-3* expression. Arrowhead marks the less-distinct caudal boundary of *Wnt-3* expression at the pt/dt border. (G) Sey/Sey embryo: very faint *Wnt-3* expression in dt forming a rostral boundary in the region of the dt/vt border and a diffuse caudal boundary in the region of the pt/dt border (borders marked with arrowheads). Scale bars, 100 μm.

**Fig. 5.** (A) Schematic map of the expression of *Pax-6* (green) at E10.5 in the +/+ mouse diencephalon (Puelles and Rubenstein, 1993). (B) Expression patterns of *Pax-6* (green), *Dlx-2* (purple) and *Wnt-3* (pink) in the E14.5 +/+ mouse diencephalon. (C) Expression patterns of *Pax-6*, *Dlx-2* and *Wnt-3* in the E14.5 Sey/Sey mouse diencephalon. AEP, anterior entopeduncular area; AH, anterior hypothalamus; CHP, choroid plexus; DT, dorsal thalamus; EMT, eminentia thalami; HCC, hypothalamic cell cord; M, mesencephalon; p1-p6, prosomeres 1-6; PEP, posterior entopeduncular area; POA, posterior preoptic area; PPO, preoptic recess; PT, pretectum; SCH, suprachiasmatic area; SPV, supraoptic/paraventricular area; TU, tuberal hypothalamus; VT, ventral thalamus.
ventrally and the ventricle is enlarged (Fig. 2C). We saw two clear constrictions in the tissue caudal to the choroid plexus (arrowheads in Fig. 2C), and three strips of low cell density associated with them. The two most rostral strips converge on the base of the choroid plexus adjacent to the most rostral constriction (Fig. 2D). We believe these two rostral strips of low cell density delineate presumptive ventral thalamus in the Sey/Sey mouse. A third more caudal strip of low cell density, coinciding with the caudal constriction, was seen in the E12.5 Sey/Sey diencephalon and in sections of the E14.5 Sey/Sey diencephalon lateral to that in Fig. 2C (as drawn in Fig. 3E). We believe that this caudal constriction and strip mark the caudal limit of the presumptive dorsal thalamus in the mutants. These conclusions are supported by data on gene expression, as described below. In E12.5 and E14.5 Sey/Sey embryos, we found neither a strip of low cell density nor a constriction between the pretectum and the mesencephalon. We also noted that the neuroepithelium rostral to the ventral thalamus is thinner than normal in the dorsoventral direction (compare Fig. 2B and 2C), the striatum (anterior to the enlarged ventricle) is much larger than normal, the cortex is smaller than normal (Fig. 2C; as described previously by Schmahl et al., 1993) and the mesencephalon appears normal in Sey/Sey embryos.

**Reduced cell numbers in E14.5 Sey/Sey diencephalon**

Regional differences in cell densities may be an indicator of segmentation, since diencephalic neuromeres appear to develop as independent units separated by cell lineage restriction boundaries (Fidgord and Stern, 1993). In the diencephalon of +/+ mice, average cell density was significantly higher in the dorsal thalamus than in both the ventral thalamus (data in Table 1; \( P<0.001 \), Student’s t-test) and pretectum (density = 8.6×10^5 cells/mm^3) (\( P<0.05 \), Student’s t-test). Cell density in the mesencephalon was not significantly different to that in the pretectum. These differences in cell density between pretectum, dorsal thalamus and ventral thalamus have not been reported previously. There were no such differences in Sey/Sey mice (densities for presumptive dorsal and ventral thalamus are in Table 1; density for pretectum = 7.2×10^5 cells/mm^3) suggesting that one important defect in the Sey/Sey embryos was a loss of variation in cell density between the dorsal and ventral thalamus. Table 1 shows the total numbers of cells in the dorsal thalamus and ventral thalamus in +/+ and Sey/Sey embryos. Whereas there was no difference in the densities of cells between the dorsal and ventral thalamus in the Sey/Sey mice, the dorsal thalamus (which is larger than the ventral thalamus in +/+ and Sey/Sey mice: Table 1, \( P<0.05 \), Student’s t-test) contained significantly more cells than the ventral thalamus in both +/+ and Sey/Sey embryos. There was a tendency for cell numbers to equilibrate in the two (ratio for dorsal:ventral thalamic cell numbers was 2.0:1 in +/+ embryos, but 1.4:1 in Sey/Sey embryos). Overall, there were fewer cells in the Sey/Sey thalamus and its volume was reduced (Table 1). It is most likely that this reduction in cell number and volume resulted from the earlier reduction in proliferative rates in the mutants, as analysed above.

