Development 126, 1903-1916 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV1390

Defects in thalamocortical axon pathfinding correlate with altered cell domains in *Mash-1*-deficient mice

Rebecca Tuttle[1,*], Yasushi Nakagawa[1,*], Jane E. Johnson[2] and Dennis D. M. O'Leary[1,‡]

[1]Molecular Neurobiology Laboratory, The Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA
[2]Center for Basic Neuroscience, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235, USA
*The first two authors contributed equally to this work
‡Author for correspondence (e-mail: dennis_oleary@qm.salk.edu)

Accepted 9 February; published on WWW 6 April 1999

SUMMARY

We have analyzed the pathfinding of thalamocortical axons (TCAs) from dorsal thalamus to neocortex in relation to specific cell domains in the forebrain of wild-type and *Mash-1*-deficient mice. In wild-type mice, we identified four cell domains that constitute the proximal part of the TCA pathway. These domains are distinguished by patterns of gene expression and by the presence of neurons retrogradely labeled from dorsal thalamus. Since the cells that form these domains are generated in forebrain proliferative zones that express high levels of *Mash-1*, we studied *Mash-1* mutant mice to assess the potential roles of these domains in TCA pathfinding. In null mutants, each of the domains is altered: the two *Pax-6* domains, one in ventral thalamus and one in hypothalamus, are expanded in size; a complementary *RPTPδ* domain in ventral thalamus is correspondingly reduced and the normally graded expression of *RPTPδ* in that domain is no longer apparent. In ventral telencephalon, a domain characterized in the wild type by *Netrin-1* and *Nkx-2.1* expression and by retrogradely labeled neurons is absent in the mutant. Defects in TCA pathfinding are localized to the borders of each of these altered domains. Many TCAs fail to enter the expanded, ventral thalamic *Pax-6* domain that constitutes the most proximal part of the TCA pathway, and form a dense whorl at the border between dorsal and ventral thalamus. A proportion of TCAs do extend further distally into ventral thalamus, but many of these stall at an aberrant, abrupt border of high *RPTPδ* expression. A small proportion of TCAs extend around the *RPTPδ* domain and reach the ventral thalamic-hypothalamic border, but few of these axons turn at that border to extend into the ventral telencephalon. These findings demonstrate that Mash-1 is required for the normal development of cell domains that in turn are required for normal TCA pathfinding. In addition, these findings support the hypothesis that ventral telencephalic neurons and their axons guide TCAs through ventral thalamus and into ventral telencephalon.

Key words: Axon guidance, Dorsal thalamus, Forebrain patterning, Globus pallidus, Hypothalamus, L1, Netrin-1, Nkx-2.1, Nkx-2.2, Pax-6, RPTPδ, Telencephalon, Transcription factors, Ventral thalamus

INTRODUCTION

Molecules on cell surfaces and in the extracellular matrix are expressed in stereotypic patterns that dictate growth patterns of axons during development (Tessier-Lavigne and Goodman, 1996). The direction and speed of axon growth as well as the degree of fasciculation are all thought to be either positively or negatively regulated by these axon guidance molecules. A major challenge in neural development is to define these molecules, the means by which they are expressed in stereotypic patterns and the ways they influence axon pathfinding.

The embryonic vertebrate brain is composed of domains that can be distinguished by morphology and patterns of gene expression (Puelles and Rubenstein, 1993; Papalopulu, 1995; Puelles, 1995; Lumsden and Krumlauf, 1996). Accumulating evidence suggests that these domains, and the borders between them, have functional consequences for axon guidance. For example, early axonal projections in the forebrain of zebrafish (Krauss et al., 1991; Macdonald et al., 1994), chick (Figdor and Stern, 1993) and mouse (Shimamura et al., 1995; Ba-Charvet et al., 1998) often correlate with gene expression domains and their borders. In addition, the pattern of retinal axon growth in embryonic rat and mouse forebrain correlates with the distribution of repulsive and stimulatory activities that appear to be restricted to expression domains of certain regulatory genes (Tuttle et al., 1998).

Recent studies provide experimental evidence supporting the assumption that regulatory genes control the patterned expression of axon guidance molecules. For example, in the zebrafish CNS, ectopic expression of Sonic Hedgehog induces the expression of the gene encoding Netrin-1a, which can act

as either an axon attractant or repellent (Lauderdale et al., 1998). In addition, ectopic expression of the *engrailed* genes, *En-1* and *En-2*, in the chick optic tectum induces the expression of the genes that encode the axon repellents, *ephrin-A2* and *ephrin-A5* (Logan et al., 1996; Shigetani et al., 1997), and influences the targeting of retinal axons that normally respond to these guidance molecules (Friedman and O'Leary, 1996; Itasaki and Nakamura, 1996).

One of the major axon tracts in the forebrain of rodents and higher mammals is formed by the thalamocortical axon (TCA) projection, which relays sensory information from the periphery to the neocortex. This projection originates from neurons of the dorsal thalamus, which in mice are generated between embryonic days (E) 10 and 13 (Angevine, 1970). By E14, the first TCAs have reached their main target, the neocortex (Bicknese et al., 1994). Enroute to the neocortex TCAs extend ventrally in the diencephalon, make a sharp turn to cross the diencephalic-telencephalic border, and then extend dorsolaterally through the ventral telencephalon towards the neocortex. While the resultant V-shaped projection, the nascent internal capsule, passes through several forebrain domains, it appears to encounter but avoid others. For example, in their ventral course through the diencephalon, TCAs turn laterally into the ventral telencephalon, rather than maintaining a straight ventral trajectory and thereby extending along the hypothalamic surface (Braisted et al., 1999). In vitro studies suggest that this pathfinding decision may be influenced by attractant and repellent activities expressed by the ventral telencephalon and hypothalamus, respectively (Braisted et al., 1999).

Other reports have described specific groups of cells in the forebrain that may help guide TCAs. In hamsters (Métin and Godement, 1996) and rats (Molnar et al., 1998), a small number of neurons in the ventral telencephalon can be retrogradely labeled from dorsal thalamus. These neurons appear to be part of a broader population recently described in mice, whose axonal projection coincides temporally and spatially with that of the oppositely extending TCAs (Braisted et al., 1999); thus, the projection of these ventral telencephalic axons could act as a scaffold to guide TCAs from dorsal thalamus into the ventral telencephalon. Another group has described a distinct population of cells in the internal capsule, which they assigned to the perireticular nucleus, and proposed to function as 'guidepost' cells for TCAs (Mitrofanis and Guillery, 1993). However, to date, there is no experimental evidence implicating either neuronal population in TCA pathfinding.

Mash-1, a basic helix-loop-helix transcription factor, is highly expressed in the proliferative zones of forebrain that give rise to the domains along the TCA pathway (Lo et al., 1991; Guillemot and Joyner, 1993; Porteus et al., 1994; Verma-Kurvari et al., 1996). *Mash-1* was first isolated as a mammalian homolog of *Drosophila* genes of the achaete-scute complex (AS-C) (Johnson et al., 1990), which are known to be involved in the development of subsets of neuroblasts (reviewed in Campuzano and Modolell, 1992; Jan and Jan, 1994). In mammals, it is expressed only in the nervous system, mainly in regions that contain dividing neuroepithelial cells (Lo et al., 1991; Guillemot and Joyner, 1993). Like AS-C, Mash-1 is required for the differentiation of subsets of neurons in the peripheral and central nervous system (Guillemot et al., 1993; Sommer et al., 1995; Cau et al., 1997; Hirsch et al., 1998).

These data led us to suspect that *Mash-1* is a critical regulator of the differentiation of cell domains that lie along the TCA pathway, and that mice deficient for *Mash-1* would exhibit defects in these domains, as well as corresponding defects in TCA pathfinding. To address this hypothesis, we analyzed TCA pathfinding in relation to cell domains in wild-type and *Mash-1*-deficient mice. We have identified several domains and molecules in wild-type mice that correlate with critical decision points in the TCA pathway. We find that, in *Mash-1*-deficient mice, these domains are absent or altered and TCA pathfinding is aberrant in a way that suggests that these defects are causally linked.

MATERIALS AND METHODS

Animals

The *Mash-1* mutant strain, in which the entire coding region of *Mash-1* has been deleted (Guillemot et al., 1993), was maintained by backcrossing to C57BL/6 mice. Heterozygote mice were mated to obtain wild-type, heterozygote and null mutant embryos. The day of insemination was designated E0; staging was confirmed by examining gross anatomical features of whole embryos (Butler and Juurlink, 1987).

For the DiI part of this study, all three genotypes were injected, examined and analyzed blind to the genotype. Since no obvious differences were noted between wild-type and heterozygote brains, only wild-type and null data are shown. The null mutant phenotypes described were apparent in all animals studied.

Anatomical and axial nomenclature

The axial nomenclature employed in this study is one that is common to studies at later developmental stages. Another nomenclature, used by Puelles (1995), reflects the true axes of forebrain at early stages of embryogenesis. In this study, sections were cut perpendicular to the base of forebrain and were referred to as 'coronal'; these sections would be defined as in-between coronal and horizontal by the other system of axial nomenclature.

In this paper, the term 'ventral telencephalon' refers to a region extending from the ventral surface of telencephalon to the lateral ventricle; this region includes medial, lateral and caudal ganglionic eminences (the ventricular and subventricular zones of ventral telencephalon), as well as structures that are ventral to and derived from them, such as the caudate-putamen, globus pallidus and amygdala.

DiI injections and analysis

Brains were fixed overnight by immersion in 4% paraformaldehyde (PFA). To anterogradely label the TCA projection, a midsagittal cut was made to separate the brain into two halves. For some brains (Figs 2-4), an additional coronal cut was made at the caudal edge of diencephalon, thereby revealing dorsal thalamus. The zona limitans intrathalamica (ZLI) that abuts dorsal and ventral thalamus provided a clear landmark for dye placement. A 0.2% solution of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; Molecular Probes) in dimethylformamide was pressure-injected into dorsal thalamus. Multiple injections were made just dorsal to the ZLI along the entire medial-to-lateral and rostral-to-caudal axes of dorsal thalamus, thereby creating a planar set of DiI injections with the objective of labeling as many TCAs as possible. To retrogradely label neurons in dorsal thalamus, a linear array of DiI injections was made along the anterior-posterior axis in the lateral part of E18 neocortex. Both types of DiI injections as well as the subsequent analyses were performed blind to the genotype; every attempt was made to make qualitatively and quantitatively equivalent injections in each set of the

three litter-matched mice (1 of each genotype). DiI-injected brains were left in fixative at 30°C for 2 weeks or longer, depending on the size of the brain. Brains were then sectioned coronally at 100 μm, counterstained with bisbenzimide (0.002% in sodium phosphate buffer; Sigma), and photographed under rhodamine (DiI) and ultraviolet (bisbenzimide) illumination.

