Published in final edited form as:

J Comp Neurol. 2004 November 22; 479(4): 399–409.

Pax6 Guides a Relay of Pioneer Longitudinal Axons in the Embryonic Mouse Forebrain

HIKMET F. NURAL and GRANT S. MASTICK*

Biochemistry Graduate Program, and Biology Department, University of Nevada, Reno, Nevada 89557

Abstract

We have characterized a system of early neurons that establish the first two major longitudinal tracts in the embryonic mouse forebrain. Axon tracers and antibody labels were used to map the axon projections in the thalamus from embryonic days 9.0–12, revealing several distinct neuron populations that contributed to the first tracts. Each of the early axon populations first grew independently, pioneering a short segment of new tract. However, each axon population soon merged with other axons to form one of only two shared longitudinal tracts, both descending: the tract of the postoptic commissure (TPOC), and, in parallel, the stria medullaris. Thus, the forebrain longitudinal tracts are pioneered by a relay of axons, with distinct axon populations pioneering successive segments of these pathways. The extensive merging of tracts suggests that axon–axon interactions are a major guidance mechanism for longitudinal axons. Several axon populations express tyrosine hydroxylase, identifying the TPOC as a major pathway for forebrain dopaminergic projections. To start a genetic analysis of pioneer axon guidance, we have identified the transcription factor Pax6 as critical for tract formation. In Pax6 mutants, both longitudinal tracts failed to form due to errors by every population of early longitudinal axons. Taken together, these results have identified potentially important interactions between series of pioneer axons and the Pax6 gene as a general regulator of longitudinal tract formation in the forebrain.

Indexing terms

Pax6; transcription factor; tyrosine hydroxylase; dopaminergic; axon guidance; knockout; embryo; thalamus; A13; zona incerta; supraoptic tract; stria medullaris

Distinct sets of projection neurons differentiate in numerous locations throughout the brain but then project their axons into a small number of common pathways. For example, in early vertebrate embryos, all longitudinal axon projections merge to form major longitudinal tracts with specific dorsal–ventral positions (Chitnis and Kuwada, 1990; Easter et al., 1994; Mastick and Easter, 1996). This raises the issue of how diverse populations of axons are guided along the same pathways.

Axons that develop later tend to join preexisting tracts rather than forming new ones (Cornel and Holt, 1992). In this way, early sets of axons may form axon scaffolds that later growing ones follow (Raper et al., 1983; Easter and Taylor, 1989; Chitnis and Kuwada, 1990; Wilson et al., 1990; Easter et al., 1994). The first neurons to grow along a pathway could be considered “pioneers” that establish a tract. Without other axons to follow, pioneers use molecular cues to navigate through the brain. In contrast, later growing axons may either follow the pioneers through selective fasciculation or independently navigate using the same cues as the pioneers. As evidence for the importance of pioneers, in several experiments, ablation of pioneer axons

*Correspondence to: Grant Mastick, University of Nevada, Biology/200, Reno, NV 89557. E-mail: gmastick@unr.edu.

or tracts results in misprojection or stalling of follower axons (Raper et al., 1984; Klose and Bentley, 1989; Chitnis and Kuwada, 1991; Pike et al., 1992; Cornel and Holt, 1992). However, there are counter-examples in which ablation of pioneers does not disrupt the pathfinding of later axons. For example, retinal axons can project throughout the forebrain if they are forced to navigate early, before the usual pioneers have developed (Cornel and Holt, 1992). Such a result suggests that both pioneers and followers can navigate along the same pathway using a common set of cues. Thus, a general regulator might be needed to establish initial axon tracts as well as to guide follower axons.

Although guidance cues for the early scaffold of pioneer axons are largely unknown, the transcription factor Pax6 has been implicated as a key regulator of forebrain axon guidance. Pax6 is expressed early in the developing dorsal forebrain, including the dorsal diencephalon, preceding major longitudinal tracts that form in this region (Walther and Gruss, 1991; Mastick et al., 1997). For example, the axons that make the tract of the postoptic commissure (TPOC) have a stereotypical projection pattern, with the pathway coinciding with Pax6 expression domains in ventral thalamus (VT). Moreover, in Pax6 mutants, TPOC axons make dramatic errors in VT and fail to pioneer this first tract (Mastick et al., 1997). Other tracts that project through VT several days later in development are also affected in Pax6 mutants. For example, thalamocortical and corticothalamic connections and mamillothalamic tracts fail to form in Pax6 mutants (Kawano et al., 1999; Pratt et al., 2000; Jones et al., 2002). The tyrosine hydroxylase-expressing (TH+) axons ascending from substantia nigra (SN) also fail to project through thalamus in Pax6 mutant mice (Vitalis et al., 2000). These similar axon defects suggest that Pax6 is a key common factor for longitudinal axon pathfinding in the forebrain.

In this study, we investigated longitudinal tract formation in the embryonic thalamus, using axon tracing to reveal a detailed map of the earliest set of longitudinal tracts. We have identified two early longitudinal tracts through the forebrain and identified specific groups of axons that contribute to those tracts. Furthermore, we investigated the role of Pax6 in the guidance of these axon systems by identifying axon errors in Pax6 mutant embryos. These findings suggest potentially important interactions between longitudinal axon populations and, furthermore, that the Pax6 gene is a general regulator of longitudinal tract formation in the forebrain.

MATERIAL AND METHODS

Mice and embryo collection

CD-1 mice were used to collect wild-type embryos. Pax6 mutant embryos were obtained from crosses between *Pax6* heterozygous mice (the *Sey-neu* allele) in an FVB background, with FVB wild-type embryos used as controls. All protocols using mice had prior approval of the Institutional Animal Care and Use Committee of the University of Nevada, Reno, in compliance with the Society for Neuroscience and Public Health Services policies on humane care and use of animals. Embryos were obtained by timed-mating, with noon of the day of the vaginal plug designated as embryonic day (E) 0.5. Pregnant females were killed by using a CO₂ chamber, and embryos were dissected out of uteri in ice-cold 0.1 M PO₄. Embryos were fixed in 4% paraformaldehyde (PFA) in 0.1 M PO₄ and stored at 4°C.