**Molecular analysis**

**Pax-6 expression**

Our whole-mount in situ hybridizations on E10.5 +/+ embryos confirmed previous studies showing Pax-6 expression in a longitudinal band through the alar plate from the region of the optic stalk to the border between the diencephalon and mesencephalon (shown schematically in Fig. 5A; Puelles and Rubenstein, 1993; Stoykova and Gruss, 1994; Stoykova et al., 1996). By E14.5, diencephalic expression was restricted to the caudal part of the ventral thalamus, the dorsal midline of the dorsal thalamus and pretectum, with a mediolateral expansion on the dorsal surface of the caudal pretectum forming a sharp caudal boundary that coincided with the diencephalon/mesencephalon border (Figs 3A,B, 5B) (\( n=12 \) embryos).

In all E14.5 Sey/Sey embryos (\( n=6 \)), Pax-6 expression was abnormal although two main features of the E14.5 +/+ pattern were seen, i.e. the transverse rostral domain and the caudally extending roofplate expression (Fig. 3C). The domain of expression in the ventral thalamus was expanded in the Sey/Sey embryos, and appeared to fill all of the ventral thalamus (reminiscent of expression at an earlier developmental age) rather than just its caudal part, as in +/+ embryos. Pax-6 expression was still detected along the dorsal midline of the dorsal thalamus and pretectum in Sey/Sey embryos, although its caudal limit did not form an expanded and sharp boundary as seen in +/+ embryos at the pretectum/mesencephalon border (compare Fig. 3A,B with 3C,D).

Parasagittal and coronal sections through these Sey/Sey whole-mounts revealed that the rostral domain of Pax-6 expression extended from the zli (the strip of low cell density between the ventral and dorsal thalamus) through the entire ventral thalamus with expression also in the emt. Expression was detected along the dorsal midline but not in the body of the dorsal thalamus (Fig. 3E). Fig. 3E shows camera lucida drawings of three equally spaced parasagittal sections with major morphological features marked (as seen with Normaski optics and in cresyl violet counterstained sections). In lateral sections the expression domain for Pax-6 in the ventral thalamus was relatively broad rostrocaudally. In more medial sections, this domain narrowed progressively. At the medial surface, labelling in the ventral thalamus was just a narrow

**Table 1. Cell densities, tissue volumes and cell numbers in dorsal thalamus (dt) and ventral thalamus (vt) in +/+ and Sey/Sey E14.5 embryos**

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<th>vt +/+</th>
<th>vt Sey/Sey</th>
<th>dt +/+</th>
<th>dt Sey/Sey</th>
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<td>Mean cell density (×10^5/mm^3)</td>
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<td>10.0</td>
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<tr>
<td>Mean volume (mm^3)</td>
<td>0.16</td>
<td>0.06</td>
<td>0.21</td>
<td>0.09</td>
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<tr>
<td>Mean cell numbers, m. (×10^5) (n=3)</td>
<td>9.8±1.0* ‡</td>
<td>4.9±0.3† ‡</td>
<td>20.3±1.5* §</td>
<td>7.0±0.1† §</td>
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Data are from 4-5 sections from each of 3 brains. Means for cell number are compared using Student’s t-test (two-tailed). Significant differences: *\( P<0.003 \); †\( P<0.03 \); ‡\( P<0.025 \); §\( P<0.01 \).
strip, but label extended a considerable distance caudally. As shown by the arrow in Fig. 3E, the tissue constriction between the dorsal thalamus and pretectum was seen clearly (shown in Fig. 3D), and labelling for Pax-6 expression extended well beyond this constriction (and the domain of Wnt-3 expression in the dorsal thalamus of the mutants, see below). For this reason, we believe that the pretectum exists in the Sey/Sey embryos.

**Dlx-2 expression**

In agreement with previous accounts of normal expression (Bulfone et al., 1993; Porteus et al., 1994), we found that Dlx-2 was expressed in two domains in E14.5 +/+ embryos (n=15 embryos) (Fig. 4A). The rostral domain (part of which is shown in Fig. 4A) coincided with the medial ganglionic eminence (mge) and surrounding structures including the anterior entopeduncular area (AEP) and posterior preoptic area (POA) (see Fig. 5B). The caudal domain extended in the alar plate from the area of the suprachiasmatic nucleus (sch) through the hypothalamic cell cord (hcc) and posterior entopeduncular area (pep) into the ventral thalamus (vt), or alar p3, and terminated abruptly at the zli (Figs 4A,B, 5B). Dlx-2 was also expressed in the tuberal hypothalamus (tu: basal p5), with a sharp caudal boundary and a more diffuse rostral boundary (Fig. 4A). The anterior and posterior domains of Dlx-2 expression were separated by a strip of Dlx-2 negative tissue corresponding to the optoemineral zone, which extends from the emt to the optic stalk (Fig. 4A; Bulfone et al., 1993). It is this Dlx-2 negative strip that, at earlier ages, is known to express Pax-6 (see Fig. 5B; Puelles and Rubenstein 1993; Stoykova et al., 1996). Thus, rostral to the ventral thalamus, expression of Pax-6 and Dlx-2 show very little if any overlap, whereas they do coincide in the ventral thalamus.