In situ hybridization

The following digoxigenin-labeled RNA probes were used: *Mash-1* (rat full-length clone; a gift from Q. Ma and D. Anderson; Johnson et al., 1990), *Pax-6* (1121-1627 of rat *Pax-6*; GenBank accession # U69644; Stoykova et al., 1996), *Nkx-2.1* (7-627 of the rat *Nkx-2.1*; X53858; Lazzaro et al., 1991), *Nkx-2.2* (mouse full length clone; a gift from S. Bertuzi; Price et al., 1992), *RPTPδ (*3′UTR of the rat clone; a gift from L. Sommer and D. Anderson; Sommer et al., 1997), and *Netrin-1* (3′UTR of the mouse clone; a gift from M. Tessier-Lavigne; Serafini et al., 1996). *Pax-6* and *Nkx-2.1* clones were obtained by RT-PCR. Hybridization using rat probes identified the same cell populations in rat and mouse.

Embryo heads were immersion-fixed in 4% PFA/PB overnight, cryoprotected in sucrose/PB and frozen. Coronal, 20 μm sections were dried onto slides, postfixed, rinsed in PBS, treated for 3 minutes with 3 μg/ml Proteinase K, fixed again and rinsed. Sections were then acetylated in 0.25% (v/v) acetic anhydrate/0.1 M triethanolamine, permeabilized in 1% Triton X-100/PBS and rinsed. Prehybridization was done for 2 hours in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt solution, 250 μg/ml yeast RNA, 500 μg/ml herring sperm DNA), followed by hybridization for 14 hours at 72°C in 1 μg/ml digoxigenin-labeled RNA probe/hybridization buffer. Sections were washed for 1 hour at 72°C in 0.2× SSC, blocked in 10% lamb serum in 0.1 M Tris pH 7.5 and 0.15 M NaCl, and incubated overnight at 4°C in a 1:5000 dilution of alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim). After washing, the color reaction was carried out for 4-12 hours in a solution containing 0.175 mg/ml NBT, 0.35 mg/ml BCIP, 1.5 mM levamisole, 0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$ and 0.1% Tween 20. The reaction was terminated in TE pH 8. Sections were then counterstained with 1 μg/ml DAPI (Sigma) and mounted in Fluoromount G (EM Sciences).

Immunohistochemistry

Tissue was fixed, sectioned and postfixed as described above. Sections were incubated in 10% lamb serum/PBS blocking solution and then incubated overnight in a 1:2000 dilution of an anti-L1 rabbit polyclonal antibody (a gift from C. Lagenaur). Sections were rinsed in PBS, incubated for 1 hour in a 1:200 dilution of biotin-conjugated anti-rabbit IgG (Vector) and rinsed again. To quench endogenous peroxidase activity, sections were incubated in 0.5% H_2O_2/PBS, rinsed in PBS and then equilibrated in TBS. The avidin-biotin reaction (Vector) was followed using 0.05% DAB (in TBS containing 0.02% hydrogen peroxide) as a substrate. Sections were then rinsed in water, counterstained with DAPI, dehydrated, cleared and mounted in DPX (EM Sciences).

RESULTS

The TCA pathway extends through domains derived from *Mash-1*-expressing neuroepithelium

Previous studies have shown that Mash-1 protein and *Mash-1* mRNA are expressed in forebrain neuroepithelium (Lo et al., 1991; Guillemot and Joyner, 1993; Porteus et al., 1994; Verma-Kurvari et al., 1996) beginning around E9.5 (Guillemot and Joyner, 1993). To provide a more detailed analysis of *Mash-1* expression in forebrain as it pertains to TCA development, in situ hybridization was performed on sections of E12.5 (data not shown) and E13.5 (Fig. 1A-C,C′) forebrain, ages when TCAs are extending from dorsal thalamus to neocortex.

The TCA projection extends through ventral thalamus and ventral telencephalon enroute to neocortex. In E13.5 brains,

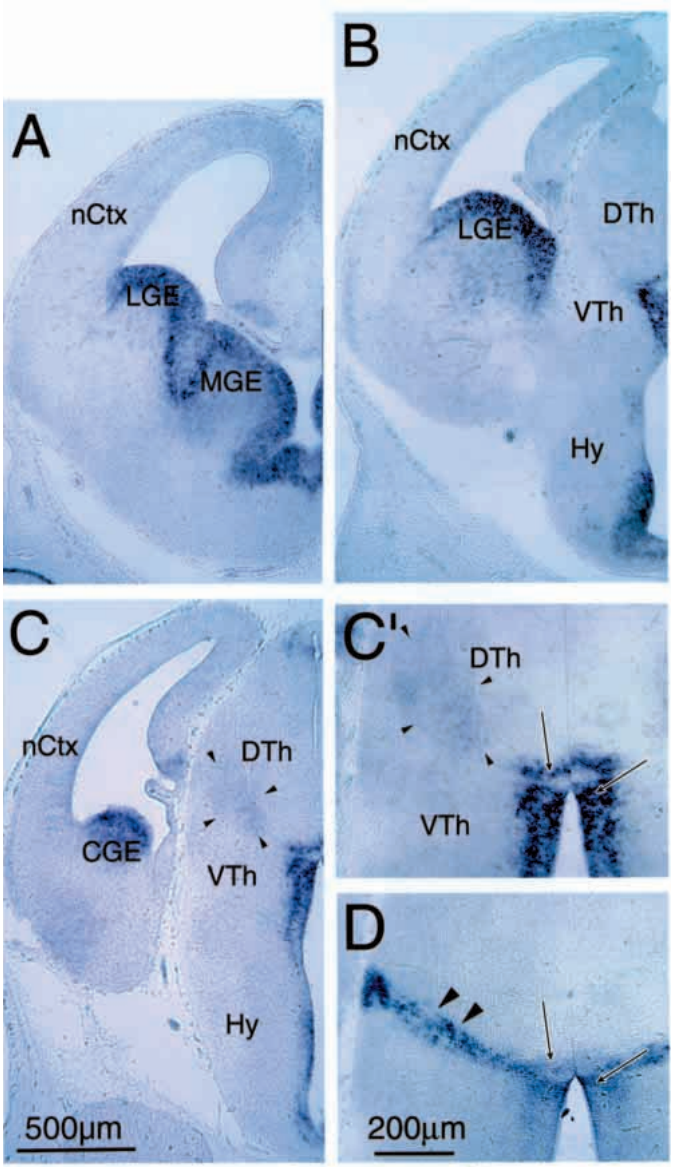

Fig. 1. TCAs extend through domains derived from *Mash-1*-expressing neuroepithelium. (A-C) A rostral (A) to caudal (C) series of coronal sections of E13.5 mouse showing *Mash-1* expression in the ventricular zone (VZ) of the medial (MGE), lateral (LGE) and caudal (CGE) ganglionic eminences, as well as the VZ of hypothalamus (Hy) and ventral thalamus (VTh). Expression in diencephalic VZ varies along the rostrocaudal and dorsoventral axes. The VZ of dorsal thalamus (DTh) does not express *Mash-1*. However, very low levels of expression are apparent in a region of DTh characterized by postmitotic neurons (arrowheads in C, and at higher magnification, C′). (D) *Nkx-2.2* expression on a section adjacent to C′ illustrating the *Nkx-2.2*-positive zona limitans intrathalamica (ZLI, double arrowheads). The VZ of the ZLI is *Mash-1*-positive (identically positioned arrows in C′,D). In this and all subsequent figures, dorsal is to the top and the midline is to the right. A, B and C are the same magnification, as are C′ and D. nCtx, neocortex.

Mash-1 was expressed in the ventricular zones (VZs) of ventral thalamus, hypothalamus and zona limitans intrathalamica (ZLI, the border between dorsal and ventral thalamus). It was also expressed in the VZs of the lateral, medial and caudal ganglionic eminences (LGE, MGE and CGE, respectively). Expression was lower in the subventricular zones (SVZs) of the LGE and MGE. Expression in neocortex was not detectable. *Mash-1* was also not detectable in the VZ of dorsal thalamus – in agreement with other studies that examined E11.5 and E12.5 mouse forebrain (Porteus et al., 1994; Verma-Kurvari et al., 1996). It was, however, expressed at low levels in the mantle zone of dorsal thalamus at E13.5 (Fig. 1C,C′), but not at E12.5 (data not shown). In all other regards, *Mash-1* expression patterns at E12.5 and E13.5 were similar. Thus, Mash-1 is likely to play a role in the differentiation of cells that lie along the TCA pathway, but is unlikely to directly affect the differentiation of cells in dorsal thalamus.

The TCA projection is severely disrupted in *Mash-1* mutant mice

To study the TCA projection in *Mash-1* mutants, DiI was injected into dorsal thalami of fixed E15 mice. The injections were localized to dorsal thalamus and were broad enough to label the majority of TCAs. In wild-type brains (Fig. 2A,A′; *n*=2 brains, 4 dorsal thalami injected), TCAs appear as a smooth, parallel array of fibers extending ventrolaterally from dorsal thalamus into ventral thalamus; as these axons approach hypothalamus, they change direction to exit diencephalon and extend dorsolaterally through ventral telencephalon into neocortex. In null mutants (Fig. 2B,B′,C,C′; *n*=3 brains, 6 dorsal thalami injected), the TCA projection is dramatically altered. In contrast to the smooth parallel array of TCAs evident in wild-type mice, the projection appears disorganized and a large number of TCAs accumulate at the dorsal surface of ventral thalamus (Fig. 2B,B′). While many TCAs extend into ventral thalamus, only a fraction of these continue along the diencephalic-telencephalic border as far as hypothalamus (Fig. 2C,C′). As in wild type, TCAs do not project into hypothalamus in null mutants. However, in striking contrast to the wild-type projection, labeled TCAs are not apparent in null mutant telencephalon.