Axon tracing

Axon tracing was carried out using the lipophilic fluorescent tracers DiI and DiO (Molecular Probes). Small dye crystals were inserted into the wall of the neural tube, using a fine tungsten needle under a dissecting microscope, followed by incubation overnight at 37°C. After removal of the skin and extra mesenchyme, embryos were bisected sagittally and mounted for microscopy in 50% glycerol/50% 4% PFA in 0.1 M PO₄ under a coverslip using coverslip fragments as spacers.

For double labeling procedures, DiI fluorescence was photoconverted to a stable product by using diaminobenzidine (DAB; McConnell et al., 1989; von Bartheld et al., 1990; Mastick and Andrews, 2001). DiI-labeled embryos were washed several times with 0.1 M PO_4 for a total of 1 hour then incubated for 30 minutes in a 0.6 mg/ml DAB solution in 0.1 M PO_4 . For photoconversion, embryos were placed under 20 $\times$ objectives with fluorescence illumination for 10–20 minutes to convert the fluorescent label to a yellow–brown DAB precipitate. Embryos were washed several times with 0.1 M PO_4 then embedded in gelatin and sectioned for antibody labeling as described in Mastick et. al. 2001.

Immunohistochemistry

For whole-mount immunohistochemistry, embryos were collected and fixed either in 2% PFA for 1 hour at room temperature for tyrosine hydroxylase (TH) antibody, or in 4% PFA overnight for β III-tubulin and Pax6 antibody labeling. Brains of embryos were bisected, and the skin and most of the mesenchyme was dissected away. Before incubation with primary antibody solution, the embryos were blocked either with slacker solution (1 $\times$ phosphate buffered saline/1% Triton/10% fetal calf serum) for β III-tubulin (Babco, 1:1,000 dilution) and TH (Chemicon 1:200 dilution) antibodies or with 4% milk TST (4% powdered milk dissolved in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Tween) for Pax6 antibody for several hours (1:1,000; Mastick and Andrews, 2001). For primary and secondary antibody dilutions and all washes, the above blocking solutions were used. Embryos were incubated with primary antibody solution for 2–3 days at 4°C with slow rotation. After rinsing with the above blocking solutions overnight, Cy3-conjugated secondary antibody (Jackson ImmunoResearch, 1:200 dilution) was applied for 2 days at 4°C with rotation. After rinsing the embryos for several hours, they were mounted under coverslips for examination under fluorescent microscope.

Antibody labeling with Pax6 (1:1,000) and TH (1:200) antibodies on sections were performed as previously described (Mastick and Andrews, 2001). The images were collected with a Leica DMR fluorescence microscope, using a Leica LEI-750 CCD video camera system and assembled into figures in Adobe Photoshop.

The percentage of Pax6-expressing supraoptic tract (SOT) neurons was calculated by dividing the number of photoconverted SOT cell bodies labeled with Pax6 by the number of photoconverted SOT cell bodies. The number of embryos used for each experiment was higher than five, unless otherwise indicated.

RESULTS

Overall patterns of axon growth through the Pax6+ ventral thalamus

For an overall view of early patterns of differentiating neurons and axon projections in the diencephalon, whole embryos were antibody labeled for neurons and for Pax6. To reveal neurons, antibody labeling for neuron-specific β III-tubulin was performed at several developmental stages (Fig. 1A–C). For this study, we focused on tracts forming in ventral thalamus and dorsal thalamus (also referred to as prethalamus and thalamus, respectively; Puelles and Rubenstein, 2003). Although the axons were too crowded to resolve individual tracts with β III-tubulin labeling, these labels revealed the overall pattern of tracts in forebrain. On E9.5, neurons and fibers were labeled around optic stalk and in ventral thalamus (VT). Neurons (Fig. 1A, white arrow) at the base of the optic stalk formed the source of TPOC (Easter et al., 1993). These axons extended around the optic stalk, then project in a bundle through the diencephalon/VT. One other heavily labeled area in E9.5 forebrain was centered in the VT, bordered both ventrally and dorsally by regions with lower densities of neurons and fibers (Fig. 1A, arrow). Neurons in VT, called A13 dopaminergic neurons, extended fibers toward dorsal thalamus (DT; Vitalis et al., 2000; Mastick and Andrews, 2001). As the embryo

develops to E10.5, TPOC and A13 axons formed the main longitudinal bundle in forebrain (Fig. 1B). Around E11.5, the density of neurons and axons increased dramatically (Fig. 1C). However, from the antibody labeling of whole-mounts, it was not possible to identify projection patterns of individual axon populations. Therefore, to determine the relationship between the TPOC axons and the later patterns of neurons, detailed analysis of tracts performed by using more specific axon tracing.

To compare the relationship between Pax6 expression and longitudinal tract formation, we performed whole-mount antibody labeling of Pax6. The longitudinal axon populations overlapped with Pax6 expression in forebrain (Fig. 1D). At E10.5, Pax6 was expressed in VT and DT as well as other forebrain regions (optic vesicle, cerebral vesicle, pretectum), consistent with previous expression studies (Walther and Gruss, 1991; Mastick et al., 1997). However, the interthalamic boundary, visible as a morphological groove under brightfield microscopy, had significantly lower Pax6 expression (Mastick et al., 1997; Fig. 1D). The expression of Pax6 in DT decreased by E11.5. Thus, the path of the axons suggests that Pax6 may function in the guidance of these axons.

These labels show that a major longitudinal axon pathway projects through the Pax6⁺ ventral thalamus. In the light of these findings, this study aims to investigate how individual tracts in the forebrain longitudinal pathway are formed and to identify common features in their guidance.

Time course of TPOC pioneer axon growth through the thalamus

TPOC originates from a cluster of neurons located at the base of the optic stalk on embryonic day 9.5 (Easter et al., 1993). TPOC axons project into VT, DT, and eventually into midbrain (Mastick and Easter, 1996). Because the TPOC is very early in development, we carried out a detailed analysis of its projection pattern as an example of longitudinal tract formation in the forebrain.

