Rapid Publication

Cortical and Thalamic Axon Pathfinding Defects in Tbr1, Gbx2, and Pax6 Mutant Mice: Evidence That Cortical and Thalamic Axons Interact and Guide Each Other

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ABSTRACT

During development, cortical areas establish precise reciprocal projections with corresponding thalamic nuclei. Pioneer axons from the cortex and thalamus first meet in the intermediate zone of the subcortical telencephalon (subpallium). Their close interactions in the subpallium suggest that they may use each other for guidance. To test this hypothesis, the development of corticothalamic and thalamocortical connections was studied in mice with mutations of transcription factor genes expressed specifically in the cortex (Tbr1), the dorsal thalamus (Gbx2), or both (Pax6). In Tbr1 mutants, cortical pioneer axons entered the subpallium at the appropriate time, but most stopped growing without entering the diencephalon. Surprisingly, thalamic axons (which do not express Tbr1) deviated into the external capsule and amygdala regions, without entering the cortex. Conversely, in most Gbx2 mutants, thalamic axons were reduced in number and grew no farther than the subpallium. Cortical axons (which do not express Gbx2) grew into the subpallium but did not enter the diencephalon. In one Gbx2−/− case, sparse thalamocortical and corticothalamic projections both developed, but in no case did one projection reach its target and not the other. In Pax6 mutants, neither corticothalamic nor thalamocortical axons reached their targets. These results suggest that thalamocortical and corticothalamic projections may not form independently. After reaching the subpallium, each projection may require a molecularly intact reciprocal projection for further guidance. This type of mechanism ensures that thalamocortical and corticothalamic axons project reciprocally. However, the exact nature of the interaction between cortical and thalamic pioneer axons remains to be elucidated. J. Comp. Neurol. 447:8–17, 2002. © 2002 Wiley-Liss, Inc.

Indexing terms: thalamocortical axons; corticothalamic axons; subplate; axon guidance

The neocortex and dorsal thalamus communicate with each other through reciprocal connections that are established during embryonic life. The earliest (pioneer) neocortical and thalamic axons grow concurrently into the subpallial telencephalon (subpallium), where they meet to form the internal capsule (Catalano et al., 1991; De Carlos and O'Leary, 1992; Miller et al., 1993). Embryonic structures within the subpallium—the lateral ganglionic eminence (LGE) and the medial ganglionic eminence (MGE)—appear to be intermediate targets for neocortical and thalamic axons (Métin and Godement, 1996; Richards et al., 1997). Cortical and thalamic axons may be guided into the subpallium by several mechanisms, including chemotraction by netrin-1, which is expressed in the LGE and MGE (Métin et al., 1997; Richards et al., 1997; © 2002 WILEY-LISS, INC.)

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CORTICAL AND THALAMIC AXONS INTERACT

Braisted et al., 2000; fasciculation on earlier-forming axons from the MGE to the neocortex and thalamus (Adams and Baker, 1995; Mézin and Godement, 1996); and chemorepulsion from the hypothalamus (Braisted et al., 1999). Specific cell groups in the subpallial telencephalon, hypothalamus, and ventral thalamus may also function as guideposts to direct the early growth of thalamocortical axons. Changes in these cell groups have been correlated with thalamocortical pathfinding errors in Mash-1 mutant mice (Tuttle et al., 1999).

After meeting in the subpallium to form the internal capsule, cortical and thalamic axons continue growing in opposite directions, toward the thalamus and neocortex, respectively. Guidance of both projections is dependent on cortical subplate neurons, which pioneer the pathway from neocortex to the internal capsule (McConnell et al., 1989). Chemical ablation of the subplate blocks thalamocortical innervation of lesioned regions (Ghosh et al., 1990; Ghosh and Shatz, 1993) and causes pathfinding errors in the reciprocal projection from neocortex to subcortical targets (McConnell et al., 1994). However, the exact role of the subplate in axon guidance remains unclear. The subplate highly expresses extracellular matrix molecules, such as neurocan, that might act as preferred substrates for thalamic axon growth (Bicknese et al., 1994; Miller et al., 1995; Fukuda et al., 1997; Kinnunen et al., 1999). Also, subplate axons projecting to the internal capsule may form a scaffold that guides thalamic axons back into the cortex (McConnell et al., 1989; Shatz et al., 1990). An extension of this idea—the "handshake hypothesis"—postulates that subplate and thalamic axons follow each other from the internal capsule to the thalamus and neocortex, respectively (Molnár and Blakemore, 1995). Consistently with this idea, neocortical and thalamic axons are closely apposed during development (Molnár et al., 1998a), and thalamic axons target ectopic subplate cells in reeler mutant mice (Yuasa et al., 1994; Molnár et al., 1998b). The cortex may also secrete diffusible factors that promote thalamic axon growth (Molnár and Blakemore, 1999).