By E14, the first TCAs have reached neocortex in wild-type mice. To determine whether TCAs eventually reach their neocortical target in *Mash-1* mutants, DiI was injected into E18 neocortex (null mutants die at birth; Guillemot et al., 1993) and dorsal thalamus was examined for the presence of retrogradely labeled neurons (for each genotype, *n*=1 brain, 2 neocortical hemispheres injected; data not shown). While a substantial number of dorsal thalamic neurons are retrogradely labeled in E18 wild-type brains, few are labeled in null mutant brains. These E15 and E18 DiI data reveal defects in the TCA projection at two points in *Mash-1* mutant forebrain: the dorsal thalamic-ventral thalamic border and the diencephalic–telencephalic border. As a consequence of these defects, and perhaps others as well, only a small fraction of the normal number of TCAs reaches neocortex by E18.

Since cell proliferation and migration can displace and distort axon bundles, the TCA projection at E15 may not reflect the initial trajectory taken by these axons. To examine the characteristics of the projection at earlier stages, DiI was injected into E13 dorsal thalamus (Figs 3, 4; *n*=2 brains, 4

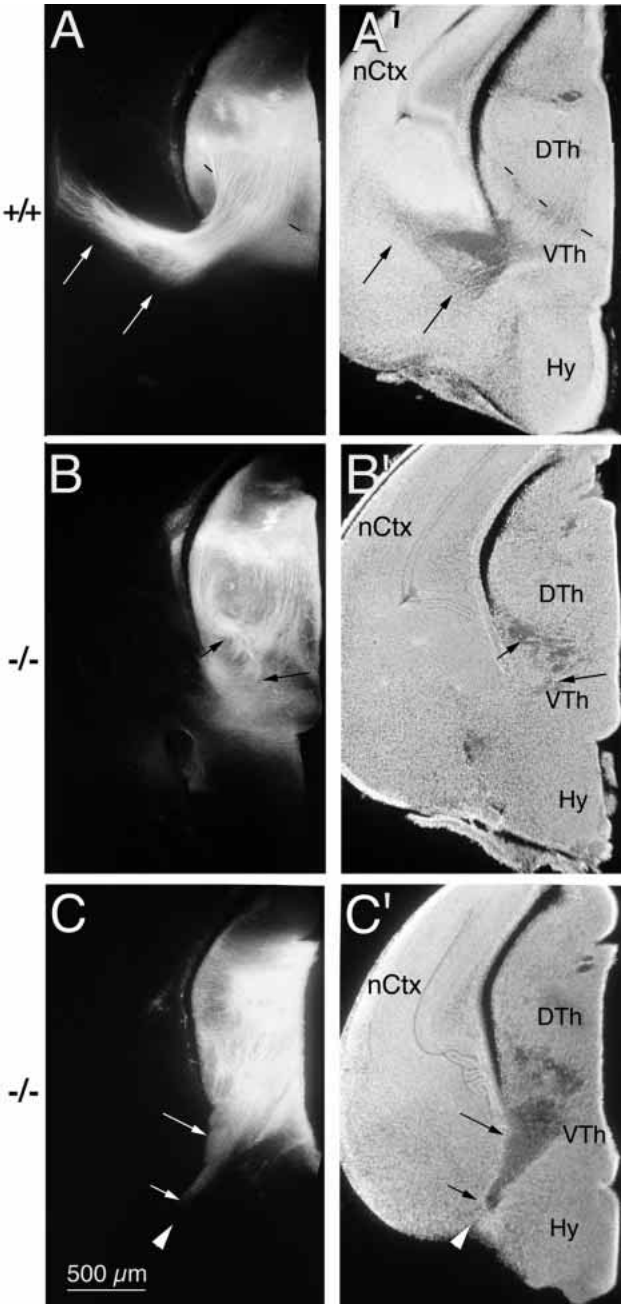

Fig. 2. Dramatic aberrancies in TCA pathfinding in E15 *Mash-1* mutant mice. In this brain, as well as those in Figs 3 and 4, dorsal thalami of fixed brains were filled with DiI (see Materials and Methods); after a couple of weeks, injected brains were sectioned coronally at 100 μm and photographed to reveal either DiI-labeled cells and processes (A,B,C) or bisbenzimide, nuclear counterstain (A′,B′,C′). Sections A and B are from similar levels; section C is further caudal. In wild-type brains (A,A′), TCAs exit DTh ventrolaterally, crossing the ZLI (dashed lines) to enter VTh. From VTh, they turn to extend laterally and dorsally through ventral telencephalon (arrows) to neocortex (nCtx). In null mutant brains (B,B′,C,C′), many TCAs appear not to enter VTh, but instead remain near the ZLI (short arrows in B,B′). Some TCAs project into VTh (e.g. long arrows in B,B′,C,C′); of these, a fraction projects further ventrally along the diencephalic-telencephalic border (short arrow in C,C′). In adjacent sections (not shown), axons are apparent at this border very near the ventral surface of forebrain (arrowhead). No TCAs were detectable in ventral telencephalon.

dorsal thalami injected for each genotype), the age when TCAs have just extended into ventral telencephalon in wild-type mice. At E13, in the dorsal part of ventral thalamus, the TCA projection is relatively unfasciculated and extends through the parenchyma (Fig. 3C). However, unlike the projection at E15, TCAs extend in a tight, straight, fascicle along the lateral surface of the ventral part of ventral thalamus to the ventral thalamic-hypothalamic-ventral telencephalic border (arrows in Fig. 3C,C′); at this point, the axons turn sharply and, unlike the E15 projection, extend in a rostral, as well as dorsolateral direction through ventral telencephalon (Fig. 3A,B). The TCA projection in E13 *Mash-1* mutants shows similar defects as at E15 (Fig. 4B,C): TCAs are not apparent in ventral telencephalon, and the TCA fascicle on the lateral surface of the ventral part of ventral thalamus is much smaller than in wild-type brains but still does not extend further ventrally than the ventral thalamic-hypothalamic border (Fig. 4C).

These E13 wild-type data demonstrate that TCAs do not turn gradually from ventral thalamus into the dorsal-most part of the mantle zone of ventral telencephalon as they appear to do in E15 embryos, but instead assume a straight, ventral trajectory to the ventral thalamic-hypothalamic border where they make an angular turn into the ventral-most part of ventral telencephalon. Once in ventral telencephalon, the axons track along a path that extends rostrally – as well as laterally and dorsally. On the contrary, the E13 null mutant data reinforce the E15 findings and further suggest that TCAs do not transiently extend into ventral telencephalon.

The TCA projection was also examined in sections of E13.5 diencephalon immunolabeled with an antibody to L1, a cell adhesion molecule (Kamiguchi and Lemmon, 1997; Brummendorf et al., 1998) expressed on TCAs (Fukuda et al., 1997) and other axons in diencephalon (Fig. 5 and data not shown). L1 immunolabeling has two advantages over DiI labeling: it is thought to label all TCAs and it reveals features within dorsal thalamus that are obscured by the intense fluorescence associated with DiI injection sites. In wild-type brains, L1 and DiI-labeled projections look similar (Fig. 5A,A′). L1-positive axons form a smooth projection of parallel fibers that are relatively unfasciculated within dorsal thalamus, are slightly more fasciculated in the dorsal part of ventral thalamus, and then gradually converge and form a tight fascicle on the lateral