To investigate the time course of TPOC axon growth, they were labeled from their source by the application of DiI crystals to the base of optic stalk at several developmental stages. At 30 somites (approximately E10), the leading TPOC axons passed the optic stalk and headed into the VT. At 32 somites, the number of fibers along the tract increased, with the longest reaching across the VT/DT boundary. Axons initially projected as a bundle around optic stalk, but by the 32-somite stage, the tract widened as axons fanned out across the surface of the VT. At 34 somites, many more axons crossed the boundary and projected toward the midbrain. Thus, the TPOC axons form one of the initial longitudinal tracts through the forebrain by early E10.5, suggesting a pioneering role for the TPOC axons.

A13 TH⁺ axons precede TPOC axons into dorsal thalamus

Along the VT pathway of the TPOC, there are Pax6-expressing neuron cell bodies (Mastick et al., 1997; Andrews et al., 2003). Previous studies showed that these neurons are fated to become the A13 dopaminergic neurons of the medial zona incerta. On the basis of the early appearance of the A13 neurons (Fig. 1) and their position within the TPOC pathway, we performed antibody labeling and axon tracing experiments to determine the spatial and temporal relationship between their axons and the TPOC.

To identify the axon projection patterns of the A13 neurons, DiI label sites were placed at several positions (dorsal, ventral, anterior, and posterior) relative to the position of the A13 neurons. Only label sites in DT labeled A13 neurons, suggesting that these neurons project caudally, that is, from VT into DT, the same direction as the TPOC axons.

To confirm the identity of these back-labeled neurons, A13 neurons were retrogradely labeled with DiI from dorsal thalamus, then the DiI fluorescence was photoconverted to allow sectioning and antibody labeling. Sections through the A13 neurons were labeled with Pax6 antibody (Fig. 3E). Pax6 was expressed in the retrogradely labeled neurons (Fig. 3E, arrows), confirming their identity.

In addition to Pax6, A13 neurons also express tyrosine hydroxylase (TH; Vitalis et al., 2000; Mastick and Andrews, 2001; Fig. 3F). Because TH is a cytoplasmic label for axons, we used TH whole-mount antibody labeling on E10.5 to directly identify the projections of these axons. The TH whole-mount antibody labeling showed that A13 axons project dorsal thalamus, agreeing with the retrograde labeling of A13 neurons with DiI (Fig. 3A,B). However, we were not able to tell if all retrogradely labeled A13 neurons were TH+, because the cytoplasmic TH labeling would be obscured with DiI labeling.

Many A13 neurons coexpress Pax6 and TH; however, the two markers did not overlap completely. To the posterior (near the VT/DT boundary), almost all TH+ neurons expressed Pax6, but more anteriorly, most of the neurons expressed only TH, although the crowded TH cytoplasmic labeling prevented accurate quantification of coexpression. The mixed expression of Pax6 and TH in early-born neurons in this region was also observed by previous studies (Vitalis et al., 2000; Mastick and Andrews, 2001). The heterogeneity might reflect a subset of early differentiating Pax6+ neurons that have not yet activated TH or vice versa.

To directly examine the relationship between the A13 axons and the TPOC, we performed double labeling of A13 neurons and TPOC with the axon tracers DiO and DiI. This labeling showed that A13 axons project in close contact with TPOC axons, sharing the same pathway (Fig. 3C). To compare the timing of A13 and TPOC axon projections through DT, a time course of A13 axon projections was made by back-labeling with DiI from DT. When the time course of A13 axons was compared with TPOC axons, it was found that A13 axons reach dorsal thalamus before TPOC axons. At 30 somites, A13 axons already crossed into DT while the leading TPOC axons were still near the optic stalk, just starting to enter VT (Figs. 2B, 3A). Thus, the descending longitudinal tract through VT is pioneered by A13 neurons, followed soon after by TPOC axons. The relationship between these axon patterns suggests a relay of axons, with distinct groups of axons pioneering successive segments of the pathway.

TPOC and A13 axons make errors in Pax6 mutants

TPOC axons make pathfinding errors in Pax6 mutants, with errors in VT, consistent with the Pax6 expression in this region (Mastick et al., 1997). Figure 4D shows the DiI labeled TPOC axons in an E11.5 mutant embryo. The initial tight part of the tract had normal growth. The tract widened when it entered to VT as in wild-type, but the mutant axons formed many loops instead of directed caudal growth. The growth of some of the axons was arrested when they encounter the VT. Some axons projected dorsally into the cerebral vesicle. However, the most profound defect in guidance was a failure of the axons to cross the VT/DT boundary, and instead, they turned dorsally when they reached the boundary.

A13 neurons are located in a Pax6 expression domain and they express Pax6 themselves. They share the same pathway with TPOC axons. Because TPOC has defects in Pax6 mutants, this finding raised the possibility that A13 axons may make guidance errors similar to the TPOC. As a test, A13 axons and their cell bodies were back-labeled from DT in mutant embryos. Very few cell bodies were labeled compared with wild-type, suggesting a failure of A13 axons to project into DT (data not shown). To determine where A13 axons project in Pax6 mutants, we performed DiI labeling at several locations. When crystals were inserted anterior to the VT/DT boundary, in the dorsal most area of VT, many neurons were back labeled in the normal location of the A13 neurons (Fig. 4C). This finding suggested that A13 axons projected dorsally

rather than crossing into DT. To examine this directly, TH antibody labeling was performed to label A13 axons (Fig. 4A,B). In wild-type, the TH⁺ A13 axons projected into DT and coursed through midbrain. In Pax6 mutants, all of the A13 axons turned dorsally at the VT/DT boundary, despite the heterogeneity in Pax6 and TH expression noted above. Thus, A13 axons and TPOC make similar errors in the absence of Pax6; suggesting common guidance mechanisms are involved in their pathfinding.

Dopaminergic neurons of substantia nigra and ventral tegmental area complex (SN-VTA) are also affected in Pax6 mutants (Vitalis et al., 2000). TH antibody labeling showed that, instead of ascending through VT (Fig. 4A), axons of the SN-VTA complex projected along the DT/pretectum boundary, avoiding DT (Fig. 4B), consistent with the axon patterns previously identified in sections of E12 embryos (Vitalis et al., 2000). It has been suggested that, in Pax6 mutants, SN-VTA neurons appear less numerous because of delayed expression of TH (Vitalis et al., 2000). This mechanism is a possible explanation for why fewer A13 fibers were labeled with TH in Pax6 mutants compared with wild-type embryos (data not shown). These results suggest that defects in these dopaminergic projections might be caused by alterations in DT, because SN-VTA axons fail to ascend into DT, whereas A13 axons fail to descend into DT.

