Development 129, 761-773 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV9814

Patterning of the basal telencephalon and hypothalamus is essential for guidance of cortical projections

Oscar Marín[1], Joshua Baker[1], Luis Puelles[2] and John L. R. Rubenstein[1]

[1]Department of Psychiatry, Nina Ireland Laboratory of Developmental Neurobiology, Langley Porter Psychiatric Institute, University of California, San Francisco, USA
[2]Departament of Morphological Sciences, School of Medicine, University of Murcia, Spain
*Author for correspondence (e-mail: jlrr@cgl.ucsf.edu)

Accepted 1 November 2001

SUMMARY

We have investigated the mechanisms that control the guidance of corticofugal projections as they extend along different subdivisions of the forebrain. To this aim, we analyzed the development of cortical projections in mice that lack *Nkx2-1*, a homeobox gene whose expression is restricted to two domains within the forebrain: the basal telencephalon and the hypothalamus. Molecular respecification of the basal telencephalon and hypothalamus in *Nkx2-1*-deficient mice causes a severe defect in the guidance of layer 5 cortical projections and ascending fibers of the cerebral peduncle. These axon tracts take an abnormal path when coursing through both the basal telencephalon and hypothalamus. By contrast, loss of *Nkx2-1* function does not impair guidance of corticothalamic or thalamocortical axons. In vitro experiments demonstrate that the basal telencephalon and the hypothalamus contain an activity that repels the growth of cortical axons, suggesting that loss of this activity is the cause of the defects observed in *Nkx2-1* mutants. Furthermore, analysis of the expression of candidate molecules in the basal telencephalon and hypothalamus of *Nkx2-1* mutants suggests that *Slit2* contributes to this activity.

Key words: *Nkx2-1*, Axon guidance, Transcription factor, Patterning, Telencephalon, Cortex, Corticofugal projection, Corticothalamic projection, Thalamocortical projection, Pyramidal tract, Cerebral peduncle, Medial ganglionic eminence, *Slit*, *Robo*, Mouse

INTRODUCTION

Axon growth and pathfinding is governed by the expression of guidance molecules in stereotypic patterns during development (Tessier-Lavigne and Goodman, 1996). Understanding the mechanisms that control wiring of the brain therefore not only requires the identification of guidance molecules and the ways they influence axon pathfinding, but also the factors that ensure the precise expression of these cues in the developing brain. In that respect, it has been suggested that the patterned expression of axon guidance molecules may be controlled by distinct sets of regulatory genes whose expression is defined by specific morphogenetic cues during a particular window of time (Wilson et al., 1993; Wilson et al., 1997). In agreement with this notion, recent studies have provided evidence supporting the role of several transcription factors in governing the patterned expression of axon guidance molecules in the developing brain and spinal cord of vertebrates (Logan et al., 1996; Shigetani et al., 1997; Ba-Charvet et al., 1998; Lauderdale et al., 1998; Matise et al., 1999; Tuttle et al., 1999).

The neocortex (isocortex) is the origin of two of the major axon tracts in the mammalian forebrain: (1) the corticothalamic projection, which originates primarily from neurons located in layer 6; and (2) the pyramidal tract, which originates from neurons in layer 5 (Jones, 1984). This latter tract comprises axons that course through the cerebral peduncle and innervate multiple subcortical targets, such as the superior colliculus, pons and spinal cord. The neocortex, on the other hand, receives most of its afferent projections from neurons of the dorsal thalamus, via the thalamocortical pathway. Because these major axonal pathways are essential for the complex integration of sensory-motor information that underlies vertebrate behaviors, understanding the mechanisms that control the development of these connections is central to the study of brain development.

Previous studies have identified pioneering axonal populations and specific cell domains that are candidates for guiding corticothalamic connections (de Carlos and O'Leary, 1992; Mitrofanis and Baker, 1993; Molnár and Blakemore, 1995; Métin and Godement, 1996; Molnár et al., 1998; Braisted et al., 1999; Molnár and Cordery, 1999; Tuttle et al., 1999; Auladell et al., 2000). By contrast, little is known about the cues that govern the guidance of major axon tracts in the cerebral peduncle as they course through the forebrain, although several molecules have been identified that cooperate in the final arrangement of corticospinal axons (Dahme et al., 1997; Cohen et al., 1998; Dottori et al., 1998; Castellani et al., 2000; Kullander et al., 2001a; Kullander et al., 2001b; Leighton et al., 2001; Yokoyama et al., 2001).

One forebrain region that appears to play a major role in the

guidance of corticofugal and thalamocortical axons is the subpallium. Neuronal populations located in the subpallium send pioneering projections to the cortex and thalamus, and it has been suggested that these axons may form a scaffold system that guides corticofugal and thalamocortical axons, respectively (Métin and Godement, 1996; Molnár et al., 1998; Braisted et al., 1999; Tuttle et al., 1999). Moreover, several guidance molecules expressed in the ventral telencephalon have been shown to affect the development of corticothalamic and thalamocortical projections (Métin et al., 1997; Richards et al., 1997; Bagnard et al., 1998; Polleux et al., 1998; Braisted et al., 2000).

Nkx2-1 (*Titf1* – Mouse Genome Informatics, MGI), a homeobox-containing gene member of the vertebrate Nkx family, is expressed in proliferative and postmitotic zones of the forebrain that give rise to a large region of the subpallium and to the hypothalamus (Price et al., 1992; Shimamura et al., 1995; Kohtz et al., 1998; Sussel et al., 1999). Homozygous mice containing a targeted disruption of the *Nkx2-1* gene die at birth and have severe morphological abnormalities in the subpallium and hypothalamus (Kimura et al., 1996; Takuma et al., 1998; Sussel et al., 1999). In the telencephalon, loss of *Nkx2-1* function causes a molecular transformation of the medial ganglionic eminence (MGE), which instead of producing its normal derivatives (e.g. globus pallidus), gives rise to LGE-like derivatives (e.g. striatum) (Sussel et al., 1999). It is conceivable that the abnormal patterning of the basal telencephalon in *Nkx2-1*-deficient mice may cause a change in the balance of guidance cues normally expressed in this region and, consequently, corresponding defects in the guidance of cortical projections. Thus, the selective nature of the basal telencephalon in *Nkx2-1* mutants provides a unique opportunity to examine the requirement of this region in providing cues that influence axon guidance.

We present evidence that, in *Nkx2-1* mutants, layer 5 cortical axons follow an abnormal course as they exit the basal telencephalon and most of them are unable to reach the cerebral peduncle. By contrast, guidance of corticothalamic and thalamocortical axons is largely normal in the absence of *Nkx2-1* function, indicating that axonal projections arising from different cortical layers are guided through at least partially independent mechanisms. In addition, when some pyramidal tract axons leave the telencephalon in *Nkx2-1* mutants, they follow an aberrant course towards the chiasmatic region, indicating that the region connecting the telencephalon and the diencephalon also constitutes a crucial decision point in the pathway of pyramidal tract axons. In vitro experiments suggest that the basal telencephalon and the rostrobasal hypothalamus contain an activity that inhibits the growth of corticofugal axons. Analysis of the expression of candidate guidance molecules in *Nkx2-1* mutants suggests that *Slit2* appears to be at least a component of this repulsive activity.

MATERIALS AND METHODS

Animals

A mouse mutant strain with a null allele of *Nkx2-1* (a gift from S. Kimura) was used in this study. This mouse strain was maintained by backcrossing to C57BL/J6 mice for more than ten generations. In addition, green fluorescent protein (GFP)-expressing transgenic mice maintained in a CD1 background (a gift of A. Nagy) were used for explant cultures experiments. For staging of embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5). PCR was performed as described by Sussel et al. (Sussel et al., 1999) to genotype offspring resulting from *Nkx2-1* heterozygous matings. Mouse colonies were maintained at UCSF in accordance with NIH and UCSF guidelines.

Immunohistochemistry

Embryos were obtained by dissection of pregnant mice at various stages of development. Embryos younger that E15.5 were anesthetized by cooling and fixed overnight in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Embryos were then cryoprotected in 30% sucrose in PBS, embedded in OCT compound, and cut frozen in the transverse or sagittal plane on a cryostat at 10 μm. E18.5 embryos were anesthetized by cooling and perfused with 4% PFA. Brains were removed and postfixed for 3 hours, cryoprotected in 30% sucrose in PBS, and cut frozen in the transverse plane on a sliding microtome at 40-50 μm. Immunohistochemistry in cryostat sections or in free-floating sections was performed as described before (Marín et al., 2000). The following primary rabbit polyclonal antibodies were used: anti-NKX2-1 (Biopat Immunotechnologies, Caserta, Italy), diluted 1:2000; anti-calbindin (Swant, Bellinzona, Switzerland), diluted 1:5000; anti-GABA (Sigma), diluted 1:2000; and anti-tyrosine hydroxylase (Pel-Freeze), diluted 1:1000.

In situ hybridization

In situ hybridization experiments were performed using ^{35}S-riboprobes on 10 μm frozen sections as described previously (Bulfone et al., 1993). The cDNA probes used in this study were for: *Dlx2* and *Dlx5* (Liu et al., 1997); *Nkx2-1* (Shimamura et al., 1995); *Nkx2-4*, kindly provided by R. Harvey; *Pax6*, kindly provided by P. Gruss; *Sim1*, kindly provided by C. Fan; *SF1*, kindly provided by H. Ingraham; *EphA4*, kindly provided by A. Nieto; *EphA7*, kindly provided by A. Wanaka; *EphB1*, *EphB2* and *EphB3*, kindly provided by M. Henkemeyer; *ephrinA2*, kindly provided by D. Wilkinson; *ephrinA5*, kindly provided by U. Drescher; *Sema6a*, kindly provided by W. D. Snyder; and *Sema3a*, *Sema3c*, *Sema3f*, *Slit1*, *Slit2*, *Robo1*, *Robo2* and *netrin 1*, kindly provided by M. Tessier-Lavigne.

DiI labeling

E13.5, E14.5 and E18.5 *Nkx2-1* homozygous and wild-type littermate embryos were perfused with 4% PFA and their brains removed and kept in fixative. Crystals of the axonal tracer 1,1′-dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR) were placed in the neocortex to label anterogradely corticofugal axons. In other cases, DiI crystals were placed in the dorsal thalamus or in the basal telencephalon to label retrogradely corticofugal neurons. Crystals of similar shape and size (100-200 μm in diameter) were used in homozygous and wild-type littermate embryos. Brains were kept in 4% PFA at room temperature for 4-8 weeks to allow the DiI to diffuse. Subsequently, brains were embedded in 4% low melting point agarose and 100 μm transversal sections were cut in a vibratome. Sections were counterstained with 50 μg/ml Hoechst 33342 (Molecular Probes).

Preparation of explants

Embryos (E12.5-14.5) were removed by Cesarean section from timed-pregnant CD1 wild-type mice mated by GFP-expressing transgenic mice. Brains were removed in ice-cold Krebs buffer as described before (Marín et al., 2000). To isolate the cortex, brains were dissected in half along the midline and the telencephalon separated from the rest of the forebrain. Pieces of dorsal cortex were cut with a fine tungsten needle. To isolate basal telencephalon and hypothalamus, brains were embedded in 4% low melting point agarose in PBS and 250 μm transversal sections were cut with a vibratome. Sections at

the appropriate levels were collected and the preoptic area and hypothalamus isolated from them and dissected into small pieces.

