Defects of Tyrosine Hydroxylase-Immunoreactive Neurons in the Brains of Mice Lacking the Transcription Factor Pax6

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In the CNS, the lack of the transcription factor Pax6 has been associated with early defects in cell proliferation, cell specification, and axonal pathfinding of discrete neuronal populations. In this study, we show that Pax6 is expressed in discrete catecholaminergic neuronal populations of the developing ventral thalamus, hypothalamus, and telencephalon. In mice lacking Pax6, these catecholaminergic populations develop abnormally: those in the telencephalon are reduced in cell number or absent, whereas those in the ventral thalamus and hypothalamus are greatly displaced and densely packed. Catecholaminergic neurons of the substantia nigra (SN) and the ventral tegmental area (VTA) do not express Pax6 protein. Nevertheless, mice lacking Pax6 display an altered pathfinding of SN–VTA projections: instead of following the route of the medial forebrain bundle ven-

trally, most of the SN–VTA projections are deflected dorsorotally at the pretectal–dorsal thalamic transition zone and in the dorsal thalamic alar plate. Moreover, some catecholaminergic neurons are displaced dorsally to an ectopic location at the pretectal–dorsal thalamic transition zone. Interestingly, from the pretectal–dorsal thalamic to the dorsal thalamic–ventral thalamic transition zones, mice lacking Pax6 display an ectopic ventral to dorsal expansion of the chemorepellant/chemoattractive molecule, Netrin-1. This may be responsible for both the altered pathway of catecholaminergic fibers and the ectopic location of catecholaminergic neurons in this region.

Key words: catecholaminergic neuron; Pax6; netrin; proliferation; adhesion; axonal pathfinding

Recently, a neuromeric model for catecholaminergic (CA) neuronal development has been proposed in several species, including lizard (Medina et al., 1994), chick (Puelles and Medina, 1994), and human (Puelles and Verney, 1998). In this model, it is proposed that permanent or transient CA (dopaminergic and noradrenergic) neurons are generated in or near the region that they occupy in the adult, rather than being generated at a few localized sources and distributed through migration (Olson and Seiger, 1972). Despite the apparent anatomical diversity of noradrenergic (NA) and dopaminergic (DA) neurons, it appears that their early specification relies on a small number of molecules. For instance, essential transcription factors such as Mash1, Phox2a, and Phox2b have been implicated in controlling the specification of all noradrenergic neurons (Pattyn et al., 1997; Hirsh et al., 1998). It appears that the two secreted molecules sonic hedgehog (SHH) and fibroblast growth factor 8 are critical for the specification of DA neurons, and the stereotypic location of most DA neurons along the anteroposterior and dorsoventral axes is defined by the integration of these two signals (Ye et al., 1998).

Gene expression studies have shown that the transcription factor Pax6 is transiently expressed in areas containing discrete CA neurons in the mesencephalon, the ventral thalamus, the hypothalamus (Stoykova and Gruss, 1994), and the olfactory bulb (Dellovade et

al., 1998). Pax6 is a member of a highly conserved gene class and encodes a transcription factor containing a paired domain and a homeodomain (Callaerts et al., 1997). The spatiotemporal expression of Pax6, from E8.5 to adulthood, suggested that Pax6 plays key roles in CNS development (Walther and Gruss, 1991). Indeed, mice lacking Pax6 display early defects in axonal pathfinding (Mastick et al., 1997), in the specification of several prosomeric transition zones (Stoykova et al., 1996; Grindley et al., 1997), in cell proliferation (Warren and Price, 1997), in the specification of motor (Ericson et al., 1997) cell subtypes, and in cell migration (Caric et al., 1997; Brunjes et al., 1998; Engelkamp et al., 1999).

In the present study, we first defined the localization of the Pax6 protein in CA [tyrosine hydroxylase-immunoreactive (TH-IR)] populations during development. We then investigated the role of Pax6 in these populations by looking at their development in mice lacking Pax6. We found that developing TH-IR neurons of the ventral thalamus [zona incerta (Zi)], hypothalamus (paraventricular nucleus), olfactory bulb, and basal telencephalon (anterior olfactory nucleus, piriform cortex, anterior amygdala, and olfactory tubercle) display high levels of Pax6 protein during a critical period of their development. Despite severe positional alterations, diencephalic and hypothalamic TH-IR neurons were identified in mice lacking Pax6, showing that Pax6 is not necessary for their specification. In contrast, TH-IR neurons were greatly reduced in number in the basal telencephalon and the remaining olfactory bulb. In addition, we found that ectopic TH-IR neurons were distributed ventrodorsally along the pretectal–dorsal thalamic transition zone and that TH-IR fibers were misguided in this zone and in the dorsal thalamic alar plate. Interestingly, this region displayed an increased and ectopic expression of the SHH-induced chemorepellant/chemoattractive molecule, Netrin-1 (Leonardo et al., 1997; Lauderdale et al., 1998), which might contribute to its having altered cues for cell migration and axonal navigation.

MATERIALS AND METHODS

Animals. The original small-eye (Pax6^{scv}) mutation arose spontaneously in a stock called “CSR” and was subsequently outcrossed. The genetic background of the small-eye strain used in this study was derived from the

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Figure 1. Determination of the neuro-meric organization of TH-IR neurons in E14.5 wild-type embryos. Sagittal sections stained for Nissl (*A*) or immunoreacted for calbindin (*B*) or calretinin (*C*) have provided the prosomeric landmarks used for the determination of the segmental organization of TH-IR groups as shown in *D–F*. *A*, The section shows constrictions in the neural wall and regions of low cell densities associated with prosomeric boundaries. Arrowheads indicate, from caudal to rostral, the isthmus constriction, the caudal limit of the posterior commissure (PC) at the mesencephalic (mes)–p1 boundary, the fasciculus retroflexus (fr) at the p1–p2 boundary (in p2), and the stria medullaris (stm) at the p3–p4 boundary. The dotted line represents the angle used for coronal sectioning. *B*, Calbindin immunoreactivity shows the posterior commissure in the roof of p1, the nucleus of the posterior commissure (NPC) in p1, the dorsal thalamus (DT) in p2 alar plate, and thalamocortical axons (tc) running through p3 alar plate. The asterisk marks a strong immunoreactive hypothalamic region in p5 and p6 basal plate. The septum (SE) and olfactory bulb (OB) are also strongly immunoreactive. *C*, Calretinin immunoreactivity shows the posterior commissure, the subthalamic nucleus (Sut) in p4 basal plate, the thalamic eminence (EMT) in p4 alar plate, and the retrochiasmatic area (RCH) in p6 basal plate. The septum (SE) is also strongly immunolabeled. *D–F*, The prosomeric boundaries (continuous black lines) and basal–alar limit (dotted lines) are deduced from the adjacent sections stained for calretinin or calbindin. Note that *B*, *C*, and *E* are alternate sections. *D*, Medial section showing a subgroup of A11 organized along the fasciculus retroflexus and the A9–A10 complex. Note that A9 is located in the basal plate and extends from mes to p2, whereas A10 is located in the floor plate and extends from the isthmus (A10i) region to p3. *E*, A more lateral section than shown in *D* showing A11 extending from mes to p2 and the diencephalic and hypothalamic groups: A13 in p3, A14 in p4, and RCH and anterior preoptic (POA) areas in p6. *F*, A lateral section shows additional groups in the hypothalamus (A14 subgroups in p5 basal and alar plates) and in the telencephalon (A15). *AB*, Anterobasal nucleus; *Is*, isthmus; *LGE*, ganglionic eminence, lateral part; *LL*, lateral lemniscal area; *MA*, mammillary region; *mes*, mesencephalon; *MGE*, ganglionic eminence, medial part; *mif*, medial longitudinal fasciculus; *mtg*, mammillotegmental tract; *OR*, optic recess; *p1–p3*, prosomeres; *r1*, rhombomere 1; *SC*, superior colliculus. Scale bar, *A–F*, 4 mm.

outbred Swiss background. The mating of Pax6^{scv}/+ (small-eye heterozygotes) was confirmed by the presence of a vaginal plug the following morning. This was designated embryonic day 0.5 (E0.5). Experiments were performed on E11.5, E12.5, E13.5, E14.5, E16.5, E17.5, and E18.5 embryos. Embryos were dissected from deeply anesthetized mothers into cold PBS on ice and examined under a dissecting microscope. Homozygous Pax6^{scv}/Pax6^{scv} embryos were easily distinguished by their absence of eyes and characteristic craniofacial phenotype of foreshortened upper jaw. From E12.5, heterozygotes (Pax6^{scv}/+) were distinguished by the characteristic appearance of their iris lacking its inferior margin (Kaufman et al., 1995). In each experiment, wild-type and Pax6^{scv}/Pax6^{scv} embryos were obtained from the same litter. Some additional experiments were also performed on embryos and postnatal and adult mice of the Swiss genetic background. Animal procedures were conducted in strict compliance with approved institutional protocols and in accordance with the provisions for animal care and use described in the *Scientific Procedures on Living Animals ACT 1986*. In all the experiments, adult mice were anesthetized with 0.3 ml 25% urethane injected intraperitoneally.