SOT axons project along the TPOC pathway and make errors in Pax6 mutants

To identify other axon populations in forebrain that may contribute to the TPOC, we examined fibers originating from the supraoptic region, because neuron antibody labels of E11.5 whole-mounts showed fibers originating in this region dorsal to the optic stalk. By analogy to zebrafish, these axons constitute the SOT, and in fish, project from neurons in the supraoptic region, caudally past the optic stalk, and merge with the TPOC, projecting both posteriorly and anteriorly (Chitnis and Kuwada, 1990; Ross et al., 1992). To map the mouse SOT axons and their relationship to the TPOC, detailed mapping of SOT was performed using axon tracers. SOT cell bodies are located between the optic vesicle and cerebral vesicle. Insertion of DiI crystals to a narrow canal between eye and cerebral vesicle labeled the whole tract. Two time points, E11.5 and E12.5, were chosen to show initial and later projection pattern of the SOT axons.

In mouse, SOT axons started to project at E11.5 coursed first ventrally toward the TPOC pathway (Fig. 5A,C), similar to the zebrafish pattern. Most of the axons turned caudally and merged with the TPOC (Fig. 5B), although a few of them turned rostrally. The descending SOT axons formed two branches (Fig. 5D). The ventral branch joins the TPOC, whereas the dorsal branch projected close to the cerebral vesicle/thalamus boundary (hemispheric sulcus). Based on the location of these dorsal descending axons, we identify these fibers as contributing to the stria medullaris thalamica (SM), which is the first dorsal longitudinal tract in the forebrain (L. Puelles, personal communication; Fortuyn, 1912; Jacobowitz, 1998). Double labeling with TPOC axons showed that the ventral branch overlaps with TPOC axons (Fig. 5B). Thus, the SOT axons start to project after the main longitudinal tract formed in forebrain. The ventral branch joins to this tract, whereas the dorsal branch contributes to the SM, the second descending longitudinal tract.

Because the SOT follows the TPOC, we tested whether SOT axons may make similar errors in Pax6 mutants. To identify the projection patterns of the SOT in Pax6 mutants, we labeled the SOT in mutant embryos with DiI. In Pax6 mutants, both SOT branches projected normally into VT, but when they encounter the VT/DT boundary, they made pathfinding errors (Fig. 5E). Axons in the dorsal branch turned dorsally at the VT/DT boundary instead of projecting into DT. Most of the axons in the ventral branch also turned dorsally at the boundary. However, a few of them entered DT, although they formed loops instead of projecting into the midbrain (Fig. 5E). Thus, most of the SOT axons made errors similar to the TPOC, with a failure to grow into or through the DT.

To examine whether SOT neurons express Pax6, we retrogradely DiI labeled SOT cell bodies, photoconverted the fluorescence and performed Pax6 antibody labeling on sections (Fig. 5F). Only 16% of the retrograde-labeled SOT cell bodies were Pax6-positive. Because most or all of the SOT axons made errors, the Pax6 effect may be mediated by Pax6 function in thalamus, i.e., a non-cell-autonomous effect on SOT axon growth.

Taken together, we found that the SOT axons start to project later in development, join TPOC and SM, and make pathfinding errors in Pax6 mutants similar to the TPOC and A13 axons. This finding suggests that SOT axons may require guidance from the earlier pioneer axons, or the SOT may independently follow the same cues as these earlier axons.

Projection patterns of SM and TZL axons in wild-type and Pax6 mutant embryos

After the TPOC is established, neurogenesis begins in the dorsal sector of VT and DT (Fig. 1C). To identify the projection patterns of these neurons, we performed DiI labeling to the dorsal aspect of the VT, close to the inter-thalamic boundary (Fig. 5H). Two other populations of axons projecting in dorsal forebrain were labeled. The first axon population, had cell bodies located just anterior to the DV/VT boundary, close to the hemispheric sulcus. Axons originating from these neurons projected ventrally along the interthalamic boundary, forming the tract of zona limitans (TZL; Puelles et al., 1992) and then turned caudally in DT to merge and descend with the TPOC. A second population of axons projected dorsal and parallel to TPOC pathway, with cell bodies located near the border of VT to cerebral vesicle, dorsal to the A13 neurons (Fig. 5I). We term these SM axons, due to their projection into the SM, with their projections roughly simultaneous to the dorsal branch of SOT axons. The TZL and SM axons appeared around E11.5, and application of DiI failed to label these axon populations at earlier time points (data not shown), consistent with the lack of neuron antibody labeling (Fig. 1A,B). TZL axons follow the preestablished TPOC, while SM axons form a longitudinal tract parallel to the TPOC.

To examine the projection patterns of TZL and SM axons in Pax6 mutants, we performed DiI labeling (Fig. 5J). Both of the tracts failed to label in Pax6 mutant embryos. This might be the result of abnormal projection of TZL and SM axons. To identify more specific defects, we carried out DiI applications in several locations. Whether crystals were inserted anterior (Fig. 5K) or posterior (data not shown) to the dorsal aspect of the DT/VT boundary, neither of the tracts were labeled, suggesting failure or delays in axon projections, defects more severe than the TPOC. Thus, Pax6 is required for projections along other longitudinal tracts, in addition to the TPOC.

DISCUSSION

This work begins to define the formation of two major longitudinal axon pathways in the developing forebrain, in both wild-type and Pax6 mutant embryos. We have identified two descending tracts, and several specific populations of axons that contribute to these tracts. Moreover, we have found Pax6 as a key regulator in longitudinal axon guidance in the forebrain, since all of the tracts identified in this study have guidance defects in the absence of Pax6.