Collagen gel co-cultures

Collagen was prepared from adult rat-tail, as described before (Lumsden and Davies, 1986). To set up the co-cultures, 25 μl of collagen solution was pipetted onto the bottom of four-well dishes (Nunc, Roskilde, Denmark) and allowed to gel for about 45 minutes. Explants were then placed onto this base and 25 μl of collagen were added on top. In most cases, collagen co-cultures consisted of three pieces (wild-type basal telencephalon or hypothalamus, GFP-expressing dorsal cortex and wild-type dorsal cortex) arranged sequentially from left to right and separated approximately by 300 μm. After a period of 45 minutes, to allow the collagen to gel, Neurobasal/B-27 medium (Gibco BRL, Life Technologies, Gaithersburg, MD) was added. Explants were cultured in a sterile incubator (37°C, 5% CO_2). In collagen cultures with isolated pieces of dorsal cortex, growth of cortical axons was similar from all sides of the explant.

Nomenclature

The telencephalon consists of pallial (cortical) and subpallial (subcortical) subdivisions. In this paper, we also refer to the subpallium as the 'ventral telencephalon' because it expresses molecular markers that are characteristic of ventral parts of the central nervous system. It should be noted, however, that topologically the subpallium is in fact rostral to the pallium (Cobos et al., 2001). The term 'basal telencephalon' is used to designate only the most ventral aspect of the subpallium, which includes derivatives from the anterior entopeduncular region and the preoptic area.

RESULTS

Cortical projections extend along two forebrain domains defined by the expression of NKX2-1

Nkx2-1 is expressed in the developing mouse forebrain from approximately embryonic day 8.5 (E8.5) (Price et al., 1992; Shimamura et al., 1995; Kohtz et al., 1998; Sussel et al., 1999). After neural tube closure, expression of NKX2-1 is restricted to two domains in the forebrain: ventral regions of the subpallium and hypothalamus (Fig. 1A). NKX2-1 is not found in cortical progenitors or in postmitotic cells derived from the cortex (Fig. 1A-C). From E12.5, corticofugal axons directed towards subcortical targets leave the cortex and transverse the developing striatum on their route to the diencephalon (de Carlos and O'Leary, 1992; Auladell et al., 2000). At this stage, NKX2-1 is expressed in both proliferative and postmitotic cells of the subpallium in a domain that includes the anlage of the globus pallidus [medial ganglionic eminence (MGE)], part of the septum, anterior entopeduncular region (AEP) and preoptic area (POa) (Fig. 1B; data not shown). In their trajectory, corticofugal axons grow through the striatum and leave the telencephalon in close proximity to the developing globus pallidus (Métin and Godement, 1996), which expresses NKX2-1. By contrast, corticofugal axons avoid entering the most ventral regions of the telencephalon, which are defined by the combined expression of NKX2-1 and Shh (Sussel et al., 1999; Olivier et al., 2001). At around E14, corticofugal axons form a tight bundle of fibers as they exit the telencephalon through the internal capsule, which is surrounded by regions of NKX2-1 expression (Fig. 1D,E). From here, cortical axons destined to the thalamus make a sharp turn caudalwards and follow straight across the ventral thalamus into the dorsal thalamus. A reciprocal route is followed by most thalamocortical axons (Métin and Godement, 1996; Molnár et al., 1998; Braisted et al., 1999; Auladell et al., 2000). By contrast, cortical axons directed towards other subcortical structures, such as the superior colliculus, pons and spinal cord, maintain their ventral course as they enter the diencephalon and later bend caudally to form the cerebral peduncle. These axons course caudally following a route parallel to the alar/basal boundary, avoiding the basal hypothalamic region (de Carlos and O'Leary, 1992), which is defined by NKX2-1 expression (Fig. 1A,C,F,G). Thus, developing corticofugal projections extend through the forebrain in close relation to regions defined by the mantle expression of NKX2-1 (Fig. 1H).

Nkx2-1 is essential for dorsoventral patterning of the ventral telencephalon and hypothalamus

Our previous work has provided evidence that disruption of *Nkx2-1* function results in re-specification of the ventral telencephalon to a more dorsal fate (Sussel et al., 1999). Although previous studies have shown hypothalamic defects in *Nkx2-1* mutants (Kimura et al., 1996; Takuma et al., 1998), it has not been established whether these defects are a consequence of abnormal dorsoventral patterning. Thus, we studied the hypothalamic expression of genes that define distinct dorsoventral positions in the hypothalamus.

At E11.5, a structure that morphologically resembles a small hypothalamus is found in *Nkx2-1* mutants (Fig. 2A-H; data not shown). To determine whether the mutant hypothalamus has molecular characteristics similar to those of the wild-type hypothalamus, we studied the expression of molecular markers whose forebrain expression is restricted to the *Nkx2-1* domain, such as the homeobox gene *Nkx2-4*, the orphan nuclear receptor *SF1* (*Nr5a1* – Mouse Genome Informatics) and *Shh*. With the exception of its most ventral aspect, expression of these factors is not detectable in the mutant hypothalamus (Fig. 2A,E,D,H; data not shown). We next analyzed the expression of *Pax6*, a paired-homeobox gene that has been shown to play an opposing role to Nkx genes in dorsoventral patterning (Ericson et al., 1997; Briscoe et al., 1999; Sussel et al., 1999; Briscoe et al., 2000; Sander et al., 2000). *Pax6* expression is restricted to alar domains in the forebrain of wild-type embryos, and therefore it is normally excluded from the hypothalamus (Fig. 2B,D) (Stoykova et al., 1996). In *Nkx2-1* mutants, *Pax6* expression is expanded ventrally into the hypothalamus, more prominently in its posterior aspect (Fig. 2F,H). In addition, the expression of the bHLH-PAS transcription factor *Sim1* is also altered in *Nkx2-1* mutants. Whereas *Sim1* expression is restricted to the alar domain of the rostral diencephalon in wild-type embryos, *Sim1* expression is expanded ventrally in *Nkx2-1* mutants (Fig. 2C,D,G,H). Nevertheless, neither *Pax6* nor *Sim1* are expressed in the most ventral aspect of the mutant hypothalamus (data not shown), suggesting that only the most ventral domain of the hypothalamus maintains some normal molecular properties in the absence of *Nkx2-1* function. Thus, the hypothalamus shows a ventral-to-dorsal shift in its molecular properties in the absence of *Nkx2-1* function.

Histological analysis at E18.5 supports the evidence for the loss of ventral structures and expansion of dorsal components in the hypothalamus of *Nkx2-1* mutants (Fig. 2I-L). For

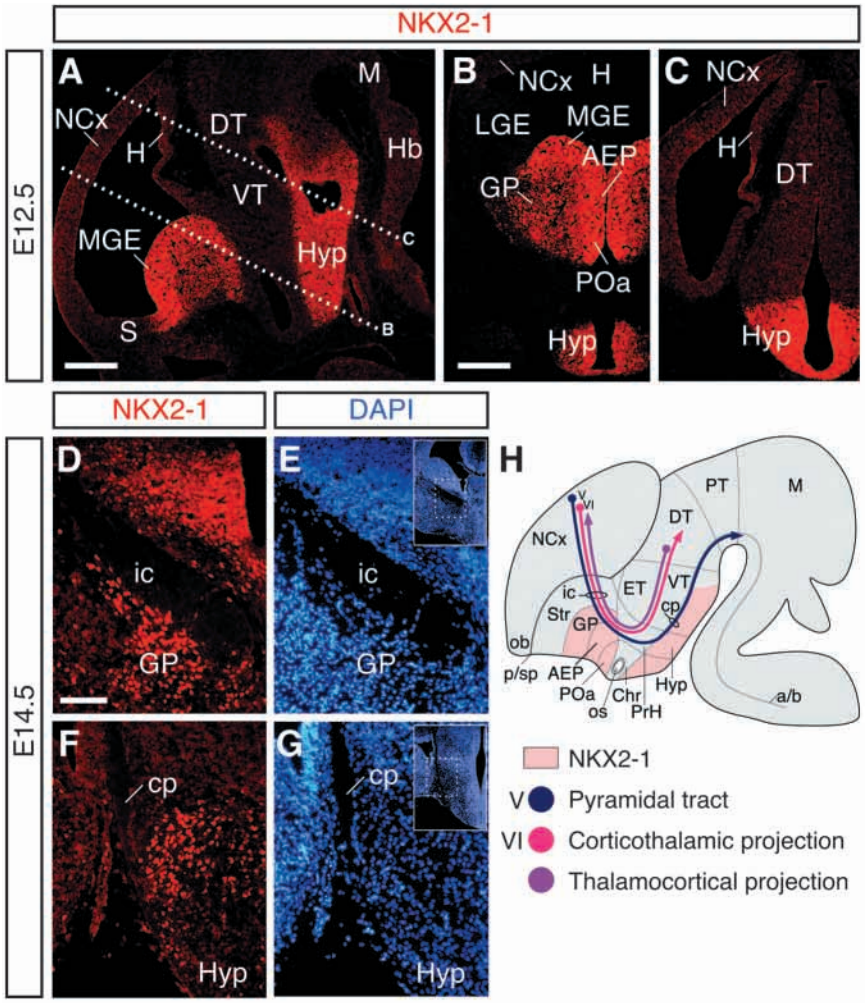

Fig. 1. Expression of NKX2-1 in the developing forebrain. (A) Sagittal section through the brain of an E12.5 embryo showing the expression of NKX2-1 in the basal telencephalic and hypothalamic primordia. The broken lines indicate the approximate levels of the sections shown in (B,C). (B,C) Coronal sections that highlight the basal telencephalon and rostral diencephalon (B) and mid-diencephalon (C) of an E12.5 embryo showing expression of NKX2-1. (D-G) High-magnification images of coronal sections through the telencephalon (D,E) and diencephalon (F,G) of an E14.5 embryo showing expression of NKX2-1 at the level of the internal capsule (D) and close to cerebral peduncle at the level of the hypothalamus (F). (E,G) DAPI counterstained images of the sections showed in (D,F). The insets in E,G indicate the level from where the images were taken. (H) Schematic drawing showing the relationship between the paths followed by pyramidal tract (blue), corticothalamic (pink) and thalamocortical (purple) axons in relation to the territories patterned by *Nkx2-1* (light pink). a/b, alar/basal boundary; AEP, anterior entopeduncular region; Chr, chiasmatic region; cp, cerebral peduncle; DT, dorsal thalamus; ET, eminentia thalami; GP, globus pallidus; H, hippocampus; Hb, hindbrain; Hyp, hypothalamus; ic, internal capsule; LGE, lateral ganglionic eminence; M, mesencephalon; MGE, medial ganglionic eminence; NCx, neocortex; ob, olfactory bulb; os, optic stalk; POa, preoptic area; PrH, peduncular region of the hypothalamus; p/sp, pallial/subpallial boundary; PT, pretectum; Str, striatum; V, layer 5 cortical neurons; VI, layer 6 cortical neurons; VT, ventral thalamus. Scale bar: 200 μm in A-C; 100 μm in D-G.

example, calbindin immunohistochemistry revealed that the mammillary hypothalamus is greatly reduced or absent in *Nkx2-1* mutants, whereas the tuberomammillary hypothalamus has an increased density of calbindin-positive cells (Fig. 2J,L). In addition, the proportion of GABAergic neurons found in the hypothalamus of *Nkx2-1* mutants is increased in relation to the non-GABAergic cell populations (Fig. 2I,K). This observation is consistent with the finding that expression of Dlx genes is preserved in the hypothalamus of *Nkx2-1* mutants (data not shown). In sum, as in the ventral telencephalon, loss of *Nkx2-1* function in the hypothalamus does not result in the complete loss of this territory, but rather in a transformation of its molecular and morphological characteristics.