Immunocytochemistry. E11.5, E12.5, and E13.5 embryos were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.6. Embryos from E14.5 to E19.5 and postnatal mice were perfused transcardially with saline followed by 4% paraformaldehyde in PB. Whole embryos or brains were post-fixed for 2–5 d in the same fixative and cryoprotected in 30% sucrose in PB. Serial coronal or sagittal sections (40 μ m) were cut on a freezing microtome and immediately processed for immunocytochemistry. In brief, sections were incubated with the primary antibodies diluted in PBS+ (0.1 M PBS with 0.2% gelatin and 0.25% Triton X-100) overnight at 4°C. Rabbit polyclonal anti-TH antibodies (1:8000, kind gift of A. Vigny, or 1:800, Profos Biotech), rabbit polyclonal anti-calretinin antibody (1:10,000; Swant), rabbit polyclonal anti-calbindin an-

tibody (1:20,000; Swant), rat monoclonal anti-L1 antibody (1:50; Roche Diagnostics), and rat monoclonal anti-NCAM antibody (1:50; Roche Diagnostics) were used. Biotinylated goat anti-rabbit and biotinylated goat anti-rat (1:200, Dako, Glostrup, Denmark) were used as secondary antibodies and revealed with a streptavidin–biotin–peroxidase complex (1:200, Amersham, Buckinghamshire, UK). Sections were then reacted with a solution containing 0.02% diaminobenzidine, 0.6% nickel ammonium sulfate, and 0.003% H₂O₂ in 0.05 M Tris buffer, pH 7.6 (DAB-Ni). From these sections, the total number of TH-IR neurons in A14 paraventricular hypothalamic nucleus (PAVH) and the diameters of randomly selected TH-IR neurons ($n = 20$) were measured in A14PAVH from E17.5 wild-type ($n = 4$) and Pax6^{scv}/Pax6^{scv} ($n = 4$) embryos.

Double Pax6 and TH immunocytochemistry. Whole embryos (E11.5, E12.5, E14.5, E16.5, E17.5, and E18.5) and dissected postnatal (P0, P2, P4, and P9) and adult brains (5 and 16 weeks old) were immediately frozen in isopentane (–40°C) and stored at –80°C until sectioning. Coronal and sagittal sections (10–14 μ m) were cut on a cryostat and processed the same day. Sections were dried at room temperature, fixed for 10 min in methanol/acetone (1:1; –20°C), dried for 15 min at room temperature, hydrated for 5 min in PBS, and blocked for 15 min in a solution containing 2% bovine serum albumin, 2% sheep serum, 7% glycerol, and 0.2% Tween 20 (BS). Sections were then incubated overnight at room temperature with three different antibodies: a rabbit polyclonal anti-TH antibody (1:5000, kind gift of A. Vigny) and two mouse monoclonal anti-PAX6 antibodies [AD1.5.6 and AD2.35; 1:50 in embryos and 1:30 in adults (Engelkamp et al., 1999)] diluted in BS. Sections were washed in PBS 0.2% Tween 20 (PBST) and incubated for 1 hr with secondary antibodies [TRITC anti-mouse antibody, 1:200 (Vector Laboratories, Burlingame, CA), and FITC anti-rabbit antibody, 1:200 (Sigma, St. Louis, MO)], diluted in PB. Sections were washed in PBST and analyzed with a Leica (Nussloch, Germany)

Table 1. Mapping of the main TH-IR groups in E14.5 mouse brain

										Secondary Prosencephalon		
										Telencephalon		
										<u>ACX</u>	<u>NCX</u> <u>ITA</u> <u>-pir^t</u> <u>-AA^t</u> <u>-OT^t</u>	<u>OB</u> <div><u>A16</u></div>
										<u>CGEL</u>	<u>LGE</u>	<u>ACB</u>
										<u>CGEM</u>	<u>MGE</u> A15d	<u>BST</u> A15d
										<u>EMT</u>	<u>AEP</u>	<div><u>A15v</u></div>
										<u>SPV</u> <div><u>A14Pavh</u></div>	<u>AH</u> A14l	<u>POP</u>
										<u>PEP</u> A14Periv	<u>HCC</u> A14Periv A14DMH	<u>SCH</u> A14Periv
										<u>MA</u>	TU	RCH
											A12	AB ^t
										p4	p5	p6

Figure 2. Pax6 protein expression in discrete developing TH-IR groups. Sections through the A8–A10 complex (*A–C*), the diencephalon and the hypothalamus (*D–F*), and the telencephalon (*G–K*) were double-immunostained with antibodies to TH (green; cytoplasmic staining) and Pax6 (red; nuclear staining). *A–C*, Absence of Pax6 and TH colocalization in the SN–VTA (A9–A10) complex and the retrorubral field (A8). *A*, The sagittal section shows a lack of Pax6 immunoreactivity in the developing SN–VTA of E12.5 embryo. Note the strong Pax6 immunolabeling of the deep mesencephalic nucleus (DPMc). *B*, The coronal section shows Pax6 immunoreactive cells (short arrows) in close proximity with TH-IR neurons of the dorsal part of the SN in E16.5 embryo. *C*, The coronal section shows Pax6 immunoreactive cells in the retrorubral field close to A8 neurons. *D*, The coronal section shows the colocalization of TH and Pax6 in A13 neurons of the zona incerta in the ventral thalamus. *E*, Higher magnification of the box shown in *D* showing individual double-immunolabeled cells (white arrows). Note the presence of TH-IR neurons (A13d) that do not express Pax6 (white arrow). *F*, The coronal section shows the lack of Pax6 immunoreactivity in A14DMH neurons of the hypothalamus. *G*, Coronal section showing Pax6-immunoreactive cells in the basal telencephalon. Pax6-immunoreactive cells are located in the anterior amygdala (large arrowhead) and the region of the piriform cortex (small arrowhead). Note Pax6 immunoreactive cells also in the cerebral cortex, hypothalamus, and ventral thalamus. *H*, Higher (Figure legend continues)

cells, a minimum of six sections taken through Zi and DMH were selected. On each section, A13 and A14DMH were identified, and the number of TH-IR neurons heavily labeled (defined as having >50% of the nucleus immunolabeled) for BrdU was estimated using 40× and 100× objectives. Only heavily labeled cells were counted because they would have been generated at the time of BrdU administration, whereas many lightly labeled cells would have been the products of further progenitor cell divisions (Gillies and Price, 1993). For each age of BrdU injection, wild-type ($n = 4$) and Pax6^{sey}/Pax6^{sey} ($n = 4$) embryos were obtained from at least two independent litters. In addition, the total number of TH-IR neurons in A13 and A14DMH was estimated from these sections in wild-type ($n = 6$) and Pax6^{sey}/Pax6^{sey} ($n = 6$) embryos.

Nissl staining and counterstaining. Complete series of parasagittal and coronal paraffin sections (10 μ m) obtained from E11.5, E12.5, E14.5, E16.5, and E18.5 wild-type and Pax6^{sey}/Pax6^{sey} embryos were Nissl-stained in a solution containing 0.05% thionin in acetic acid, pH 5.5.