In E14.5 Sey/Sey embryos, Dlx-2 was also expressed in two domains (n=6 embryos). Expression in the rostral domain appeared normal (not shown). In the caudal domain expression appeared normal in alar tissue corresponding to the sch, hcc, pep and in the basal plate in the tuberal hypothalamus (Fig. 4C,E), and expression boundaries were similar to those in +/+ embryos. By contrast, Dlx-2 expression was abnormal in the ventral thalamus (Fig. 4D). It was very weak and its caudal boundary appeared faded and diffuse in wholemounts (compare Fig. 4D with 4B). The appearance of the staining in the ventral thalamus in Fig. 4D resulted from the superimposition of very weak, low contrast but specific label from different depths. Indeed, the labelling in the ventral thalamus was so weak that it was not seen clearly in sectioned material.

Overall, these data add additional weight to the conclusion that the ventral thalamus, defined morphologically and on the basis of Pax-6 and Dlx-2 expression, is present but in an abnormal form in Sey/Sey embryos.

**Wnt-3 expression**

In E14.5 +/+ embryos (n=15 embryos), Wnt-3 was expressed in a single domain coinciding with the dorsal thalamus (Fig. 4F), as described previously by Salinas and Nusse (1992). Expression was strongest in ventricular cells of the dorsal midline (Fig. 4F), from where it extended ventrally. Thus, at E14.5 Wnt-3 expression in the dorsal thalamus was strongest dorsally, where Pax-6 expression persisted, but extended into ventral regions where Pax-6 would have been expressed at earlier ages (Fig. 5A). In all Sey/Sey embryos (n=6 embryos), Wnt-3 expression was always much weaker than in +/+ embryos (Fig. 4G; reactions were carried out with Sey/Sey and +/+ embryos together in the same tubes). In the mutants, the weak staining was still confined to the dorsal thalamus. Strong dorsal midline expression of Wnt-3, characteristic in the +/+ embryos (Fig. 4F), was absent in Sey/Sey embryos (Fig. 4G).

Based on these findings and the morphology of the E14.5 Sey/Sey diencephalon, we concluded that the dorsal thalamus was present in the mutants. As described above, it was clear that the caudal extension of Pax-6 expression in the Sey/Sey mutants extended caudal to the morphological features that distinguished the caudal border of the dorsal thalamus (Fig. 3). In addition, careful comparison of Pax-6 and Wnt-3 expression indicated that the domain of Pax-6 expression extended caudal to the caudal limit of Wnt-3 expression in the Sey/Sey embryos. Therefore, we believe that the pretectum is also present in the mutants. Our main conclusions on the anatomy and molecular characteristics of the E14.5 Sey/Sey diencephalon are summarized in Fig. 5C.

**DISCUSSION**

Pax-6 is expressed throughout the alar diencephalon of early (E10.5) embryos. Our first major finding was that proliferative rates in the early (E10.5) Sey/Sey diencephalon are significantly reduced in alar regions, but not in basal regions or the mesencephalon where Pax-6 is not expressed. Results from older embryos suggest that the reduced diencephalic proliferative rate in the E10.5 mutants leads to a depletion of the proliferating precursor pool at E12.5 and a reduction in total diencephalic cell numbers at E14.5. In both the +/+ and Sey/Sey diencephalon, very few proliferating cells were found at E14.5, indicating that proliferation terminates in this region at a similar time in the wild-types and mutants. We found no evidence for an altered rate of cell death in the diencephalon of the mutants.

This result of a loss of function of Pax-6 in the diencephalon is remarkably similar to the consequence for telencephalic development of creating a null-mutation of the winged helix transcription factor, brain factor 1 (BF-1). Null-mutation of BF-1 results in reduction in the size of the cerebral hemispheres as a consequence of earlier reduction in telencephalic proliferative rates (Xuan et al., 1995). As is the case in the telencephalon of BF-1 knock-outs, it is not yet certain whether the reduced proliferation in the diencephalon of Sey/Sey embryos is a consequence of a reduced pool of precursors or a lengthening of the cell cycle. Two observations suggest that, for Pax-6, the latter is more likely. While the LIs were reduced by about a third in the diencephalon of Sey/Sey E10.5 embryos, there was a relatively larger reduction in the numbers of cells actually undergoing mitosis at the apical surface of the neuroepithelium. This suggests that S-phase may be longer in the mutants, and hence the rate of cell division slower.

