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A role for *Pax6* in the normal development of dorsal thalamus and its cortical connections

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SUMMARY

The transcription factor *Pax6* is widely expressed throughout the developing nervous system, including most alar regions of the newly formed murine diencephalon. Later in embryogenesis its diencephalic expression becomes more restricted. It persists in the developing anterior thalamus (conventionally termed "ventral" thalamus) and pretectum but is downregulated in the body of the posterior (dorsal) thalamus. At the time of this downregulation, the dorsal thalamus forms its major axonal efferent pathway via the ventral telencephalon to the cerebral cortex. This pathway is absent in mice lacking functional Pax6 (small eye homozygotes: *Sey*/*Sey*). We tested whether the mechanism underlying this defect includes abnormalities of the dorsal thalamus itself. We exploited a new transgenic mouse ubiquitously expressing green fluorescent protein tagged with tau, in which axonal tracts are clearly visible, and co-cultured dorsal thalamic explants from *Pax6*$^{+/+}$ or *Pax6*$^{Sey/Sey}$ embryos carrying the transgene with wild-type tissues from other regions of the forebrain. Whereas *Pax6*$^{+/+}$ thalamic explants produced strong innervation of wild-type ventral telencephalic explants in a pattern that mimicked the thalamocortical tract in vivo, *Pax6*$^{Sey/Sey}$ explants did not, indicating a defect in the ability of mutant dorsal thalamic cells to respond to signals normally present in ventral telencephalon. *Pax6*$^{Sey/Sey}$ embryos also showed early alterations in the expression of regulatory genes in the region destined to become dorsal thalamus. Whereas in normal mice *Nkx2.2* and *Lim1/Lhx1* are expressed ventral to this region, in the mutants their expression domains are throughout it, suggesting that a primary action of *Pax6* is to generate correct dorsoventral patterning in the diencephalon. Our results suggest that normal thalamocortical development requires the actions of *Pax6* within the dorsal thalamus itself.

Key words: Confocal microscopy, Diencephalon, Dorsoventral patterning, Green fluorescent protein; *Lim1/Lhx1*, *Nkx2.2*, Organotypic co-cultures, Small eye mice, Tau, Thalamocortical axons, Transgenic mice

INTRODUCTION

The forebrain (prosencephalon) develops from the anterior-most region of the neural tube. Recent studies have revealed that the underlying organisation of the prosencephalon and of more caudal segmented regions of the neural tube may share common properties. On the basis of tissue morphology and patterns of gene expression, the Prosomeric Model (Puelles and Rubenstein, 1993; Rubenstein et al., 1994) was generated to provide a framework for understanding the morphological development of the prosencephalon. The Prosomeric Model subdivides the forebrain into six transverse domains called prosomeres, lying perpendicular to the anteroposterior (AP) axis of the neural tube, and four longitudinal histogenic domains, lying parallel to the AP axis of the neural tube (from dorsal to ventral: roofplate, alar plate, basal plate and floorplate). The diencephalon develops in prosomeres 1-3 (p1-p3; p1 is the most posterior), which are identified by morphological constrictions in the tissue and the expression domains of regulatory genes including *Pax6*, *Dlx2* and *Wnt3* (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. In relation to the AP axis of the CNS, which bends sharply in the brain, the dorsal thalamus develops posterior to the ventral thalamus. In this report, we retain the conventional nomenclature of "ventral thalamus" and "dorsal thalamus" wherever possible, although we sometimes apply the synonymous terms "anterior thalamus" and "posterior thalamus" (e.g. where the terms are embedded in a discussion of the ventral and dorsal longitudinal domains in the neural tube). The dorsal thalamus is a pivotal forebrain structure, receiving sensory inputs from the periphery and communicating via thalamocortical axons with the cerebral cortex.

The mechanisms that pattern the diencephalon and control the later development of structures such as the dorsal thalamus and its thalamocortical connections are poorly understood. In this study, we tested the role of the transcription factor, *Pax6*, in these processes. Pax6 contains two DNA-binding motifs, a paired domain and a paired-like homeodomain. The

spatiotemporal pattern of its expression, extending from embryonic day 8.5 (E8.5) until adulthood in many structures including the diencephalon, suggests that it plays a role in key stages of forebrain development (Walther and Gruss, 1991; Mansouri et al., 1994; Stoykova and Gruss, 1994). Mutations of *Pax6* result in the small eye (*Sey*) phenotype (Hill et al., 1991). Homozygotes (*Pax6*$^{Sey/Sey}$) show defects in numerous forebrain structures, including the diencephalon, and die shortly after birth (Hogan et al., 1986; Schmahl et al., 1993; Quinn et al., 1996; Stoykova et al., 1996; Caric et al., 1997; Grindley et al., 1997; Mastick et al., 1997; Warren and Price, 1997). Previous studies of the expression domains of regulatory genes have indicated that AP patterning of the diencephalon is relatively normal and that the dorsal thalamus is present in *Pax6*$^{Sey/Sey}$ embryos (Stoykova et al., 1996; Grindley et al., 1997; Mastick et al., 1997; Warren and Price, 1997). Kawano et al. (1999) have shown that, despite the presence of the dorsal thalamus in homozygous small eye rat embryos, thalamocortical connections do not form. Dorsal thalamic axons normally traverse the ventral thalamus and ventral telencephalon to reach the cerebral cortex whereas in the mutants they traverse the ventral thalamus but not the ventral telencephalon.

It has been suggested that the absence of thalamocortical axons in *Pax6*$^{Sey/Sey}$ embryos is due to abnormalities of *Pax6*-expressing cell clusters that lie along their path and would normally guide them (Kawano et al., 1999). The full extent of dorsal thalamic abnormalities in the absence of Pax6 has never been clearly defined, however, and it is equally possible that *Pax6*$^{Sey/Sey}$ dorsal thalamic axons have an intrinsic inability to respond to appropriate growth and guidance factors. We tested this hypothesis by taking dorsal thalamic explants from *Pax6*$^{Sey/Sey}$ embryos in which all the cells were marked with green fluorescent protein (GFP) tagged with tau and confronting them with wild-type ventral telencephalon, the region through which thalamocortical axons normally grow. The results indicated autonomous defects in the responses of dorsal thalamic axons to ventral telencephalic factors. Normal dorsal thalamus downregulates *Pax6* expression at the time when thalamocortical axons start to form (Puelles and Rubenstein, 1993; Stoykova and Gruss, 1994; Stoykova et al., 1996; Mastick et al., 1997; Warren and Price, 1997; Kawano et al., 1999; Auladell et al., 2000; our unpublished observations), and we went on to examine whether other regulatory genes might act as intermediates between Pax6 and the responsiveness of the dorsal thalamus. We describe an early abnormality in dorsoventral (DV) patterning of the *Pax6*$^{Sey/Sey}$ thalamus that may underlie defects in the responses of thalamocortical axons.

MATERIALS AND METHODS

Generation of transgenic mice expressing tau-GFP

Transgenic mice carrying a ubiquitously expressed GFP tagged with tau were generated. The GFP variant mmgfp6 cDNA (a gift from J. Haseloff; Siemering et al., 1996; Zernika-Goetz et al., 1997) was fused at its 5′ end to the 3′ end of a cDNA encoding bovine tau (Callahan and Thomas, 1994). The fusion cDNA was inserted into the mammalian expression vector, pCAGiP (a gift from I. Chambers), allowing the CAG promoter to drive expression of tau-GFP and puromycin resistance (Niwa et al., 1991). This construct was introduced into embryonic stem cells and the transgenic mouse line TgTP6.3 was derived by germline transmission. In these animals tau-GFP is expressed throughout the developing CNS including thalamus (Pratt et al., 2000). The tau tag tethers GFP to the microtubule-containing cytoskeleton, resulting in clear labelling of axons including those of the thalamocortical pathway (Fig. 2A).

Breeding

Embryonic development was assumed to have begun at midnight on the night of mating. Experiments were carried out on E10.5, E11.5, E12.5, E13.5, E14.5 E15.5, E17.5 and E19.5 embryos from matings between *Pax6*$^{Sey/+}$ mice, some of which carried the *tau-GFP* transgene (generating *tau-GFP:Pax6*$^{+/+}$ or *tau-GFP:Pax6*$^{Sey/Sey}$ embryos). *Pax6*$^{Sey/Sey}$ embryos were easily distinguished by their absence of eyes and shortened snout (Hill et al., 1991). Age-matched controls were *Pax6*$^{+/+}$ littermates from these matings and embryos from matings between *Pax6*$^{+/+}$ mice, some of which carried the *tau-GFP* transgene.

Organotypic cocultures

Cultures were carried out following methods in Lotto and Price (1999). Organotypic explants (300 μm thick) containing the regions of interest (dorsal thalamus, cerebral cortex, ventral telencephalon or hypothalamus) were dissected from coronal slices of E14.5 brains (as illustrated in Fig. 2A-D) and arranged on collagen-coated inserts (Costar, UK). Dorsal thalamic explants were from *tau-GFP:Pax6*$^{+/+}$ or *tau-GFP:Pax6*$^{Sey/Sey}$ embryos; other tissues were from *Pax6*$^{+/+}$ or *Pax6*$^{Sey/Sey}$ embryos that did not carry the transgene. Dorsal thalamus was recognized under transmitted and epifluorescence illumination, guided by anatomical landmarks known to correspond to gene expression boundaries defining the dorsal thalamus in both wild-type and mutants (Fig. 2A-E; described in Results). The left and right dorsal thalamus were left joined dorsally in culture and their cut edges (red dotted lines in Fig. 2B,D, just anterior to the border between dorsal and ventral thalamus) were placed against different tissues on each side. To recreate the topography of intact brain as much as possible, these cut edges of dissected thalamus were placed in contact with either the medial edge of dissected ventral telencephalon or the dorsal edge of dissected hypothalamus (Fig. 2B,D). Three days after being placed in serum-free medium, cultures were fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS), stained with the nuclear counterstain TO-PRO3 (Molecular Probes, USA) and mounted in 1:1 glycerol:PBS containing 10% Vectashield (Vector Laboratories, UK). Images of the cultures were obtained by combining serial optical sections acquired with a Leica confocal microscope. The following co-culture combinations were tested: *Pax6*$^{+/+}$ ventral telencephalon with either *tau-GFP:Pax6*$^{+/+}$ thalamus (n=25) or *tau-GFP:Pax6*$^{Sey/Sey}$ thalamus (n=21); *Pax6*$^{+/+}$ cortex with either *tau-GFP:Pax6*$^{+/+}$ thalamus (n=21) or *tau-GFP:Pax6*$^{Sey/Sey}$ thalamus (n=21); *Pax6*$^{+/+}$ hypothalamus with either *tau-GFP:Pax6*$^{+/+}$ thalamus (n=6) or *tau-GFP:Pax6*$^{Sey/Sey}$ thalamus (n=6); *Pax6*$^{Sey/Sey}$ ventral telencephalon with *tau-GFP:Pax6*$^{+/+}$ thalamus (n=4).