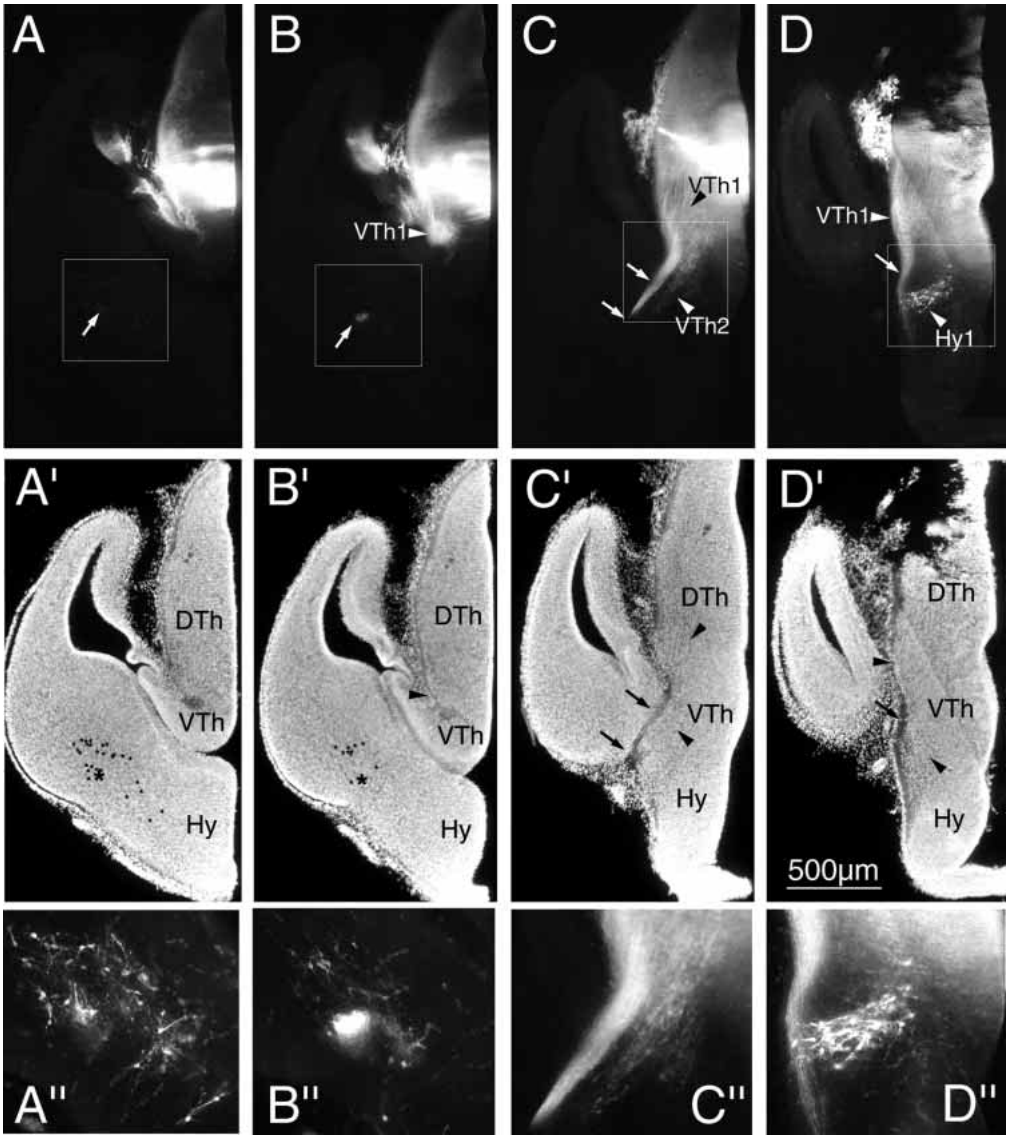

Fig. 3. Developing dorsal thalamic/habenular afferents and efferents in E13 wild-type forebrain. A rostral-to-caudal (A-D) series of 100 μm sections from a single brain photographed to reveal cells and processes labeled by DiI injections into DTh (A-D), bisbenzimide counterstain (A′-D′), or higher magnifications of boxed areas in A-D (A″-D″; all same magnification); in this series of serial sections, one section, between B and C, and a second, between C and D, are not shown. Anterogradely labeled TCAs can be seen coursing ventrally and laterally in diencephalon (C). Within VTh, TCAs are more fasciculated in the ventral part (short arrows in C,C′) where they course along the lateral surface, extending only as far as the hypothalamic border (bottom arrow in C,C′). At this point, TCAs extend laterally into ventral telencephalon (short arrows in A,B, or identically positioned asterisks in A′,B′). Another, more caudal projection (short arrows in D,D′) is likely to be composed mainly of retrogradely labeled axons. Four domains of neurons were retrogradely labeled by the DiI injections: two ventral thalamic (VTh1 and VTh2), one hypothalamic (Hy1; arrowheads in B-D and B′-D′), and one ventral telencephalic (VTel1; dots in A′,B′). TCAs extend through VTh1 and VTel1, but not VTh2 and Hy1.

surface of the ventral part of ventral thalamus. In *Mash-1* mutants, many L1-positive axons form a dense knot between the dorsal thalamus and ventral thalamus (Fig. 5B,B′). This abnormal axonal formation is reminiscent of the Probst bundles seen in animals that fail to form a corpus callosum (Ozaki and Wahlsten, 1993). Ventral to the Probst-like bundle, in the dorsal part of ventral thalamus, the L1-positive axon projection is much more fasciculated than in the wild type. In addition, rather than narrowing gradually to a single fascicle at the ventrolateral surface of ventral thalamus, the L1-positive axon projection narrows at a discrete point – between the dorsal and ventral halves of ventral thalamus. Those L1-positive axons that extend past this point are in a single fascicle that is considerably narrower than the one in the wild type.

In summary, DiI axonal tracing and L1 immunohistochemistry reveal that the TCA projection in *Mash-1* mutants is disrupted at least at three discrete points along its pathway: the dorsal thalamic-ventral thalamic border, the border between the ventral and dorsal halves of ventral thalamus, and the diencephalic-telencephalic border.

A ventral telencephalic projection to dorsal thalamus is missing in *Mash-1* mutants

In addition to anterogradely labeling TCAs, DiI injections into dorsal thalamus retrogradely label axons and cell bodies of neurons projecting to dorsal thalamus, or through it, to the more dorsally located habenula, pretectum and superior colliculus. The largest contribution of afferents to dorsal thalamus is from neocortex. However, neocortical axons do not reach dorsal thalamus until about E14.5 (data not shown); at E13.5 they have not yet reached diencephalon. Globus pallidus neurons, which reside in the medial part of ventral telencephalon, project to both dorsal thalamus and habenula. In ventral thalamus, the reticular thalamic nucleus and zona incerta project to dorsal thalamus (Ricardo, 1981; Jones, 1985), and the ventral lateral geniculate nucleus projects to pretectum and superior colliculus (Jones, 1985). Within hypothalamus, the entopeduncular nucleus projects to both dorsal thalamus and habenula (van der Kooy

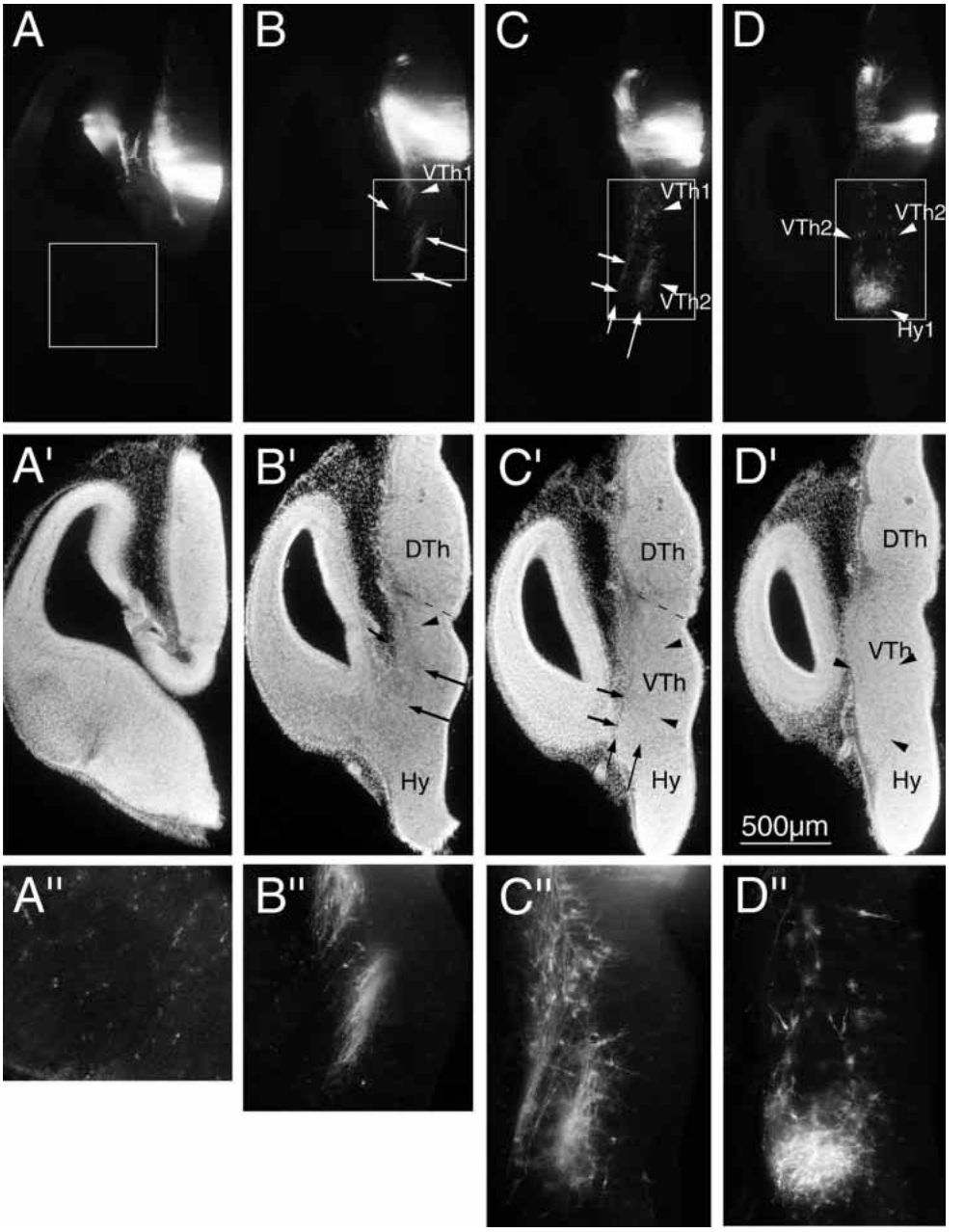

Fig. 4. TCA pathfinding is perturbed and cell domains are altered in E13 *Mash-1* mutant forebrain. A rostral-to-caudal (A-D) series of 100 μm sections from a single brain, a littermate of the wild-type brain in Fig. 3; in this series of serial sections, one section, between A and B, is not shown. Photographs reveal cells and processes labeled by DiI injections into DTh (A-D), bisbenzimide counterstain (A′-D′), or higher magnifications of boxed areas in A-D (A″-D″). A bundle of DiI-labeled axons is apparent coursing along the lateral surface of VTh (short arrows in B,B′,C,C′) to the VTh-Hy border (bottom short arrow in C,C′). A second, more medial axon fascicle (long arrows in B,B′,C,C′) is mainly retrogradely labeled axons from neurons in domain, Hy1; this bundle is much smaller in wild-type brains. No retrogradely labeled neurons, or anterogradely labeled axons are apparent in null mutant ventral telencephalon (A,A″). In contrast, the three populations of diencephalic neurons that were retrogradely labeled in the wild type were also labeled in the null mutant (arrowheads in B-D and B′-D′). Population Hy1 is expanded in size. Most DiI-labeled axons course over the ZLI (dashed line) at its extreme lateral edge; in contrast, in the wild-type retrogradely labeled axons from VTh1, for example, course straight dorsally into DTh (see Fig. 3C,D).