Abnormal guidance of pyramidal tract axons in the ventral telencephalon and hypothalamus of *Nkx2-1* mutants

Analysis of patterning defects present in *Nkx2-1* mutants suggests that the ventral telencephalon and the hypothalamus of these mice have acquired molecular characteristics that are different from the equivalent wild-type territories. As corticofugal and thalamocortical projections grow in close relation to the territories patterned by NKX2-1 (Fig. 1), we wondered whether the transformation of these domains could affect the course of these tracts in *Nkx2-1* mutants.

To study cortical projections in *Nkx2-1* mutants, DiI crystals were inserted into the parietal cortex of fixed brains from E14.5 mice. In wild-type brains, corticofugal axons coursed through the internal capsule and arrived at the junction between the telencephalon and the diencephalon (n=8, Fig. 3A). Some of the leading axons at this stage continued into the dorsal thalamus, whereas the remaining joined the developing cerebral peduncle (data not shown). DiI deposits in the dorsal cortex of *Nkx2-1* mutants consistently labeled a large number of corticofugal axons (n=8). In *Nkx2-1* mutants, cortical axons grew normally into the internal capsule (data not shown), suggesting that guidance of corticofugal axons is normal to this point. At the telencephalic-diencephalic boundary, however, the majority of corticofugal axons did not maintain their original course, but instead extended towards the pial surface of the telencephalon (Fig. 3E, arrow). The remaining labeled axons entered the diencephalon normally (Fig. 3E, arrowhead) and grew into the dorsal thalamus (data not shown).

Analysis of DiI deposits in the parietal cortex of *Nkx2-1* mutant mice at E18.5 revealed that the guidance defect of corticofugal projections is even more prominent at this stage. As in earlier stages, corticofugal axons reached the internal capsule normally in *Nkx2-1* mutants. Moreover, corticothalamic axons followed a normal route at the telencephalic-diencephalic boundary, turning caudally after

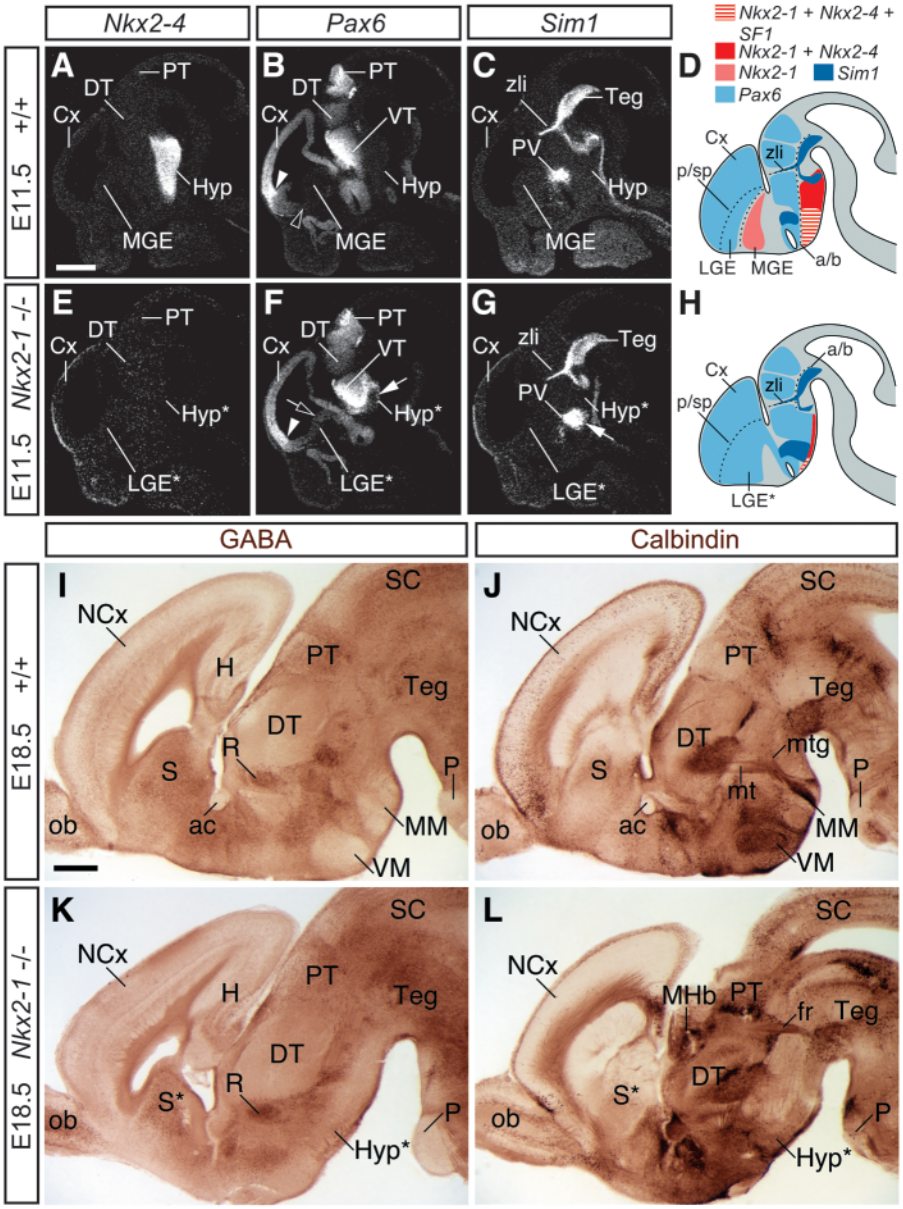

Fig. 2. Patterning defects in the diencephalon of *Nkx2-1* mutant mice. (A-H) Serial sagittal sections through the brain of E11.5 wild-type (A-C) and *Nkx2-1* mutant embryos (E-G) showing mRNA expression of *Nkx2-4* (A,E), *Pax6* (B,F) and *Sim1* (C,G). The white arrowhead indicates the pallial-subpallial boundary, whereas the black (with white border) arrowhead denotes the LGE-MGE boundary. Note ventral expansion of *Pax6* in the telencephalon (black arrows) and of *Pax6* and *Sim1* in the diencephalon (white arrow). (D,H) Schematic drawings summarizing the expression of pattern of *Nkx2-1*, *Nkx2-4*, *Pax6*, *SF1* and *Sim1* in the forebrain of wild-type and *Nkx2-1* mutant embryos. (I-L) Serial sagittal sections through the brain of E18.5 wild-type (I,J) and *Nkx2-1* mutant fetuses (K,L) showing immunohistochemistry for GABA (I,K) and calbindin (J,L). a/b, alar-basal boundary; ac, anterior commissure; DT, dorsal thalamus; fr, fasciculus retroflexus; H, hippocampus; Hb, hindbrain; Hyp, hypothalamus; Hyp*, mutant hypothalamus; ic, internal capsule; LGE, lateral ganglionic eminence; LGE*, mutant lateral ganglionic eminence; MGE, medial ganglionic eminence; MGE*, mutant medial ganglionic eminence; MHb, medial habenula; MM, mammillary hypothalamus; mt, mammillothalamic tract; mtg, mammillotegmental tract; NCx, neocortex; ob, olfactory bulb; p/sp, pallial/subpallial boundary; P, pontine nuclei; PT, pretectum; PV, paraventricular hypothalamic region; R, reticular nucleus; S, septum; S*, mutant septum; SC, superior colliculus; Teg, mesencephalic tegmentum; VM, ventromedial hypothalamic nucleus; VT, ventral thalamus; zli, zona limitans intrathalamica. Scale bar: 400 μm in A-C,E-G; 500 μm in I-L.

leaving the telencephalon and growing longitudinally through the ventral thalamus to reach the dorsal thalamus (*n*=12; Fig. 3B-D,F,H,I). By contrast, cortical axons directed to the cerebral peduncle failed to enter the diencephalon and instead extended toward the pial surface (Fig. 3B-D,F,H,I). In most cases, labeled axons in this ectopic peduncle stopped at the surface of the telencephalon, while a few labeled axons extended inside the telencephalic stalk or caudally into the amygdala (data not shown). By contrast, DiI crystal deposits in the cortex of *Nkx2-1* mutants revealed a normal pattern of retrogradely labeled cells in the dorsal thalamus (Fig. 3B,C,H), suggesting that thalamocortical connections develop normally in *Nkx2-1* mutants. Immunohistochemical detection of 5-HT and calretinin, two markers of thalamocortical fibers at this stage, confirmed this finding (data not shown).

To confirm that the ectopic bundle consists of axons originally directed towards the cerebral peduncle (pyramidal tract axons, which originate primarily in cortical layer 5) (Jones, 1984) and not of corticothalamic axons (which originate in cortical layer 6) (Jones, 1984), we used retrograde DiI labeling to identify the neurons that extend their axons into this ectopic projection. We inserted small DiI crystals in the surface of the telencephalic stalk of *Nkx2-1* mutants, in the region of the ectopic peduncle, and found that retrogradely labeled cells were primarily confined to cortical layer 5 (*n*=3) (Fig. 3G). DiI placements in the dorsal thalamus, however, confirmed that corticothalamic projections are essentially normal in *Nkx2-1* mutants and that they originate from cortical layer 6, as in control mice (*n*=5) (Fig. 4). Thus, corticothalamic and thalamocortical projections develop normally in the absence of *Nkx2-1* function, whereas pathfinding of pyramidal tract axons is severely disrupted in the telencephalon of *Nkx2-1* mutants.