In situ hybridisation

RNA probes were labelled with digoxigenin, as described by the manufacturer (Roche Diagnostics, UK). The *Sonic hedgehog (Shh), Pax6, Lim1/Lhx1, Nkx2.2* and *Dlx2* plasmids were provided by J. Rubenstein, the *Netrin 1* plasmid by M. Tessier-Lavigne, the *Hbnf* plasmid by T. Vogt and L. Amet and the *VMAT2* plasmid by R. Edwards. Sense probes were synthesised for controls. Embryos were dissected from anaesthetised mothers (0.3 ml of 25% urethane in sterile saline, i.p.) in phosphate-buffered saline (PBS) at 4°C and fixed for 3-12 hours in 4% paraformaldehyde + 0.2 mM egtazic acid (EGTA) at 4°C. The embryos were washed in PBS, dehydrated in ethanol and cleared in xylene for 3 hours. The embryos were then wax embedded, coronally sectioned at 6 μm and collected on TESPA-coated slides. In situ hybridisations were performed as described by Wilkinson (1992). Whole-mount in situ hybridisations (for *Pax6*,

Nkx2.2 and *Netrin 1*) were performed on whole embryos or on 150-200 μm-thick sections, as described by Shimamura et al. (1994).

Pharmacological treatments

Clorgyline (NBI, 30 mg/kg), a specific inhibitor of monoamine oxidase A activity, was administered intraperitoneally to pregnant dams at E17.5. Embryos were sacrificed 3 hours following injection.

Immunocytochemistry for serotonin, calretinin and Nkx2.2

5-HT immunocytochemistry was performed using a rat monoclonal anti-5-HT antibody (1:50; Harlan-SeraLab). Calretinin immunocytochemistry was performed using rabbit polyclonal antibodies (1:10,000; Swant). Embryos were transcardially perfused with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Brains were removed and postfixed 2-5 days in the same fixative and cryoprotected in 30% sucrose in PB. Serial coronal sections (50 μm) were cut on a freezing microtome and processed as described previously (Cases et al., 1996). Nkx2.2 immunocytochemistry was performed on paraffin-embedded sections. Sections were dewaxed and rehydrated, transferred to a glass container containing 10 mM sodium citrate pH 5.5 and antigens were retrieved by boiling the sections in a microwave (med-high for 15 min). Tissue sections were then transferred to PBS. Non-specific binding sites were blocked by using 2% (v:v) normal sheep serum and 2% (w:v) bovine serum albumin (BSA) in PBS. The excess blocking solution was carefully removed and tissue sections were incubated overnight at room temperature with a 1:30 dilution of mouse monoclonal anti-Nkx2.2 antibody (74.5A5, Developmental Studies Hybridoma Bank, USA). Tissue sections were washed three times in PBS, then incubated for 2 hours with a 1:200 dilution of biotinylated secondary sheep anti-mouse IgG (Amersham, UK). After washing with PBS, tissue sections were incubated with a streptavidin-biotin complex (Amersham, UK) for 2 hours, washed again in PBS and developed as described previously (Cases et al., 1996).

Anatomical analyses

E15.5, E17.5 and E19.5 embryos were processed for wax sectioning as described above and counterstained with Haematoxylin and Eosin or Nissl-stained. We used the nomenclature for anatomical structures described by Paxinos et al. (1991). To reveal the thalamocortical tract with dioctadecyl tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, USA), E13.5, E15.5. and E17.5 embryos were fixed with paraformaldehyde, small crystals of DiI were placed in the dorsal thalamus and, after diffusion, sections were cut with a vibratome.

RESULTS

Lack of thalamocortical projections in *Pax6$^{Sey/Sey}$* embryos

Kawano et al. (1999) described a failure of thalamocortical axonal development in homozygous small eye rat embryos and, as illustrated in Fig. 1, we found a similar defect in *Pax6$^{Sey/Sey}$* mouse embryos. We labelled dorsal thalamic neurons and their projections with antibodies to 5-HT following treatment with clorgyline, a specific inhibitor of the degradative enzyme monoamine oxidase A, as described by Cases et al. (1998) and Vitalis et al. (1998). In E17.5 clorgyline-treated wild-type embryos, we observed immunolabelled thalamocortical sensory neurons and their axons running from the dorsal thalamus to the cortical subplate (Fig. 1A) through the internal capsule (Fig. 1B). In E17.5 clorgyline-treated *Pax6$^{Sey/Sey}$* embryos, we were unable to detect 5HT-immunolabelled axons in the subplate and internal capsule (Fig. 1C,D). Fig. 1E,F shows staining for calretinin in sections of E17.5 wild-type and *Pax6$^{Sey/Sey}$* embryos. In wild-type embryos, staining was detected in the cell bodies and axonal processes of discrete dorsal thalamic nuclei and in the thalamocortical tract (Arai et al., 1994; Frassoni et al., 1998). In *Pax6$^{Sey/Sey}$* embryos, staining was greatly decreased in the dorsal thalamus, labelled cells were absent from the thalamic midline and there was a loss of immunolabelled thalamocortical axons. We also demonstrated absence of thalamocortical projections in *Pax6$^{Sey/Sey}$* embryos using DiI. As shown in Fig. 1G,H, from E15.5 onwards DiI placed in the dorsal thalamus diffused to the cerebral cortex in wild type (Fig. 1G) but did not get further than the medial part of the ventral telencephalon in the mutants (Fig. 1H shows at high magnification, an area in a mutant equivalent to that indicated by an asterisk in Fig. 2C).

Analysis of thalamocortical growth in culture

The aim of these experiments was to test whether or not axons from dorsal thalamus in *Pax6$^{Sey/Sey}$* embryos are able to respond to growth and/or guidance signals along their path to the cortex. Fig. 2A shows that the thalamocortical tract is clearly visible in our transgenic mice expressing tau-GFP on a wild-type background (*tau-GFP:Pax6$^{+/+}$*). Axons originate from the dorsal thalamus, enter the ventral thalamus and turn at the hypothalamus into the ventral telencephalon, which they penetrate to reach the cortex (Fig. 2A). This tract is not seen in *tau-GFP:Pax6$^{Sey/Sey}$* embryos (Fig. 2C). To test the ability of thalamic axons to innervate tissue encountered along the normal thalamocortical pathway, we cultured explants of *tau-GFP:Pax6$^{+/+}$* and *tau-GFP:Pax6$^{Sey/Sey}$* thalamus in contact with wild-type ventral telencaphalon, hypothalamus or cerebral cortex (Fig. 2F-M; results are shown on a grey tint, which makes axons clearer). Dorsal thalamic explants were removed from sections appearing similar to that shown in Fig. 2A,C, as illustrated in Fig. 2B,D. Analysis of *Dlx2*, *Hbnf* and *Pax6* expression patterns (Figs 2E, 4C-F) and previous descriptions of gene expression patterns (Stoykova et al., 1996; Warren and Price, 1997; Kawano et al., 1999), helped us to define tissue landmarks and to make accurate dissections.

Dorsal thalamus from *tau-GFP:Pax6$^{+/+}$* embryos produced prolific outgrowth into wild-type ventral telencephalon, extending well over half-way through the telencephalic explants, in 76% of cultures (typical examples are shown in Fig. 2F,G). In 62% of these cultures large numbers of thalamic axons curved often quite sharply in a dorsal direction. These features, illustrated in Fig. 2F,G are reminiscent of the in vivo trajectory of thalamic axons as they grow through the internal capsule and turn dorsally towards the neocortex (Fig. 2A). In marked contrast, none of the cultures of *tau-GFP:Pax6$^{Sey/Sey}$* dorsal thalamus with wild-type ventral telencephalon showed these features. In 60% of these cultures thalamic axons did enter the medial part of the ventral telencephalon, but in almost all these cases (86%) not even a single thalamic axon reached anywhere near half-way through the telencephalic explant. A typical example is shown in Fig. 2H. In none of these cultures, nor in the 14% of cases where longer thalamic axons were seen (Fig. 2I illustrates the culture in which there was most growth), was there any evidence of a consistent turning of the thalamic axons in a dorsal direction. Repeated observations of tau-GFP-labelled wild-type and *Pax6$^{Sey/Sey}$* axons in some of the cultures during their 3 days in vitro revealed that the major features of