We recently found that thalamocortical and corticothalamic projections are lacking in mice mutant for Tbr1, a T-box transcription factor gene expressed in the cortex but not the thalamus of embryonic mice (Hevner et al., 2001). Tbr1 is strongly expressed in early-born cortical neurons, including layer 6, subplate, and Cajal-Retzius cells (Bulfone et al., 1995; Hevner et al., 2001). In Tbr1 mutants, early-born neurons do not differentiate appropriately, cortical layers are malformed, many cortical efferent and afferent projections grow to inappropriate targets, and olfactory projection neurons are lacking (Bulfone et al., 1998; Hevner et al., 2001).

We have also reported thalamocortical axon defects in mutant mice lacking the Gbx2 homeobox gene (Miyashita-Lin et al., 1999). Gbx2 is expressed at early stages of dorsal thalamic development. Because Gbx2 is not expressed in the cortex, it is likely that intrinsic properties of the corticothalamic fibers should be normal. Therefore, this mouse provides a useful experimental system to test the role of the thalamocortical fibers in supporting the growth of the corticothalamic tract.

To investigate the hypothesis that thalamic and cortical axons interact and rely on each other for guidance cues, we have performed three types of studies. First, we have extended our analysis of thalamic and cortical axon development in Tbr1 and Gbx2 mutant mice during the embryonic period, using DiI tracing to label axons and thus determine when and where each projection initially shows abnormal pathfinding. Second, we have used two-color axon tracing with DiA and DiQ to determine whether corticothalamic and thalamocortical axons overlap in the internal capsule of control and Tbr1 mutant brains. Third, we have studied Pax6 (SeyNeu) mutant mice to test the prediction that defects in either the thalamocortical or the corticothalamic projection will be associated with corresponding defects in the reciprocal projection. Thalamocortical defects have been observed in Pax6 mutant rats (Kawano et al., 1999) and mice (Pratt et al., 2000), but no corticothalamic defects have been reported previously. Pax6 is expressed in many forebrain areas, including the cortex and thalamus, and Pax6 mutants show defective development of most if not all forebrain structures (Schmahl et al., 1993; Stoykova and Gruss, 1994; Mastick et al., 1997; Stoykova et al., 1997; Warren and Price, 1997; Pratt et al., 2000). The results of our studies support the hypothesis that thalamic and cortical axons associate in the internal capsule and exchange guidance cues for each other.

### MATERIALS AND METHODS

#### Animals

We used mouse mutant strains with null alleles of Tbr-1 (Bulfone et al., 1998; Hevner et al., 2001), Gbx-2 (Wassarman et al., 1997; Miyashita-Lin et al., 1999), and Pax-6 (SeyNeu, Schmahl et al., 1993; Sander et al., 1997). Animals were genotyped by polymerase chain reaction as described in the papers referenced. For timed pregnant matings, the morning on which the vaginal plug was found was considered embryonic day (E) 0.5. Mouse colonies were maintained in accordance with NIH guidelines, and protocols were approved by the IACUC of UCSF. Pregnant females were killed by cervical dislocation with chloral hydrate anesthesia (400 mg/kg, i.p.). Neonatal pups were killed by transcardial perfusion with fixative while they were under anesthesia by hypothermia.