Despite the prominent defect found in the guidance of layer 5 projections at the level of the telencephalic stalk, DiI deposits in the parietal cortex labeled a small bundle of axons in the cerebral peduncle of some *Nkx2-1* mutants (*n*=7/12; Fig. 3H). These results suggest that, at least in some cases, some cortical axons are able to enter the cerebral peduncle normally. In wild-type brains, corticofugal axons in the cerebral peduncle grew

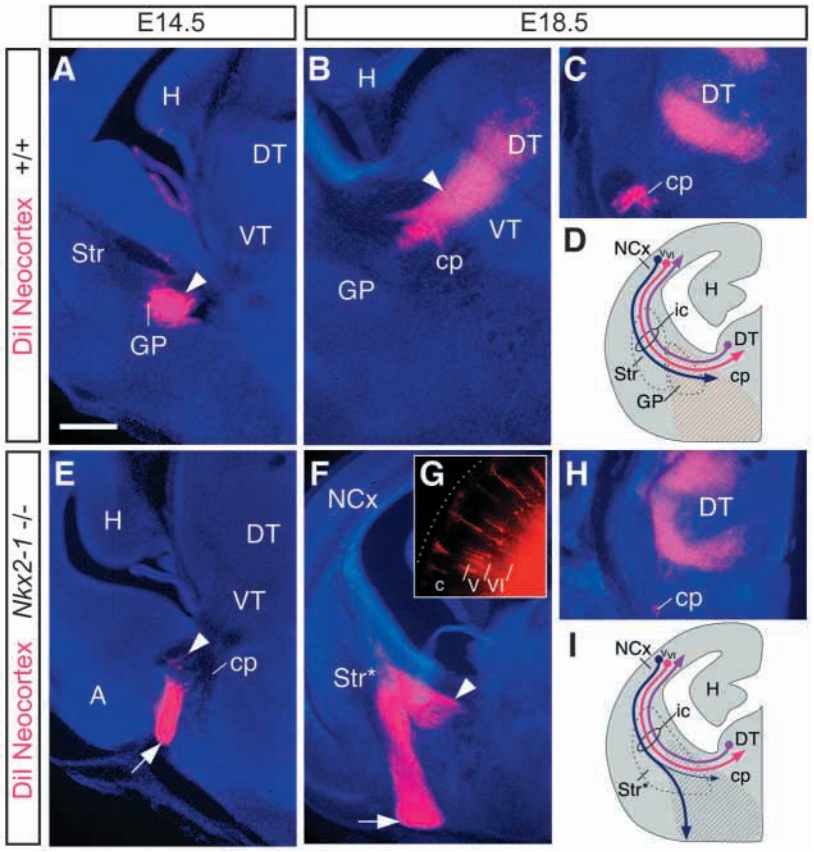

Fig. 3. Pathfinding defects of layer 5 cortical projections in the telencephalon of *Nkx2-1* mutants. Coronal sections through the telencephalon of E14.5 (A,E) or E18.5 (B,C,F,G,H) wild-type (A-C) or *Nkx2-1* mutant mice (E-H) showing anterogradely labeled fibers (A-C,E,F,H) and retrogradely labeled thalamic cells (B,C,H) after DiI crystal placements in the neocortex. Arrowheads point to corticofugal fibers following a normal course, whereas arrows indicate corticofugal fibers abnormally projecting towards the surface of the telencephalon. (G) Coronal section through the telencephalon of an E18.5 *Nkx2-1* mutant mice showing retrogradely labeled cells in the cortex after a DiI crystal placement in the ventral surface of the telencephalon (at the approximate location of the arrow in F). The dotted line indicates the surface of the cortex. (D,I) Schematic drawings showing the paths followed by pyramidal tract (blue), corticothalamic (pink) and thalamocortical (purple) axons in wild-type (D) and *Nkx2-1* mutant mice (I). The hatched areas (D,I) indicate the presumptive territory patterned by *Nkx2-1* function. A, amygdala; c, cortical plate; cp, cerebral peduncle; DT, dorsal thalamus; GP, globus pallidus; H, hippocampus; ic, internal capsule; NCx, neocortex; Str, striatum; Str*, mutant striatum; V, layer 5 cortical neurons; VI, layer 6 cortical neurons; VT, ventral thalamus. Scale bar: 200 μm in A,E; 400 μm in B,C,F,H; 100 μm in G.

ventrally through the peduncular region of the hypothalamus until they reached the diencephalic alar/basal boundary, where they turned caudalwards towards the mesencephalon (Fig. 5A,C; see also Fig. 1H). By making this 90° turn, pyramidal tract axons avoid entering the chiasmatic region of the hypothalamus. In *Nkx2-1* mutants, however, most pyramidal tract cortical axons failed to turn caudally and, instead, grew into the chiasmatic region (Fig. 5D,F). Compared with wild-type brains, very few cortical axons reached the mesencephalon and rhombencephalon in the mutants (Fig. 5B,E). Thus, in *Nkx2-1* mutant mice, most layer 5 cortical projections either fail to leave the telencephalon (Fig. 3) or fail to turn caudally once they reach the hypothalamus (Fig. 5).

The basal telencephalon and the hypothalamus contain a repellent activity for the growth of cortical axons

The previous experiments suggest that abnormal guidance of layer 5 cortical projections occurs in *Nkx2-1* mutants because patterning defects in the basal telencephalon and hypothalamus lead to alterations in the signals that direct this axon tract. In *Nkx2-1* mutants, cortical axons invade the telencephalic stalk (the most ventral aspect of the subpallium, which includes derivatives from the AEP and POa) and the chiasmatic region, two regions that cortical axons normally do not enter. These observations suggest that the basal telencephalon and the hypothalamus may

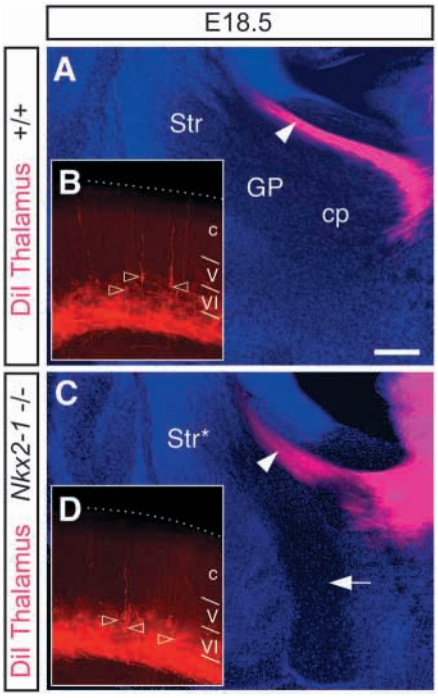

Fig. 4. Normal pathfinding of layer 6 cortical projections in *Nkx2-1* mutants. Coronal sections through the telencephalon of E18.5 wild-type (A,B) or *Nkx2-1* mutant mice (C,D) showing the path followed by corticothalamic connections (A,C; white arrowheads) and retrogradely labeled cortical cells (B,D; open arrowheads) after DiI crystal placements in the dorsal thalamus. (C) The arrow points to the poor-cell region containing the abnormal tract formed by layer 5 cortical axons (not labeled in this experiment) present in *Nkx2-1* mutants (see Fig. 3F). c, cortical plate; cp, cerebral peduncle; GP, globus pallidus; Str, striatum; Str*, mutant striatum; V, layer 5 cortical neurons; VI, layer 6 cortical neurons. Scale bar: 200 μm in A,C; 100 μm in B,D.

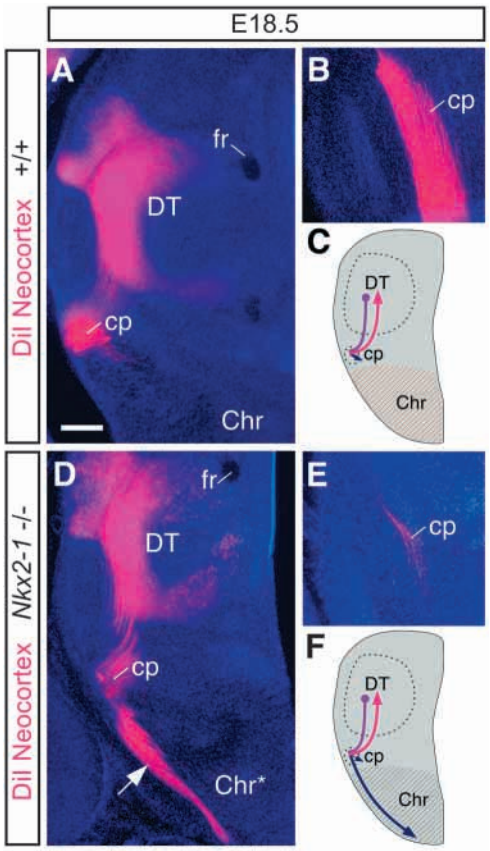

Fig. 5. Pathfinding defects of layer 5 cortical projections in the diencephalon of *Nkx2-1* mutants. Coronal (A,D) and horizontal (B,E) sections through the diencephalon (A,D) or pons (B,E) of E18.5 (A,B,D,E) wild-type (A,B) or *Nkx2-1* mutant mice (D,E) showing anterogradely labeled fibers (A,B,D,E) and retrogradely labeled thalamic cells (A,D) after DiI crystal placements in the neocortex. The arrow indicates corticofugal fibers that follow an abnormal rostral path into the chiasmatic region of the hypothalamus. (C,F) The paths followed by pyramidal tract (blue), corticothalamic axons (pink) and thalamocortical axons (purple) in wild-type (C) and *Nkx2-1* mutant mice (F). The hatched area indicates the presumptive territory patterned by *Nkx2-1* function. Chr, chiasmatic region; Chr*, mutant chiasmatic region; cp, cerebral peduncle; DT, dorsal thalamus; fr, fasciculus retroflexus. Scale bar: 400 μm in A,B,D,E.

Table 1. Expression of axon guidance molecules in the basal telencephalon of *Nkx2-1* mutant mice at E13.5

Protein	Expression
EphA4	No change
EphA7	No change
EphrinA2	No change
EphrinA5	Reduced
EphB1	No change
EphB2	Reduced
EphB3	Reduced
Sema3A	Increased/expanded
Sema3C	Reduced
Sema3F	Increased/expanded
Sema6A	No change
Slit1	Increased/expanded
Slit2	Reduced or absent
Netrin1	No change

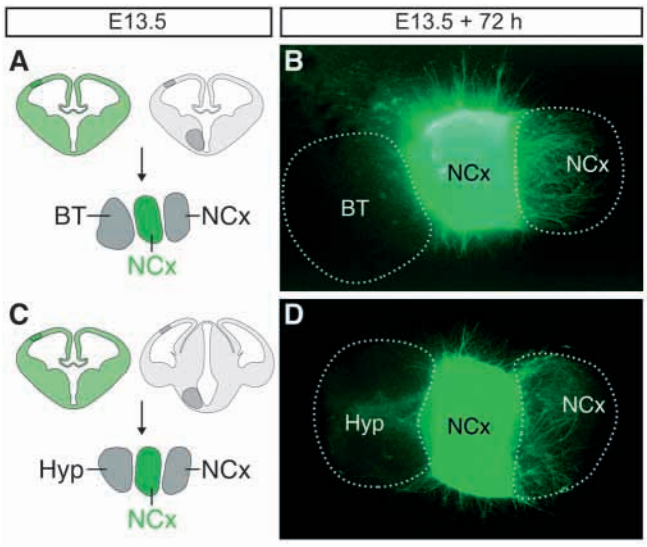

Fig. 6. The basal telencephalon and hypothalamus contain a non-permissive activity for cortical axons. (A,C) Experimental paradigm for cortical-basal telencephalic (A) and cortical-hypothalamic (C) co-cultures in three-dimensional collagen matrices. Green slices and explants are derived from E13.5 GFP transgenic embryos, whereas gray slices and explants are derived from wild-type littermate embryos. (B,D) Explants after 72 hours in culture showing immunohistochemistry for GFP. The broken lines indicate the location of the non-GFP expressing explants. BT, basal telencephalon; Hyp, hypothalamus; NCx, neocortex.

contain a repulsive or non-permissive activity for cortical axons, and that the loss of this activity in *Nkx2-1* mutants may divert these axons from their normal course. To test this hypothesis, we carried out collagen co-culture assays using explants from E13.5-E14.5 mice, ages at which cortical axons are extending though the subpallium and diencephalon in vivo. Wild-type explants of the dorsal cortex obtained from GFP-expressing transgenic mice were co-cultured adjacent to explants of the dorsal cortex and explants of the basal telencephalon, both obtained from littermates (Fig. 6A). In most cases, cortical axons grew into the adjacent cortical explant, but avoided growing into the basal telencephalic explant (n=26/30; Fig. 6B). Co-cultures using explants from the hypothalamus (Fig. 6C), instead of the basal telencephalon, showed similar results (n=30/38; Fig. 6D). Most cortical explants cultured alone in collagen as a control showed symmetrical axonal outgrowth (n=10/13; data not shown). Therefore, both the basal telencephalon and the hypothalamus contain an activity that prevents the ingrowth of cortical axons.