In situ hybridization. E11.5, E12.5, E13.5, E14.5, E16.5, and E19.5 wild-type and Pax6^{sey}/Pax6^{sey} embryos were dissected in PBS, fixed, and cryoprotected overnight in 4% paraformaldehyde–30% sucrose. Sections (80–100 μ m thick) were obtained on a freezing microtome, washed in PBS 0.1% Tween 20 (PTW), dehydrated for 20 min in methanol, and rehydrated in PTW before hybridization. Hybridization was performed as described in Henrique et al. (1995). Briefly, sections were treated with proteinase K (10 mg/ml) for 10 min, rinsed in PTW, fixed for 20 min in 4% paraformaldehyde–0.2% glutaraldehyde, rinsed in PTW, rinsed in the hybridization medium (50% formamide, 1.3× SSC, 50 mM EDTA, 0.2% Tween 20, 10% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 100 mg/ml heparin) at room temperature until the sections settled, and rinsed in the hybridization medium (HM) at 65°C before hybridization. Sections were then hybridized overnight at 65°C with digoxigenin-labeled (Roche Diagnostics) riboprobes for Netrin-1 (kind gift of M. Tessier-Lavigne; *EcoRI*, T3: antisense; *SacI*, T7: sense) or Pax6 (kind gift of S. Saule; *PstI*, T3: antisense; *HindIII*, T7: sense). The following day, sections were rinsed in HM (2 × 30 min, 65°C), washed in a 1:1 mixture of HM and MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5, 0.1% Tween 20) for 10 min at 65°C and 15 min at room temperature, incubated for 1 hr in MABT with 2% blocking reagent (Roche Diagnostics), and incubated for 4 hr in MABT with 2% blocking reagent and 20% heat-treated sheep serum (MABT+), and finally incubated overnight with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:2000, Roche Diagnostics) in MABT+. Sections were washed in MABT for 4 hr and in a mixture of 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂, and 0.1% Tween 20 for 20 min before the enzymatic color detection with the nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Roche Diagnostics).

Nomenclature. On the basis of gene expression domains and anatomical features (constrictions in the neural wall and regions of low cell density), the brain has been subdivided into neuromeres. The rhombomeric and mesencephalic organizations have been described by Lumsden (1990), Krumlauf (1994), and Guthrie (1996), and the prosomeric organization has been described in Rubenstein et al. (1994) and Puelles (1995). Eight consecutive rhombomeres (r1–r8), the isthmus (Is), and the mesencephalon (mes) are identified in the rhombencephalon and midbrain. Note that rhombomere 1 and isthmus are represented as a single entity (r1–Is) in this scheme. According to the prosomeric model, the forebrain is subdivided into six transverse domains called prosomeres (p1–p6). The diencephalon develops in prosomeres 1–3 (p1–p3), and the secondary prosencephalon (hypothalamus, preoptic areas, and its hyper-alar extension, the telencephalon) develops in p4–p6. In addition, these transverse domains are subdivided dorsoventrally into roof plate, alar plate, basal plate, and floor plate or prechordal plate (from p4) (Shimamura et al., 1995). Telencephalic organization refers also to the work of Fernandez et al. (1998). Our anatomical description refers to the atlas of the developing rat brain (Paxinos, 1991), the atlas of the mouse brain (Franklin and Paxinos, 1995), and the chemoarchitectonic atlas of the developing mouse brain (Jacobowitz and Abbott, 1998). To describe the permanent TH-IR cell groups (A1–A17), we have mainly used the nomenclature of Hokfelt et al. (1984) and Jacobowitz and Abbott (1998). The description of the distribution of TH-IR neurons in the hypothalamus and preoptic regions also refers to the work of Ruggiero et al. (1984) and Foster (1994). A14 complex was subdivided into subgroups relative to their main anatomical locations. In addition, some transient TH-IR neuronal populations have already been described in the developing CNS (Jaeger and Joh, 1983; Verney et al., 1988; Nagatsu et al., 1990).

RESULTS

Neuromeric location of TH-immunoreactive groups in E14.5 wild-type embryos

So far, no description of the neuromeric location of permanent and transient TH-IR groups is available in developing mice despite the increasing references to the neuromeric organization of the brain (Bulfone et al., 1993; Rubenstein et al., 1994; Puelles, 1995; Shimamura et al., 1995, 1997). In this study we first provide a comprehensive neuromeric location of the different TH-IR groups in E14.5 wild-type mice. At this age, most of the permanent TH-IR groups (A1–A17) (Hokfelt et al., 1984) occupy their definitive position, some transient TH-IR groups are detected, and the neuromeric limits are still visible. The topological landmarks necessary for the description of the neuromeric organization were obtained by studying alternate sections stained for Nissl (Fig. 1*A*) or for several differentiation markers, principally, the two calcium-binding proteins calbindin (Fig. 1*B*) and calretinin (Fig. 1*C*), which display complementary immunoreactive patterns [Jacobowitz and Abbott (1998) and Fig. 1]. The neuromeric location of the main discrete TH-IR groups is shown in Figure 1*D–F* and detailed in Table 1. The description of TH-IR groups that did or did not display Pax6 immunoreactivity in wild-type mice is presented within this framework (see below and Table 1).

Colocalization of Pax6 and TH immunoreactivities

TH-immunoreactive neurons displaying Pax6 immunoreactivity (Table 1)

Pax6 immunoreactivity was detected in three dopaminergic groups of the forebrain. In the alar plate of the ventral thalamus, a subpopulation (~40%) of TH-IR neurons of the zona incerta (A13) displayed a strong and transient Pax6 immunoreactivity from E12.5 to P9 (Fig. 2*D,E*). This subpopulation corresponds to the body of A13. In the hypothalamus, TH-IR neurons of the magnocellular part of the hypothalamic paraventricular nucleus displayed transient Pax6 immunoreactivity from E14.5 to P2 (A14PAVH in Table 1; data not shown). TH-IR neurons of the supraoptic nucleus (A15v) display also a transient Pax6 immunoreactivity from P0 to P9 (Table 1; data not shown). In the telencephalon, transient TH-IR neurons located in the anterior olfactory nucleus (A16AON) displayed Pax6 immunoreactivity from E14.5 to E18.5 (Table 1; data not shown). Transient TH-IR neurons of the piriform cortex, the olfactory tubercle, and the anterior amygdala display Pax6 immunoreactivity from E14.5 to E18.5 (Fig. 2*G–I*). In the olfactory bulb (A16OB), TH-IR external tufted cells displayed Pax6 immunoreactivity from E14.5 and TH-IR periglomerular interneurons from E18.5 (Fig. 2*J,K*, Table 1).

TH-immunoreactive neurons not displaying Pax6 immunoreactivity (Table 1)

Noradrenergic (A1–A7) and adrenergic (C1–C3) neurons of the brainstem never displayed Pax6 immunoreactivity (Table 1). Dopaminergic neurons of the ventral tegmental area (A10i, A10m, A10p1, A10p2, and A10p3) in the floor plate, of the substantia nigra (A9m, A9p1, and A9p2), and of the retrorubral field (A8) in the basal plate, and of A11 complex (A11m, A11p1, A11p2) in the alar plate did not display Pax6 immunoreactivity throughout development (Fig. 2*A–C*). In the hypothalamus, the TH-IR groups listed below did not display Pax6 immunolabeling at any stage of development: the lateral hypothalamic nucleus (A14l), the medial preoptic area (POA and A14d), the arcuate nucleus (A12). In the telencephalon, TH-IR neurons of the bed nucleus of the stria terminalis (A15d) did not display Pax6 immunoreactivity. Al-

magnification of *G* showing individual double-labeled neurons at the level of the anterior amygdala. *I*, Higher magnification of *G* showing individual double-labeled neurons at the level of the piriform cortex (arrows). *J*, *K*, Coronal sections of the olfactory bulb. *J*, TH-IR external tufted cells display a strong Pax6 immunostaining in E15.5 embryo (small arrow). *K*, Both TH-IR periglomerular neurons and TH-IR external tufted cells in A16 display Pax6 immunoreactivity at P0. Scale bar: *A*, *G*, 6 mm; *B*, *C*, 4 mm; *D*, *F*, *J*, 1 mm; *E*, 0.25 mm; *H*, 0.12 mm; *I*, 0.5 mm; *K*, 5 mm.

Figure 3. Determination of the presumptive neuromeric organization of TH-IR groups in E14.5 Pax6^{sey}/Pax6^{sey} embryos. Nissl-staining (*A*) and immunolabeling for calbindin (*B*) or calretinin (*C*) have provided prosomeric landmarks used for the determination of the segmental organization of TH-IR groups (*D*). *A*, The sagittal section shows constrictions in the neural wall and regions of low cell densities associated with neuromeric boundaries. From caudal to rostral, arrowheads point to the isthmus constriction and the presumptive p1–p2, p2–p3, and p3–p4 boundaries. Thin arrows point to the presumptive p2–p3 and p3–p4 boundaries. Arrows point to the presumptive p1–p2, p2–p3, p3–p4, and p4–p5 boundaries. The dotted line represents the angle used for coronal sectioning. *B*, The sagittal section immunoreacted for calbindin shows a normal medial longitudinal fasciculus (*mlf*), retrochiasmatic (*RCH*) and anterobasal (*AB*) areas, and septum (*SE*). The white star indicates the lack of clustering of the presumptive dorsal thalamus and the lack of thalamocortical axons. *C*, Sagittal section immunoreacted for calretinin shows normal immunoreactivity in the thalamic eminence (*EMT*), the stria medullaris (*sm*), and the subthalamic nucleus (*Sut*) in p4. *D*, The sagittal section immunoreacted for TH shows numerous groups and complexes: *A11*, *A9–A10*, and in anterobasal and preoptic (*POA*) areas. The limit between *mes* and *p1* is not indicated because this neuromeric limit is altered in the mutant (Mastick et al., 1997). Scale bar: *A–D*, 4 mm.

though no colocalization of TH and Pax6 was observed in TH-IR neurons of the dorsal medial hypothalamic nucleus (Fig. 2*F*, A14DMH), Pax6 was expressed in the neuroepithelium of A14DMH during its period of genesis (from E9.75 to E12.5; see below).