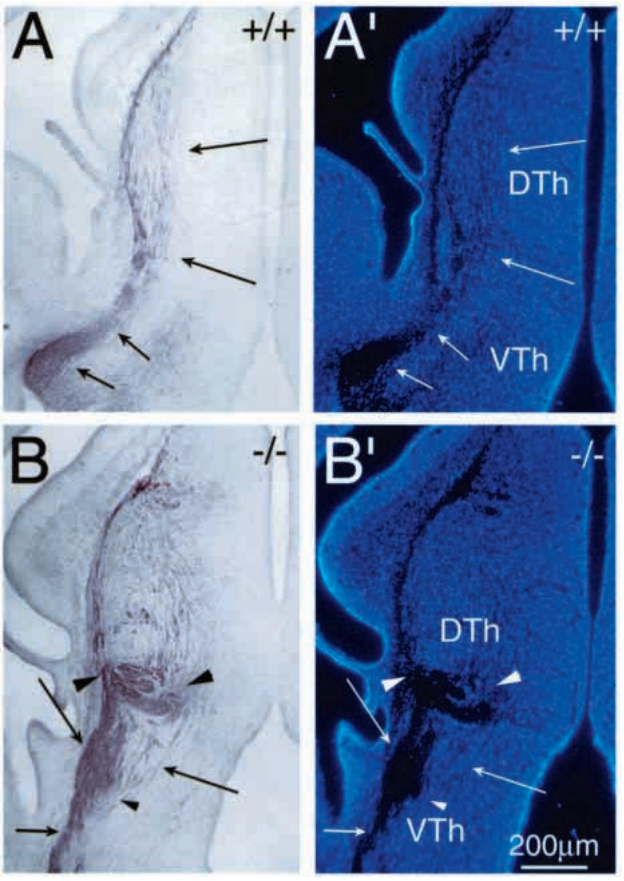

Fig. 5. TCA pathfinding defects in *Mash-1* mutant diencephalon detected by L1 immunostaining. Coronal sections of E13.5 wild-type (A,A′) and null mutant (B,B′) diencephalon immunolabeled with an antibody to L1 (A,B), or DAPI counterstained (A′,B′). In the wild type, L1-positive axons extend ventrally in smooth, parallel arrays (long arrows) that are least fasciculated in DTh, slightly more fasciculated in the dorsal part of VTh and then gradually narrow to form a single dense fascicle at the lateral surface of the ventral half of VTh (short arrows in A,A′). In null mutants, many L1-positive axons accumulate in a dense, acellular knot at the border between VTh and DTh (arrowheads in B,B′). In the dorsal half of VTh, L1-positive axons are much more fasciculated than in the wild type (long arrows in B,B′). At a point midway along the dorsal-ventral axis of VTh, L1-positive axons appear to stall (small arrowheads in B,B′). On the lateral surface of the ventral half of VTh, the L1-positive axon fascicle is much smaller than the one in the wild type (small arrow in B,B′).

and Carter, 1981). Thus, depending on the developmental timing of these projections, neurons in these nuclei could be retrogradely labeled by DiI injections into E13 dorsal thalamus.

In the present study, DiI injections into E13 dorsal thalamus retrogradely label neurons in four domains in diencephalon and ventral telencephalon (Fig. 3; Table 1). Two of these were in ventral thalamus and were thus designated VTh1 and VTh2 (Fig. 3B,C,C″); the border between VTh1 and 2 was obscured by axonal labeling. A broad domain in ventral telencephalon, VTel1, extends to the diencephalic-telencephalic border and is likely to be globus pallidus (Fig. 3A″,B″). The fourth domain, Hy1, is in the dorsolateral part of hypothalamus. The TCA projection extends through VTh1 and VTel1. VTh2 lies outside but immediately adjacent to the TCA pathway; in the wild type, Hy1 is close, but not adjacent to the TCA pathway. In null mutant animals, retrogradely labeled neurons are not observed in ventral telencephalon (Fig. 4A,A″). However, the retrogradely labeled neurons in the three diencephalic domains are apparent. In fact, domain Hy1 is actually expanded in size and extends further dorsally than in the wild type (Fig. 4D,D″; e.g. compare Figs 3D and 4D). Because the VTh1-VTh2 border is not clear in the wild-type DiI data, it is not possible to determine with certainty whether the size of these domains is altered.

These in vivo data describe four domains of neurons that are retrogradely labeled from dorsal thalamus. The cell bodies and axons of neurons of two of these domains, VTel1 and VTh1, lie within the TCA pathway, and may therefore provide a growth substrate for TCAs. The null mutant data supports this hypothesis: retrogradely labeled neurons in VTel1 are missing in null mutants, resulting either from their loss or failure to project into dorsal thalamus. Therefore, the failure of TCAs to project into ventral telencephalon, as well as the observed TCA pathfinding defects within ventral thalamus, could result from changes to these neurons or their axonal projection, respectively.

Ventral telencephalic cell domain that projects to dorsal thalamus is absent in *Mash-1* mutants

The data presented above suggest that, in *Mash-1* mutants, changes in a domain of retrogradely labeled neurons in ventral telencephalon might mediate defects in TCA pathfinding. To further characterize this domain, we examined the expression of *Nkx-2.1* and *Netrin-1* in E13.5 brains (Figs 6, 7; Table 1). *Nkx-2.1* encodes a homeodomain-containing transcription factor (Guazzi et al., 1990). In agreement with previous studies (Shimamura et al., 1995; Olsson et al., 1997; Kohtz et al., 1998), *Nkx-2.1* is expressed in MGE, but not in LGE in wild-type brains (Fig. 6A-C); within the mantle zone of ventral telencephalon, *Nkx-2.1* is expressed in the globus pallidus, which is likely to be derived from MGE (Olsson et al., 1997), and surrounding scattered cells (Fig. 6B,C). The *Nkx-2.1* expression domain within the mantle zone and the domain, VTel1, of retrogradely labeled ventral telencephalic neurons appear to coincide (compare Figs 6C and 3A′,B′). *Netrin-1* encodes a secreted protein that can have positive effects on axon growth in vitro (Kennedy et al., 1994; Serafini et al., 1994; Métin et al., 1997; Richards et al., 1997) and in vivo (Serafini et al., 1996; Mitchell et al., 1996). In agreement with previous studies (Métin et al., 1997), we show that *Netrin-1* is expressed throughout the mantle zone of ventral telencephalon (Fig. 6D). In addition, our data demonstrate that, like the retrogradely labeled ventral telencephalic neurons, these *Nkx-2.1* and *Netrin-1* expression domains extend caudally to the diencephalic-telencephalic border, and are associated with the TCA projection both at the border (Fig. 7A,A′,B) and within ventral telencephalon (Fig. 7E). The *Nkx-2.1* and *Netrin-1* expression patterns at E13 (data not shown) are the same as those seen at E13.5. These data demonstrate that, upon entry into ventral telencephalon, TCAs encounter the caudal tip of the *Nkx-2.1*-positive, VTel1 domain, and then take a lateral and rostral path through it before entering the more laterally located *Nkx-2.1*-negative striatum.

In *Mash-1* mutants, the *Nkx-2.1*-positive globus pallidus is not apparent (Fig. 6F,G). However, *Nkx-2.1*-positive MGE and MGE-derived mantle zone are still apparent (Fig. 6 E-G), although considerably shrunken (compare Fig. 6A-C with 6E-G). *Netrin-1* expression is not detected in the mantle zone in the medial part of ventral telencephalon (Fig. 6H). Importantly, *Nkx-2.1* and *Netrin-1* are also not detectable at the diencephalic-telencephalic border where TCAs normally enter ventral telencephalon (Fig. 7C,D). The diminished expression of these markers, and the reduced size of the MGE-derived mantle zone, suggest that Mash-1 is required for the differentiation of these cells, perhaps including the neurons whose axons project into dorsal thalamus. These findings, combined with the mutant TCA phenotype, support the hypothesis that the MGE-derived cells that lie within and surround the TCA pathway in the medial part of ventral telencephalon are required for TCAs to turn sharply, cross the diencephalic-telencephalic border, and extend through the medial part of ventral telencephalon.

Alterations in *Pax-6* expression domains in *Mash-1* mutant diencephalon correlate with defects in the TCA projection

We show that, in *Mash-1* mutants, many TCAs form a knot at the dorsal surface of ventral thalamus. We therefore hypothesized that either the ZLI or ventral thalamic cell populations immediately ventral to it are altered in the absence of Mash-1 protein. This hypothesis is supported by *Mash-1* in situ data (Fig. 1; also see Porteus et al., 1994; Verma-Kurvari et al., 1996). To test this hypothesis, we used in situ hybridization on sections of E13.5 brains to identify genes expressed in the ZLI or ventral thalamus. Three genes, *Nkx-2.2*, *Pax-6* and *RPTPδ*, met these criteria (Fig. 8; Table 1). The *RPTPδ* expression data are described in the following section. *Nkx-2.2* encodes a homeodomain-containing transcription factor (Price et al., 1992), and *Pax-6* encodes a transcription factor that has both a homeodomain and a paired domain (Walther and Gruss, 1991). Cells of the ZLI express *Nkx-2.2* (Price et al., 1992; Kitamura et al., 1997). In lateral