Abnormal expression of axon guidance molecules in the forebrain of *Nkx2-1* mutants

As the territories patterned by *Nkx2-1* contain an activity that inhibits the growth of cortical axons, it is conceivable that loss of this chemorepellent activity in *Nkx2-1* mutants may account for the defects observed in the guidance of layer 5 cortical axons. We employed a candidate molecule approach to identify genes encoding axon guidance proteins whose expression is altered in the basal telencephalon of *Nkx2-1* mutants. First, we analyzed the expression different members of the semaphorin family, which has been previously described to affect the

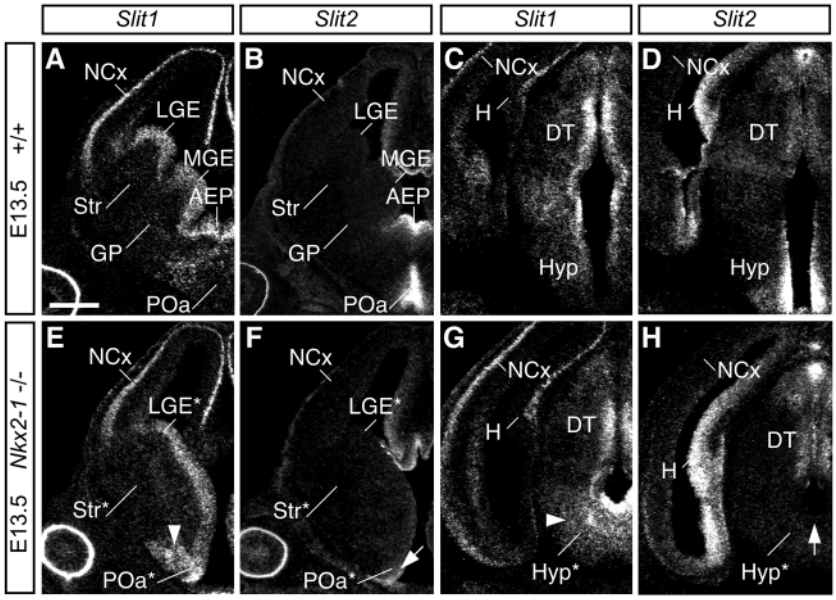

Fig. 7. Expression of *Slits* is altered in the forebrain of *Nkx2-1* mutants. Serial coronal sections through the telencephalon (A,B,E,F) and diencephalon, and caudal telencephalon (C,D,G,H) of E13.5 wild-type (A-D) and *Nkx2-1* (E-H) mutant embryos showing the expression of *Slit1* (A,C,E,G) and *Slit2* (B,D,F,H). Dorsal is upwards and the midline is to the right in all panels. Arrowheads point to regions of increased *Slit1* expression, whereas arrows denote regions where *Slit2* expression is either lost or decreased. AEP, anterior entopeduncular region; DT, dorsal thalamus; GP, globus pallidus; H, hippocampus; Hyp, hypothalamus; Hyp*, mutant hypothalamus; LGE, lateral ganglionic eminence; LGE*, mutant lateral ganglionic eminence; MGE, medial ganglionic eminence; NCx, neocortex; POa, preoptic area; POa*, mutant preoptic area; Str, striatum; Str*, mutant striatum. Scale bar: 500 μm.

guidance of cortical and thalamic axons (Bagnard et al., 1998; Polleux et al., 1998; Leighton et al., 2001). Expression of *Sema3a*, *Sema3c*, *Sema3f* and *Sema6a* was maintained in the basal telencephalon of *Nkx2-1* mutants (Table 1). Next, we examined the expression of *netrin 1*, because this molecule has been implicated in the guidance of both corticofugal and thalamocortical axons (Métin et al., 1997; Richards et al., 1997; Braisted et al., 2000). In situ hybridization analysis showed that expression of *netrin 1* is also preserved in the subpallium of *Nkx2-1* mutants (Table 1). Then, we analyzed the expression of Eph tyrosine kinase receptors and their ligands, the ephrins, as previous studies have demonstrated a role for these molecules in the guidance of cortical axons (Henkemeyer et al., 1996; Orioli et al., 1996; Dottori et al., 1998; Kullander et al., 2001a; Kullander et al., 2001b; Leighton et al., 2001; Yokoyama et al., 2001). Interestingly, expression of *EphB2*, *EphB3* and *ephrinA5* was reduced in the forebrain of *Nkx2-1* mutants (Table 1). Nevertheless, analysis of layer 5 cortical projections in *EphB2*/*EphB3* double mutants (n=8; O. M., M. Henkemeyer and J. L. R. R., unpublished) and in *ephrinA5* mutants (n=4; O. M., J. Holmberg, J. Frisen and J. L. R. R., unpublished) revealed no obvious defects. Thus, loss of *EphB2*/*EphB3* or *ephrinA5* alone does not seem to recapitulate the defects found in *Nkx2-1* mutant mice.

Slit proteins constitute another family of extracellular matrix molecules that possess chemorepulsive activity for growing axons in a variety of systems (Brose and Tessier-Lavigne, 2000). Like semaphorins, Eph/ephrins and netrin, Slits are also expressed in the forebrain in a pattern that may affect the growth of cortical axons (Yuan et al., 1999; Marillat et al., 2001) (Bagri et al., 2002). Recently, it has been reported that loss of Slit2 function in mice produces a dramatic defect in the guidance of cortical axons (Bagri et al., 2002). Interestingly, this defect is strikingly similar to that found in *Nkx2-1* mutants, as in the absence of Slit2 most cortical projections destined to the cerebral peduncle course abnormally to the ventral surface of the telencephalon. Interestingly, we found that expression of *Slit1* and *Slit2* in the forebrain of *Nkx2-1* mutants was significantly altered. The most remarkable finding was that *Slit2* expression in the AEP and POa domains of the ventral telencephalon was almost completely absent in the mutant (Fig. 7B,F). The alteration in *Slit1* expression was less unexpected because, in line with other markers that expand ventrally in the telencephalon (see Fig. 2) (Sussel et al., 1999), *Slit1* expression was found throughout the entire VZ of the subpallium in *Nkx2-1* mutants, including the POa, a region that normally lacks *Slit1* expression (Fig. 7A,E). Moreover, the number of postmitotic *Slit1*-expressing cells in the mantle of the POa was increased compared with wild-type mice (Fig. 7A,E).

Analysis of the expression of *Slit1* and *Slit2* in the hypothalamus showed similar results; expression of *Slit1* was increased, whereas expression of *Slit2* was severely reduced compared with wild-type littermates (Fig. 7C,D,G,H). Thus, the telencephalic stalk region and the hypothalamus of *Nkx2-1* mutants lack expression of *Slit2*, but have increased *Slit1* expression. In situ hybridization analysis revealed that there are no obvious differences in the expression of the *Slit* receptors *Robo1* and *Robo2* in the cortex of wild-type and *Nkx2-1* mutant mice (data not shown), showing that cortical neurons should be competent to respond to *Slit* in *Nkx2-1* mutants.

Abnormal guidance of ascending dopaminergic projections in *Nkx2-1* mutants

Our previous experiments show that efferent forebrain fibers tha contribute to the cerebral peduncle (e.g. layer 5 cortical projections) are misrouted in the basal telencephalon and hypothalamus of *Nkx2-1* mutants, and that loss of *Slit2* expression is correlated with this phenotype. We reasoned that afferent forebrain fibers could also be affected, particularly as *Slit2* function is required for the guidance of rostral-growing fibers in the hypothalamus, such as the mesotelencephalic dopaminergic projections (Bagri et al., 2002). Thus, we studied the distribution of dopaminergic fibers using antibodies against tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine synthesis. In both control and *Nkx2-1* mutant mice, dopaminergic fibers from the substantia nigra/ventral tegmental area coursed longitudinally in the medial forebrain bundle, which lies ventromedial to the cerebral peduncle. In normal mice, dopaminergic fibers turned dorsally as they approach the telencephalic stalk, and then entered the

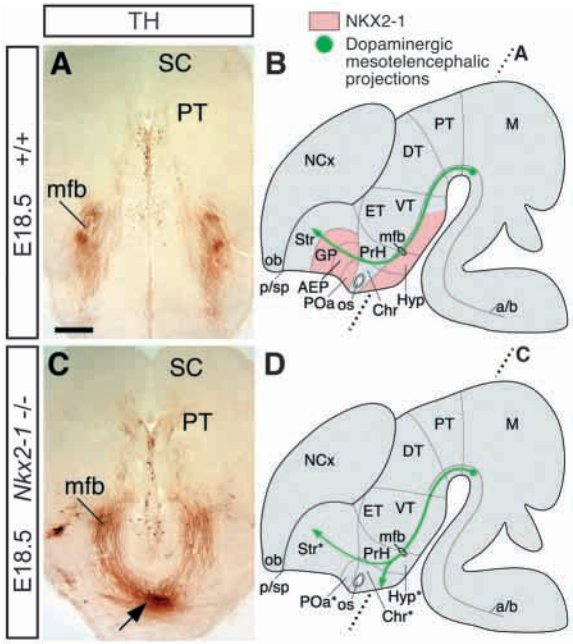

Fig. 8. Distribution of mesotelencephalic dopaminergic fibers is altered in the diencephalon of *Nkx2-1* mutants. (A,C) Sections horizontal to the diencephalon of E18.5 wild-type (A) and *Nkx2-1* mutant (C) fetuses showing immunohistochemistry against tyrosine hydroxylase (TH). Caudal is upwards and rostral is downwards in both panels. The arrow points to the region where TH-positive fibers abnormally converge towards the rostral midline. (B,D) The path followed by dopaminergic mesotelencephalic axons (green) in wild-type (B) and *Nkx2-1* mutant mice (D). The pink domains indicate the territories patterned by *Nkx2-1* function. a/b, alar/basal boundary; AEP, anterior entopeduncular region; Chr, chiasmatic region; Chr*, mutant chiasmatic region; cp, cerebral peduncle; DT, dorsal thalamus; ET, eminentia thalami; GP, globus pallidus; H, hippocampus; Hb, hindbrain; Hyp, hypothalamus; Hyp*, mutant hypothalamus; ic, internal capsule; M, mesencephalon; mfb, medial forebrain bundle; NCx, neocortex; POa, preoptic area; POa*, mutant preoptic area; PrH, peduncular region of the hypothalamus; p/sp, pallial/subpallial boundary; PT, pretectum; ob, olfactory bulb; os, optic stalk; SC, superior colliculus; Str, striatum; Str*, mutant striatum; V, layer 5 cortical neurons; VI, layer 6 cortical neurons; VT, ventral thalamus. Scale bar: 400 μm.

subpallium. In *Nkx2-1* mutants, however, a large number of TH-positive fibers failed to enter the telencephalon and instead coursed directly into the chiasmatic region, a region that they normally avoid (Fig. 8). This is the same error that the efferent corticofugal fibers make (Fig. 5D, Fig. 9B). Although many axons take an aberrant path, many are successful at entering the telencephalon and innervating the striatum in *Nkx2-1* mutants (data not shown) (Sussel et al., 1999). Thus, *Nkx2-1* function is required for the proper trajectories of major efferent and afferent fibers of the cerebral peduncle.