#### DiI tracing

Embryos were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) with 4% sucrose. Neonatal mice were anesthetized by hypothermia and perfused transcardially. Brains were removed, sagi-

### TABLE 1. Brains Studied by DiI Tracing

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1 Studied by DiA and DiQ (not DiI) injections.
Fig. 1. *Tbr1*−/− cortical axon growth is deficient in the internal capsule between E14.5 and E16.5. **A–D**: Dil labeling from the cortex. At E14.5, cortical pioneer axons reached the internal capsule (ic; green arrowheads) in *Tbr1* mutant (B) and control (A) mice and were tipped by large complex growth cones (insets). By E16.5, cortical axons reached the ventral thalamus (vt) in controls (C), but, in *Tbr1* mutants (D), cortical axons terminated in the internal capsule (red arrowhead), without entering the diencephalon. (The blue fluorescence is DAPI counterstain to show cell nuclei.) The dorsal thalamus (dt) contained retrogradely labeled neurons and fibers in controls (C, inset) but not in mutants. **E–G**: Dil labeling from the internal capsule at E14.5. In controls (E, confocal image), cortical pioneer neurons (labeled retrogradely with Dil; red) expressed Tbr1 protein (shown by double labeling with Tbr1 immunohistochemistry; green) and occupied the subplate (sp) or deep part of the cortical plate (cp). In *Tbr1* mutants (F), pioneer neurons were scattered in the cortex, and no distinct subplate was visible. Retrograde labeling in the dorsal thalamus was the same in *Tbr1* mutants (G) and controls (not shown). ad, Apical dendrite; iz, intermediate zone; mz, marginal zone; svz, subventricular zone; vz, ventricular zone. Scale bar = 0.5 mm in A (applies to A–D,G), 100 μm for insets, 100 μm in F, 20 μm in E.
tally hemisected, further dissected if necessary to reveal the internal capsule or thalamus, postfixed overnight, and injected with DiI (“large crystal” preparation), DiA, or DiQ (all from Molecular Probes, Eugene, OR). Crystals of tracer (100–200 μm) were placed in the cortex, internal capsule, or dorsal thalamus using an insect pin (Fine Science Tools). Different cortical regions and thalamic nuclei were injected with DiI, although the presumptive barrel cortex and ventrobasal complex were targeted most frequently. Brains were stored in fixative or in PBS with 0.1% azide (instead of fixative, to preserve immunoreactivity). After storage for 4–8 weeks in the dark at room temperature, brains were rinsed in PBS, embedded in 4% low-melting-point agarose in PBS, and sectioned coronally at 80–100 μm on a Vibratome or a Leica VT1000 microscope. Sections were counterstained with DAPI (Molecular Probes) to label DNA fluorescently, coverslipped in 30% sucrose in PBS, and examined by epifluorescence microscopy. Photographic slides were digitally scanned and assembled into montages and figures using Adobe Photoshop. DiI fluorescence (red) was digitally converted to white to enhance contrast. A list of experiments is presented in Table 1.

**Immunohistochemistry**

Thick vibratome sections (80–100 μm) were preincubated for 1 hour at 4°C in blocking solution (PBS containing 5% normal goat serum and 1 mg/ml bovine serum albumin) and then incubated overnight with antibody Tbr-C (directed against the C-terminal peptide of Tbr1; Hsueh et al., 2000) in blocking solution. Sections were rinsed three times for 5 minutes each in PBS and incubated for 4 hours at room temperature with goat anti-rabbit antibody conjugated to Alexa 594 (Molecular Probes). Sections were again rinsed three times for 5 minutes each and coverslipped with Vectashield (Vector, Burlingame, CA).

**RESULTS**

**Thalamic axon pathfinding errors occur prior to cortical axon pathfinding errors in Tbr1 mutant mice**

We previously reported that connections between the thalamus and cortex were defective in Tbr1 mutant brains around the time of birth (Hevner et al., 2001). Thalamic axons projected to the external capsule or the amygdala rather than the cortex, and cortical axons ended in the internal capsule region rather than projecting to the thalamus. In the present study, we further examined the development of these pathways to establish when and where the first defects are detectable.

The growth of cortical pioneer axons was examined at E14.5, when the fibers normally just enter the intermediate zone of the LGE (striatal anlage). At this stage, Tbr1 mutants were indistinguishable from controls (Fig. 1A, B). Thus, early-born cortical neurons retained the functions of axon growth and guidance to the subplate in Tbr1 mutants.

By E16.5, in normal mice, corticothalamic axons grew through the subplate (forming the internal capsule) and reached the ventral thalamus (Fig. 1C). However, Tbr1–/– cortical efferent axons slowed or stopped their growth in the midportion of the subplate (Fig. 1D). Furthermore, whereas DiI labeling of the cortex retrogradely labeled dorsal thalamic neurons in normal mice (inset in Fig. 1C), Tbr1 mutants lacked this property (Fig. 1D), indicating that thalamic axons had not reached the cortex.