An overview of defects in Pax6^{sey}/Pax6^{sey} embryos

From E10.5 to E14.5, Pax6^{sey}/Pax6^{sey} embryos displayed a delay in their growth. A marked difference in their crown-rump length was observed at E11.5 (wild type: 4.8 ± 0.3 mm, $n = 15$; Pax6^{sey}/Pax6^{sey}: 3.8 ± 0.33 mm, $n = 15$). By E14.5, wild-type and Pax6^{sey}/Pax6^{sey} embryos displayed no significant difference in their crown-rump length (wild type: 11.7 ± 0.07 mm, $n = 30$; Pax6^{sey}/Pax6^{sey}: 11.1 ± 0.1 mm, $n = 30$). By E17.5, brain weights were similar in wild-type and Pax6^{sey}/Pax6^{sey} embryos (wild type: 0.76 ± 0.07 gm, $n = 30$; Pax6^{sey}/Pax6^{sey}: 0.74 ± 0.06 gm, $n = 25$). Some brain regions in Pax6^{sey}/Pax6^{sey} embryos have been shown to display higher than normal cell densities (Schmahl et al., 1993; Caric et al., 1997), and there may be hypertrophy of brain regions in response to an increased SHH expression. This may compensate for the reduction of some structures such as the olfactory bulbs and, for example, the decrease of the cortical thickness.

TH-IR groups that did or did not display Pax6 immunoreactivity were described in Pax6^{sey}/Pax6^{sey} embryos within the same framework used above (see Fig. 3 for a general overview at E14.5). We observed several alterations in both Pax6-expressing TH-IR populations and TH-IR neurons that did not express Pax6, such as SN and VTA neurons. Noradrenergic (A1–A7) and adrenergic (C1–C3) neurons and mesencephalic dopaminergic neurons of A8, which did not express Pax6 (Table 1), displayed no delay and appeared normally organized in Pax6^{sey}/Pax6^{sey} embryos. The following description will focus on TH-IR groups displaying alterations.

Defects in Pax6-immunoreactive components of the incerto-hypothalamic axis

The incerto-hypothalamic axis (Bjorklund et al., 1975) includes TH-IR neurons of A11–A14. In this structure in normal animals,

TH-IR neurons appear fused together along an axis extending from the mesencephalon to the anterior hypothalamus. In this structure, TH-IR neurons are arranged either in discrete nuclei (A12, A13, A14PAVH, and A14DMH) or in a periventricular position (A11, A14Periv). A13 and A14PAVH express Pax6 transiently during development, whereas A11, A12, A14DMH, A14Periv, and A14I do not express Pax6.

Cell generation

In E11.5 wild-type embryos, it was possible to identify scattered TH-IR neurons in the ventral thalamus at the level of the primordium of A13 and a few medium-sized TH-IR neurons of the A14 complex in p4 and p5. These cells were more numerous by E12.5 (Fig. 4*A*). In Pax6^{sey}/Pax6^{sey} embryos, the A13 and A14 primordia appeared with a 2 d delay (Fig. 4*A,B*), and when they first appeared, they contained fewer TH-IR neurons than in wild-type embryos (50% reduction estimated). This delay did not persist. By E17.5, the total numbers of TH-IR profile counts in mutants and wild types were similar in A13 ($n = 600 \pm 65$, from six wild types; $n = 524 \pm 60$, from six mutants; values are means $\pm$ SEM), in A14DMH ($n = 510 \pm 40$, from six wild types; $n = 486 \pm 60$, from six mutants), and in A14PAVH ($n = 40 \pm 4$, from four wild types; $n = 38 \pm 3$, from four mutants). Because the mean diameters of TH-IR neuronal cell bodies were similar in wild-type (A13, $n = 10 \mu\text{m} \pm 0.7$; A14DMH, $n = 11 \mu\text{m} \pm 0.7$; A14PAVH, $n = 12 \mu\text{m} \pm 0.9$; values are means $\pm$ SEM from 30 TH-IR neurons from four wild types) and Pax6^{sey}/Pax6^{sey} embryos (A13, $n = 11 \mu\text{m} \pm 0.6$; A14DMH, $n = 10.5 \mu\text{m} \pm 1.0$; A14PAVH, $n = 11.5 \mu\text{m} \pm 0.4$, values are means $\pm$ SEM from 30 TH-IR neurons from four mutants), this indicates that there is no difference in TH-IR cell number in these structures.

Although Pax6 is expressed in differentiated TH-IR neurons of A13 and A14PAVH but not A14DMH, Pax6 is expressed during the time of genesis of all of these TH-IR populations. We have investigated a possible delay in cell generation of the cells destined for these groups in Pax6^{sey}/Pax6^{sey} embryos. Cell proliferation was studied by analyzing BrdU incorporation into S-phase cells and

Figure 4. Delay in the appearance of a TH phenotype in A13 and A14DMH is not caused by a cell proliferation defect. *A, B*, Sagittal sections of E12.5 wild-type (*A*) and Pax6^{sey}/Pax6^{sey} (*B*) embryos immunolabeled for TH. *A*, Arrow points to the A13 and A14 primordia. *B*, The black asterisk indicates the presumptive location of the A13 and A14 primordia in the mutant; note the lack of TH-IR neurons in these regions. *C–F*, Double immunolabeling for TH and BrdU of E17.5 wild-type (*C, D*) and Pax6^{sey}/Pax6^{sey} embryos (*E, F*) at the level of A13. BrdU was injected on E10.5. *D, F*, Arrows point to TH–BrdU double-labeled neurons. *G, H*, Histograms showing similar mean percentages ($\pm$ SEM) of TH-IR neurons darkly labeled for BrdU in A13 (*G*) and A14DMH (*H*) in wild-type (white bars) and Pax6^{sey}/Pax6^{sey} (black bars) E17.5 embryos after injections of BrdU on E9.75–E12.5. Scale bar: *A, B*, 2 mm; *C, E*, 0.5 mm; *D, F*, 0.1 mm.

visualizing them at E17.5 using anti-BrdU and anti-TH antibodies (Fig. 4*C–F*). To identify the embryonic stages at which the neurons of these nuclei are generated, a single injection was applied at four different developmental stages: E9.75, E10.5, E11.5, and E12.5. The number of BrdU–TH-positive cells and TH-positive cells was determined on coronal sections at E17.5. The labeling index was calculated as the percentage of the total number of TH-positive cells that were BrdU–TH-positive. In wild-type embryos, A13 and A14DMH are generated from E9.75 to E11.5 with a peak at E10.5 (Fig. 4*G, H*, white bars). In Pax6^{sey}/Pax6^{sey} embryos, the labeling index after each injection was unchanged and no delay was ob-

served (Fig. 4*G, H*, black bars), suggesting that cell generation is unaffected in A13 and A14DMH.

Positional alterations

In wild-type embryos, A13, A14DMH, and A14PAVH were populated by large TH-IR neurons and appeared fused with each other from E14.5 (Figs. 1*F*, 5*A, C*). In Pax6^{sey}/Pax6^{sey} embryos, A13, A14DMH, and A14PAVH appeared greatly disjoined and abnormally shaped (Fig. 5*E–G*). In wild-type embryos, three distinct A13 subgroups were observed from E16.5 (Hokfelt et al., 1984): a dorsal group (A13d), a lateral group (Fig. 5*B, A13L*), and a medial group

Figure 5. Alterations of the incerto-hypothalamic axis in E18.5 Pax6^{sey}/Pax6^{sey} embryos. Coronal sections are shown for wild-type (*A–D*) and Pax6^{sey}/Pax6^{sey} (*E–H*) embryos and are organized from caudal (*top*) to rostral (*bottom*). *A–C*, The components of the incerto-hypothalamic axis, A13, A14PAVH, and A14DMH appear fused together in wild-type embryos. The medial forebrain bundle is also indicated in *A–D* and *F–H* (large unlabeled arrows). *A*, Arrow indicates TH-IR neurons of the A14DMH complex. *B*, TH-IR neurons of A13 are divided into three distinct groups: a dorsal group (A13d), a lateral group (A13L), and a medial group A13 (A13). *C*, Arrow indicates TH-IR neurons of the paraventricular hypothalamic nucleus (A14PAVH). *D*, Rostral section at the level of the anterior commissure showing TH-IR neurons located in the medial preoptic nucleus (MnPo), the striato-hypothalamic nucleus (StHy), and the anterobasal region (AB). *E–G*, In Pax6^{sey}/Pax6^{sey} embryos, the components of the incerto-hypothalamic axis are completely disjointed and display abnormally high packing of the neurons. *E*, Arrows indicate A13 and A14DMH. Open arrows indicate abnormally located TH-IR fibers originating from the SN–VTA. *F*, Arrows indicate A13 and A14DMH. Projections from A14DMH to the area of the arcuate nucleus–median eminence are abnormally highly fasciculated. *G*, *H*, Arrows indicate the location of A14PAVH, StHy, the anterior medial preoptic nucleus (AMPo), MnPo, and AB. Scale bar: *A–H*, 2 mm.