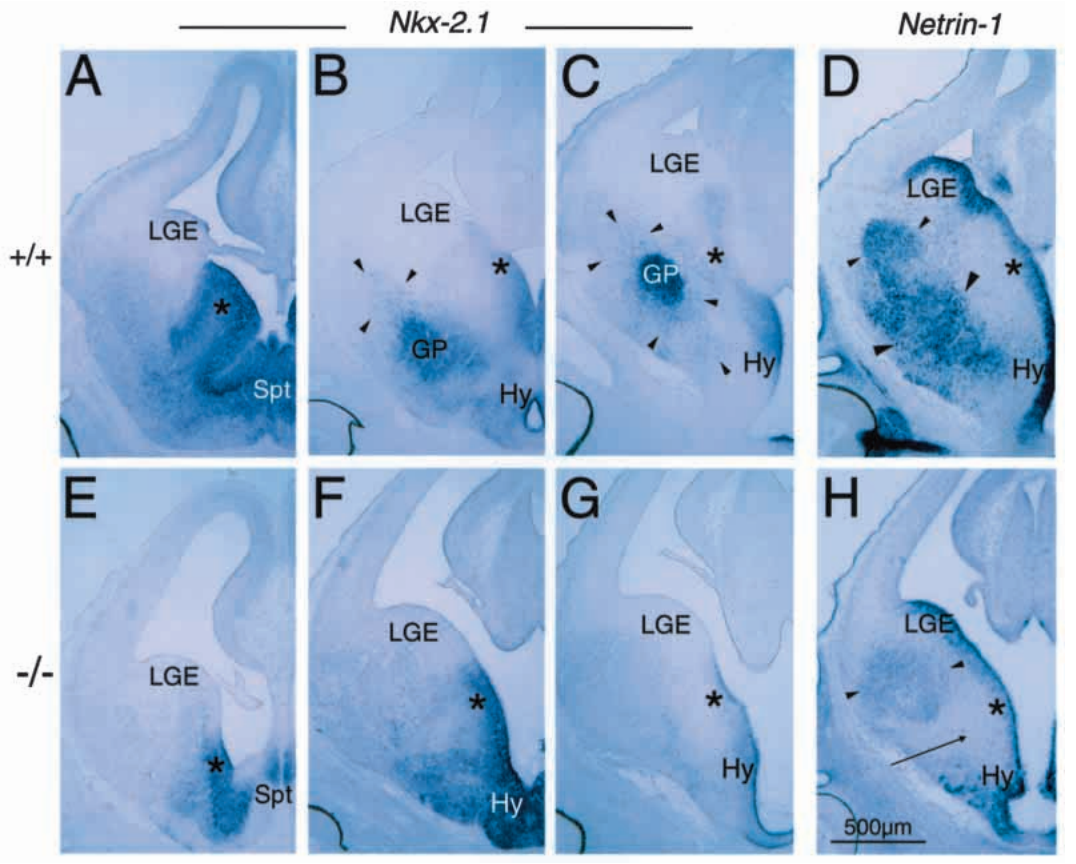

Fig. 6. *Nkx-2.1*- and *Netrin-1*-expressing ventral telencephalic cells in the TCA pathway appear to be missing in *Mash-1* mutants. *Nkx-2.1* in situ hybridization in a rostral-to-caudal series of coronal sections of E13.5 wild-type (A-C) and null mutant (E-G) forebrain. (D,H) *Netrin-1* in situ hybridization in wild-type (D) and null mutant (H) forebrain. In wild-type ventral telencephalon, *Nkx-2.1* is expressed in the VZ and SVZ of MGE (asterisks) and septum (Spt), but not in LGE. In addition, *Nkx-2.1* is expressed in the mantle zone of MGE, particularly in the globus pallidus (GP); this expression domain is characterized by a central region where most, if not all, cells are *Nkx-2.1*-positive and a surrounding region of scattered *Nkx-2.1*-positive cells (arrowheads in B,C). In the wild type, *Netrin-1* is expressed throughout the mantle zone of ventral telencephalon in a domain localized laterally beneath the LGE (small arrowheads) and in a larger domain, with patchy expression (large arrowheads), located beneath MGE (asterisk). *Netrin-1* is also expressed in the VZs of LGE, MGE and Hy. In the null mutant, the *Nkx-2.1*-positive GP is not apparent and the MGE is reduced in size. Also, *Netrin-1* expression is not detectable in the mantle zone underlying the medial part of MGE (long arrow), but is detectable laterally in what appears to be striatum (small arrowheads). Sections A-C and E-G are each 100 μm apart; section D and H are approximately at the same level as B and F, respectively.

diencephalon, we identified two *Pax-6* expression domains: one in dorsolateral ventral thalamus just ventral to the ZLI and the second in dorsolateral hypothalamus (Fig. 8B). These two domains correspond to the retrogradely labeled neuron domains, VTh1 and Hy1, respectively, and are therefore referred to by the same nomenclature (compare Fig. 8B and D). Within domain VTh1, *Pax-6* expression is high ventrally (VTh1-v) and low dorsally (VTh1-d).

In *Mash-1* mutants, the ZLI appears as a discontinuous array of *Nkx-2.2*-positive cells (Fig. 8E); the largest of these discontinuities coincides with the position of the Probst-like bundle described above. These disruptions could be a secondary effect of the aberrant accumulation of TCAs. Also in null mutants, *Pax-6* domains, VTh1 and Hy1, are expanded along the dorsoventral axis (compare Fig. 8B and 8F); the expansion of domain Hy1 in null mutants correlates with the increased number of retrogradely labeled neurons in this domain (compare Figs 3D and 4D, and also Fig. 8D and H). Within the dorsal aspect of VTh1, the level of *Pax-6* expression is much higher than in wild type. These results indicate that in the absence of Mash-1 protein, domain VTh1 is altered such that many TCAs are unable to project into it.

Alterations in *RPTPδ* expression domains in *Mash-1* mutant diencephalon correlate with defects in TCA pathfinding

Another ventral thalamic marker used in this study is *RPTPδ*, which codes for a transmembrane receptor-type protein tyrosine phosphatase (Krueger et al., 1990; Mizuno et al., 1993) expressed in embryonic diencephalon and neocortex (Sommer et al., 1997). In E13.5 wild-type ventral thalamus, *RPTPδ* is expressed immediately medial to the TCA fascicle, in domain VTh2 and weakly in the medial part of VTh1 (Fig. 8C). *RPTPδ* expression is graded, with a high ventral to low dorsal distribution. Interestingly, TCA fasciculation increases in parallel with *RPTPδ* expression levels (compare Fig. 8B′ and C). In the ventral half of VTh2, where *RPTPδ* expression levels are highest, the TCAs are in a single, thick fascicle located at the lateral surface of the domain. *RPTPδ* and *Pax-6* expression are complementary (compare Fig. 8B and C). The caudal ZLI is also positive for *RPTPδ* (data not shown); however, at more rostral levels, including the region where TCAs cross the ZLI and grow into ventral thalamus, the ZLI is not *RPTPδ*-positive (Fig. 8C).

RPTPδ expression is altered in null mutants (Fig. 8G). As

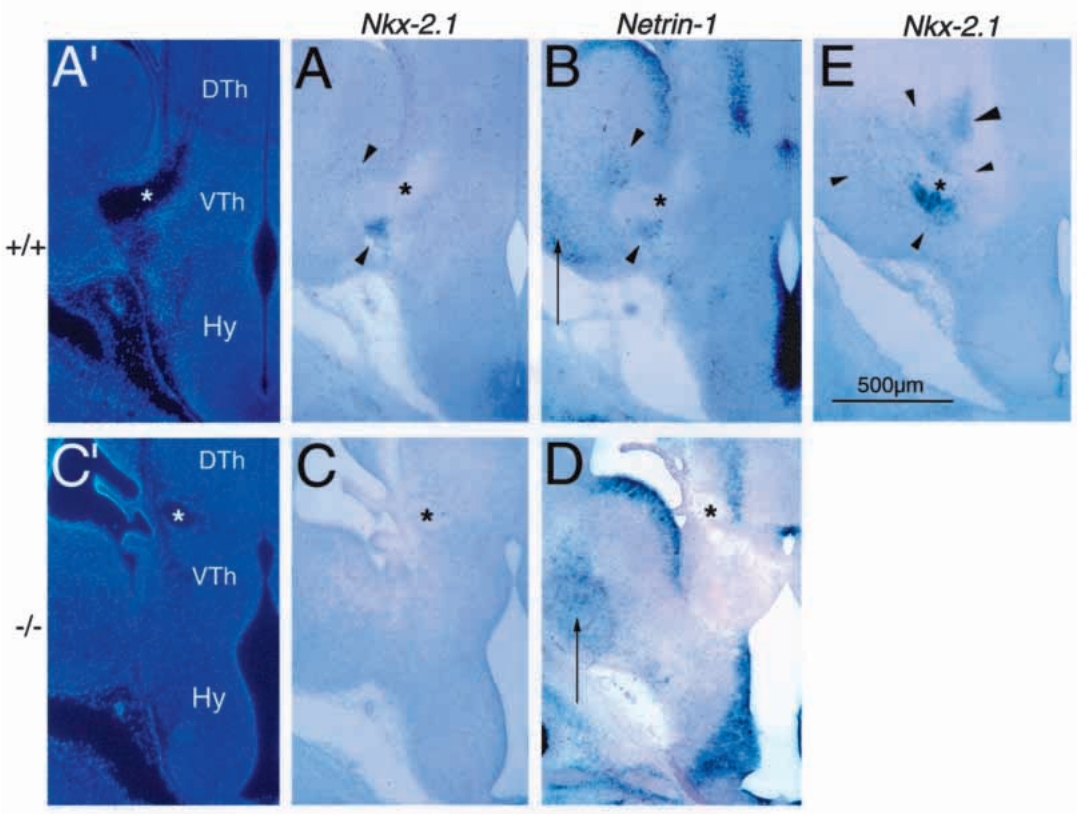

Fig. 7. The *Nkx-2.1*- and *Netrin-1* expression domain, VTel1, extends to the diencephalic border, is associated with TCAs and is not detectable in *Mash-1* mutants. *Nkx-2.1* (A,C,E) and *Netrin-1* (B,D) in situ hybridization on coronal sections of E13.5 wild-type (A,B,E) and null mutant (C,D) forebrain. A′ and C′ are DAPI counterstaining of A and C, respectively. All of these sections are caudal to those shown in Fig. 6; E is from a level between Fig. 6C and Fig. 7A. In wild-type ventral telencephalon, the caudal tip of the *Nkx-2.1*- and *Netrin-1*-positive expression domain VTel1 (illustrated in Fig. 6), is associated with the TCA bundle (asterisk in A,A′,B) at the diencephalic-telencephalic border (arrowheads in A,B). In *Mash-1* mutants, this *Nkx-2.1*-, *Netrin-1*-positive, internal capsule-associated domain is not apparent (C,D, respectively). In contrast, *Netrin-1* is expressed in the more laterally located striatum (long arrow). (E) After entering ventral telencephalon, the TCA projection extends rostrally and laterally through the rostral expansion of the *Nkx-2.1*-positive domain (small arrowheads) that underlies *Nkx-2.1*-positive MGE (large arrowhead). Asterisks in C,C′ and D indicate the position of the Probst-like axon bundle.