DISCUSSION

In this study, we provide evidence that appropriate patterning of the basal telencephalon and hypothalamus is required for the establishment of some of the major efferent and afferent projections that interconnect the telencephalon with the rest of the brain (Fig. 9A,B). Loss of *Nkx2-1* homeobox gene function causes a molecular transformation of the basal telencephalon and hypothalamus, which results in the enlargement of the LGE and the alar prethalamic diencephalon, respectively (present study) (Sussel et al., 1999). Consequently, most layer 5 cortical projections fail to project out of the telencephalon, and instead course abnormally to the surface of the telencephalic stalk region (AEP). Moreover, most of those corticofugal axons that are able to enter the hypothalamus grow towards the chiasmatic region, instead of caudally (Fig. 9A,B). Likewise, many afferent dopaminergic fibers show similar pathfinding errors in the hypothalamus (Fig. 9A,B).

In contrast to the cerebral peduncle defects, corticothalamic and thalamocortical projections develop normally in the absence of *Nkx2-1* function. Together with our in vitro observations that both the basal telencephalon and the hypothalamus contain an activity that prevents the growth of cortical axons, these findings suggest that loss of a chemorepellent activity in *Nkx2-1* mutants may account for the guidance defects of layer 5 cortical axons. This notion is supported by the fact that the basal telencephalon and hypothalamus of *Nkx2-1* mutants lack expression of *Slit2*, an extracellular matrix molecule that has been recently demonstrated to play a key role in the guidance of cortical projections (Bagri et al., 2002). Insights from these experiments are discussed below.

Role of the ventral telencephalon and hypothalamus in the guidance of corticofugal and thalamocortical projections

Previous studies have analyzed the early development of corticofugal and thalamocortical projections, and have identified pioneering axonal populations, potential intermediate targets and critical guidance decision points for these axons (Blakemore and Molnár, 1990; Ghosh et al., 1990; Shatz et al., 1990; de Carlos and O'Leary, 1992; Miller et al., 1993; Mitrofanis and Baker, 1993; Molnár and Blakemore, 1995; Métin and Godement, 1996; Molnár et al., 1998; Braisted et al., 1999; Molnár and Cordery, 1999; Tuttle et al., 1999; Auladell et al., 2000; Braisted et al., 2000). One general insight derived from these studies is that multiple cell domains distributed along the length of each of these axonal pathways are involved in the guidance of the axons to their target.

It has been previously shown that the subpallium plays a prominent role in the guidance of corticofugal and thalamocortical axons. Specifically, the mantle regions of the LGE and MGE act as intermediate targets for corticofugal and thalamocortical axons, respectively (Métin and Godement, 1996). Moreover, the initial trajectories of corticofugal and thalamocortical axons into the subpallium appear to be pioneered by transient projections from the ganglionic eminences. For example, projections from the mantle of the MGE to the dorsal thalamus seem to constitute a substrate for the growth of thalamocortical axons into the telencephalon (Mitrofanis and Baker, 1993; Métin and Godement, 1996; Molnár et al., 1998; Braisted et al., 1999; Tuttle et al., 1999). In addition, a projection from the mantle of the LGE to the cortex may be used by corticofugal fibers as a guiding axonal scaffold in their growth toward the internal capsule (Métin and Godement, 1996). Thus, the mantle regions of the LGE and the

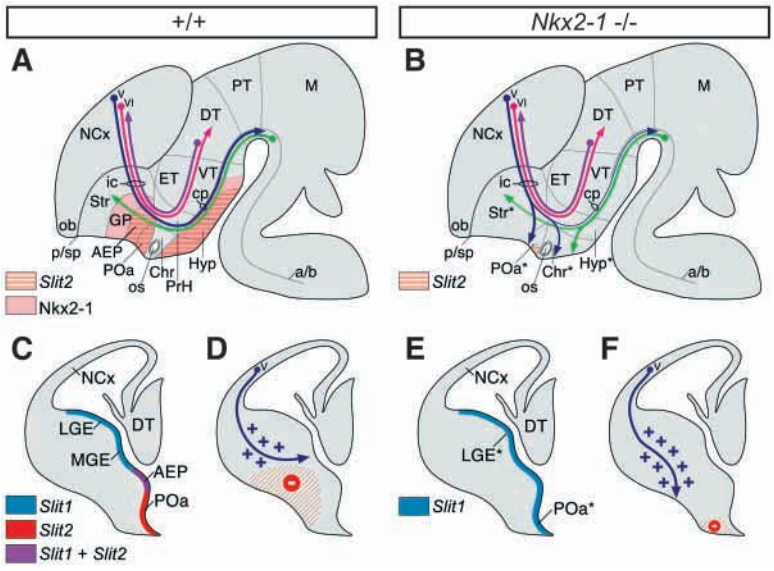

Fig. 9. A model for the guidance of cortical projections in *Nkx2-1* mutant mice. (A,B) The paths followed by pyramidal tract (blue), corticothalamic (pink), thalamocortical (purple) and dopaminergic mesotelencephalic (green) axons in wild-type (A) and *Nkx2-1* mutant mice (B). The broken lines indicate the territories patterned by *Nkx2-1* function. (C-F) The expression of *Slit* genes in the ventricular zone of the subpallium in wild-type (C) and *Nkx2-1* mutant mice (E), and the hypothesized consequences of their function on the guidance of layer 5 cortical axons in wild-type (D) and *Nkx2-1* mutant mice (F). Blue positive signs indicate a permissive territory, whereas negative signs and red hatching denote a non-permissive territory. a/b, alar/basal boundary; AEP, anterior entopeduncular region; Chr, chiasmatic region; Chr*, mutant chiasmatic region; cp, cerebral peduncle; DT, dorsal thalamus; ET, eminentia thalami; GP, globus pallidus; H, hippocampus; Hb, hindbrain; Hyp, hypothalamus; Hyp*, mutant hypothalamus; ic, internal capsule; M, mesencephalon; NCx, neocortex; POa, preoptic area; PrH, peduncular region of the hypothalamus; p/sp, pallial/subpallial boundary; PT, pretectum; ob, olfactory bulb; os, optic stalk; Str, striatum; Str*, mutant striatum; V, layer 5 cortical neurons; VI, layer 6 cortical neurons; VT, ventral thalamus.

MGE are permissive territories for the growth of corticofugal and thalamocortical axons.

In the present study, we have identified a subpallial telencephalic subdomain as an important territory in the guidance of cortical axons destined to the cerebral peduncle (pyramidal tract axons). This telencephalic domain is located just outside the mantle of the MGE [the globus pallidus; VTel1 domain of Tuttle et al. (Tuttle et al., 1999)], and consists of the mantle regions of the AEP and POa. Our in vitro experiments demonstrated that this region constitutes a non-permissive substrate for cortical axons. Thus, the subpallium consists of two different domains that are essential for the guidance of corticofugal and thalamocortical projections: (1) a permissive territory that these axons can traverse (the mantle zone of the LGE and MGE); and (2) a non-permissive territory that these axons normally avoid (the mantle zone of the AEP and POa) (Fig. 9D).

Another novel finding derived from the analysis of *Nlx2-1* mutants is that corticofugal axons derived from different cortical layers respond differently to cues present in the basal telencephalon. Thus, whereas a large number of layer 5 axons fail to reach the diencephalon in *Nkx2-1* mutants, layer 6 axons follow a normal path to the dorsal thalamus. This suggests that different mechanisms regulate the guidance of layer 5 and layer 6 axons as they course through the ventral telencephalon. Alternatively, layer 6 axons may be equally sensitive to cues present in the telencephalic stalk area, but additional mechanisms may compensate for their loss in *Nkx2-1* mutants. For example, it has been hypothesized that corticothalamic and thalamocortical axons use each other as guidance scaffolds (Molnár et al., 1998). As thalamocortical projections develop normally in *Nkx2-1* mutants, their presence in the internal capsule may be sufficient to ensure the guidance of cortical projections to the thalamus.

It has been previously suggested that the mantle of the MGE contains a cell domain that constitutes a permissive substrate for the growth of thalamocortical axons [VTel1 domain (Braisted et al., 1999; Tuttle et al., 1999); perireticular nucleus (Mitrofanis and Baker, 1993; Métin and Godement, 1996; Molnár et al., 1998)]. This domain is defined by the expression of *Nkx2-1* and *netrin 1* (Tuttle et al., 1999), and seems to facilitate the extension of thalamic axons into the telencephalon by two different mechanisms: (1) by providing a growth-promoting environment to thalamic axons, which may consist at least in part of netrin 1 activity (Braisted et al., 2000); and (2) by providing an axonal scaffold through its transient projections to the dorsal thalamus (Mitrofanis and Baker, 1993; Métin and Godement, 1996; Molnár et al., 1998; Braisted et al., 1999; Tuttle et al., 1999). As this domain is located in the region of the basal telencephalon patterned by *Nkx2-1*, it was anticipated that these neurons would not be present in *Nkx2-1* mutants. However, a similar cell domain is present in *Nkx2-1* mutants, as identified by location, *netrin 1* expression and projections to the dorsal thalamus (O. M., J. B., L. P. and J. L. R. R., unpublished), suggesting that patterning of this cell domain is not dependent on *Nkx2-1* function. The correlation between the presence of these cells and normal pattern of thalamocortical projections in *Nkx2-1* mutants reinforces the notion that this cell domain may play an important role in the guidance of thalamocortical axons (Braisted et al., 1999; Tuttle et al., 1999; Braisted et al., 2000).

Analysis of *Nkx2-1* mutants shows that the hypothalamus is also a crucial region for the guidance of pyramidal tract axons. The hypothalamus, which contains a non-permissive activity for the growth of cortical axons, is normally avoided by pyramidal tract axons in their route toward the mesencephalon. The presence of this repulsive activity may be required to ensure that pyramidal tract axons turn appropriately into a longitudinal path roughly parallel to the alar/basal boundary, avoiding entering the basal hypothalamus. Interestingly, in vitro experiments suggested that the hypothalamus is also repulsive for thalamocortical axons, preventing them from growing excessively ventrally in their route towards the telencephalon (Braisted et al., 1999). Transformation of the hypothalamus in *Nkx2-1* mutants, however, does not impair thalamocortical projections, perhaps owing to the endurance of other repellent cues for thalamocortical axons in the mutant hypothalamus.