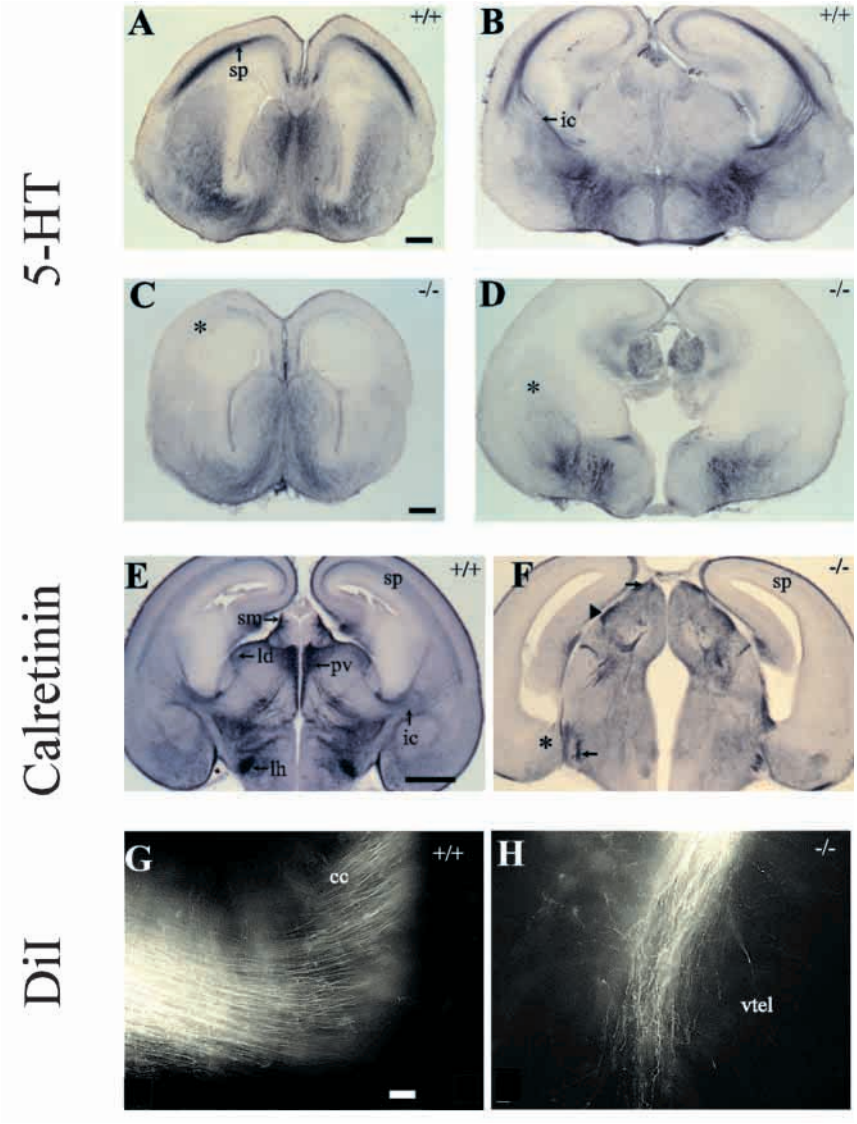

Fig. 1. Thalamocortical defects in *Pax6*$^{sey/Sey}$ embryos. (A-D) 5-HT immunolabeling in clorgyline-treated E17.5 *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos. (A,B) *Pax6*$^{+/+}$ embryos: 5-HT immunolabelled sensory thalamocortical axons at the level of (A) the subplate (sp), and (B) the internal capsule (ic). (C,D) *Pax6*$^{Sey/Sey}$ embryos: loss of 5-HT-immunolabelled sensory axons at the level of (C) the subplate and (D) the internal capsule (* marks expected position of axons in C and D). (E,F) Calretinin immunolabelling in E17.5 *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos. (E) *Pax6*$^{+/+}$ embryo: calretinin labelling in the stria medullaris (sm) of the epithalamus, the laterodorsal (ld), paraventricular (pv) and other midline nuclei of the dorsal thalamus and in the lateral hypothalamus (lh). Note calretinin immunopositive fibres extending to/from the pv and ld through the internal capsule (ic) into the subplate (sp) of the developing cortex. (F) *Pax6*$^{Sey/Sey}$ embryo: residual calretinin labelling in lateral regions of the epithalamus (upper arrow), dorsal thalamus (arrowhead) and hypothalamus (lower arrow) and loss of labelling in the midline nuclei. Note loss of calretinin immunopositive fibres at the level of the internal capsule (*marks expected position of fibres). (G,H) High-power views showing the furthest extent of DiI labelling from the dorsal thalamus at E15.5 in (G) *Pax6*$^{+/+}$ and (H) *Pax6*$^{Sey/Sey}$ embryos. Label is in the cerebral cortex (cc) in wild type but has got no further than the medial part of the ventral telencephalon (vtel) in mutants; the mutant axons are more disorganised than those in the wild type. The area in H corresponds to that marked by an asterisk in Fig. 2C. Scale bars, A-F, 500 μm; G,H, 50 μm.

innervation were established quickly, within 1-2 days of the start of culture, and there was less progression between 2 and 3 days. It is unlikely, therefore, that the defective innervation from the *tau-GFP:Pax6*$^{Sey/Sey}$ thalamic explants can be explained simply by a slowing of development.

In all cultures where *tau-GFP:Pax6*$^{+/+}$ dorsal thalamus was placed next to wild-type hypothalamus, thalamic axons coursed around its edge and failed to innervate it (Fig. 2J), in agreement with previous findings on the repulsive nature of the hypothalamus for dorsal thalamic axons (Braisted et al., 1999). The same result was obtained in all cases where *tau-GFP:Pax6*$^{Sey/Sey}$ dorsal thalamic explants were used (Fig. 2L). When *tau-GFP:Pax6*$^{+/+}$ dorsal thalamus was placed with wild-type cortex, most cultures showed large numbers of thalamic axons wrapping around its edges and upper and lower surfaces (Fig. 2K). This pattern has been described before and is due to the inability of the early cortical layers to receive thalamic innervation (Molnar and Blakemore, 1991; Goetz et al., 1992). In 95% of these cultures, thalamic axons extended distances equivalent to at least half-way round the circumference of the cortical explants. When *tau-GFP:Pax6*$^{Sey/Sey}$ dorsal thalamus was cultured with wild-type cortex, the pattern of thalamic axonal growth was similar to that with *tau-GFP:Pax6*$^{+/+}$ dorsal thalamus (Fig. 2M), although in many cases it appeared that there were fewer thalamic axons. In 92% of these cultures large numbers of axons extended distances equivalent to well over half-way round the circumference of the cortical explants, as occurred with *tau-GFP:Pax6*$^{+/+}$ dorsal thalamus.

Several observations indicate that the inability of the *Pax6*$^{Sey/Sey}$ dorsal thalamus to generate normal innervation of ventral telencephalon is not caused by a non-specific inability to grow. First, *Pax6*$^{Sey/Sey}$ dorsal thalamic explants are capable of producing many long axons when co-cultured with wild-type cortex, as do *Pax6*$^{+/+}$ thalamic explants (Rennie et al., 1994). The apparent reduction in the numbers of mutant thalamic axons in these co-cultures is compatible with previous observations that mutant dorsal thalamus contains fewer neurons (proliferation in the early mutant diencephalon is reduced; Warren and Price, 1997), although it could conceivably be due to a defective response by some thalamic neurons to cortex-derived growth-promoting factors. Second, analysis of nuclear morphology with TO-PRO3 showed that