(A13) (Fig. 5*B*). In Pax6^{sey}/Pax6^{sey} embryos, only two subgroups were identified in A13; they were displaced laterally from the third ventricle and appeared as a ventral round-shaped group (Fig. 5*E,F*) and as a dorsal group (Fig. 5*F*). The presumptive A14PAVH was observed more rostrally, as a small nucleus densely packed with few TH-IR neurons (Fig. 5*G*). The presumptive A14DMH group was laterally displaced and organized as an ovoid-shaped nucleus (Fig. 5*E,F*).

Cellular segregation

In wild-type embryos, the structures of the incerto-hypothalamic axis, Zi, DMH, and PAVH are each composed of several neuronal groups with different phenotypes, such as TH, neurotensin, or vasopressin neurons. In these structures, TH-IR neurons are mixed with other cell types that do not express TH (Fig. 6*A*). In Pax6^{sey}/Pax6^{sey} embryos, TH-IR neurons constituting A13, A14DMH, and A14PAVH appeared more highly clustered (Fig. 6*B*). As described above, the total number of TH-IR neurons in A13, A14DMH, and A14PAVH are the same in the wild-type and Pax6^{sey}/Pax6^{sey} embryos (Fig. 6*C–F*); however, the volume occupied by A13 and A14DMH was smaller in Pax6^{sey}/Pax6^{sey} embryos (Fig. 6*C*). Interestingly, although the mean cellular density was similar between wild-type and Pax6^{sey}/Pax6^{sey} embryos (Fig. 6*D*), the cellular density of TH-IR neurons in these structures was higher in Pax6^{sey}/Pax6^{sey} embryos (Fig. 6*F*), indicating a higher segregation of

TH-IR neurons in these structures as estimated by the increased percentage of TH-IR neurons within them (Fig. 6*E*). TH-IR neurons were more clustered or less mixed with cell types that did not express TH. This suggests altered adhesive properties of cells composing the Zi and DMH in Pax6^{sey}/Pax6^{sey} embryos.

Defects of TH-immunoreactive neurons in the telencephalon of Pax6^{sey}/Pax6^{sey} embryos

In wild-type embryos, from E14.5, TH-IR neurons were observed at the level of the bed nucleus (Fig. 7*A*, A15d) and the anterior olfactory nucleus (A16AON) (Nagatsu et al., 1990) (Fig. 8*C*). In Pax6^{sey}/Pax6^{sey} embryos, A15d was greatly reduced in cell number (Fig. 7*C*), whereas A16AON was absent by E14.5 (Fig. 8*G*). In wild-type embryos, TH-IR neurons were also observed in the olfactory bulb as soon as E16.5 (Fig. 8*D*). Based on their age, large soma size, and the location in the developing glomerular layer, these neurons probably correspond to external tufted cells. By E18.5, a large number of TH-IR neurons were observed in the glomerular layer of the olfactory bulb, corresponding to both external tufted cells and the earliest population of periglomerular interneurons. In Pax6^{sey}/Pax6^{sey} embryos, from E16.5, only a few lightly labeled TH-IR neurons were observed at the level of the residual olfactory bulb (Fig. 8*G*). Evidence for the development of a residual olfactory structure is provided by calretinin and calbindin immunoreactivities (Fig. 8*E,F*). In this structure, TH-IR

Figure 6. Increased cellular segregation of TH-IR neurons in A13 and A14DMH of mice lacking Pax6. TH was revealed with fluorescein-coupled antibodies (green in *A* and *B*), and nuclei were revealed on the same sections with propidium iodide (red in *A* and *B*). Pictures show the addition of two confocal images acquired simultaneously with a two-channel excitation beam. *A*, In A13 in the wild-type embryo, TH-IR neurons were mixed with non-TH-IR neurons. *B*, In Pax6^{sey}/Pax6^{sey} embryos, TH-IR neurons of A13 appeared more densely clustered and more segregated from the non-TH-IR neurons. *C*, Histogram shows the estimated volume occupied by TH-IR neurons in A14DMH and A13 in wild-type (white bars) and Pax6^{sey}/Pax6^{sey} (black bars) embryos. *D*, Histogram shows that the mean cell density of propidium-positive nuclei in A14DMH and A13 was similar in wild-type (white bars) and Pax6^{sey}/Pax6^{sey} (black bars) embryos. *E*, Histogram shows a significant increase of the percentage of TH-IR neurons compared with the total number of propidium-positive nuclei in A14DMH and A13 of Pax6^{sey}/Pax6^{sey} embryos. *F*, Histogram shows a significant increase in the density of TH-IR neurons in A14DMH and A13 in Pax6^{sey}/Pax6^{sey} embryos. *C–F*, Significant differences with Student's *t* test between groups are indicated: **p* < 0.05; ***p* < 0.01. Scale bar: *A*, *B*, 0.75 mm.

neurons were scattered but differentiated: they were large with angular shapes and short processes probably corresponding to the external tufted cells (Fig. 8*H*). The reduction in the mutant of TH-IR neurons in A15d (Fig. 7*C*) and A16 (data not shown) persisted in older Pax6^{sey}/Pax6^{sey} embryos. No small TH-IR neurons corresponding to the periglomerular interneurons were observed in older Pax6^{sey}/Pax6^{sey} embryos.

In wild-type embryos, from E16.5, TH-IR neurons were observed at the level of the strio-hypothalamic nuclei (Fig. 5*D*). In Pax6^{sey}/Pax6^{sey} embryos, despite the lack of the anterior commissure, TH-IR neurons were observed at the presumptive level of the strio-hypothalamic nucleus (Fig. 5*H*).

In addition, transient TH-IR neurons were observed in the piriform cortex (Fig. 7*A, B*, near A15v) from E14.5 to E18.5 and in the anterior amygdala (data not shown) and olfactory tubercle (data not shown) from E16.5 to E18.5 in wild-type embryos. In Pax6^{sey}/Pax6^{sey} embryos, TH-IR neurons were rare, round, and pale ($n = 15 \pm 4$, values are mean $\pm$ SEM from four mutants; $n = 90 \pm 10$, values are mean $\pm$ SEM from four wild types) at the presumptive level of the anterior amygdala. By E18.5, TH-IR neurons were not detected, and no pyknotic profiles were observed in this region. This suggests that these neurons were generated and had progressively lost their ability to maintain TH expression. TH-IR neurons were never observed in the presumptive olfactory

Figure 7. Defects of the telencephalic TH-IR neurons in Pax6^{sey}/Pax6^{sey} embryos. Coronal sections through the basal telencephalon and hypothalamus of E14.5 (*A, C*) and E18.5 (*B, D*) wild-type (*A, B*) and Pax6^{sey}/Pax6^{sey} (*C, D*) embryos. *A*, The section shows both the transient TH-IR neurons of the piriform cortex (*pir*) and the permanent TH-IR groups of *A15v* and *A15d* in continuation with *A14d*. Note the location of the medial forebrain bundle (*mfb*). *B*, High magnification of the *pir*–*A15v* area. *C*, The section shows a reduced *A15d* still in continuation with *A14d*. The black star indicates the lack of *pir*–*A15v* at the presumptive level of the rhinal fissure. *D*, The lack of *pir*–*A15v* persists in older age embryos (black star). The large asterisk indicates the abnormal swirl of TH-IR fibers at the medial forebrain bundle (*mfb*). Scale bar: *A–E*, 2 mm.

tubercle, the piriform cortex (Fig. 7*C,D*), and the neighboring hypothalamic *A15v* (Fig. 7*C*) and at any age studied in Pax6^{sey}/Pax6^{sey} embryos.