Guidance molecules underlying the patterning of cortical projections in the forebrain

As discussed above, corticofugal axons extend along several telencephalic and diencephalic domains in their route towards the midbrain, approaching some territories and avoiding others. These observations suggest that diffusible or contact-mediated guidance cues exist in several potential guidance decision points within the forebrain to control the pathfinding of cortical axons. The molecular nature of these signals is only beginning to be revealed.

The early steps in the guidance of neocortical axons, including both corticothalamic and pyramidal tract axons, appear to be controlled by common mechanisms. For example, two class 3 semaphorin proteins, Sema3a and Sema3c, regulate the initial extension of cortical axons toward the cortical white matter. Semaphorins appear to determine the initial direction of cortical axons through a complex mechanism involving repulsion from the marginal zone and attraction from the subventricular zone (Bagnard et al., 1998; Polleux et al., 1998). Corticofugal axons are subsequently attracted laterally towards the internal capsule by a mechanism that involves netrin 1 (Métin et al., 1997; Richards et al., 1997), which is prominently expressed in the mantle of the LGE and MGE (Serafini et al., 1996; Livesey and Hunt, 1997; Métin et al., 1997; Richards et al., 1997).

As shown here, a crucial decision point in the guidance of corticofugal fibers in located at the telencephalic/diencephalic boundary. Corticofugal fibers enter the peduncle systematically at the same location, and subsequently split into the corticothalamic and pyramidal tract projections. Recently, genetic analysis of mice carrying loss-of-function alleles for *Slit1*, *Slit2* or both genes has demonstrated that these proteins are involved in guiding the exit of corticofugal fibers from the telencephalon by preventing their entry into the basal telencephalon (Bagri et al., 2002). Loss of Slit1 function does not result in a profound abnormality in the guidance of corticofugal axons, whereas loss of Slit2 produces a phenotype that is rather similar to that found in *Nkx2-1* mutants. Thus, in *Slit2* mutant mice, corticofugal axons fail to enter the peduncle normally, and instead follow an abnormal course toward the surface of the telencephalon (Bagri et al., 2002). Of note, although *Slit2* is expressed in the ventricular zone, analysis of *Slit2* mutant mice suggests that Slit2 protein is likely to be localized in the mantle zone, where it can exert an effect on the guidance of cortical projections (Bagri et al., 2002). It has been proposed that Slit2 protein may become localized to the mantle zone by virtue of being expressed by radial glial cells, whose long processes may deliver Slit2 protein to more superficial regions (Bagri et al., 2002).

Two important differences exist between the phenotypes observed in *Slit2* and *Nkx2-1* mutant mice. First, a population of neocortical axons aberrantly crosses the midline at the level of the anterior commissure in *Slit2* mutants, indicating that Slit proteins are also involved in guidance across the midline (Bagri et al., 2002). This phenotype has not been observed in *Nkx2-1* mutant mice, where the misguided corticofugal axons stop ipsilaterally close to the midline but do not cross it. Second, although to a minor extent, corticothalamic projections also appear to follow an abnormal course in *Slit2* mutant mice (Bagri et al., 2002), they are normal in *Nkx2-1* mutants. Although further genetic experiments may be required to clarify these differences, the ventral expansion of *Slit1* expression in the telencephalon of *Nkx2-1* mutants could explain them. Thus, ectopic Slit1 expression at the midline of the basal telencephalon may prevent cortical axons from approaching the midline. Moreover, the increased levels of *Slit1* expression in the basal telencephalon may help corticothalamic axons to follow their normal path, but may not prevent pyramidal axons from taking an abnormal course. If this hypothesis were correct, these results would indicate that corticothalamic and pyramidal tract axons respond differentially to Slit1 and Slit2 or that both Slit proteins have different biological properties in vivo (e.g. different diffusion capabilities). In agreement with this notion, it has been suggested that different Slit cleavage fragments may have different cell association characteristics and therefore different extents of diffusion and binding properties in vivo (Brose et al., 1999). Accordingly, recent in vitro experiments have demonstrated that different Slit2 proteolytic fragments elicit different responses in different types of axons (Ba-Charvet et al., 2001; Battye et al., 2001; Chen et al., 2001).

Additional evidence suggesting that the guidance abnormalities found in *Nkx2-1* mutants are at least partially due to loss of Slit2 expression arises from the comparison of hypothalamic defects in *Nkx2-1* and *Slit2* mutants. As in the telencephalon, the hypothalamus of *Nkx2-1* mutants lacks *Slit2* and has an expanded *Slit1* expression domain. In both *Slit2* and *Nkx2-1* mutants, pyramidal tract fibers follow abnormal paths as they approach the hypothalamus (this study) (Bagri et al., 2002). Similarly, ascending dopaminergic projections make similar pathfinding errors as they course through the diencephalon in both *Slit2* and *Nkx2-1* mutants (this study) (Bagri et al., 2002). By contrast, thalamocortical fibers course ventrally into the hypothalamus of *Slit2* mutants (Bagri et al., 2002), a defect that was not observed in *Nkx2-1* mutants. It is possible that this abnormality may be compensated in *Nkx2-1* mutants by the increased levels of *Slit1* expression. In conclusion, the results obtained from the analysis of *Nkx2-1* and Slit mutants suggest that although Slit1 and Slit2 may be functionally redundant proteins in vitro, they most likely play different biological roles in the guidance of cortical and thalamic axons in vivo.

Although lack of Slit2 function in the basal telencephalon and hypothalamus of *Nkx2-1* mutants may be the primary cause of the abnormal guidance of pyramidal tract axons, additional factors may also contribute to this phenotype. Thus, a normal balance of attractive/permissive and repulsive/non-permissive cues may be required to determine the normal exit-point of pyramidal tract axons from the telencephalon and to ensure their normal transition through the forebrain (Fig. 9).

Nkx2-1 regulation of hypothalamic patterning

Previous studies on mice containing a targeted disruption of the *Nkx2-1* gene have demonstrated that loss of *Nkx2-1* function causes prominent morphological abnormalities in the basal telencephalon and hypothalamus (Kimura et al., 1996; Takuma et al., 1998). More recently, the molecular mechanisms that underlie the telencephalic defects observed in *Nkx2-1* mutant mice were determined (Sussel et al., 1999). Specifically, loss of *Nkx2-1* function results in the molecular repatterning of the MGE into a LGE-like tissue. Consequently,

derivatives from the telencephalic region patterned by *Nkx2-1* do not develop normally and, in turn, there is an expansion of the territories derived from the LGE, such as the striatum (Sussel et al., 1999). We have extended these observations and found that *Nkx2-1* plays a similar role in patterning the hypothalamus. Thus, *Nkx2-1* expression in the hypothalamic anlage is required to maintain molecular characteristics of the developing hypothalamus (e.g. *Nkx2-4* and *SF1* are reduced to a small region near the ventral midline) and to repress molecular characteristics of dorsal alar fates (e.g. *Pax6* and *Sim1* are expanded ventrally). The role of *Nkx2-1* in the specification of ventral fates in the hypothalamus is further supported by the described functions of other Nkx genes in the vertebrate central nervous system. For example, *Nkx2-2* or *Nkx6-1* mutant mice also have ventral to dorsal transformations (Briscoe et al., 1999; Sander et al., 2000). Moreover, the *Drosophila* homolog of *Nkx2-1*, *ventral nervous system defective* (*vnd*), is required to specify ventral column identity (Chu et al., 1998; McDonald et al., 1998; Weiss et al., 1998). Interestingly, although other genes of the Nkx family are expressed in the hypothalamus in a pattern almost identical to *Nkx2-1*, such as *Nkx2-4* (compare Fig. 1A with Fig. 2A), loss of *Nkx2-1* function is enough to alter the dorsoventral patterning of this region.

The internal capsule traverses the NKX2-1-positive regions of the MGE, demonstrating that *Nkx2-1* expression alone does not define the tissues that are non-permissive for these axons. Instead, a subdomain of the *Nkx2-1* region contains the repellent agent(s) (e.g. *Slit2*). Thus, it is probable that *Nkx2-1* alone does not directly regulate *Slit2*, but rather, that it contributes to the regulation of another transcription factor(s) that restrict *Slit2* expression to the AEP/POa region.

Concluding remarks

In the present study, we have identified two forebrain regions, the basal telencephalon and the hypothalamus, that play a crucial role in patterning one of the most prominent tracts in the brain, the pyramidal tract. The mechanisms that underlie the guidance of cortical axons through these two regions seem to be similar, suggesting that a single patterning event, mediated by the homeobox gene *Nkx2-1*, is necessary to establish the appropriate exit of pyramidal tract axons from the telencephalon, as well as their normal path through the extra-telencephalic forebrain. Furthermore, *Nkx2-1* patterning appears to be essential for the development of other fiber tracts that follow a course similar to the pyramidal tract axons, although in an opposite direction, such as the ascending dopaminergic mesotelencephalic projections. Thus, *Nkx2-1* function is required to ensure the precise expression of signals that control the guidance of some of the major afferent and efferent projections of the telencephalon.

We are very grateful to M. Henkemeyer and J. Frisen for providing the *EphB2/EphB3* and *ephrinA5* mutant mice, respectively, to A. Bagri for his help with the collagen co-culture experiments, and to M. Tessier-Lavigne, S. Garel, A. Bagri, A. Plump and S. Pleasure for comments and discussions. This work was supported by the research grants to J. L. R. R. from Nina Ireland, NARSAD, NIDA (R01DA12462) and NIMH (RO1 MH49428-01, RO1 MH51561-01A1 and K02 MH01046-01). O. M. is a NARSAD Young Investigator Award recipient and a UC Davis MIND Institute Scholar.

REFERENCES

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

Ba-Charvet, K. T., von Boxberg, Y., Guazzi, S., Boncinelli, E. and Godement, P. (1998). A potential role for the OTX2 homeoprotein in creating early 'highways' for axon extension in the rostral brain. *Development* **125**, 4273-4282.

Ba-Charvet, K. T., Brose, K., Ma, L., Wang, K. H., Marillat, V., Sotelo, C., Tessier-Lavigne, M. and Chédotal, A. (2001). Diversity and specificity of actions of Slit2 proteolytic fragments in axon guidance. *J. Neurosci.* **21**, 4281-4289.

Bagnard, D., Lohrum, M., Uziel, D., Püschel, A. W. and Bolz, J. (1998). Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. *Development* **125**, 5043-5053.

Bagri, A., Marín, O., Plump, A. S., Mak, Y., Pleasure, S. J., Rubenstein, J. L. R. and Tessier-Lavigne, M. (2002). Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. *Neuron* (in press).

Battye, R., Stevens, A., Perry, R. L. and Jacobs, J. R. (2001). Repellent signaling by Slit requires the leucine-rich repeats. *J. Neurosci.* **21**, 4290-4298.

Blakemore, C. and Molnár, Z. (1990). Factors involved in the establishment of specific interconnections between thalamus and cerebral cortex. *Cold Spring Harb. Symp. Quant. Biol.* **55**, 491-504.