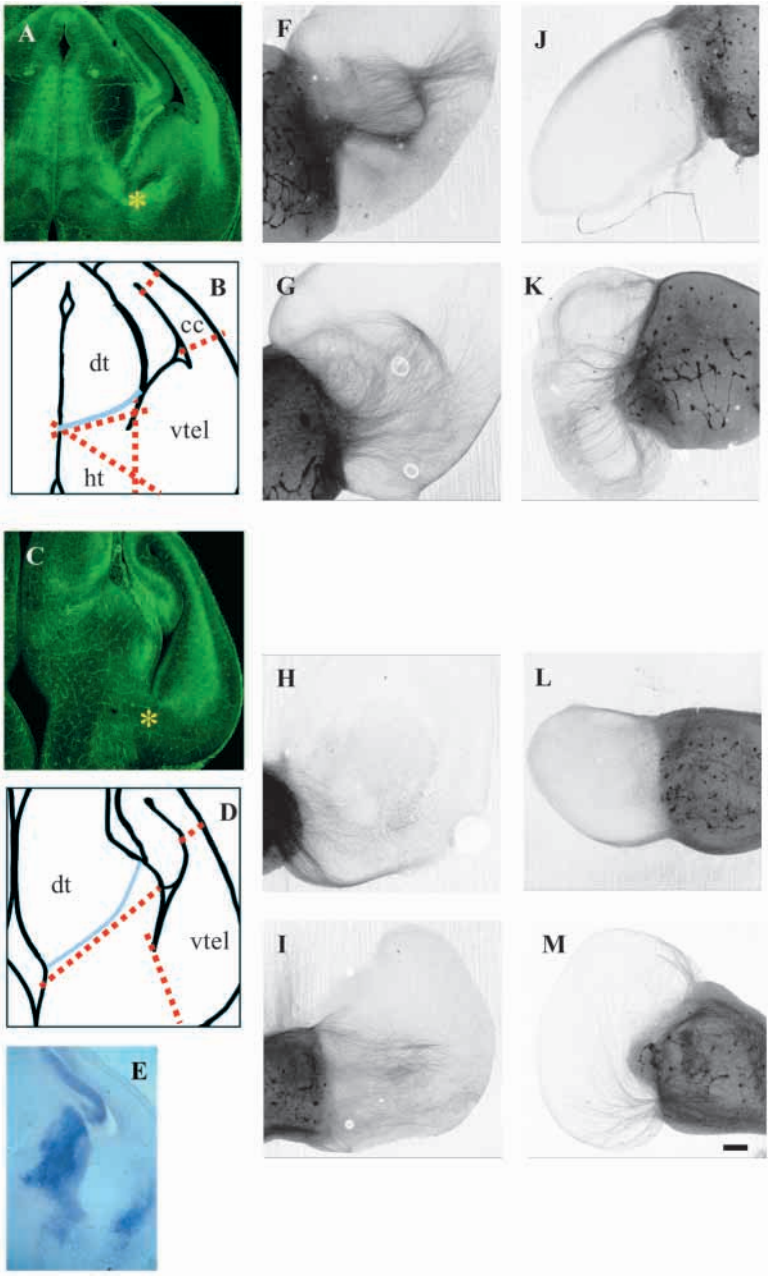

Fig. 2. *Pax6*$^{Sey/Sey}$ thalamic explants exhibit defective tract formation, as shown by organotypic co-culture of *tau-GFP:Pax6*$^{+/+}$ or *tau-GFP:Pax6*$^{Sey/Sey}$ dorsal thalamic (dt) explants with wild-type explants of ventral telencephalon (vtel), hypothalamus (ht), or cerebral cortex (cc). (A,C) Fluorescence images of coronal sections of E14.5 *tau-GFP:Pax6*$^{+/+}$ and *tau-GFP:Pax6*$^{Sey/Sey}$ brains similar to those from which explants were dissected. (A) *tau-GFP:Pax6*$^{+/+}$ embryo showing strong tau-GFP labelling of thalamic axons as they turn sharply to avoid the hypothalamus and enter the ventral telencephalon to form the internal capsule (*). (C) *tau-GFP:Pax6*$^{Sey/Sey}$ embryo, showing that the strong tau-GFP label seen in *tau-GFP:Pax6*$^{+/+}$ embryos is lacking from the position normally occupied by the internal capsule (*). (B,D) Schematic representations of sections shown in A,C with red dotted lines indicating locations of cuts used to isolate the tissues used in these experiments (dt, vtel, ht, and cc) from coronal slices. Panels A-D show only one side but note that the left and right dorsal thalamus were left joined dorsally in culture and their cut edges, just anterior to the border with ventral thalamus, were placed against different tissues on each side. The border between the ventral and dorsal thalamus, as defined by tissue landmarks and the expression of genes such as *Dlx2* and *Pax6*, is shown with a blue line. (E) In situ hybridization on a 200 μm-thick vibratome section (pink counterstain) of a *Pax6*$^{Sey/Sey}$ E14.5 embryo corresponding to the section in C,D, showing expression of *Pax6* (purple) in the thalamus, where it acts as a marker of ventral thalamus even in the mutants (Stoykova et al., 1996; Warren and Price, 1997), and telencephalon. (F-M) Grey tint presentation of fluorescence images showing representative examples of co-cultures of (F,G,J,K) *tau-GFP:Pax6*$^{+/+}$ thalamus or (H,I,L,M) *tau-GFP:Pax6*$^{Sey/Sey}$ thalamus with (F-I) wild-type ventral telencephalon, (J,L) wild-type hypothalamus or (K,M) wild-type cerebral cortex. Scale bar, 200 μm.

under all conditions used here almost all cells in both *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ dorsal thalamic explants remain viable. Third, in other experiments in which E14.5 dorsal thalamic explants were dissociated and cultured for 3 days in serum-free medium, *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ cells survived and grew neurites equally well (reported in preliminary form by Edgar et al., 1999). Therefore, the inability of the *Pax6*$^{Sey/Sey}$ dorsal thalamus to generate normal ventral telencephalic innervation is likely to be due to its inability to respond to growth and guidance cues in that region.

Although the main focus of this work was the dorsal thalamus and its axons, we carried out a few experiments to gain a better idea of the status of the ventral telencephalon in the mutants. In cultures of *tau-GFP:Pax6*$^{+/+}$ dorsal thalamus with *Pax6*$^{Sey/Sey}$ ventral telencephalon we observed prolific innervation by long axons, indicating that the mutant ventral telencephalon does not lack the ability to stimulate and receive dorsal thalamic innervation (Fig. 3A). Innervation did not appear normal, however, and many axons turned in abnormal ways in the mutant ventral telencephalon (compare Fig. 3A with Fig. 2F,G). We probed the *Pax6*$^{Sey/Sey}$ ventral telencephalon for expression of *Netrin 1*, a potential candidate for a chemoattractant of thalamocortical axons (Braisted et al., 1999, 2000), and found similar expression as in wild-types (Fig. 3B,C). Although the *Pax6*$^{Sey/Sey}$ ventral telencephalon may be permissive for incoming thalamic axons, it is quite possible that defects in this region contribute to the lack of thalamocortical innervation in small eye homozygotes.

Altered expression of dorsal thalamic markers in *Pax6*$^{Sey/Sey}$ embryos

Previous studies of the major subdivisions of the diencephalon in small eye homozygotes, using molecular markers other than those applied here, all identified a region equivalent to dorsal thalamus in the mutants (Stoykova et al., 1996; Grindley et al., 1997; Warren and Price, 1997; Kawano et al., 1999). Major molecular defects of this region were not described. By probing *Pax6*$^{Sey/Sey}$ embryos for expression of two mRNAs that normally mark dorsal thalamus, we obtained evidence of such defects. The mRNAs examined encode the vesicular monoamine transporter type 2 (*VMAT2*; Lebrand et al., 1999)

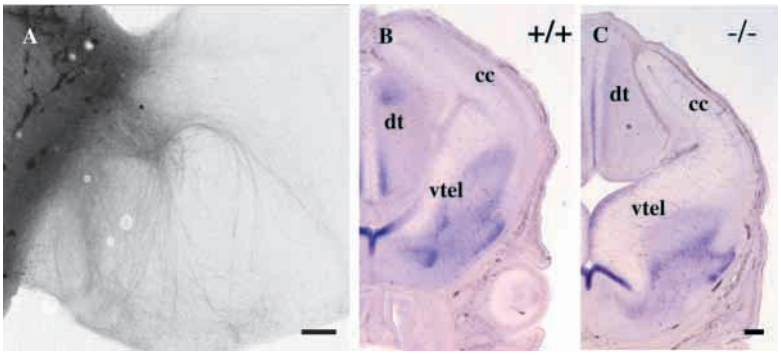

Fig. 3. The nature of the ventral telencephalon in *Pax6*$^{Sey/Sey}$ embryos. (A) Organotypic co-culture of *tau-GFP:Pax6*$^{+/+}$ dorsal thalamic explant with explant of *Pax6*$^{Sey/Sey}$ ventral telencephalon. (B,C) In situ hybridizations showing *Netrin 1* expression in the ventral telencephalon of *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ E14.5 embryos. Abbreviations as in Fig. 2. Scale bar, 200 μm.

and the secreted molecule heparin binding neurotrophic factor (*Hbnf*; Bloch et al., 1992; Rauvala et al., 1994). In E15.5 and E17.5 *Pax6*$^{+/+}$ embryos, *VMAT2* was expressed in dorsal thalamic nuclei (Fig. 4A; Lebrand et al., 1999). In age-matched *Pax6*$^{Sey/Sey}$ embryos there was a severe loss of *VMAT2* expression in the body of the dorsal thalamus, with only very faint residual expression barely detectable posterodorsally (arrow in Fig. 4B). In *Pax6*$^{+/+}$ embryos aged E13.5 or more, *Hbnf* was expressed throughout the entire body of the dorsal thalamus, with a sharp anterior boundary at the border with the ventral thalamus, the zona limitans intrathalamica (zli; arrow in Fig. 4C), and in the epithalamus. In *Pax6*$^{Sey/Sey}$ embryos aged E13.5 or more there was a loss of *Hbnf* expression in the dorsal thalamus (Fig. 4D). Expression was retained posterodorsally in the epithalamus (arrow in Fig. 4D). In these experiments we used *Dlx2* expression to mark the posterior ventral thalamic border at the zli, which it does even in *Pax6*$^{Sey/Sey}$ mice (Fig. 4E,F; Warren and Price, 1997), and showed that this boundary is well-separated from residual *Hbnf* expression in *Pax6*$^{Sey/Sey}$ epithalamus (Fig. 4D). The tissue between the posterior boundary of *Dlx2* expression and the epithalamus is labelled dorsal thalamus (dt) in Fig. 4C-F.

Ectopic expression of *Lim1/Lhx1* in the *Pax6*$^{Sey/Sey}$ dorsal thalamus

Since *Pax6* is downregulated in the dorsal thalamus as thalamocortical axons begin to form (around E12.5-E13.5; Auladell et al., 2000), it is possible that the incompetence of *Pax6*$^{Sey/Sey}$ dorsal thalamic axons to navigate the ventral telencephalon is an indirect defect caused via more persistent changes in other regulatory genes. In *Pax6*$^{Sey/Sey}$ embryos aged E13.5 or more, we found a striking abnormality of *Lim1/Lhx1* expression in the thalamus (Fig. 5). In wild-type embryos, *Lim1/Lhx1* was expressed in ventral thalamic nuclei but was excluded from the dorsal thalamus (Fig. 5A,C,D), whereas in mutants it was expressed throughout both the ventral and the dorsal thalamus (Figs 5B,E,F). In these experiments, we confirmed the location of the zli by staining adjacent sections for expression of *Dlx2*. As described before by Mastick et al. (1997), *Lim1/Lhx1* expression was lost from the pretectum in mutants (Fig. 5D,F).