Defects in TH-immunoreactive groups not expressing Pax6

Defects in the SN–VTA (*A9–A10*) and *A11* complex

In wild-type embryos, from E11.5 to E13.5, TH-IR neurons of the primordium of the ventral tegmental area (*A10i*, *A10m*, *A10p1*, *A10p2*, and *A10p3*) and of the substantia nigra (*A9m*, *A9p1*, and *A9p2*) migrate radially from their proliferative zones to more superficial positions (Kawano et al., 1995). These cells are shown in Figure 9*A*. In Pax6^{sey}/Pax6^{sey} embryos, by E11.5, TH-IR neurons of *A9m* and *A9p1* and of *A10m* and *A10p1* were normally radially organized, suggesting that they were normally migrating to their ventral positions (data not shown). At this age, TH-IR neurons of *A10p2* and *A10p3*, in the p2–p3 floor plate of the mutants, were less numerous than in wild type (75% reduction estimated), probably because of a delayed TH expression. By E12.5, although sagittal mediolateral sections of wild-type embryos showed that the oldest TH-IR neurons of *A9p1* and *A9p2* were oriented caudorostrally in p1 and p2, in Pax6^{sey}/Pax6^{sey} embryos, very few radially oriented TH-IR neurons were observed on medial-most sections in mes, p1, p2, and p3. Strikingly, in mediolateral and lateral sections, TH-IR neurons of *A9* were abnormally positioned along the presumptive p1–p2 transition zone (Fig. 9, compare *E* with *A*).

In wild-type embryos, the number of radially oriented TH-IR neurons detected in the vicinity of the ventricular surface gradually decreased by E13.5. By E14.5, most *A9* and *A10* neurons had reached their final locations in more superficial positions of the ventral floor plate and basal plate, respectively, and were oriented parallel to the ventral pial surface. From E16.5, TH-IR neurons of the *A9* complex displayed their characteristic “inverted fountain” pattern (Hanaway et al., 1971; Kawano et al., 1995). This arrangement was even more striking in embryos of older stages (Fig. 9*B*).

Strikingly, in Pax6^{sey}/Pax6^{sey} embryos, from E16.5, defects in the topography of *A9* neurons were accentuated at the p1–p2 border, and in p2, *A9* did not show its characteristic inverted fountain organization (Fig. 9, compare *F* and *B*). On sagittal sections, TH-IR neurons accumulated abnormally at the p1–p2 border in Pax6^{sey}/Pax6^{sey} embryos (Fig. 9, compare *C* with *G* and *D* with *H*). Taken together, these results suggest defects in the migration of TH-IR *A9* and *A10* neurons in the mutant.

TH-IR fiber pathway alterations in Pax6^{sey}/Pax6^{sey} embryos

In wild-type embryos, by E11.5, nigrostriatal and mesocortical fibers originating from *A9* and *A10* followed the pathway of the medial forebrain bundle (*mfb*) in mes, p1, p2, p3, and p4 basal plate. By E14.5, nigrostriatal fibers terminated in the lateral portion of the caudate-putamen (Fig. 10*A*), whereas mesocortical fibers continued rostrally to reach the prefrontal cortex and the striatum by E15.5. In addition, a few TH-IR fibers originating from *A10* were observed running along the fasciculus retroflexus toward the epithalamus (Skagerberg et al., 1984). By E18.5, the caudate-putamen and the globus pallidus were homogeneously labeled, and a denser band of terminals was visible under the external capsule (Fig. 10*K*). Mesocortical fibers emerged from *mfb* and entered the olfactory tubercle or ramified into the ventral lateral part of the nucleus accumbens (Fig. 10*K*). The remaining mesocortical TH-IR fibers turned dorsally to enter the medial, prefrontal, and anterior cingulate cortices (Verney et al., 1982; Voorn et al., 1988; present study).

In Pax6^{sey}/Pax6^{sey} embryos, by E11.5, most TH-IR fibers did not follow the pathway of *mfb*. Fibers were misguided along the presumptive p1–p2 transition zone where they followed a straight ventrodorsal course (Fig. 10*B,C*). A few fibers originating from the more rostral and ventromedial neurons of the *A9–A10* complex followed the pathway of the presumptive *mfb* (Fig. 10*D*), although they only reached p4 by E12.5. By E14.5, misguided TH-IR fibers looped in the roof of p2, plunged mediolaterally after the presump-

Figure 8. Delay and diminution in the number of A16 neurons in the anterior olfactory nucleus and the residual olfactory structure of Pax6^{sey}/Pax6^{sey} embryos. Sagittal (*A–C*) and coronal (*D–H*) sections are shown for E14.5 (*A–C*, *E–G*) and E16.5 (*D*, *H*) wild-type (*A–D*) and Pax6^{sey}/Pax6^{sey} (*E–H*) embryos. Alternate sections are immunostained for calbindin (*A*, *E*), calretinin (*B*, *F*), or TH (*C*, *F*). *A*, Calbindin immunoreactivity labels short axon cells of the olfactory bulb. *B*, Calretinin immunoreactivity labels mitral and tufted cells of the olfactory bulb. *C*, Arrows indicate neurons of A16 in the anterior olfactory bulb in A16AON. *D*, Section showing TH-IR external tufted cells in the olfactory bulb of E16.5 wild-type embryo. *E*, Calbindin immunoreactivity strongly labels cells that may correspond to short axon cells. *F*, Calretinin immunoreactivity strongly labels cells that may correspond to the mitral and tufted cells of the remaining olfactory bulb. *G*, TH-IR neurons are absent in the remaining olfactory structure of E14.5 Pax6^{sey}/Pax6^{sey} embryo. *E–G*, Arrow points to the residual olfactory structure. *H*, High magnification shows scattered TH-IR neurons with short processes (arrows) in the residual olfactory bulb of E16.5 Pax6^{sey}/Pax6^{sey} embryo. Scale bar: *A–C*, *E–G*, 2 mm; *D*, *H*, 1 mm.

tive p2–p3 border, and turned rostroventrally in p3 basal plate to reach and follow the pathway of the presumptive mfb in p4 and p5 (Fig. 10*E–J*). In addition, few TH-IR fibers looped rostrally in presumptive p2 alar plate (Fig. 10*E*, *G*). In their ascending and descending courses, TH-IR fibers appeared abnormally highly fasciculated (Fig. 10*H*). At the presumptive level of the internal capsule and the optic tract, TH-IR fibers swirled just before they entered the caudate putamen (Fig. 7*D*). From E18.5, at least some TH-IR fibers reached the same rostral levels as observed in wild-type embryos, although the number of terminals was greatly reduced, particularly in the olfactory tubercle (Fig. 10*L*).

General fiber pathway alterations in Pax6^{sey}/Pax6^{sey} embryos

Using the neuronal cell adhesion molecules NCAM and L1 as general markers of most axonal pathways, we analyzed whether alterations of TH-IR axons in the presumptive diencephalon were a selective defect of catecholaminergic fibers or a general defect of all ascending and descending fibers.

NCAM (from E11.5 to E13.5) and L1 (from E14.5) immunoreactivities revealed most of the fiber pathways traveling in the diencephalon. In wild-type embryos, the posterior, pretectal, and tectal commissures, the fasciculus retroflexus, and the stria medullaris were labeled with NCAM (Fig. 11*B*) or L1 (Fig. 11*E*). In addition, the zona limitans intrathalamica (Zli) at the p2–p3 boundary displayed NCAM immunoreactivity from E11.5 to E13.5 (Fig. 11*B*).

In Pax6^{sey}/Pax6^{sey} embryos, fibers traveling medially followed a normal trajectory in the basal plate of the rhombencephalon, mes, p1, p2, and p3, whereas fibers located laterally in basal plate and alar plate were misguided at the presumptive p1–p2 transition zone and in p2 alar plate (Fig. 11*D*, *F*). In p2, most of the L1 immunoreactive fibers were highly fasciculated into several straight and parallel bundles (Fig. 11*F*). In the roof of p2, most fibers looped and descended at the presumptive p2–p3 limit.

