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 Pax6Sey/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, Pax6Sey/Sey explants did not, indicating a defect in the ability of mutant dorsal thalamic cells to respond to signals normally present in ventral telencephalon. Pax6Sey/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
abnormality in dorsoventral (DV) patterning of the überorganisation (Sev) (Hill et al., 1991). Homozygotes (Pax6Sev/Sev) show defects in numerous forebrain structures, including the diencephalon, and die shortly after birth (Hogan et al., 1986; Schmah 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 Pax6Sev/Sev 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 Pax6Sev/Sev 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 Pax6Sev/Sev 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 Pax6Sev/Sev 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 thalamic axons downregulate 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; 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 Pax6Sev/Sev 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 tag were generated. The GFP variant mngfp6 CDNA (a gift from J. Haseloff; Siemer 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 TgP6.3 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+/+ mice, some of which carried the tau-GFP transgene (generating tau-GFP:Pax6+/+ or tau-GFP:Pax6Sev/Sev embryos). Pax6Sev/Sev 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:Pax6Sev/Sev embryos; other tissues were from Pax6+/+ or Pax6Sev/Sev 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 thalami 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:Pax6Sev/Sev thalamus (n=21); Pax6+/+ cortex with either tau-GFP:Pax6+/+ thalamus (n=21) or tau-GFP:Pax6Sev/Sev thalamus (n=21); Pax6+/+ hypothalamus with either tau-GFP:Pax6+/+ thalamus (n=6) or tau-GFP:Pax6Sev/Sev thalamus (n=6); Pax6Sev/Sev 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 Ntrin 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 egtagic 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-montage 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 diodacetyl tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, USA), E13.5, E15.5 and E17.5 embryos were fixed with paraformaldehyde and 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 Pax6Sey/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 Pax6Sey/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 Pax6Sey/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 Pax6Sey/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 Pax6Sey/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 Pax6Sey/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 thalami in Pax6Sey/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:Pax6Sey/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:Pax6Sey/Sey thalami in contact with wild-type ventral telencephalon, 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, Hmfn and Pax6 expression patterns (Figs 2E, 4C-E) 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 thalami 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:Pax6Sey/Sey dorsal thalami 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 Pax6Sey/Sey axons in some of the cultures during their 3 days in vitro revealed that the major features of
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:Pax6Sey/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. 2I), 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:Pax6Sey/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:Pax6Sey/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 Pax6Sey/Sey dorsal thalamic explants to generate normal innervation of ventral telencephalon is not caused by a non-specific inability to grow. First, Pax6Sey/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
under all conditions used here almost all cells in both Pax6+/+ and Pax6Sey/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 Pax6Sey/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 Sey/Sey 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 Pax6Sey/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)
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 Pax6Sey/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 (zi; arrow in Fig. 4C), and in the epithalamus. In Pax6Sey/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 zi, which it does even in Pax6Sey/Sey mice (Fig. 4E,F; Warren and Price, 1997), and showed that this boundary is well-separated from residual Hbnf expression in Pax6Sey/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 Pax6Sey/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 Pax6Sey/Sey dorsal thalamic axons to navigate the ventral telencephalon is an indirect defect caused via more persistent changes in other regulatory genes. In Pax6Sey/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 (Figs 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 zi 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 Pax6Sey/Sey diencephalon

We examined expression of two genes, Shh and Nkx2.2,
Fig. 5. Lim1/Lhx1 expression in Pax6Sey/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 Pax6Sey/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.

Role of Pax6 in thalamic development

Implicated in DV patterning of the central nervous system. The expression of Shh in Pax6+/+ and Pax6Sey/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 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.

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 (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).

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.
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 (Hbfn, 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 thalami 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, is 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 Pax6Sey/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 Pax6Sey/Sey embryos. Roles for Pax6 in regulating adhesive properties have been elegantly demonstrated in the eye by analysis of Pax6Sey/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, RPTPβ, 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 Pax6Sey/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 of Pax6Sey/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 Pax6Sey/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 Pax6Sey/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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