Altered expression of *Shh* and *Nkx2.2* in *Pax6*$^{Sey/Sey}$ diencephalon

We examined expression of two genes, *Shh* and *Nkx2.2*,

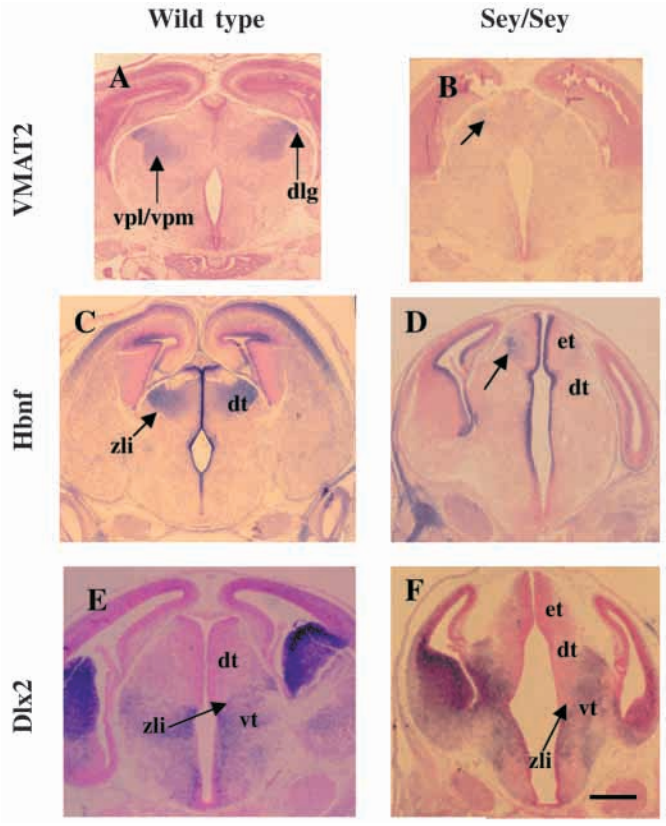

Fig. 4. Expression of dorsal thalamic markers in *Pax6*$^{Sey/Sey}$ embryos. In situ hybridisation on coronal sections of (A,B) E17.5 and (C-F) E15.5 *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos for (A,B) *VMAT2* expression, (C,D) *Hbnf* expression and (E,F) *Dlx2* expression. (A) *Pax6*$^{+/+}$ embryo: *VMAT2* expression in dorsal thalamic nuclei including the ventral posteromedial (vpm) and ventral posterolateral (vpl) and dorsolateral geniculate (dlg). (B) *Pax6*$^{Sey/Sey}$ embryo: loss of *VMAT2* expression in the dorsal thalamus, with only very faint residual expression (arrow). (C) *Hbnf* expression in *Pax6*$^{+/+}$ embryo: there is strong expression in the dorsal thalamus as well as at other sites including the epithalamus, cells lining the 3rd and lateral ventricles, the basal telencephalon and developing cortical plate. (D) *Hbnf* expression in *Pax6*$^{Sey/Sey}$ embryos: there is a severe loss of Hbnf expression in the body of the dorsal thalamus with only slight residual expression in the epithalamus (arrow). (E,F) *Dlx2* expression in sections corresponding to those in C and D respectively. Even in *Pax6*$^{Sey/Sey}$ embryos, expression is restricted to ventral thalamus (Stoykova et al., 1996; Warren and Price, 1997), making this a useful marker of the border between dorsal and ventral thalamus. zli, zona limitans intrathalamica; dt, dorsal thalamus; et, epithalamus; vt, ventral thalamus. Scale bar, 500 μm.

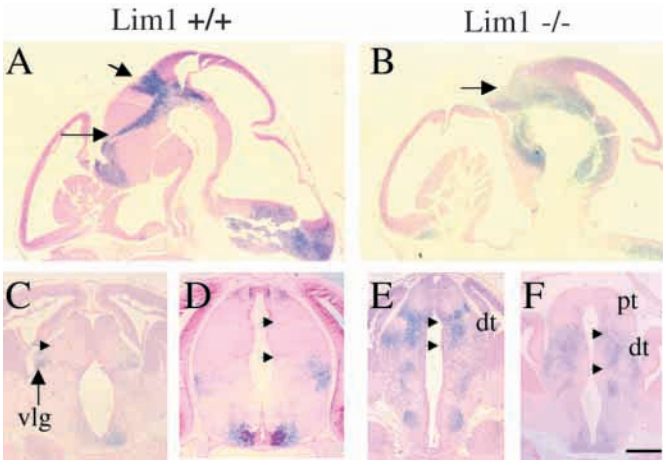

Fig. 5. *Lim1/Lhx1* expression in *Pax6*$^{Sey/Sey}$ embryos. In situ hybridisation on (A,B) sagittal and (C-F) coronal sections of (A,B) E13.5 and (C-F) E15.5 *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos. (A) In wild type, *Lim1/Lhx1* is expressed in the pretectum (top arrow), ventral to the dorsal thalamus and around the zli (bottom arrow). (B) In mutants, *Lim1/Lhx1* expression extends over the body of the dorsal thalamus (arrow). (C) Rostral and (D) caudal sections of wild type embryos: *Lim1/Lhx1* expression is restricted to specific nuclei in the ventral thalamus, including the ventrolateral geniculate nucleus (vlg) rostrally. (E) Rostral and (F) caudal sections of mutant embryos: a large band of ectopic *Lim1/Lhx1* expression is visible within the body of the dorsal thalamus (dt) at all rostrocaudal levels. Note the absence of label in the pretectum (pt). In the ventral thalamus, *Lim1/Lhx1* expression is distorted and no longer restricted to discrete areas. The borders of the dorsal thalamus are marked with arrowheads; the position of the border between dorsal and ventral thalamus was confirmed by probing for *Dlx2* expression in nearby sections. Scale bar, 500 μm.

implicated in DV patterning of the central nervous system. The expression of *Shh* in *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos, in whole-mount (Fig. 6A,B) and sectioned embryos (not shown), confirmed the findings of others (Barth et al., 1995; Shimamura et al., 1995; Grindley et al., 1997). In both wild-type and mutant embryos at all ages studied (E10.5, E13.5 and E15.5), *Shh* was expressed along the AP axis of the ventral neural tube with a dorsal deflection at the zli (Fig. 6A,B). As reported by Grindley et al. (1997), the domain of expression at the ventral end of the zli (the region between the short lines in Fig. 6A,B) was slightly broader in age-matched mutants, extending a little further into the presumptive dorsal thalamus.

In situ hybridizations for *Nkx2.2* in E10.5 *Pax6*$^{+/+}$ embryos showed expression along the AP axis in a longitudinal band adjacent to *Shh* expression (not shown; described before by Shimamura et al., 1995; summarized in Fig. 8). In E13.5 *Pax6*$^{+/+}$ embryos, the domain of *Nkx2.2* expression remained tightly restricted around the zli and did not encroach on the body of the dorsal (posterior) thalamus (Fig. 6C; Shimamura et al., 1995; Kitamura et al., 1997). In age-matched *Pax6*$^{Sey/Sey}$ embryos, *Nkx2.2* expression extended to include the body of the dorsal thalamus (Fig. 6D).

Immunocytochemical analysis of Nkx2.2 expression confirmed these findings. At E10.5, diencephalic expression of Nkx2.2 was restricted to a band of cells adjacent to *Shh* expression in both *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos. A difference between diencephalic expression in *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos emerged between E11.5 and E12.5, with cells labelled for Nkx2.2 dispersed in the body of the presumptive dorsal thalamus in the mutants but not in the wild-type embryos. This difference persisted at older ages. At E17.5 (Fig. 7), many cells expressing Nkx2.2 in the mutants were present throughout the dorsal thalamus and the pretectum, right up to their dorsal edge (between the arrows in Fig. 7B), whereas Nkx2.2-positive cells were confined to the ventral edge of these regions in wild type (Fig. 7A). The density of ectopic labelled cells in the mutants was greatest in the anterior and ventral parts of the dorsal thalamus.

The results of our work are summarized in Fig. 8. Our gene expression studies indicate a respecification of the presumptive posterior (dorsal) thalamus in prosomere 2 in *Pax6*$^{Sey/Sey}$ embryos. In this respecification the dorsal thalamus takes on molecular characteristics normally restricted to tissue ventral and anterior to it, changes that are indicated by arrows in Fig.

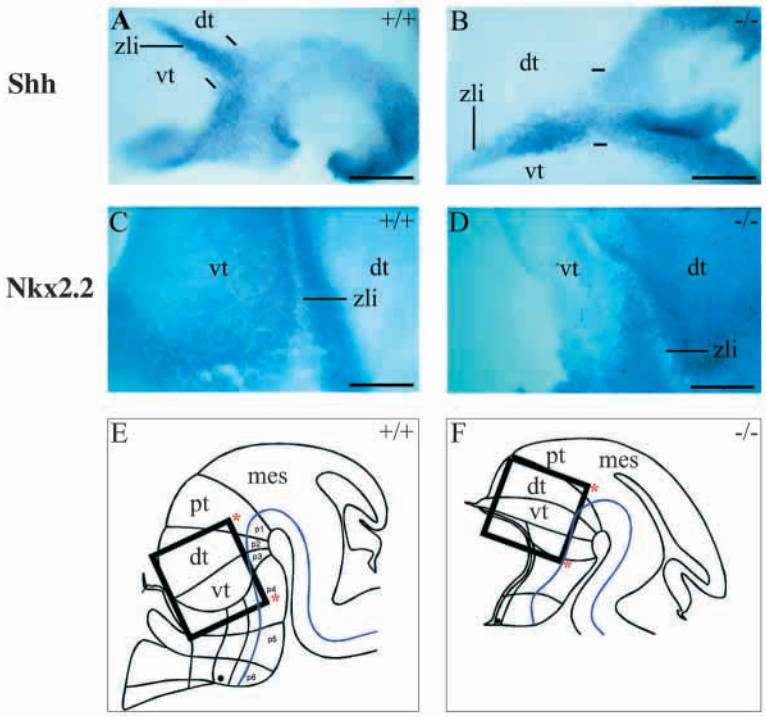

Fig. 6. In situ hybridisations on dissected embryos at E13.5. (A) *Pax6*$^{+/+}$ embryo: *Shh* expression along the AP axis of the ventral neural tube and along the zli. (B) *Pax6*$^{Sey/Sey}$ embryo: a similar pattern is seen, but note slight expansion of the domain of *Shh* expression around the zli (described by Grindley et al., 1997; compare distance between black bars in A and B). (C) *Pax6*$^{+/+}$ embryo: *Nkx2.2* expression flanks the zli and is confined to a narrow strip along its posterior edge. (D) *Pax6*$^{Sey/Sey}$ embryo: abnormal *Nkx2.2* expression is seen throughout the body of the dorsal thalamus. The black boxes in the schematics shown in E and F indicate where panels C and D were taken from; the bases of the panels are indicated by red asterisks in E and F. Abbreviations as in Fig. 4; mes, mesencephalon. Scale bars A,B, 500 μm; C,D, 250 μm.