Previous work has indicated that Hox genes control rates of proliferation in other regions of the CNS (Dolle et al., 1993; Condie and Capecchi, 1994). Clearly, correct regionally specific regulation of cell proliferation is crucial in the developing forebrain, where adjacent regions proliferate with very different characteristics to generate structures as different in
size as the diencephalon and the telencephalon. It is likely that genes such as Pax-6 and BF-1, that are selectively expressed in discrete regions in the diencephalon and telencephalon, play an essential role in generating these differences. It is interesting that the rate of cell proliferation is higher in the diencephalon than the mesencephalon of wild-type mice. This may reflect the independent development of these adjacent regions.

Our anatomical and molecular evidence indicates that, despite these early proliferative defects, distinct and recognizable diencephalic regions (ventral thalamus, dorsal thalamus and pretectum), corresponding to their counterparts in wild-type embryos, do emerge in Sey/Sey embryos, in agreement with a recent report by Stoykova et al. (1996). These findings indicate that Pax-6 is neither the master regulator of, nor even an essential permissive agent for, diencephalic regionalization. However, it is probable that Pax-6 does have roles in fine-tuning aspects of regional differentiation. Certainly, although present, the ventral and dorsal thalamus and the pretectum of Sey/Sey embryos are strikingly abnormal. Among the abnormalities that we noted were a loss of the normal difference in cell density between the ventral and dorsal thalamus, a reduction in the difference in cell numbers between them, a reduced expression of Dlx-2 in the ventral thalamus and of Wnt-3 in the dorsal thalamus, and a loss of a clear diencephalon/mesencephalon border.

Reduced Dlx-2 expression in the ventral thalamus might reflect the fact that normal Pax-6 expression is required for normal Dlx-2 expression in this area. The downregulation of Wnt-3 expression in the dorsal thalamus may be a direct or indirect consequence of the loss of normal Pax-6 expression in this region. There is clear evidence for interactions among homeobox genes and between homeobox genes and wingless-type genes in other species, including Drosophila (DiNardo and O’Farrell, 1987; Ingham et al., 1988; Hidalgo and Ingham, 1990; Cohen, 1990; Heemskerk et al., 1991) and zebrafish (Krauss et al., 1992), and they have been hypothesized in mouse (McMahon et al., 1992; Bulfone et al., 1993; Iler et al., 1995).

The expression of Pax-6 itself in Sey/Sey embryos was interesting. In rostral diencephalon, there was an increased domain of Pax-6 expression. In caudal diencephalon, there was a loss of the normal transverse stripe of Pax-6 expression at the diencephalon/mesencephalon border. The alteration in the level of Pax-6 transcripts in Sey/Sey embryos suggests that a functional Pax-6 protein is required (directly or indirectly) for the expression of its own gene in the forebrain. In vitro binding studies have demonstrated that Pax-6 can recognize sites within its own promoter (Plaza et al., 1993). The abnormal expression of Pax-6 in caudal pretectum coincides with a loss of morphological markers of the diencephalon/mesencephalon border, and suggests that Pax-6 may be involved in the formation of this border. In a similar vein, injection of antibodies against the Pax-b protein in zebrafish disrupts formation of the constriction at the midbrain/hindbrain border (Krauss et al., 1992).

The Small-eye phenotype studied here results from a point mutation that introduces a stop codon between the paired box and the homeobox. Since the several alleles of Small-eye have similar phenotypes, it is very likely that any truncated Pax-6 protein that is made in the homozygotes is highly unstable and afunctional (Hill et al., 1991). However, at present we cannot exclude the possibility that the abnormalities in forebrain development that we have described here result from a loss of specifically the Pax-6 homeodomain.

In conclusion, normal Pax-6 expression is needed for normal proliferation in the diencephalon, and for diencephalic regions to develop some of their important cellular and molecular attributes and specific distinguishing features. However, a degree of diencephalic regionalization can occur without normal Pax-6 expression. Thus, if there is a master regulator of diencephalic regionalization, it is unlikely to be Pax-6. We suggest that the major roles of Pax-6 in the diencephalon are in the control of cell proliferation and the later differentiation of some features of its three main regions.

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