The axon populations identified in this study are summarized in Figure 6. Several features stand out. First, each population of axons comes from a group of neurons located in a distinct region. Each group of neurons also has distinct genetic identities. For instance, A13 neurons express Pax6 and TH, along with Isl1 and Lim1 (Mastick and Andrews, 2001), while the TPOC neurons do not express any of these markers but instead expresses R-cadherin (Andrews and Mastick, 2003). Second, although the axons originate from diverse regions of the forebrain, their trajectories merge to form only two longitudinal tracts (at least up to E12). TPOC, the

first longitudinal tract, consists of TPOC axons originating from base of the optic stalk, A13 axons originating from neurons actually lying along the pathway of the TPOC, and later in development, the majority of the SOT axons also join the TPOC. The SM forms as a second, more dorsal, longitudinal tract, apparently pioneered by axons originating from supraoptic neurons. The SOT axons first form the SOT posterior to the optic stalk. Subsequently, a branch of SOT axons contributes to the SM. SOT axons make choices, in that some of them follow the TPOC pathway while other diverges to follow the SM pathway. Axons extending from the dorsal aspect of VT project into TZL initially then join to TPOC. Thus, distinct sets of axons chose to follow a relatively small number of longitudinal pathways through forebrain, establishing two major longitudinal tracts. Interestingly, all of the axon populations identified in this study, aside from the SN-VTA axons from ventral midbrain, are descending, suggesting a general descending tendency for pioneer axons.

Axons follow common pathways, meaning that later growing axons join preexisting tracts rather than establishing new ones (present study; Cornel and Holt, 1992; Easter et al., 1994). The first axons to grow out are called pioneers. Pioneer axons may lay down pathways for later growing axons. Previous studies showed that ablation of pioneers may result in misprojection or arrest of the growth of followers. In grasshopper, a specific set of pioneer neurons are needed for later axons to make a proper turn en route (Bastiani et al., 1984; Jacobs and Goodman, 1989). In fish central nervous system, a dorsal spinal cord pathway has abnormal projection in the absence of pioneering axons. However, in related experiments, there are results contradicting these pioneer–follower relationships. Retinal axons follow the pathway of TPOC, but they do not need TPOC axons for their guidance to their target (Cornel and Holt, 1992). These results indicate that followers need pioneers for guidance, at least in some cases. Future experiments are required to identify whether pioneer–follower relationships play a role in the formation of forebrain longitudinal tracts.

A potential strategy for establishing a longitudinal tract is to have one set of dedicated pioneer axons, projecting early enough to pioneer the entire pathway. Instead, we observe here that a relay of axons cooperates to link projections along a common pathway. This is a relay in the sense that several groups along the pathway pioneer different segments of the pathway. The apparent relay pattern suggests that specific pioneer–follower relationships may be important for longitudinal axon guidance. For instance, A13 axons are the first axons to cross the VT/DT boundary. These axons may be needed for later growing populations such as the TPOC and SOT to project into DT. On the other hand, the TPOC may be needed in the initial segments of the pathway guiding axons through VT. Therefore, the major longitudinal pathway is pioneered by relay of axons, with distinct groups pioneering successive segments of the pathway.

These forebrain tracts are potentially a good system to test pioneer–follower relationships. The tracts analyzed in this study have relatively simple projection patterns. They have similar defects in Pax6 mutants, suggesting shared guidance mechanisms. Manipulations in pioneer tracts such as TPOC may reveal a role of these tracts in the guidance of followers.

Another common feature of these tracts is that they are located in the alar plate. Previous studies identified that the TPOC axons respect the alar/basal boundary, not crossing it (Mastick and Easter, 1996). Other tracts joining the TPOC pathway respect the alar boundary as well. Moreover, ventral tracts located in the basal plate, such as mamillotegmental tract, do not join these dorsal pathways (Mastick and Easter, 1996). Thus, the alar/basal boundary may be analogous to the ventral and dorsal midlines in acting as a barrier to specific axon populations. This suggests regional, alar plate-specific cues or guidance mechanisms. Pax6 is needed to guide all descending forebrain axons.

Axons make errors in Pax6 mutants

All of the early forebrain axon populations make pathfinding errors in Pax6 mutant embryos (Fig. 6B). The common feature of these is that they normally project through the Pax6⁺ VT. In Pax6 mutants, we have observed two major errors in the development of these tracts. First, the majority of axons turn dorsally at the VT/DT boundary, instead of projecting into DT. Second, some axons arrest in growth. The first discovered defect in forebrain longitudinal axons was the TPOC (Mastick et al., 1997). TPOC axons fail to project into DT, instead they make a dorsal turn at the DT/VT boundary. When we observed the other early forebrain axon populations, we discovered similar errors in pathfinding. A13 axons and SOT, although they have normal initial development, do not project into the DT but turn dorsally at the boundary. TZL and SM also have defects in mutants, which might be more profound than for other tracts. Aside from these two examples, it is striking that the initial segments of axon trajectories appear normal but extensive defects start in VT. Thus, Pax6 is not required for all aspects of pathfinding. These results suggest a requirement for Pax6, with severe guidance defects in several aspects of the axon trajectories.

Several possibilities can explain the axon errors in Pax6 mutants. Pax6 is expressed in the neuroepithelia in the VT and DT, as well as in subpopulations of neurons in these regions. This suggests that Pax6 may function on any of multiple levels to regulate axon guidance. Pax6 is involved in differentiation and patterning (Stoykova et al., 1996). The location of the axon errors would be consistent with failure of normal differentiation of VT, DT, and/or the interthalamic boundary tissue. Recent studies in thalamocortical axons in Pax6 mutants revealed that normal differentiation of DT is required for proper growth of these axons (Pratt et al., 2000). Another possibility is that Pax6 might regulate molecular cues required for axon guidance. The loss of Pax6 could result in the altered attractant or repellent cues. For example, loss of attractant(s) from DT may be one reason that axons cannot project into DT. Further investigations are needed to address these possibilities.

Changes in molecular cues under regulation of Pax6 may cause defects

Recent studies showed that Pax6 regulates expression of the cell adhesion molecule R-cadherin (Andrews and Mastick, 2003). R-cadherin expression overlaps with Pax6 expression in VT. In the absence of Pax6, R-cadherin is missing from VT, thus correlating with the longitudinal axon errors. The role of R-cadherin in longitudinal axon guidance has been tested extensively for the TPOC (Andrews and Mastick, 2003). Both in vitro and in vivo experiments showed that R-cadherin promotes growth of TPOC axons. Restoration of R-cadherin into Pax6 mutants, by means of electroporation and whole mouse embryo culture, rescued defects in TPOC axon outgrowth, suggesting that R-cadherin provides a growth-promoting substrate in forebrain. Together, this evidence suggests that Pax6 influences axon growth through the regulation of R-cadherin in this segment of the pathway. Pax6-regulation of region-specific adhesive properties suggests a general mechanism to translate regional patterning information into axon projection patterns.