Altered expression of the chemorepellent/chemoattractive molecule Netrin-1 in Pax6^{sey}/Pax6^{sey} embryos

In Pax6^{sey}/Pax6^{sey} embryos, from E11.5, the developmental expression of Netrin-1 was roughly normal in the rhombencephalic and mesencephalic floor plate, along the floor of the fourth ventricle and along the wall of the lateral ventricle (Fig. 12). From E13.5, Netrin-1 was normally expressed in the striatum and from E14.5 in the vicinity of the SN–VTA complex (Livesey and Hunt, 1997) (Fig. 12*A*, *C*). However, an abnormally high and expanded expression of Netrin-1 was observed from the presumptive p1–p2 transition zone to the p2–p3 transition zone. Instead of being expressed in the ventral part of the diencephalic basal plate and in the Zli (Fig. 12*C*), Netrin-1 expression was expanded in all of the basal plate and the most ventral part of the presumptive alar plate (Fig. 12*D*). This altered Netrin-1 expression persisted and was correlated with increased and expanded expression of SHH reported previously (Grindley et al., 1997) (data not shown). The comparison of the pattern of Netrin-1 expression with TH-IR immunoreactivity (compare Figs. 12*D* and 10*E*, *H*) showed that TH-IR fibers and neurons seemed orientated abnormally toward the increased and ectopic Netrin-1 expression located at the pretectal–dorsal thalamic transition zone.

DISCUSSION

Our results in normal mice are in good agreement with previous comparative analyses on catecholaminergic systems in sauropsids (Medina et al., 1994) and humans (Puelles and Verney, 1998). The main TH-IR neurons clearly arise independently along the whole brain axis. Table 1 shows the resulting topological map of these groups. This mosaic pattern strongly suggests that this phenotype is generated by the combinatorial effects of regionally expressed transcription factors, such as Pax6, and diffusible morphogens such as SHH or FGF8. Differences between groups of TH-IR neurons may be caused by differences in the factors they express and the signals they receive.

It has been suggested that Pax6 could be a good candidate for controlling the proliferation, specification, or maintenance of discrete CA populations (Stoykova and Gruss, 1994; Dellovade et al., 1998). Our study indicates that discrete CA populations in the diencephalon, the hypothalamus, and the basal telencephalon express Pax6, either permanently or transiently. By analyzing mice lacking Pax6, we show that Pax6 is not necessary for the specification and time of generation of diencephalic and hypothalamic DA neurons but is needed for the normal packing and segregation of these cells. The lack of Pax6 leads also to a virtual absence of

Figure 9. Developmental defects of the SN–VTA complex in Pax6^{se/y}/Pax6^{se/y} embryos. *A*, Sagittal section showing the developing SN–VTA complex of E12.5 wild-type embryo. Arrowheads indicate the radially oriented TH-IR neurons from *mes* to *p3*. Note that TH-IR neurons of A10 in *p3* (A10*p3*) display a less intense immunoreactivity. *B*, Coronal section of E18.5 wild-type embryo showing the characteristic topographical inverted fountain-like organization of the SN–VTA complex. *C*, *D*, Medial (*C*) and lateral (*D*) sagittal sections of E18.5 wild-type embryo showing the organization of the SN–VTA complex. *E*, Sagittal section of the developing SN–VTA complex in E12.5 Pax6^{se/y}/Pax6^{se/y} embryo showing the abnormal topographical organization of TH-IR neurons in *p1* and *p2* (small arrowheads). Small arrows point to A10*p3*; large arrowheads point to radially migrating TH-IR neurons. *F*, The coronal section shows the abnormal topographical organization of the SN–VTA complex in an E18.5 Pax6^{se/y}/Pax6^{se/y} embryo. *B*, *F*, Curved arrows emphasize the topographical organization of dopaminergic neurons of the SN and the main direction of their neuropils. *G*, *H*, Medial (*G*) and lateral (*H*) sagittal sections of E18.5 Pax6^{se/y}/Pax6^{se/y} embryo. The arrows point to TH-IR neurons abnormally located along the *p1*–*p2* border and in *p2*. *C*, *D*, The black star indicates the lack of TH-IR neurons in wild-type embryo at the corresponding location where ectopic TH-IR neurons are seen in the mutant. Scale bar: *A*, *E*, 2 mm; *B*, *F*, 1 mm; *C*, *D*, *G*, *H*, 0.5 mm.

TH-IR neurons in the basal telencephalon. We also describe non-cell autonomous defects among DA neurons of the SN–VTA complex: some are abnormally located, and the medial forebrain bundle, the major ascending pathway of DA neurons, is misrouted.

Dopaminergic populations expressing Pax6

Diencephalic and hypothalamic TH-IR neurons: cell adhesion defect

The neuroepithelium of prosomeres 3, 4, and 5 expresses Pax6 during the time of genesis of diencephalic and hypothalamic TH-IR neurons. Our study indicates that differentiated TH-IR neurons of A13 and A14PAVH continue to express Pax6 until the first postnatal days, whereas A14DMH does not. In mice lacking Pax6, these populations differentiate but display a 1–2 d delay in their appearance. Because previous studies have shown abnormally low proliferative rates in the entire diencephalic alar plate of mice lacking Pax6 (Warren and Price, 1997), we looked for a delay in the genesis of these TH-IR groups. Our results clearly indicate no significant delay in genesis in A13 and A14DMH and no reduction in cell number of TH-IR neurons in A13, A14PAVH, and A14DMH.

In mice lacking Pax6, A13, A14PAVH, and A14DMH, TH-IR neurons display an increase in cell density, suggesting altered adhesive properties. Previous studies have suggested that Pax6 regulates the expression of adhesion molecules (Stoykova et al., 1997; Meech et al., 1999). In mice lacking Pax6, there is a loss of

R-cadherin expression in areas in which this gene is normally coexpressed with Pax6. Moreover, it has been shown that the segregation normally observed in aggregates of cortical and striatal cells in an *in vitro* assay is lost in mice lacking Pax6 (Stoykova et al., 1997). This could be explained by a model in which loss of Pax6 disrupts the adhesive mechanisms involving R-cadherin, thereby increasing cell mixing and leading to some of the morphological disruptions observed. Interestingly, TH-IR neurons in A13, A14PAVH, and A14DMH do not display a particular scattering or increased cell mixing, as might be expected, but paradoxically they are more densely packed in roundish cell clusters. We suggest that the selective loss of some adhesion molecules (such as R-cadherins) may alter the balance between heterophilic and homophilic interactions in such a way that some cells may have reduced ability to adhere to other types of cells and may have a tendency to adhere more strongly to cells of their own type.

Telencephalic populations: cell migration/maintenance defect

In the olfactory bulb, Pax6 is expressed from E15.5 in TH-IR external tufted cells and from E18.5 in TH-IR periglomerular interneurons. In mice, external tufted cells are born between E13 and E18 (Hinds, 1968a,b) and proliferate in the ventricular zone of the olfactory bulb. In mice lacking Pax6, we observe rare TH-IR neurons in the region of the olfactory structure, and their onset of TH expression and morphology correspond to those expected for external tufted cells. This suggests that Pax6 is important for the

Figure 10. Alterations of TH-IR fibers pathway in Pax6^{scy}/Pax6^{scy} embryos. Sagittal (A–J) and coronal (K, L) sections are shown for wild-type (A, K) and Pax6^{scy}/Pax6^{scy} (B–J, L) embryos. Embryos were sectioned at E11.5 (B–D), E14.5 (A, E–J), and E18.5 (K, L). Large arrows indicate the direction of the fibers. A, Large arrow indicates the direction of the medial forebrain bundle (mfb), the major fiber pathway originating from TH-IR neurons of the SN–VTA complex. B, The sagittal section shows early alterations of TH-IR fiber pathway. C, D, Small arrowheads point to growth cones. C, Higher magnification of area outlined in B, showing that most of the TH-IR fibers are abnormally deflected dorsally after the presumptive pretectal–dorsal thalamic boundary. D, Higher magnification of area outlined in B shows that some TH-IR fibers are not deflected dorsally. E, A lateral section shows a high number of fibers from the SN–VTA complex misguided in the diencephalic alar plate (arrow). F, A mediolateral section of Pax6^{scy}/Pax6^{scy} embryos indicating neurons of the SN and their projections. Arrow indicates some TH-IR neurons of the SN that are not misguided. G, A medial section shows TH-IR fibers looping in the roof of the diencephalon (arrow). H, I, J, Higher magnifications of E, G, and F, respectively. H, Reconstruction of TH-IR fiber pathway. K, The coronal section shows the main projecting areas of TH-IR fibers, the striatum (ST), the nucleus accumbens (ACB), and the olfactory tubercle (OT). L, TH-IR fibers terminated normally in the striatum and the nucleus accumbens of Pax6^{scy}/Pax6^{scy} embryo. The black star indicates a lack of terminals in the olfactory tubercle. Scale bar: A, E–G, K, J, 4 mm; B, 2 mm; C, H–J, 1 mm; D, 0.5 mm.

specification of external tufted cells in the olfactory bulb. Proliferation defects may account for the low number of external tufted cells. Alternatively, because mice lacking Pax6 fail to develop a nasal olfactory epithelium, this dramatic reduction could be attributable to the lack of induction by primary olfactory afferents (McLean and Shipley, 1988; Baker and Farbman, 1993; Cigola et al., 1998).