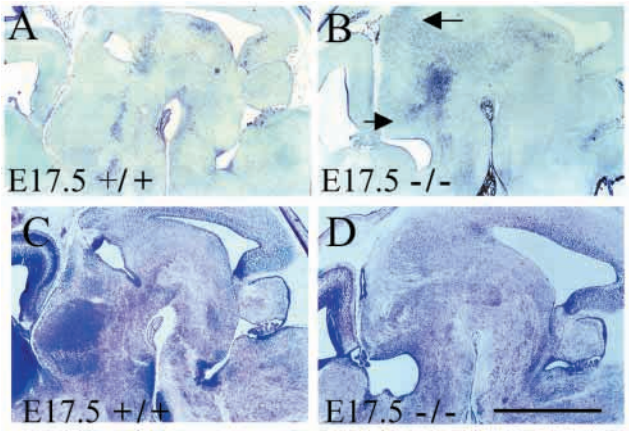

Fig. 7. Illustrates the results of immunocytochemistry for Nkx-2.2 in parasagittal sections of E17.5 *Pax6*$^{+/+}$ and *Pax6*$^{Sey/Sey}$ embryos. (A,B) Immunochemistry and (C,D) corresponding Nissl sections. In mutants labelled cells are found throughout the dorsoventral extent of the diencephalon, even along its dorsal edge (between the arrows in B). In wild type embryos they are confined in a strip running ventral to the posterior thalamus and its surrounding structures. Scale bar, 1 mm.

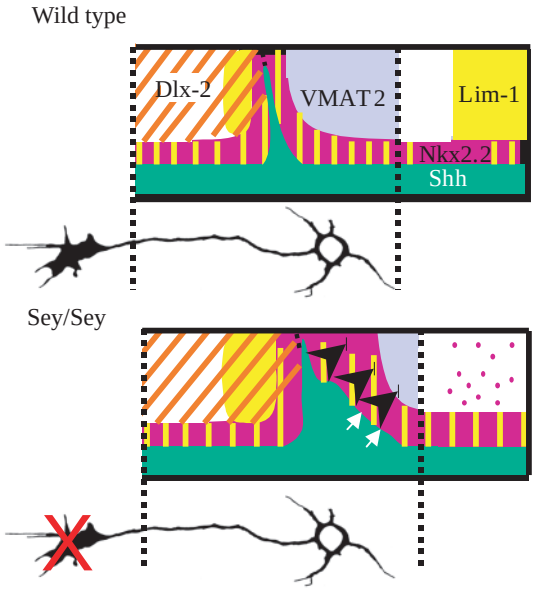

Fig. 8. Summary diagram of our main findings. Gene expression: dark blue, *Shh*; red (areas and dots, the latter indicating less dense label), *Nkx2.2*; yellow (areas and vertical bars, the latter used where there is overlap), *Lim1/Lhx1*; light blue, *VMAT2*; orange cross-hatching, *Dlx2*. Arrows and arrowheads indicate the main changes that form the focus of this study. For clarity, the schematics provide a qualitative summary only, they do not reflect the shape of the diencephalon nor are they to scale. The neuron below each schematic represents a thalamocortical axon, the red cross indicating incompetence to respond to ventral telencephalic growth and guidance factors.

8. For comparison with work in other regions of the central nervous system, this respecification may be more easily described as a ventralization than an anteroventralization, since elsewhere *Shh* and *Nkx2.2* are expressed ventrally and not, as is the case at and around the zli, in alar regions.

DISCUSSION

The thalamocortical tract fails to form normally in *Pax6*$^{Sey/Sey}$ mice. In the wild type, the tract projects from the dorsal thalamus and travels through the ventral thalamus, avoids the hypothalamus, turns sharply into the ventral telencephalon (forming the internal capsule), and then reaches its target in the cerebral cortex (Tuttle et al., 1999; Braisted et al., 1999; Auladell et al., 2000). In *Pax6*$^{Sey/Sey}$ mice, the dorsal thalamus does project a tract but it fails to enter the cortex and appears to become stalled en route from the ventral thalamus to the ventral telencephalon (Kawano et al., 1999; present study).

A comparison of timetables for *Pax6* gene expression in developing forebrain (Stoykova et al., 1996; Grindley et al., 1997; Mastick et al., 1997; Warren and Price, 1997; Kawano et al., 1999) and thalamocortical tract formation (Tuttle et al., 1999; Braisted et al., 1999; Auladell et al., 2000) suggests several locations at which *Pax6* could influence the developing thalamocortical tract. *Pax6* is expressed at many points along the thalamocortical pathway at times coinciding with critical events in thalamocortical tract formation including the dorsal thalamus, where *Pax6* expression coincides with neurogenesis between E10 and E13 (Angevine, 1970), the ventral thalamus, the hypothalamus, the ventral telencephalon and the cerebral cortex. The failure of the thalamocortical tract to form in the absence of *Pax6* function could reflect a requirement for *Pax6* at one or more of these locations.

We present new evidence that supports the hypothesis that a structure equivalent to the dorsal thalamus emerges in the absence of *Pax6* but that its subsequent differentiation and ability to project a thalamocortical tract does require *Pax6* function.

Pax6 is not required for formation of the dorsal thalamus but is required for its correct differentiation

Previous studies of the embryonic diencephalon in *Pax6*$^{Sey/Sey}$ mice showing relatively normal AP expression patterns of *Dlx*, *Otx*, *Emx Wnt* genes, *Prox1*, *Gbx2*, *Mash1* and *Pax6* have indicated that diencephalic AP patterning is not greatly disturbed (Stoykova et al., 1996; Warren and Price, 1997; Grindley et al., 1997). These gene expression studies have suggested that the embryonic *Pax6*$^{Sey/Sey}$ diencephalon possesses correlates of the histogenetic regions that in the wild type give rise to major diencephalic structures. Despite obvious distortions in the shape of the embryonic *Pax6*$^{Sey/Sey}$ diencephalon (Stoykova et al., 1996), which may in part be due to defects of cell proliferation in this region (Warren and Price, 1997), morphological features reminiscent of those that distinguish diencephalic regions in the wild type have been described. Equivalents of prosomeres 1-3 and their main components, the ventral and dorsal thalamus and the pretectum, have been identified on the basis of gene expression and morphology in the mutants and named according to their wild-type counterparts (Stoykova et al., 1996; Mastick et al., 1997; Warren et al., 1997; Kawano et al., 1999; present study).

The alterations we observe in the expression patterns of several genes (*Hbnf*, *VMAT2*, *Lim1/Lhx1*, and *Nkx2.2*) are

superimposed on these structures and reflect a failure of the *Pax6*$^{Sey/Sey}$ dorsal thalamus to differentiate normally in the absence of *Pax6* expression. These molecular patterning defects indicate that the differentiation program of the *Pax6*$^{Sey/Sey}$ dorsal thalamus is disrupted during the period when it fails to make appropriate connections with the central cortex.

Pax6 is required within the dorsal thalamus for thalamocortical tract formation

Pax6 and dorsal thalamic tract projection

From observations of the *Pax6*$^{Sey/Sey}$ brain alone, it is conceivable that the thalamocortical tract defect results from an autonomous defect in the dorsal thalamus itself, or from defects in the environment through which the thalamocortical axons navigate, or a combination of the two. To address this issue, we show that explants of mutant dorsal thalamus are defective in their ability to project a tract capable of correct navigation through wild-type ventral telencephalon. This defect may be limited to this specific aspect of thalamocortical growth since *Pax6*$^{Sey/Sey}$ thalamic axons innervate wild-type cortex and avoid wild-type hypothalamus, as do wild-type thalamic axons. These new data provide direct evidence that *Pax6* expression is required within the dorsal thalamus itself, regardless of whether *Pax6* expression is also required at other locations.

Because our thalamic explants contained some ventral thalamus and epithalamus as well as dorsal thalamic tissue, it is formally possible that our results could be explained by the lack of essential signalling to axons *en passant* (Wang and Tessier-Lavigne, 1999). This possibility would demand that adjacent tissues instruct axons leaving the dorsal thalamus in ventral telencephalic navigation. The zli or the ventral thalamus would be possible candidates for this type of interaction (Mastick et al., 1997; Braisted et al., 1999; Tuttle et al., 1999). The evidence, however, is against such a possibility. Wild-type thalamic explants in our cultures exhibit similar features of axon outgrowth to cultures using dorsal thalamic explants prepared from embryonic brain at similar ages but with surrounding tissues removed. These features include avoidance of the hypothalamus, attraction towards the ventral telencephalon and penetration of the cortex (Molnar and Blakemore, 1991, Goetz et al., 1992; Braisted et al., 1999, 2000). Since these 'pure' dorsal thalamic explants have been isolated from potential instruction by cells lying outside the dorsal thalamus, such instructions are unlikely to be important for these features of thalamocortical tract formation.

The design and interpretation of the explant experiments depends on correct identification of dorsal thalamus in mutant brain for explant isolation. Given the disturbed anatomy of the mutant diencephalon, it was critical that in spite of these abnormalities the dorsal thalamic explants isolated from wild-type and mutant brain were equivalent. We argue above that the *Pax6*$^{Sey/Sey}$ embryo does possess a structure corresponding to the dorsal thalamus which can be identified by its morphology and gene expression patterns. The problem of equivalence reduces to choosing explants in which axons projecting from *Pax6*$^{Sey/Sey}$ and wild-type dorsal thalamus have equal opportunities to interact with target tissues. The major defect in thalamocortical tract formation in mutants appears to occur as axons attempt to navigate from the ventral thalamus into the ventral telencephalon (Kawano et al., 1999; present study). There is no evidence that axons originating from the dorsal thalamus become stalled in the ventral thalamus or that they fail to avoid the hypothalamus. We therefore selected explants that centered on the dorsal thalamus but also contained some ventral thalamic and epithalamic tissue. This ensured that dorsal thalamus was present and enabled us to position our explants to recreate the topography of intact brain more accurately than if we had attempted to dissect smaller pieces of tissue with resulting loss of anatomical landmarks. Since the ventral thalamus/ventral telencephalon transition zone, where thalamocortical defects in the *Pax6*$^{Sey/Sey}$ mice occur, was absent from our thalamic explants, axons from *Pax6*$^{Sey/Sey}$ or wild-type were able to navigate to the edge of the thalamic explant where they were confronted by the target tissue. The observation that thalamic axons responded to the target tissues in ways reminiscent of their behavior in vivo supported the accuracy of our culture system.