Alternatively, Pax6 may affect axon guidance by regulating the responsiveness of axons to molecular cues, through a cell-autonomous function of Pax6 in neurons. A subpopulation of A13 neurons and SOT neurons express Pax6. In the absence of Pax6, gene expression pattern in these neurons could be altered, setting up an internal cause for pathfinding errors. For instance, *Lim1* is expressed in wild-type but not Pax6 mutant A13 neurons (Mastick and Andrews, 2001). Because *Lim1* has been implicated in regulating axon guidance in motor neurons (Kania et al., 2000), Pax6 activation of *Lim1* may be critical for A13 axon projections. This possibility suggests that misspecification of neurons may cause axon projection errors.

Pax6 is emerging as a broad regulator of longitudinal axon guidance in the forebrain. In forebrain, there are other examples of Pax6 function in axon guidance. Thalamocortical (TC) axons, which grow later in development, also have guidance defects in Pax6 mutants. In wild-type, the TC tract projects from DT, transiting VT to destinations in the cerebral cortex (Braisted et al., 1999; Tuttle et al., 1999; Auladell et al., 2000; Pratt et al., 2000). In Pax6 mutant mice, the TC axons project through DT but become arrested in VT and fail to reach the cortex. Explant experiments suggest that Pax6 in DT is required for thalamocortical tract formation (Pratt et al., 2000). The reciprocal corticofugal axonal projections from cortex to thalamus are also dependent on Pax6 (Jones et al., 2002). The SN axon population is another example of a disrupted pathway in Pax6 mutants (Vitalis et al., 2000). Instead of ascending through VT, mutant SN axons project into the DT alar plate. So far, Pax6 is required for all of the longitudinal axons examined in the thalamus, suggesting a key role for this gene in establishing tracts in the forebrain.

Other guidance cues, including several secreted guidance proteins, have been implicated in longitudinal tract formation in the forebrain. For example, the Slit family of cues is required for the proper development of several tracts in the forebrain such as corticofugal, thalamocortical, and callosal fiber tracts, as well as the initiation of the optic tract (Bagri et al., 2002; Plump et al., 2002). Netrin-1 is another guidance molecule for forebrain longitudinal tracts, with functions for optic tract and thalamocortical projections (Deiner and Sretavan, 1999). In Pax6 mutants, Netrin expression is abnormally high and dorsally expanded, suggesting regulation by Pax6 (Vitalis et al., 2000). Analysis of these guidance cues along with Pax6 may elucidate general mechanisms of longitudinal axon guidance in the developing brain.

Acknowledgements

We thank Dr. Luis Puelles and Dr. Steven Easter for their helpful suggestions on characterization of axon populations and tracts; Gracie Andrews for advice on antibody labeling and photoconversion techniques; Todd Farmer for his contributions in developing whole-mount antibody labeling protocols; Amy Altick for her useful suggestions about experiments and comments on the article.

Grant sponsor: National Institutes of Health; Grant number: HD38069 (to G.S.M.); Grant sponsor: University of Nevada (to H.F.M.).

LITERATURE CITED

- Andrews GL, Mastick GS. R-cadherin is a Pax6-regulated, growth-promoting cue for pioneer axons. *J Neurosci* 2003;23:9873–9880. [PubMed: 14586016]
- Andrews GL, Yun K, Rubenstein JL, Mastick GS. Dlx transcription factors regulate differentiation of dopaminergic neurons of the ventral thalamus. *Mol Cell Neurosci* 2003;23:107–120. [PubMed: 12799141]
- Auladell C, Perez-Sust P, Super H, Soriano E. The early development of thalamocortical and corticothalamic projections in the mouse. *Anat Embryol (Berl)* 2000;201:169–179. [PubMed: 10664178]
- Bagri A, Marin O, Plump AS, Mak J, Pleasure SJ, Rubenstein JL, Tessier-Lavigne M. Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. *Neuron* 2002;33:233–248. [PubMed: 11804571]
- Bastiani MJ, Raper JA, Goodman CS. Pathfinding by neuronal growth cones in grasshopper embryos. III. Selective affinity of the G growth cone for the P cells within the A/P fascicle. *J Neurosci* 1984;4:2311–2328. [PubMed: 6481449]
- Braisted JE, Tuttle R, O'Leary DD. Thalamocortical axons are influenced by chemorepellent and chemoattractant activities localized to decision points along their path. *Dev Biol* 1999;208:430–440. [PubMed: 10191056]
- Chitnis AB, Kuwada JY. Axonogenesis in the brain of zebrafish embryos. *J Neurosci* 1990;10:1892–1905. [PubMed: 2355256]