To label cortical pioneer neurons retrogradely, DiI crystals were placed in the midportion of the internal capsule in E14.5 and E15.5 brains. In controls, many neurons in the subplate and deep portion of the cortical plate were labeled retrogradely (Fig. 1E) as well as rare cells in the marginal zone (Meyer et al., 1998; Molnár et al., 1998a,b). The cortical pioneer neurons expressed Tbr1 protein, as determined by double labeling with Tbr1 immunohistochemistry and DiI from the internal capsule (Fig. 1E). This was consistent with our previous results showing that Tbr1 protein is expressed in subplate neurons marked by the expression of goll-lacZ transgene (Hevner et al., 2001). In Tbr1 mutants, DiI-labeled cortical pioneer neurons appeared normal in number and morphology, although there was no distinction between the subplate and the cortical plate (Fig. 1F). A few neurons in the marginal zone of Tbr1 mutants were also labeled retrogradely from the internal capsule. Projections from the thalamus to the internal capsule were also labeled retrogradely in mutants (Fig. 1G) and were indistinguishable from controls (not shown).

DiI labeling of thalamic axons (Fig. 2) showed a pathfinding abnormality in the internal capsule region of the subpallium at E14.5, when cortical axon pathfinding still appeared normal. This was surprising in that Tbr1 is not expressed in thalamic neurons or in the internal capsule (Bulfone et al., 1995; Puelles et al., 2000; Hevner et al.,

Fig. 2. Defective thalamocortical axon pathfinding in the internal capsule of E14.5 Tbr1 mutant mice. A–D: DiI labeling from the thalamus. In control mice (A), thalamic axons grew straight through the internal capsule (ic) at E14.5 and reached the subplate (sp) layer of cortex (ctx) by E16.5 (C). In Tbr1 mutants, thalamic axons deviated laterally through the middle of the internal capsule (arrowhead) at E14.5 (B) and grew into the external capsule (ec) instead of the cortex at E16.5 (D). Scale bar = 0.5 mm.
Control thalamic axons grew straight through the internal capsule and turned dorsally toward the cortex (Fig. 2A), but \textit{Tbr1}\textsuperscript{−/−} thalamic axons turned midway through the internal capsule and curved laterally toward the external capsule (arrowhead, Fig. 2B). By E16.5, when control thalamic axons innervated the subplate layer of cortex (Fig. 2C), \textit{Tbr1}\textsuperscript{−/−} axons ended in the internal capsule (Fig. 2D). At later ages, up to postnatal day (P) 1.5, mutant thalamic axons remained mainly in the internal capsule, the external capsule, and the amygdalar region (Hevner et al., 2001) but did not innervate cortex.

To elucidate molecular defects that may underlie this phenotype, we examined the expression of some candidate thalamocortical axon guidance molecules in \textit{Tbr1}\textsuperscript{−/−} mutants by immunohistochemistry, including the IgCAMs \textit{L1} (Fukuda et al., 1997) and \textit{TAG-1} (Wolfer et al., 1994), and the proteoglycans chondroitin sulfate (Bicknese et al., 1994) and \textit{neurocan} (Miller et al., 1995; Fukuda et al., 1997), but their expression levels appeared unchanged (data not shown). The lack of thalamocortical and corticothalamic projections was consistently observed in occipital, parietal, temporal, and sensorimotor cortex but not in the prefrontal or cingulate cortex regions. In these areas, thalamocortical projections were preserved, as confirmed by calretinin immunohistochemistry as well as DiI tracing (data not shown).

\textbf{Corticothalamic and thalamocortical projections are absent or proportionally reduced in \textit{Gbx2} mutant mice}

We previously reported that thalamic axons do not innervate the cortex of \textit{Gbx2} mutant mice by the time of birth in most cases (Miyashita-Lin et al., 1999). Here we show that the reciprocal projection from cortex to thalamus is also defective in \textit{Gbx2} mutants.