Pax6 and the ventral telencephalon

We also show that *Pax6*$^{Sey/Sey}$ ventral telencephalon maintains normal expression patterns of netrin 1, a molecule implicated in thalamocortical tract formation (Braisted et al., 2000), and is permissive to wild-type thalamic axons. Therefore although we cannot rule out the possibility that defects in the ventral telencephalon contribute to the lack of thalamocortical innervation in *Pax6*$^{Sey/Sey}$ embryos, we find no evidence that such defects can account for the *Pax6*$^{Sey/Sey}$ thalamocortical phenotype wholly.

Roles of *Pax6* in the genetic cascade which programs the development of dorsal thalamus

Regulation of transcription factors and secreted morphogens by *Pax6*

Work on the developing spinal cord and hindbrain has implicated Pax6, together with the secreted morphogen Shh, the transcription factor Nkx2.2 and members of the LIM/LHX family of homeodomain proteins in DV patterning and neuronal fate determination in these posterior regions (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994, 1995; Appel et al., 1995; Marti et al., 1995; Tanabe et al., 1995; Ericson et al., 1995, 1996, 1997; Osumi et al., 1997; Briscoe et al., 1999). *Pax6* may play a similar role in the developing diencephalon. It is possible that *Nkx2.2* expression ventral and anterior (at the zli) to the posterior thalamus of normal embryos is induced by Shh, which diffuses from ventral territory and from the zli, and directly or indirectly repressed by Pax6 in dorsal territory. The accumulation of *Nkx2.2*-positive cells in the posterior thalamus in *Pax6*$^{Sey/Sey}$ embryos older than E10.5 could result from Shh induction in the absence of a constraining influence from Pax6. Our observation that densities of ectopic labelling for *Nkx2.2* in the mutants were highest at the ventral and anterior parts of the posterior thalamus could be explained because these are the parts closest to the expression domain of *Shh*. Another likely contributing factor is that, as first reported by Grindley et al. (1997), the domain of *Shh* expression is slightly expanded at the ventral end of the zli. It is interesting that the normal downregulation of *Pax6* in the developing wild-type posterior thalamus is not followed by upregulation of expression of *Nkx2.2* in this region. This argues against the persistence of a simple reciprocal relationship between levels of *Pax6* and *Nkx2.2*

expression throughout the period of posterior thalamic development. While such a relationship may play an important role in early patterning of this region, other factors such as the levels of Shh protein or repressive molecules in the posterior thalamus may constrain the dorsal edge of the domain of *Nkx2.2* later on in normal development. It is possible that ectopic expression of *Nkx2.2* in the posterior thalamus in *Pax6*$^{Sey/Sey}$ embryos contributes to generating ectopic expression of *Lim1/Lhx1* in this region, as in more caudal regions of the neural tube (Ericson et al., 1997; Briscoe et al., 1999).

Regulation of cell surface properties by *Pax6*

The cerebral cortex, like the dorsal thalamus, exhibits *Pax6* expression in ventricular zone cells during neurogenesis and downregulates *Pax6* expression in postmitotic cells. The expression of Pax6 in proliferating cortical neurons influences their postmitotic gene expression patterns and cell surface properties with the maintenance of this genetic pattern becoming independent of the presence of Pax6 (Warren et al., 1999). Work on the developing cerebral cortex has implicated *Pax6* in regulating members of the cadherin family of cell adhesion molecules (Stoykova et al., 1997) and the trk family of neurotrophin receptors (Warren et al., 1999). In vitro aggregation assays (Stoykova et al., 1997) and transplantation experiments (our unpublished results) have established that the adhesive properties of cortical cells are altered in *Pax6*$^{Sey/Sey}$ embryos. Roles for *Pax6* in regulating adhesive properties have been elegantly demonstrated in the eye by analysis of *Pax6*$^{Sey/Sey}$ ↔ wild-type chimeras (Quinn et al., 1996; Collinson et al., 2000). *Pax6* has also been implicated in axon pathfinding in the tract of the postoptic commissure (Mastick et al., 1997) and spinal cord (Ericson et al., 1997) as well as in the thalamocortical tract (Tuttle et al, 1999; Kawano et al., 1999; present study). Consistent with its roles elsewhere in the CNS, Pax6 probably controls thalamocortical tract formation by regulating the cell surface properties of dorsal thalamic cells and influencing the navigation choices made by their growth cones.

Clarification of the molecular mechanisms of Pax6 action will be facilitated as the tally of molecules implicated in thalamocortical tract formation increases. At present these include cell surface receptors DCC, Neogenin, Robo1, Robo2, RPTPδ, and LAMP, and secreted molecules Netrin 1, Slit1 and Slit2 (Ringsted et al., 1999; Braisted et al., 2000; Tuttle et al., 1999; Mann et al., 1998). A recent study by Braisted et al. (2000) has demonstrated that Netrin 1 promotes the growth of thalamocortical axons through the ventral telencephalon and so it is possible that abnormalities in *Pax6*$^{Sey/Sey}$ embryos may be contributed to by defects of receptors mediating its attractant effects, DCC and Neogenin. Both are expressed in the normal dorsal thalamus. Our preliminary evidence, however, suggests that DCC is still expressed in the dorsal thalamus of *Pax6*$^{Sey/Sey}$ embryos (T. V., unpublished observations). Given that defects of thalamocortical axons are less severe in loss-of-function mutation of Netrin 1 than in *Pax6*$^{Sey/Sey}$ embryos and that blocking Netrin 1 in vitro does not abolish thalamic axon attraction to ventral telencephalon (Braisted et al., 2000), it is hard to explain the small eye defects on the basis of a loss of Netrin 1 responsiveness alone. Other perhaps as yet unidentified signalling systems are likely involved.

Conclusion

The requirement for Pax6 in the dorsal thalamus is lifted by the time its cells become postmitotic. It is possible that *Pax6* has direct effects on the expression of cell surface molecules involved in receptor/ligand interactions, cell adhesion or axon pathfinding before it is downregulated. However, our findings that the defective responses of *Pax6*$^{Sey/Sey}$ dorsal thalamic axons coincide with persistent changes in the expression patterns of regulatory genes other than *Pax6* from the early stages of dorsal thalamic development allows that these defects may result indirectly from the early absence of Pax6 from this region. Genes such as *Lim1/Lhx1* or *Nkx2.2* whose expression is influenced by *Pax6* may have more direct effects, for example up- or downregulating the expression of effector molecules. Whether direct or indirect, we conclude that *Pax6* plays an essential role in the development of the dorsal thalamus and the ability of its axons to respond to key growth and guidance cues.

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REFERENCES

Angevine, J. B. Jr. (1970) Time of neuron origin in the diencephalon of the mouse. An autoradiographic study. *J. Comp. Neurol.* **139**, 129-187.

Appel, B., Korzh, V., Glasgow, E., Thor, S., Edlund, T., Dawid, I. B. and Eisen, J. S. (1995). Motoneuron fate specification revealed by patterned LIM homeobox gene expression in embryonic zebrafish. *Development* **121**, 4117-4125.

Arai, R., Jacobowitz, D. M. and Deura, S. (1994). Distribution of calretinin, calbindin-D28k and parvalbumin in the the rat thalamus. *Brain Research Bulletin* **33**, 595-615.

Auladell, C., Perez-Sust, P., Super, H. and Soriano, E. (2000) The early development of thalamocortical and corticothalamic projections in the mouse. *Acta Embryol.* **201**, 169-179.

Barth, K. A. and Wilson, S. W. (1995). Expression of zebrafish *nk2.2* is influenced by *sonic hedgehog/vertebrate hedgehog-1* and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121**, 1755-1768.

Bloch, B., Normand, E., Kovesdi, I. and Bohlen, P. (1992). Expression of HBNF gene in the brain of fetal, neonatal and adult rat: an in situ hybridisation study. *Dev. Brain Res.* **70**, 267-278.

Braisted, J. E., Tuttle, R. and O'Leary, D. D. M. (1999). Thalamocortical axons are influenced by chemorepellent and chemoattractant activities localized to decision points along their path. *Dev. Biol.* **208**, 430-440.

Braisted, J. E., Catalano, S. M., Stimac, R., Kennedy, T. E., Tessier-Lavigne, M., Shatz, C. J. and O'Leary, D. D. M. (2000) Netrin-1 promotes thalamic growth and is required for proper development of the thalamocortical projection. (2000). *J. Neurosci.* **20,** 5792-5801.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L. and Ericson J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* **398**, 622-627.

Bulfone, A., Puelles, L., Porteus, M. H., Frohman, M. A. and Rubenstein, J. L. R. (1993). Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2 and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J. Neurosci.* **13**, 3155-3172.

Callahan, C. A. and Thomas, J. B. (1994). Tau-β-galactosidase, an axon targeted fusion protein. *Proc. Natl. Acad. Sci. USA* **91**, 5972-5976.

Caric, D., Gooday, D., Hill, R. E., McConnell, S. K. and Price, D. J. (1997). Determination of the migratory capacity of embryonic cortical cells lacking the transcription factor Pax6. *Development* **124**, 5087-5096.

Cases, O., Vitalis, T., Seif, I., De Maeyer, E., Sotelo, C. and Gaspar, P.