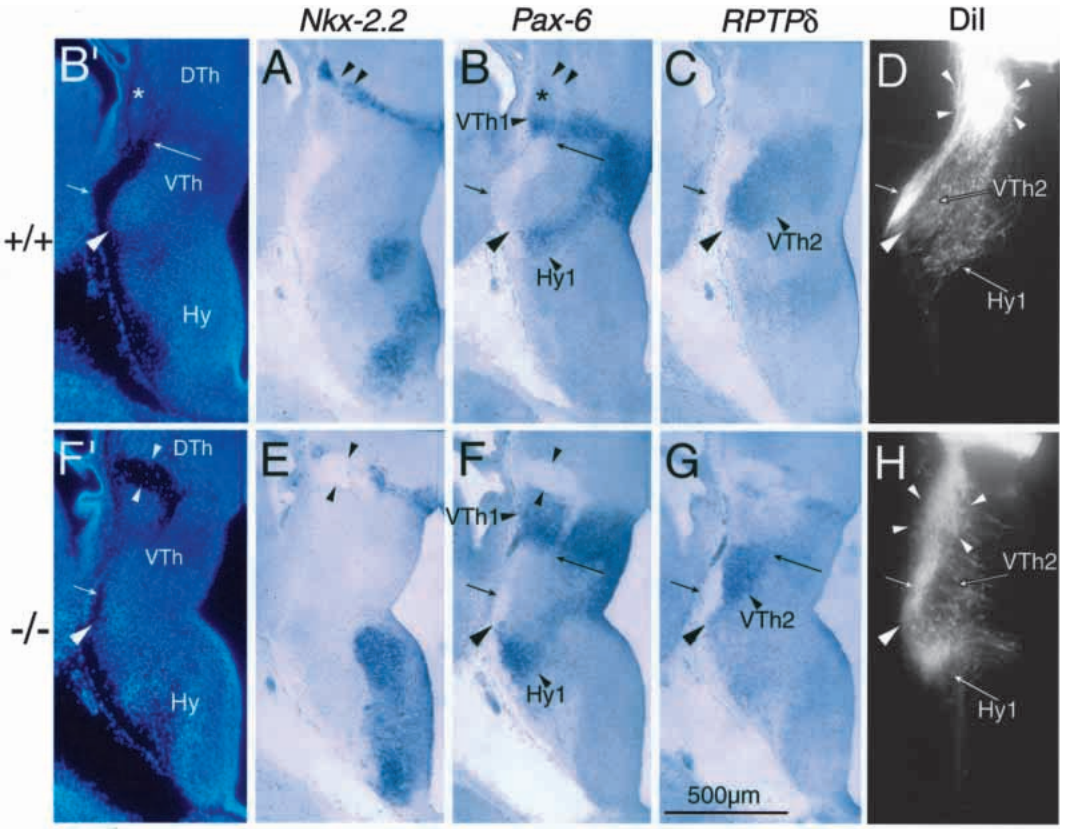

Fig. 8. Aberrant cell domains in *Mash-1* mutant diencephalon. In situ hybridization of *Nkx-2.2* (A,E), *Pax-6* (B,F) and *RPTPδ* (C,G) on 20 μm coronal sections of E13.5 wild-type (A-C) and null mutant (E-G) diencephalon. Sections for each genotype are from a single brain; no two sections are more than 40 μm apart. B′ and F′ are DAPI counterstaining of B and F, respectively. D and H are 100 μm sections from E13 wild-type and null mutant brains, respectively, that had DiI injected into DTh; these sections were scaled for comparison with the E13.5 images. In DAPI-labeled wild-type sections (B′), the TCA projection appears relatively unfasciculated in DTh, slightly more fasciculated after crossing the *Nkx-2.2*-positive ZLI (double arrowheads in A,B) to enter domain, VTh1. VTh1 can be divided into dorsal and ventral subdomains of low (asterisks in B and B′), and high *Pax-6* expression, respectively; both contain retrogradely labeled neurons (small arrowheads in D). At the ventral border of VTh1 (long arrows in B,B′), TCAs aggregate into a thick bundle (short arrows in B,B′,C) that runs along the lateral surface of domain VTh2, characterized by *RPTPδ* expression (small arrowhead in C) and a group of retrogradely labeled neurons (black-outlined arrow in D). *RPTPδ* and *Pax-6* exhibit roughly reciprocal patterns of expression. A third domain, Hy1, is located in the dorsolateral hypothalamus, and is characterized by *Pax-6* expression (small arrowhead in B) and a population of retrogradely labeled neurons (long white arrow in D). In the null mutant, the Probst-like bundle of TCAs (arrowheads in E,F,F′) coincides with a large discontinuity in the normally linear array of *Nkx-2.2*-positive ZLI cells (E). As in the wild type, *RPTPδ* and *Pax-6* exhibit reciprocal patterns of expression. Thus, while the *Pax-6* expression domains, VTh1 and Hy1, are expanded along the dorsal-ventral axis, the *RPTPδ* expression domain, VTh2, is reduced. A bundle of TCAs, thinner than in the wild type, is juxtaposed to the lateral surface of VTh2 (short arrows in F,F′,G,H), and extends to the VTh2-Hy1 border (large arrowheads in B,B′,C,D,F,F′,G,H).

in the wild type, *RPTPδ* and *Pax-6* expression are complementary. Thus, while *Pax-6* expression domains are expanded in null mutants, the *RPTPδ* expression domain is reduced, being restricted to the ventral half of ventral thalamus. *RPTPδ* expression in this domain is high and, in contrast to the wild type, appears to no longer be graded, but instead drops off sharply at its dorsal border. This border of *RPTPδ* expression correlates precisely with the point at which many L1-labeled axons appear to stop (Fig. 5B,B′). As in the wild type, a fascicle of TCAs is located on the surface of the *RPTPδ* domain. *RPTPδ* is also expressed in the ZLI; however, expression in null mutants extends further rostrally than in the wild type, where it appears as a thin band underlying, and sometimes overlying, the Probst-like bundle (Fig. 8G). Thus, *RPTPδ* expression in both wild-type and null mutant ventral thalamus suggests that *RPTPδ*-positive cells, and possibly *RPTPδ* itself, inhibit TCA growth.

DISCUSSION

In this study, we show that mice lacking Mash-1 protein exhibit a dramatically altered TCA projection. In these Mash-1 mutants, many TCAs fail to project out of the dorsal thalamus; those that do so exhibit abnormal growth within the ventral thalamus and fail to project into the ventral telencephalon. These findings, summarized in Table 1 and Fig. 9, suggest that cells in these regions play a critical role in TCA pathfinding and that Mash-1 is required for their normal differentiation. This study further suggests that multiple cues distributed along the length of an axon pathway are involved in the guidance of axons to their target. Our work suggests, in agreement with previous studies (Figdor and Stern, 1993; Macdonald et al., 1994), that axons alter their growth behavior at gene expression boundaries. The implication of this latter finding, at least with respect to TCA pathfinding, is that axon guidance molecules are expressed within discrete gene expression domains and do not diffuse beyond them. However, axon guidance molecules may come to be distributed outside of their domains of gene expression if they are present on axons extended outside of the domain by expressing neurons. For example, neurons in each of the four domains defined in this study, extend axons into dorsal thalamus.

The pathfinding mechanisms addressed in this study are distinct from those proposed in the ‘handshake’ hypothesis of Molnar et al (1998). A basic tenet of this hypothesis is that

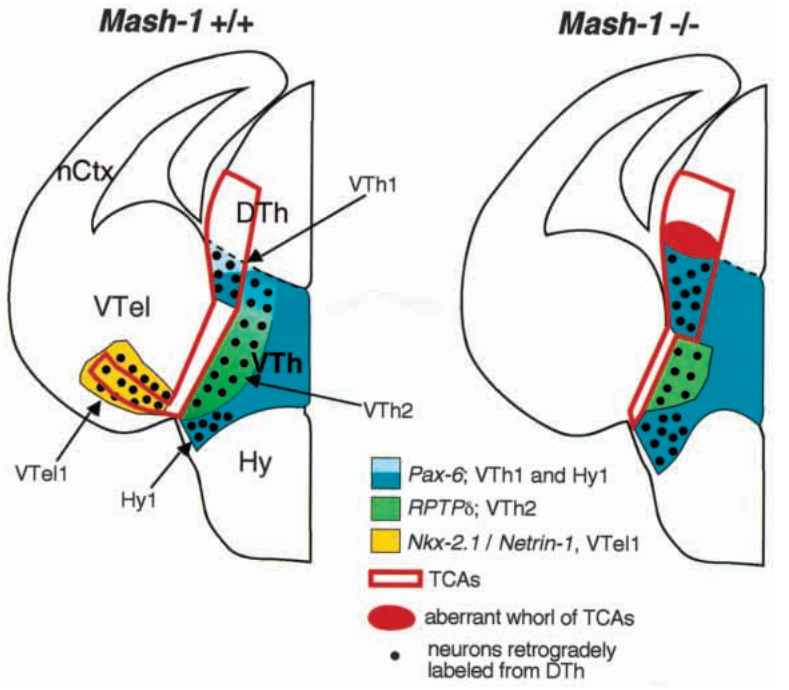

Fig. 9. Schematic representation of the thalamocortical (TCA) projection and associated cell domains in E13 wild-type and *Mash-1* mutant forebrain. In the wild type, four domains along the proximal half of the TCA pathway can be distinguished by their expression of *Pax-6* (blue, domains VTh1 and Hy1), a gradient of *RPTPδ* (green; domain VTh2 and the medial part of VTh1) and *Nkx-2.1* or *Netrin-1* (both yellow, domain VTel1); see Table 1 for more detail. Each of the four domains contains neurons (black dots) whose axons (not shown) project to or through DTh. The TCA projection (red outline) extends through domain VTh1, and then gradually narrows to a single fascicle alongside domain VTh2; the degree of fasciculation correlates with the level of *RPTPδ*. At the border between hypothalamus and ventral thalamus, TCAs make a sharp turn laterally into VTel1, a large domain that extends rostrally and laterally from the diencephalic-telencephalic border. In the null mutant, the *Pax-6* domains, VTh1 and Hy1, are expanded in size, and the *RPTPδ* domain, VTh2, is reduced. Most dramatically, the *Netrin-1, Nkx-2.1* domain, VTel1, is missing, along with its associated, retrogradely labeled neurons. Many TCAs form a knot at the dorsal surface of VTh1 (red oval). Other TCAs encounter a growth barrier at the border between VTh1 and 2, which, unlike in the wild type, is represented by an abrupt increase in *RPTPδ* expression. The axons that reach the ventral thalamic-hypothalamic-ventral telencephalon border appear to pause or halt since none are found in ventral telencephalon.

corticofugal and thalamocortical axons meet in the internal capsule (i.e. ventral telencephalon) and use each other as guidance scaffolds along the remainder of their trajectories. The four cell domains that we have identified lie along the proximal portion of the TCA pathway and are traversed by TCAs before they come into contact with corticofugal axons in the ventral telencephalon. Therefore, the defects that we find in TCA pathfinding in *Mash-1* mutants are independent of any potential interactions with corticofugal axons, or possible defects in corticofugal projections.