In contrast to external tufted cells, periglomerular cells are born from E18 and arise along the anterior subventricular zone (Hinds, 1968a,b; Betarbet et al., 1996). In mice lacking Pax6, no TH-IR periglomerular interneurons are observed in late embryos or neonatal pups. Interestingly, Pax6^{scy}/+ heterozygote mice display a dramatic and specific decrease of TH-IR periglomerular interneurons, whereas external tufted cells are preserved. This reduction has been correlated to a progressive diminution in primary afferents (Dellovade et al., 1998).

Pax6 is strongly and transiently expressed in all TH-IR neurons of the piriform cortex, olfactory tubercle, and anterior amygdala. Recently, it has been suggested that cells populating these structures may be derived in part from a transient structure, the intermediate telencephalic territory (ITA), located at the transition zone between the neocortex and the lateral ganglionic eminence. Pax6 is expressed (from E12.5 to E14.5) in both proliferating cells and cells located near or in migrating neurons of the lateral cortical migratory stream derived from ITA (our unpublished results). When cells reach their targets, most of them express Pax6 during the formation of the different structures of the basal telencephalon. In mice lacking Pax6, ITA is dramatically altered: radial glial fascicles do not form at the cortical–ganglionic eminence transition zone and the expression of R-cadherin and the extracellular matrix molecule tenascin-C is lost (Stoykova et al., 1997). Interestingly, cellular migration in the lateral cortical migratory stream occurs in

Pax6^{scy}/Pax6^{scy} embryos, although cells fail to stop in their final locations in the basal telencephalon and continue to migrate to the pial surface of the brain (Brunjes et al., 1998). We observe that the transient TH-IR neurons of the piriform cortex, the anterior amygdala, and the olfactory tubercle are decreased in number and fail to maintain TH expression in Pax6^{scy}/Pax6^{scy} embryos. We suggest that the absence of TH immunoreactivity in these cells may be because of the failure of TH induction or maintenance of TH expression in these migrating neurons that do not recognize a “stop signal” in the basal telencephalon.

Defects in catecholaminergic populations not expressing Pax6

Although TH-IR neurons of the SN–VTA complex never display Pax6 immunoreactivity, we show in mice lacking Pax6 an abnormal location of TH-IR neurons and an altered pathway of catecholaminergic fibers along the pretectal–dorsal thalamic transition zone and in the alar plate of the dorsal thalamus. The abnormal location of TH-IR neurons might be caused by either an ectopic genesis induced by altered expression of morphogenetic molecules or an altered migratory behavior induced by changes in the navigational environment.

Ectopic genesis of TH-IR neurons

Pax6^{scy}/Pax6^{scy} mice display an early abnormal ventral to dorsal expansion of the signaling secreted morphogen SHH and the SHH-induced gene, the winged helix transcription factor hepatocyte nuclear factor 3 β (HNF-3 β) at the level of the pretectal–dorsal thalamic transition zone and in the alar plate of the dorsal thalamus (Grindley et al., 1997) (our unpublished observation).

Figure 11. Alterations of specific fiber pathways in Pax6^{sc}/Pax6^{sc} embryos. Sagittal sections are shown for E12.5 wild-type (*A*, *B*) and Pax6^{sc}/Pax6^{sc} (*C*, *D*) embryos. Nissl-stained sections (*A*, *C*) are shown in parallel with sections immunoreacted for NCAM (*B*, *D*). *A*, *C*, The sagittal section shows constrictions and regions of low cell densities associated with prosomeric boundaries. *A*, *C*, Arrows indicate, from caudal to rostral, the mes–p1 boundary and the p1–p2 boundary. *B*, NCAM immunoreactivity reveals ascending and descending fibers of the posterior commissure (*pc*), the fasciculus retroflexus (*fr*), the stria medullaris (*sm*), and thalamic axons. A cellular labeling also reveals the zona limitans intrathalamica (*zli*). *D*, A remaining *pc* is distinguishable in p1 but most of the fiber tracts are misguided in the diencephalon (*white arrow-head*). *E*, *F*, Sagittal sections immunoreacted for L1 are shown for E18.5 (*E*), wild-type, and (*F*) Pax6^{sc}/Pax6^{sc} embryos. *E*, The sagittal section shows the posterior commissure (*pc*) in the caudal part of the pretectum and the fasciculus retroflexus (*fr*) at the pretectal–dorsal thalamic transition zone. *F*, The sagittal section shows aberrant fiber pathways in the pretectal and dorsal thalamic alar plate (between the arrows). Note that fibers traveling in the lower part of the basal plate and in the floor plate maintain a normal trajectory. Scale bar: *A*–*D*, 4 mm; *E*, *F*, 8 mm.

There is evidence that HNF-3 β , SHH, and FGF8 create induction sites for TH-IR neurons along the dorsoventral axis (Sasaki and Hogan, 1994; Hynes et al., 1995; Wang et al., 1995; Ye et al., 1998). It is possible that the early ectopic SHH and HNF-3 β expression domains in Pax6^{sc}/Pax6^{sc} mice induce ectopic catecholaminergic neurons.

Changes in navigational environment

Clearly a complex set of attractive and repulsive guidance molecules is provided in the environment. Ectopic expression of SHH or FGF8 could induce an ectopic expression of guidance cues, leading to the misrouting of growth cones or defects in cellular

Figure 12. Alteration of Netrin-1 expression in the diencephalon of Pax6^{sc}/Pax6^{sc} embryos. Coronal (*A*, *C*) and sagittal (*B*, *D*) sections of E14.5 (*B*, *D*) and E16.5 (*A*, *C*) wild-type (*A*, *B*) and Pax6^{sc}/Pax6^{sc} (*C*, *D*) embryos. *A*, In the mesencephalon, Netrin-1 is expressed at the level of the SN–VTA complex. *B*, The sagittal section shows Netrin-1 expression in the floor of the fourth ventricle and in the basal plate of p1, p2, and p3. Note also a weak expression along the zona limitans intrathalamica (*Zli*). *C*, The coronal section shows a normal Netrin-1 expression at the level of the SN–VTA complex. *D*, In the diencephalon, Netrin-1 expression is increased and expanded dorsally. Arrows indicate the dorsal expansion in the ventral and dorsal thalamic alar plates. *mes*, Mesencephalon. Scale bar: *A*, *C*, 4 mm; *B*, *D*, 2 mm.

migration. For instance, it has been shown recently that ectopic expression of Hedgehog molecules induces ectopic Netrin-1 expression in the CNS of zebrafish embryos (Lauderdale et al., 1998). In mice lacking Pax6, the early ventral to dorsal expansion of SHH (Grindley et al., 1997) coincides with the ventral to dorsal expansion of Netrin-1. Netrin-1 is a laminin-related secreted protein with critical roles in axon guidance (Leonardo et al., 1997) and cell migration (Przyborski et al., 1998; Bloch-Gallego et al., 1999) that induces either attractive or repulsive responses, depending on the netrin receptor expressed. Normally, TH-IR neurons of the SN–VTA complex migrate in two phases: first, radially along tenascin-bearing glial processes, and second, tangentially, giving the SN its characteristic inverted fountain shape (Hanaway et al., 1971; Kawano et al., 1995). In Pax6^{sey}/Pax6^{sey} embryos, the radial migration of SN–VTA neurons occurs normally (our unpublished results). Later, the rostral pretectal SN–VTA neurons are disoriented at the pretectal–dorsal thalamic transition zone near the expansion of Netrin-1 expression, and ectopic TH-IR neurons in the dorsal thalamus are disoriented and appear to be migrating away from the expanded Netrin-1 expression.

Nearly all SN–VTA projections are also misrouted at the pretectal–dorsal thalamic transition zone. Instead of following the medial forebrain bundle ventrally, SN–VTA projections are deflected rostrorodorsally away from the expanded Netrin-1 expression. Taken together, we speculate that Netrin-1 has a chemorepellant activity on both the tangential migration and the pathfinding of SN–VTA neurons.

In conclusion, our study indicates that Pax6 is directly or indirectly involved in the adhesion and migration of discrete catecholaminergic populations and the maintenance of their phenotype. Second, Pax6 has a primordial role in determining the correct navigational environment for early diencephalic axonal pathfinding.

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