- Chitnis AB, Kuwada JY. Elimination of a brain tract increases errors in pathfinding by follower growth cones in the zebrafish embryo. *Neuron* 1991;7:277–285. [PubMed: 1873031]
- Cornel E, Holt C. Precocious pathfinding: retinal axons can navigate in an axonless brain. *Neuron* 1992;9:1001–1011. [PubMed: 1281416]
- Deiner MS, Sretavan DW. Altered midline axon pathways and ectopic neurons in the developing hypothalamus of netrin-1- and DCC-deficient mice. *J Neurosci* 1999;19:9900–9912. [PubMed: 10559399]
- Easter SS Jr, Taylor JS. The development of the *Xenopus* retinofugal pathway: optic fibers join a pre-existing tract. *Development* 1989;107:553–573. [PubMed: 2612377]
- Easter SS Jr, Ross LS, Frankfurter A. Initial tract formation in the mouse brain. *J Neurosci* 1993;13:285–299. [PubMed: 8423474]
- Easter SS Jr, Burrill J, Marcus RC, Ross LS, Taylor JS, Wilson SW. Initial tract formation in the vertebrate brain. *Prog Brain Res* 1994;102:79–93. [PubMed: 7800834]
- Fortuyn D. Die Ontogenie der Kerne des Zwischenhirns beim Kaninchen. *Arch Anat Physiol Anat Abt* 1912;18:303–352.
- Jacobowitz, D. Chemoarchitectonic atlas of the developing mouse brain. Boca Raton, FL: CRS Press; 1998.
- Jacobs JR, Goodman CS. Embryonic development of axon pathways in the *Drosophila* CNS. II. Behavior of pioneer growth cones. *J Neurosci* 1989;9:2412–2422. [PubMed: 2545837]
- Jones L, Lopez-Bendito G, Gruss P, Stoykova A, Molnar Z. Pax6 is required for the normal development of the forebrain axonal connections. *Development* 2002;129:5041–5052. [PubMed: 12397112]
- Kania A, Johnson RL, Jessell TM. Coordinate roles for LIM homeobox genes in directing the dorsoventral trajectory of motor axons in the vertebrate limb. *Cell* 2000;102:161–173. [PubMed: 10943837]
- Kawano H, Fukuda T, Kubo K, Horie M, Uyemura K, Takeuchi K, Osumi N, Eto K, Kawamura K. Pax-6 is required for thalamocortical pathway formation in fetal rats. *J Comp Neurol* 1999;408:147–160. [PubMed: 10333267]
- Klose M, Bentley D. Transient pioneer neurons are essential for formation of an embryonic peripheral nerve. *Science* 1989;245:982–984. [PubMed: 2772651]
- Mastick GS, Andrews GL. Pax6 regulates the identity of embryonic diencephalic neurons. *Mol Cell Neurosci* 2001;17:190–207. [PubMed: 11161479]
- Mastick GS, Easter SS Jr. Initial organization of neurons and tracts in the embryonic mouse fore- and midbrain. *Dev Biol* 1996;173:79–94. [PubMed: 8575640]
- Mastick GS, Davis NM, Andrew GL, Easter SS Jr. Pax-6 functions in boundary formation and axon guidance in the embryonic mouse forebrain. *Development* 1997;124:1985–1997. [PubMed: 9169845]
- McConnell SK, Ghosh A, Shatz CJ. Subplate neurons pioneer the first axon pathway from the cerebral cortex. *Science* 1989;245:978–982. [PubMed: 2475909]
- Pike SH, Melancon EF, Eisen JS. Pathfinding by zebrafish motoneurons in the absence of normal pioneer axons. *Development* 1992;114:825–831. [PubMed: 1618146]
- Plump AS, Erskine L, Sabatier C, Brose K, Epstein CJ, Goodman CS, Mason CA, Tessier-Lavigne M. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. *Neuron* 2002;33:219–232. [PubMed: 11804570]
- Pratt T, Vitalis T, Warren N, Edgar JM, Mason JO, Price DJ. A role for Pax6 in the normal development of dorsal thalamus and its cortical connections. *Development* 2000;127:5167–5178. [PubMed: 11060242]
- Puelles L, Rubenstein JL. Forebrain gene expression domains and the evolving prosomeric model. *Trends Neurosci* 2003;26:469–476. [PubMed: 12948657]
- Puelles L, Sanchez MP, Spreafico R, Fairen A. Prenatal development of calbindin immunoreactivity in the dorsal thalamus of the rat. *Neuroscience* 1992;46:135–147. [PubMed: 1594097]
- Raper JA, Bastiani MJ, Goodman CS. Guidance of neuronal growth cones: selective fasciculation in the grasshopper embryo. *Cold Spring Harb Symp Quant Biol* 1983;48(Pt 2):587–598. [PubMed: 6586377]

- Raper JA, Bastiani MJ, Goodman CS. Pathfinding by neuronal growth cones in grasshopper embryos. IV. The effects of ablating the A and P axons upon the behavior of the G growth cone. *J Neurosci* 1984;4:2329–2345. [PubMed: 6481450]
- Ross LS, Parrett T, Easter SS Jr. Axonogenesis and morphogenesis in the embryonic zebrafish brain. *J Neurosci* 1992;12:467–482. [PubMed: 1371313]
- Stoykova A, Fritsch R, Walther C, Gruss P. Forebrain patterning defects in Small eye mutant mice. *Development* 1996;122:3453–3465. [PubMed: 8951061]
- Tuttle R, Nakagawa Y, Johnson JE, O'Leary DD. Defects in thalamocortical axon pathfinding correlate with altered cell domains in Mash-1-deficient mice. *Development* 1999;126:1903–1916. [PubMed: 10101124]
- Vitalis T, Cases O, Engelkamp D, Verney C, Price DJ. Defect of tyrosine hydroxylase-immunoreactive neurons in the brains of mice lacking the transcription factor Pax6. *J Neurosci* 2000;20:6501–6516. [PubMed: 10964956]
- von Bartheld CS, Cunningham DE, Rubel EW. Neuronal tracing with DiI: decalcification, cryosectioning, and photoconversion for light and electron microscopic analysis. *J Histochem Cytochem* 1990;38:725–733. [PubMed: 2185313]
- Walther C, Gruss P. Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development* 1991;113:1435–1449. [PubMed: 1687460]
- Wilson SW, Ross LS, Parrett T, Easter SS Jr. The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. *Development* 1990;108:121–145. [PubMed: 2351059]

Abbreviations

CF	cephalic flexure
CV	cerebral vesicle
DT	dorsal thalamus
M	midbrain
OP	optic stalk
SM	stria medullaris
SN	substantia nigra
SOT	supraoptic tract
TH	tyrosine hydroxylase
TPOC	tract of the postoptic commissure
TZL	tract of the zona limitans

VT

ventral thalamus

VTA

ventral tegmental area

Fig. 1.