(1996). Lack of barrels in the somatosensory cortex of monoamine oxidase A-deficient mice: a role of a serotonin excess during the critical period. *Neuron* **16**, 297-307.

Cases, O., Lebrand, C., Giros, B., Vitalis, T., De Mayer, E., Caron, M. G., Price, D. J., Gaspar, P. and Seif, I. (1998). Plasma membrane transporters of serotonin, dopamine and norepinephrine mediate serotonin accumulation in atypical locations in the developing brain of monoamine oxidase A knock-outs. *J. Neuorosci.* **18**, 6914-6927.

Collinson, J. M., Hill, R. E. and West, J. D. (2000). Different roles for Pax6 in the optic vesicle and facial epithelium mediate early morphogenesis of the murine eye. *Development* **127**, 945-956.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mphler, J., McMaho, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Edgar, J. M., Asavaritikrai, P. and Price, D. J. (1999). Cell death and the neurotrophic theory in developing thalamus. *Soc. Neurosci. Abstr.* **25**, 705.5.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning along the rostrocaudal axis of the neural tube. *Cell* **81**, 747-756.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of sonic hedgehog signalling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signalling. *Cell* **90**, 169-180.

Frassoni, C., Arcelli, P., Selvaggio, M. and Spreafico, R. (1998). Calretinin immunoreactivity in the developing thalamus of the rat: A marker of early generated thalamic cells. *Neuroscience* **83**,1203-1214.

Goetz, M., Novak, N., Bastmeyer, M. and Bolz, J. (1992). Membrane-bound molecules in rat cerebral cortex regulate thalamic innervation. *Development* **116**, 507-519.

Grindley, J. C., Hargett, L., Hill, R. E., Ross, A. and Hogan, B. L. M. (1997). Disruption of Pax6 function in mice homozygous for the Pax6Sey-1NEU mutation produces abnormalities in the early development and regionalization of the diencephalon. *Mech. Dev.* **64**, 111-126.

Hill, R. E., Favor, J., Hogan, B. L. M., Ton, C. C. T., Saunders, G. F., Hanson, I. M., Prosser, J., Jordan, T., Hastie, N. D. and van Heyningen, V. (1991). Mouse small eye results from mutations in a paired-like homeobox-containing gene. *Nature* **354**, 522-525.

Hogan, B. L. M., Horsburgh, G., Cohen, J., Hetherington, C. M., Fischer, G. and Lyon, M. F. (1986). Small eye (Sey): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. *J. Embryol. Exp. Morph.* **97**, 95-110.

Kawano, H., Fukuda, T., Kubo, K., Horie, M., Uyemura, K., Takeuchi, K., Osumi, N., Eto, K. and Kawamura, K. (1999) Pax-6 is required for thalamocortical pathway formation in fetal rats. *J. Comp. Neurol.* **408**, 147-160.

Kitamura, K., Miura, H., Yanazawa, M., Miyashita, T. and Kato, K. (1997) Expression patterns of *Brx1* (*Rieg* gene), *Sonic hedgehog*, *Nkx2.2*, *Dlx1* and *Arx* during zona limitans intrathalamica and embryonic ventral lateral geniculate nuclear formation. *Mech. Dev.* **67**, 83-96.

Krauss, S., Concordet, J. P. and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.

Lebrand, C., Cases, O., Werhle, R., Blakely, R., Edwards, J. and Gaspar, P. (1999). Transient developmental expression of monoamine transporters in the rodent brain. *J. Comp Neurol* **401**, 506-524.

Lotto, R. B. and Price, D. J. (1999). Thalamus and cerebral cortex: organotypic culture and co-culture. In *The neuron in tissue culture. Ed. Haynes, L.* John Wiley and Sons Ltd., Chichester. pp. 518-524.

Mann, F., Zhukareva, V., Pimenta, A., Levitt, P. and Bolz, J. (1998). Membrane associated molecules guide limbic and non-limbic thalamocortical projections. *J. Neurosci.* **18**, 9409-9419.

Mansouri, A., Stoykova, A. and Gruss, P. (1994). Pax genes in development. *J. Cell Sci.* **18**, 35-42.

Marti, E., Bumcrot, D. A., Takada, T. and McMahon, A. P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types. *Nature* **375**, 322-325.

Mastick, G. S., Davis, N. M., Andrews, G. L. and Easter, Jr, S. S. (1997). Pax6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. *Development.* **124**, 1985-1997.

Molnar, Z. and Blakemore, C. (1991). Lack of regional specificity for connections formed between thalamus and cortex in co-culture. *Nature* **351**, 475-477.

Niwa, H., Yamamura, K. and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-199.

Osumi, N., Hirota, A., Ohuchi, H., Nakafuku, M., Iimura, T., Kuratani, S., Fujiwara, M., Noji, S. and Eto, K. (1997). Pax6 is involved in specification of the hindbrain motor neuron subtype. *Development*, **124**, 2961-2972.

Paxinos, G., Tork, E., Tecott, L. H. and Valentino, K. L. (1991). *Atlas of the Developing Rat Brain.* Academic Press.

Pratt, T., Sharp, L., Nichols, J., Price, D. J. and Mason, J. O. (2000). Embryonic stem cells and transgenic mice ubiquitously expressing a tau tagged green fluorescent protein. *Dev. Biol.* (in press).

Puelles, L. and Rubenstein, J. L. (1993). Expression patterns of homeobox and other putative regulatory genes in the embryonic mouse forebrain suggest a neuromeric organization. *Trends Neurosci* **16**, 472-479.

Quinn, J. C., West, J. D. and Hill, R. E. (1996). Multiple functions for *Pax6* in mouse eye and nasal development. *Genes. Dev.* **10**, 435-446.

Rauvala, H., Vanhala, A., Castren, E., Nolo, R., Raulo, E., Merenmies, J. and Panula, P. (1994). Expression of HB-GAM (heparin-binding growth-associated molecules) in the pathways of developing axonal processes in vivo and neurite outgrowth in vitro induced by HB-GAM. *Dev. Brain Res.* **79**, 157-176.

Rennie, S., Lotto, R. B. and Price, D. J. (1994) Growth-promoting interactions between the murine neocortex and thalamus in organotypic co-cultures. *Neuroscience* **61**, 547-564.

Ringstedt, T., Braisted, J. E., Brose, K., Kidd, T., Goodman, C. S., Tessier-Lavigne, M. and O'Leary, D. D. M. (1999). Slit repulsion influences thalamocortical and retinal axon pathfinding in the diencephalon. *Society for Neuroscience Abstract* **25**, 305. 4.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M., et al. (1994) Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of Sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Rubenstein, J. L. R., Martinez, S., Shimamura, K. and Puelles, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* **266**, 578-580.

Schmahl, W., Knoediseder, M., Favor, J. and Davidson, D. (1993). Defects of neuronal migration and the pathogenesis of cortical malformations are associated with small eye (sey) in the mouse, a point mutation at the Pax-6 locus. *Acta Neuropathol* **86**, 126-135.

Shimamura, K., Hirano, S., McMahon, A. P. and Takeichi, M. (1994). *Wnt1*-dependent regulation of local E-cadherin and αN-catenin expression in the embryonic mouse brain. *Development* **120**, 2225-2234.

Shimamura, K., Hartigan, D. J., Martinez, S., Puelles, L. and Rubenstein, J. L. R. (1995). Longitudinal organisation of the anterior neural plate and neural tube. *Development.* **121**, 3923-3933.

Siemering, K. R., Golbik, R., Sever, R. and Haseloff, J. (1996). Mutations that suppress the thermosensitivity of green fluorescent protein. *Curr. Biol.* **6**, 1653-1663.

Stoykova, A. and Gruss, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14**, 1395-1412.

Stoykova, A., Fritsch, R., Walther, C. and Gruss, P. (1996). Forebrain patterning defects in Small eye mutant mice. *Development* **122**, 3453-3465.

Stoykova, A., Gotz, M. and Price, J. (1997). Pax6-dependent regulation of adhesive patterning, R-cadherin expression and boundary formation in developing forebrain. *Development* **124**, 3765-3777.

Tanabe, Y., Roelink, H. and Jessell, T. M. (1995). Induction of motor neurons by sonic hedgehog is independent of floor plate differentiation. *Curr. Biol.* **5**, 651-658.

Tuttle, R., Nakagawa, Y., Johnson, J. E. and O'Leary, D. D. M. (1999). Defects in thalamocortical axon pathfinding correlate with altered cell domains in Mash-1-deficient mice. *Development* **126**, 1903-1916.

Vitalis, T., Cases. O., Callebert, J., Lanay, G. M., Price, D. J., Seif, I. and

Gaspar, P. (1998). Effects of monoamine oxidase A inhibition on barrel formation in the mouse somatosensory cortex. *J. Comp. Neurol.* **393**, 169-184.

Walther, C. and Gruss, P. (1991). *Pax6*, a murine paired box gene, is expressed in the developing CNS. *Development* **113**, 1435-1449.

Wang, H. and Tessier-Lavigne, M. (1999). En passant neurotrophic action of an intermediate axonal target in the developing mammalian CNS. *Nature* **401**, 765-769.

Warren, N. and Price, D. J. (1997). Roles of Pax6 in murine diencephalic development. *Development* **124**, 1573-1582.

Warren, N., Caric, C., Pratt, T., Clausen, J. A., Asavaritikrai, P., Mason, J. O., Hill, R. E. and Price D. J. (1999). The transcription factor, Pax6, is required for cell proliferation and differentiation in the cerebral cortex. *Cerebral Cortex* **9**, 627-635.

Wilkinson, D. G. (1992). In Situ Hybridization. A Practical Approach. *The Practical Approach Series.* Senior Editors: D. Rickwood and B. D. Hames. Oxford: IRL Press.

Zernika-Goetz, M., Pines, J., McLean hunter, S., Dixon, J. P. C., Siemering, K. R. and Haseloff, J. (1997). Following cell fate in the living mouse embryo. *Development* **124**, 1133-1137.