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

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

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

Briscoe, J., Pierani, A., Jessell, T. M. and Ericson, J. (2000). A homeodomain protein code specifies progenitor cell identity and neural fate in the ventral neural tube. *Cell* **101**, 435-445.

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* **96**, 795-806.

Brose, K. and Tessier-Lavigne, M. (2000). Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. *Curr. Opin. Neurobiol.* **10**, 95-102.

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

Castellani, V., Chédotal, A., Schachner, M., Faivre-Sarrailh, C. and Rougon, G. (2000). Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. *Neuron* **27**, 237-249.

Chen, J. H., Wen, L., Dupuis, S., Wu, J. Y. and Rao, Y. (2001). The N-terminal leucine-rich regions in Slit are sufficient to repel olfactory bulb axons and subventricular zone neurons. *J. Neurosci.* **21**, 1548-1556.

Chu, H., Parras, C., White, K. and Jiménez, F. (1998). Formation and specification of ventral neuroblasts is controlled by vnd in Drosophila neurogenesis. *Genes Dev.* **12**, 3613-3624.

Cobos, I., Shimamura, K., Rubenstein, J. L. R., Martínez, S. and Puelles, L. (2001). Fate map of the avian anterior forebrain at the 4 somite stage, based on the analysis of quail-chick chimeras. *Dev. Biol.* (in press).

Cohen, N. R., Taylor, J. S., Scott, L. B., Guillery, R. W., Soriano, P. and Furley, A. J. (1998). Errors in corticospinal axon guidance in mice lacking the neural cell adhesion molecule L1. *Curr. Biol.* **8**, 26-33.

Dahme, M., Bartsch, U., Martini, R., Anliker, B., Schachner, M. and Mantei, N. (1997). Disruption of the mouse L1 gene leads to malformations of the nervous system. *Nat. Genet.* **17**, 346-349.

de Carlos, J. A. and O'Leary, D. D. (1992). Growth and targeting of subplate axons and establishment of major cortical pathways. *J. Neurosci.* **12**, 1194-1211.

Dottori, M., Hartley, L., Galea, M., Paxinos, G., Polizzotto, M., Kilpatrick,

T., Bartlett, P. F., Murphy, M., Köntgen, F. and Boyd, A. W. (1998). EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. *Proc. Natl. Acad. Sci. USA* **95**, 13248-13253.

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

Ghosh, A., Antonini, A., McConnell, S. K. and Shatz, C. J. (1990). Requirement for subplate neurons in the formation of thalamocortical connections. *Nature* **347**, 179-181.

Henkemeyer, M., Orioli, D., Henderson, J. T., Saxton, T. M., Roder, J., Pawson, T. and Klein, R. (1996). Nuk controls pathfinding of commissural axons in the mammalian central nervous system. *Cell* **86**, 35-46.

Jones, E. G. (1984). Laminar distribution of cortical efferent cells. In *Cerebral Cortex. Vol 1: Cellular Components of the Cerebral Cortex* (ed. A. Peters and E. G. Jones), pp. 521-553. New York: Plenum Press.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10**, 60-69.

Kohtz, J. D., Baker, D. P., Corte, G. and Fishell, G. (1998). Regionalization within the mammalian telencephalon is mediated by changes in responsiveness to Sonic Hedgehog. *Development* **125**, 5079-5089.

Kullander, K., Croll, S. D., Zimmer, M., Pan, L., McClain, J., Hughes, V., Zabski, S., DeChiara, T. M., Klein, R., Yancopoulos, G. D. et al. (2001a). Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. *Genes Dev.* **15**, 877-888.

Kullander, K., Mather, N. K., Diella, F., Dottori, M., Boyd, A. W. and Klein, R. (2001b). Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. *Neuron* **29**, 73-84.

Lauderdale, J. D., Pasquali, S. K., Fazel, R., van Eeden, F. J., Schauerte, H. E., Haffter, P. and Kuwada, J. Y. (1998). Regulation of netrin-1a expression by hedgehog proteins. *Mol. Cell. Neurosci.* **11**, 194-205.

Leighton, P. A., Mitchell, K. J., Goodrich, L. V., Lu, X., Pinson, K., Scherz, P., Skarnes, W. C. and Tessier-Lavigne, M. (2001). Defining brain wiring patterns and mechanisms through gene trapping in mice. *Nature* **410**, 174-179.

Liu, J. K., Ghattas, I., Liu, S., Chen, S. and Rubenstein, J. L. R. (1997). Dlx genes encode DNA-binding proteins that are expressed in an overlapping and sequential pattern during basal ganglia differentiation. *Dev. Dyn.* **210**, 498-512.

Livesey, F. J. and Hunt, S. P. (1997). Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and cerebellar development. *Mol. Cell. Neurosci.* **8**, 417-429.

Logan, C., Wizenmann, A., Drescher, U., Monschau, B., Bonhoeffer, F. and Lumsden, A. (1996). Rostral optic tectum acquires caudal characteristics following ectopic engrailed expression. *Curr. Biol.* **6**, 1006-1014.

Lumsden, A. G. and Davies, A. M. (1986). Chemotropic effect of specific target epithelium in the developing mammalian nervous system. *Nature* **323**, 538-539.

Marillat, V., Cases, O., Nguyen Ba-Charvet, K. T., Tessier-Lavigne, M., Sotelo, C. and Chédotal, A. (2001). Spatio-temporal expression patterns of slit and robo genes in the rat brain. *J. Comp. Neurol.* (in press).

Marín, O., Anderson, S. A. and Rubenstein, J. L. R. (2000). Origin and molecular specification of striatal interneurons. *J. Neurosci.* **20**, 6063-6076.

Matise, M. P., Lustig, M., Sakurai, T., Grumet, M. and Joyner, A. L. (1999). Ventral midline cells are required for the local control of commissural axon guidance in the mouse spinal cord. *Development* **126**, 3649-3659.

McDonald, J. A., Holbrook, S., Isshiki, T., Weiss, J., Doe, C. Q. and Mellerick, D. M. (1998). Dorsoventral patterning in the Drosophila central nervous system: the vnd homeobox gene specifies ventral column identity. *Genes Dev.* **12**, 3603-3612.

Métin, C. and Godement, P. (1996). The ganglionic eminence may be an intermediate target for corticofugal and thalamocortical axons. *J. Neurosci.* **16**, 3219-3235.

Métin, C., Deléglise, D., Serafini, T., Kennedy, T. E. and Tessier-Lavigne, M. (1997). A role for netrin-1 in the guidance of cortical efferents. *Development* **124**, 5063-5074.

Miller, B., Chou, L. and Finlay, B. L. (1993). The early development of thalamocortical and corticothalamic projections. *J. Comp. Neurol.* **335**, 16-41.

Mitrofanis, J. and Baker, G. E. (1993). Development of the thalamic reticular and perireticular nuclei in rats and their relationship to the course of growing corticofugal and corticopetal axons. *J. Comp. Neurol.* **338**, 575-587.

Molnár, Z. and Blakemore, C. (1995). How do thalamic axons find their way to the cortex? *Trends Neurosci.* **18**, 389-397.

Molnár, Z. and Cordery, P. (1999). Connections between cells of the internal capsule, thalamus, and cerebral cortex in embryonic rat. *J. Comp. Neurol.* **413**, 1-25.

Molnár, Z., Adams, R. and Blakemore, C. (1998). Mechanisms underlying the early establishment of thalamocortical connections in the rat. *J. Neurosci.* **18**, 5723-5745.

Olivier, C., Cobos, I., Pérez Villegas, E. M., Spassky, N., Zalc, B., Martínez, S. and Thomas, J. L. (2001). Monofocal origin of telencephalic oligodendrocytes in the anterior entopeduncular area of the chick embryo. *Development* **128**, 1757-1769.

Orioli, D., Henkemeyer, M., Lemke, G., Klein, R. and Pawson, T. (1996). Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. *EMBO J.* **15**, 6035-6049.

Polleux, F., Giger, R. J., Ginty, D. D., Kolodkin, A. L. and Ghosh, A. (1998). Patterning of cortical efferent projections by semaphorin-neuropilin interactions. *Science* **282**, 1904-1906.

Price, M., Lazzaro, D., Pohl, T., Mattei, M. G., Rüther, U., Olivo, J. C., Duboule, D. and Di Lauro, R. (1992). Regional expression of the homeobox gene Nkx-2.2 in the developing mammalian forebrain. *Neuron* **8**, 241-255.

Richards, L. J., Koester, S. E., Tuttle, R. and O'Leary, D. D. (1997). Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. *J. Neurosci.* **17**, 2445-2458.

Sander, M., Paydar, S., Ericson, J., Briscoe, J., Berber, E., German, M., Jessell, T. M. and Rubenstein, J. L. R. (2000). Ventral neural patterning by Nkx homeobox genes: Nkx6.1 controls somatic motor neuron and ventral interneuron fates. *Genes Dev.* **14**, 2134-2139.

Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C. and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87**, 1001-1014.

Shatz, C. J., Ghosh, A., McConnell, S. K., Allendoerfer, K. L., Friauf, E. and Antonini, A. (1990). Pioneer neurons and target selection in cerebral cortical development. *Cold Spring Harb. Symp. Quant. Biol.* **55**, 469-480.

Shigetani, Y., Funahashi, J. I. and Nakamura, H. (1997). En-2 regulates the expression of the ligands for Eph type tyrosine kinases in chick embryonic tectum. *Neurosci. Res.* **27**, 211-217.

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

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

Sussel, L., Marín, O., Kimura, S. and Rubenstein, J. L. R. (1999). Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. *Development* **126**, 3359-3370.

Takuma, N., Sheng, H. Z., Furuta, Y., Ward, J. M., Sharma, K., Hogan, B. L., Pfaff, S. L., Westphal, H., Kimura, S. and Mahon, K. A. (1998). Formation of Rathke's pouch requires dual induction from the diencephalon. *Development* **125**, 4835-4840.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-1133.

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

Weiss, J. B., Von Ohlen, T., Mellerick, D. M., Dressler, G., Doe, C. Q. and Scott, M. P. (1998). Dorsoventral patterning in the Drosophila central nervous system: the intermediate neuroblasts defective homeobox gene specifies intermediate column identity. *Genes Dev.* **12**, 3591-3602.

Wilson, S. W., Placzek, M. and Furley, A. J. (1993). Border disputes: do boundaries play a role in growth-cone guidance? *Trends Neurosci.* **16**, 316-323.

Wilson, S. W., Brennan, C., Macdonald, R., Brand, M. and Holder, N. (1997). Analysis of axon tract formation in the zebrafish brain: the role of territories of gene expression and their boundaries. *Cell Tissue Res.* **290**, 189-196.

Yokoyama, N., Romero, M. I., Cowan, C. A., Galvan, P., Helmbacher, F., Charnay, P., Parada, L. F. and Henkemeyer, M. (2001). Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. *Neuron* **29**, 85-97.

Yuan, W., Zhou, L., Chen, J. H., Wu, J. Y., Rao, Y. and Ornitz, D. M. (1999). The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. *Dev. Biol.* **212**, 290-306.