Early longitudinal axons project through the Pax6⁺ thalamic region. Wild-type whole-mount embryos labeled with β -III tubulin (A–C) or Pax6 antibody (D). **A:** Two main clusters of neurons and fibers were labeled in anterior forebrain at embryonic day (E) 9.5. TPOC (white arrow) and A13 neurons (open arrow) appear very early in forebrain development. **B:** By E10.5, increased numbers of TPOC and A13 axons project through VT. **C:** Many more axons and neurons were labeled by E11.5, with the labeling becoming too crowded to resolve individual tracts. However, a new tract (SM) formed at the border between CV and VT (arrowhead), and axons project from the supraoptic region can be seen. **D:** Pax6 antibody labeling shows

expression of Pax6 in forebrain at E10.5. Pax6 expression overlaps with TPOC in VT. Dashed lines, DT/VT boundary. For abbreviations, see list. Scale bar = 200 μm in D (applies to A–D).

Fig. 2.

Time course of TPOC axon growth through the forebrain. **A:** Schematic representation of TPOC projection pattern. The gray line represents axons; the circle indicates cell bodies. **B–D:** TPOC neurons are located at the base of the optic stalk, and TPOC axons were labeled with DiI in embryos at different developmental stages. **B:** At 30 somites (som), the leading TPOC axons were in the VT. **C:** At 32 somites, the first TPOC axons entered DT. The majority of the axons were close to the VT/DT boundary but had not crossed yet. **D:** At 34 somites, more axons crossed the boundary, with the longest projecting into midbrain. Dashed lines, DT/VT boundary; asterisks, location of DiI crystals. For abbreviations, see list. Scale bar = 200 μ m in D (applies to B–D).

Fig. 3.

A13 axons precede TPOC axons across the interthalamic boundary. **A:** Cell bodies of A13 axons retrogradely labeled with DiI from DT. Inset shows the location of DiI crystal (red dot) and angle of section (red dashed line) for E and F. **B,C:** TH antibody labeling showed that TH⁺ cell bodies were distributed over VT (arrowhead in B), whereas the source of TPOC was TH⁻ (B); the longest TH⁺ projections reached DT by the 30-somite (som) stage (C). **D:** Anterograde labeling of TPOC with DiI (red) combined with retrograde labeling of A13 axons with DiO (green). A13 neurons were located in the TPOC pathway; thus, these two axon populations share the TPOC tract. **E:** Section. A13 neurons retrogradely labeled with DiI and photoconverted (gray), then labeled with Pax6 antibody. Arrows indicate Pax6 expression in A13 neurons. **F:** A subset of the A13 neurons were double labeled with Pax6 and TH ($n = 2$; arrows). Dashed lines, interthalamic boundary; asterisks, location of DiI and DiO crystals. For abbreviations, see list. Scale bars = 50 μm in C (applies to A,C), 100 μm in B, 150 μm in D, 10 μm in E,F.

Fig. 4.

A13 and TPOC axons make errors in Pax6 mutants. **A,B:** Whole-mount antibody labeling against TH revealed projection pattern of A13 axons and SN in wild-type (+/+) and Pax6 mutant (-/-) embryos. **A:** A13 axons projected through DT in wild-type (embryonic day [E] 10.5). SN-VTA complex, located in midbrain floor plate, extended their axons rostrally through VT (A). **B:** In Pax6 mutants, A13 axons did not project into DT but instead turned dorsally at the interthalamic boundary. SN-VTA axons failed to project into DT. **C:** DiI labeling from dorsal region of VT, anterior to interthalamic boundary. Inset shows location of the label site. Cell bodies of A13 axons retrogradely labeled from this dorsal location confirmed that A13 axons did not project into DT but rather turned dorsally at the boundary. **D:** Errors in guidance of TPOC axons are shown by DiI labeling. Similar to A13 axons, TPOC axons failed to cross the inter-thalamic boundary and instead turned dorsally along it. Some of the TPOC axons projected dorsally into CV. Dashed lines, DT/VT boundary. Scale bar = 400 μm in D (applies to A-D).

Fig. 5.

Supraoptic tract (SOT) axons project along TPOC pathway and make errors in Pax6 mutants.

A: Location of crystals and schematic representation of SOT. The blue line indicates the angle of section in F. **B,C:** SOT axons started to project at embryonic day (E) 11.5, coursed ventrally and merged with the TPOC. DiI labeling of SOT at E11.5 (B) and E12.5 (C) shows the projection pattern of SOT. SOT axons formed two branches. The ventral branch joined TPOC and the dorsal branch projected close to the cerebral vesicle–thalamus boundary, merging with SM. **D:** Labeling of SOT with DiO (green) and TPOC with DiI (red). SOT axons and TPOC axons had an overlapping projection pattern. **E:** Double labeling of SOT cell bodies with DiI (brown) and Pax6 (green; n = 2). A subpopulation of SOT neurons expressed Pax6 (white and green arrowheads), whereas some photo-converted SOT neurons did not express Pax6 (white arrowheads) and some Pax6⁺ cells were not back-labeled with DiI (green arrowhead). **F:** DiI label of SOT axons in Pax6 mutant embryo. The majority of SOT axons turned dorsally at the boundary, although a few axons projected into DT and formed loops. Dorsal tracts, including the stria medullaris (SM) and tract of the zona limitans (TZL) are disrupted in Pax6 mutants. **G:** Schematic representation of projection pattern TZL (blue) and SM (pink). **H:** Anterograde labeling of TZL and retrograde labeling of SM with DiI from dorsal aspect of VT anterior to interthalamic boundary. TZL projected along interthalamic boundary and turned caudally into

DT, while SM projected through DT parallel to thalamus and CV boundary. **I:** Labeling of TPOC with DiI (red) and SM and TZL with DiO (green). **J:** Higher magnification of boxed area in B. TZL axons merged with TPOC in DT. **K:** DiI labeling from dorsal region of VT in Pax6 mutants. Both TZL and SM failed to be labeled, but A13 neurons were back-labeled. Dashed lines, interthalamic boundary; asterisks, location of DiI and DiO crystals. For abbreviations, see list. Scale bars = 500 μm in B (applies to B–D,F), 10 μm in E, 500 μm in J, 500 μm in H (applies to H,I,K).

Fig. 6.

Pax6 is a common regulator of several distinct axon populations in the forebrain. **A,B:** Schematic map of tracts and their sources in the forebrain of wild-type (A) and Pax6 mutant (B) embryos. The gray area represent tracts, and colored lines indicate different populations of axons projecting along those tracts. For abbreviations, see list.