Alterations in ventral telencephalic cell populations in *Mash-1* mutants

We demonstrate that the TCA pathway extends through VTel1, a domain in the medial part of ventral telencephalon that extends to the diencephalic-telencephalic border, expresses *Nkx-2.1* and *Netrin-1*, and contains neurons that project into dorsal thalamus. In *Mash-1* mutants, VTel1 is not detectable with any of these three markers and TCAs fail to project beyond the diencephalic-telencephalic border. These results suggest that this domain is missing in *Mash-1* null mutants, an

Table 1. Cell domains in E13 mouse forebrain that lie within or along the TCA pathway are altered in *Mash-1* mutants

Cell domains	Anatomical correlate	Gene expression data				DiI data		Null mutant phenotype
		Pax-6	Nkx-2.1	RPTPδ	Netrin-1	Neurons labeled from DTh	Part of TCA pathway	
Ventral thalamus								
VTh1-d	*	low	–	–	–	yes	yes	expanded; incr.Pax-6
VTh1-v	*	+	–	–	–	yes	yes	expanded
VTh2	*	–	–	+	–	yes	no	contracted
Hypothalamus								
Hy1	EP	+	–	–	–	yes	no	expanded
Telencephalon								
VTel1	GP	low	+	low	+	yes	yes	absent

EP, entopeduncular nucleus; GP, globus pallidus; *, tentative (see legend); +, highly expressed; –, not detected

Anatomical correlates for domains Hy1 and VTel1 were determined by location, axonal projection patterns, and gene expression patterns. However, the anatomical identification of VTh1 and VTh2 is more difficult than that of Hy1 and VTel1, since the nuclei that make up this part of the ventral thalamus, the ventral lateral geniculate nucleus, the reticular thalamic nucleus, and zona incerta, are difficult to distinguish at these stages of development and there are discrepancies in the literature. Because of our uncertainty, we prefer not to assign specific nuclei to VTh1 and VTh2. Our DiI and gene expression data suggest that these four domains may be divisible into two or more subdomains. For example, as indicated, domain VTh1 seems to be divisible into dorsal and ventral subdomains based on Pax-6 expression. In addition, the E13.5 RPTPδ expression data show that, while the lateral part of VTh1 is negative, the medial part is weakly positive.

interpretation consistent with the reduced size of the medial part of the ventral telencephalon. It is possible, however, that VTel1 cells are present but substantially altered such that they no longer express *Nkx-2.1* or *Netrin-1* or project their axons into dorsal thalamus. In either case, though, our findings suggest that VTel1 provides a substrate for TCA growth in ventral telencephalon and that the axons of VTel1 neurons, whose pathway through ventral thalamus is coincident with the TCA pathway (this study; Braisted et al., 1999), help guide TCAs through ventral thalamus.

The apparent absence of VTel1 in *Mash-1* mutants may underlie the inability of TCAs to grow into the telencephalon. This hypothesis is supported by in vitro data showing that dorsal thalamic axons are attracted by the medial aspect of ventral telencephalic tissue (Braisted et al., 1999). In addition, the defects in TCA guidance within the ventral thalamus may be due, in part, to the absence of axons projecting from VTel1 to dorsal thalamus. These findings also suggest that, in wild-type forebrain, Netrin-1 may influence TCA growth into and through the MGE-derived mantle zone; if Netrin-1 is associated with the axons of VTel1 neurons that extend into dorsal thalamus, then it might also have some influence on TCA growth through ventral thalamus.

This broad domain of retrogradely labeled VTel1 neurons has been described in another DiI study of embryonic mouse forebrain (Braisted et al., 1999). In addition, other groups have described a very small number of ventral telencephalic neurons in E11.5 hamster (Métin and Godement, 1996) and E14 rat (Molnar et al., 1998) that are retrogradely labeled from dorsal thalamus; at least some of these neurons are likely to be located within domain VTel1. The difference in the number of neurons retrogradely labeled may be the result of large differences in the amount of DiI injected. In another study, Mitrofanis and colleagues (Mitrofanis and Guillery, 1993) describe 'perireticular' cells located in the internal capsule near the diencephalic-telencephalic border. However, since the markers used to identify this population are only expressed postnatally, it is difficult to compare it with VTel1.

Alterations within the dorsal part of ventral thalamus of *Mash-1* mutants

In wild-type brains, the segment of the TCA projection within the dorsal thalamus is slightly less fasciculated than the more distal part of the projection just ventral to the ZLI; this suggests that the dorsal part of ventral thalamus may be slightly more inhibitory for TCA growth than the dorsal thalamus. In null mutants, the response of TCAs to the dorsal thalamic-ventral thalamic border is more dramatic in that many TCAs are unable to extend into ventral thalamus; those that do are much more fasciculated in the dorsal half of ventral thalamus. These data suggest that, in *Mash-1* mutants, the dorsal part of ventral thalamus is more inhibitory for TCA growth than in the wild type. In the null mutant, the *Pax-6* expression domain, VTh1, is expanded along the dorsoventral axis. In addition, the level of *Pax-6* expression within at least the dorsal part of this domain (VTh1-d) is elevated. Thus, changes in *Pax-6* expression may lead to changes in axon guidance molecules within VTh1. To date, the only other example of changes in *Pax-6* expression affecting axon pathfinding is the failure of dorsally extending tpoc axons ('tract of the postoptic commissure', which originates from neurons at the ventral midline of diencephalon) to cross the ZLI in *Sey* mice, which are null for *Pax-6* (Mastick et al. 1997). These authors proposed that this phenotype was caused by the absence of a population of cells at the ZLI that facilitate the crossing of tpoc axons. Thus, it is possible that the same *Pax-6* expression domain has opposite effects on the guidance of thalamocortical and tpoc axons.

Role of the *RPTPδ*-positive domain in regulating TCA growth

We showed that *RPTPδ* is expressed in a ventral thalamic domain, VTh2, that TCAs extend over, but not into, in a tight fascicle. It has previously been shown in vitro (Bray et al., 1980) and in vivo (Tuttle et al., 1998) that axons can extend over inhibitory regions but tend to do so in fascicles. Interestingly, we found that *RPTPδ* is expressed in a gradient, with its level of expression increasing in parallel with the degree of fasciculation of TCAs. In the null, this gradient is absent; instead, TCAs extending ventrally in the ventral thalamus encounter a sudden change in *RPTPδ* expression levels – from undetectable to high. Coincident with this change is an ectopic accumulation of TCAs, giving the appearance that they have confronted a repulsive domain. Furthermore, within the neocortex, *RPTPδ* is expressed transiently in the cortical plate (Y. N. and D. D. M. O'L., unpublished data), at a stage when it appears to express inhibitors for TCA growth (Tuttle et al., 1995; Emerling and Lander, 1996) and TCAs contact but do not extend into it (Bicknese et al., 1994). Together, these findings suggest that the *RPTPδ*-positive domain, VTh2, is inhibitory for TCA growth, and that within this domain, RPTPδ, or other molecules with a similar graded expression, are mediating these concentration-dependent effects on TCA growth and fasciculation.

The only evidence that RPTPδ can directly affect axon growth is a preliminary report that the extracellular domain promotes axon growth from chick forebrain neurons (Wang and Bixby, 1998). However, in *Drosophila* a number of receptor protein tyrosine phosphatases have been shown to be required for the guidance of specific axons (Desai et al., 1996; Krueger et al., 1996); these phosphatases are present on axons and are thought to act cell autonomously. However, ligands for phosphatases might also transduce signals upon binding phosphatases, leading to a response in the ligand-expressing cells (growth cones of TCAs in this instance). Such a bidirectional signaling mechanism has been proposed for a receptor tyrosine phosphatase, RPTPß, and its interacting molecule, contactin (Peles et al., 1995; reviewed in Holland et al., 1998), and for the EphB subfamily of receptor tyrosine kinases and their transmembrane ephrin-B ligands (Henkemeyer et al., 1996; Holland et al., 1996; Bruckner et al., 1997). The latter example is particularly relevant since axons that express ephrin-B ligands and form specific forebrain commissures, show defective pathfinding in mice deficient for their receptors, EphB2 and EphB3, at positions where the axons would normally encounter cells expressing these receptors (Henkemeyer et al., 1996; Orioli et al., 1996).

Hypothalamic repulsive activity

During development TCAs do not extend into the hypothalamus (this study; Braisted et al., 1999), suggesting that the hypothalamus is repulsive for TCAs. Recent in vitro

studies support this hypothesis (Braisted et al., 1999). Interestingly, the hypothalamus has also been shown to be repulsive for retinal ganglion cell axons (Tuttle et al., 1998), indicating that this region is repulsive for multiple populations of axons.

In *Mash-1* mutants, as in the wild type, TCAs extend only as far as the ventral thalamic-hypothalamic border. This suggests that although *Mash-1* is expressed in the hypothalamic VZ, it is not required for expression of the repulsive activity. This conclusion is supported by in vitro assays demonstrating that the retinal ganglion cell axon repulsive activity in hypothalamus (which may not be the same as the repulsive activity affecting TCAs) is intact in *Mash-1* mutants (R. T., J. E. J. and D. D. M. O'L., unpublished data).

Mash-1 regulation of forebrain patterning

Our data demonstrate that Mash-1 is required for normal patterning in developing forebrain. We show that, in the absence of Mash-1, each of the four cell domains that we have identified based on patterns of gene expression and retrograde labeling are altered or missing. In the diencephalon, the *Pax-6* expression domains are expanded and the *RPTPδ* domain is contracted. In ventral telencephalon, VTel1, distinguished by *Nkx-2.1* and *Netrin-1* expression and neurons retrogradely labeled from the dorsal thalamus, is not detected. These findings indicate that Mash-1 is involved in regulating these domains, likely by influencing the generation or differentiation of their constituent neurons.

We are grateful to Trisha Savage for genotyping the embryos, and to D. Anderson, S. Bertuzi, C. Lagenaur, Q. Ma, L. Sommer and M. Tessier-Lavigne for providing reagents. We thank Noelle Dwyer and Elise Lamar for their very helpful comments on the manuscript. Y. N. was supported by the Human Frontier Science Program and Uehara Memorial Foundation. This work was supported by NIH grants NS31558 (D. D. M. O.) and NS32817 (J. E. J.).

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