DiI injections in the E14.5 thalamus showed that \textit{Gbx2}\textsuperscript{−/−} mutants had a marked decrease in the number of thalamic axons projecting to the internal capsule (Fig. 3B, arrowhead). The length of \textit{Gbx2}\textsuperscript{−/−} axons was also reduced. \textit{Gbx2}\textsuperscript{−/−} mutants have severe defects of thalamic growth, nucleus formation, and region-specific gene expression as well (Miyashita-Lin et al., 1999). By E16.5, thalamic axons innervated the subplate layer of cortex in controls (Fig. 3C), but in the mutants the axons ended in the middle of the subpallium (Fig. 3D). Also, retrogradely labeled neurons were absent from the cortex of \textit{Gbx2} mutants after thalamic DiI injections, suggesting that there was a defect of corticothalamic projections.

The growth of cortical pioneer axons and their pathfinding into the subpallial telencephalon were intact in \textit{Gbx2} mutants at E14.5 (Fig. 4A,B). However, at E16.5, \textit{Gbx2}\textsuperscript{−/−} cortical axons failed to grow into the thalamus, although projections through the cerebral peduncle were intact...
Fig. 5. Thalamocortical and corticothalamic connections are absent in most Gbx2 mutant mice at E18.5. A: Dil labeling from the thalamus at E18.5. Thalamic axons appeared tangled (arrowheads) in the internal capsule (ic) and did not enter the cortex (ctx). There was no retrograde labeling of neurons in the cortex. Asterisk indicates tissue artifact. B,C: Dil labeling from the cortex at E18.5. Gbx2–/– cortical axons followed a tortuous course through the internal capsule. Some axons grew into the cerebral peduncle (cpd), but none entered the ventral thalamus (vt), nor were retrogradely labeled neurons present in the dorsal thalamus (dt). chp, Choroid plexus; lv, lateral ventricle. Scale bar = 0.5 mm in A (applies to A–C), 0.5 mm in B.

Fig. 6. Proportional reduction of corticothalamic and thalamocortical connections in a Gbx2 mutant brain. A,B: Dil labeling from the thalamus at E18.5. Thalamocortical projections were present but decreased in a Gbx2 mutant (B) compared with control (A) brain. Retrograde labeling of cortical neurons was also less in the mutant (B; arrowheads) than in the control brain (A; arrowheads). C,D: Dil labeling from the cortex (ctx) at E18.5. The dorsal thalamus (dt) contained fewer retrogradely labeled neurons in the Gbx2 mutant (D; arrowheads) than in the control (C; arrowheads), and the ventral thalamus (vt) contained fewer axons in the mutant. The cerebral peduncle (cpd) was unchanged. ic, Internal capsule. Scale bar = 0.5 mm.

Thalamocortical and cortical projections are closely apposed in the internal capsule in normal and Tbr1 mutant brains

In normal and reeler mutant rodents, thalamic and cortical axons are closely apposed in the internal capsule (Molnár et al., 1998a,b). To determine whether thalamic and cortical axons occupy separate compartments in the internal capsule or grow near each other in Tbr1–/– embryos, we used two-color fluorescent tracing with DiA and DiQ. By injecting one tracer in the thalamus and the other tracer in the cortex, it was possible to distinguish cortical and thalamic axons in the internal capsule.

In control as well as Tbr1–/– mice, cortical and thalamic axons showed considerable overlap in the internal capsule (Fig. 7). The extent of overlap varied from experiment to experiment, probably reflecting variability in the exact locations of tracer injections. In controls, cortical and thalamic axons appeared to run parallel and adjacent to each other as observed on confocal microscopy (Fig. 7E). In Tbr1 mutants, cortical and thalamic axons were likewise...
closely apposed and occupied the same fiber bundles (Fig. 7F). This indicates that axon pathfinding errors in Tbr1 mutant mice did not result from a lack of overlap between thalamic and cortical axon populations.

**Thalamocortical and corticothalamic projections are both defective in Pax6 (Sey<sup>enu</sup>) mutant mice**

Thalamic axons do not enter the cortex of Pax6 mutant rats (Kawano et al., 1999) or mice (Pratt et al., 2000). We hypothesized that corticothalamic projections might likewise be deficient in Pax6 mutants, if cortical and thalamic axons use each other as guides for growth from the internal capsule to their reciprocal targets. These studies were carried out using the Sey<sup>enu</sup> allele of Pax6 (Schmahl et al., 1993).

DiI injections in the thalamus confirmed that thalamic axons did not enter the cortex of Pax6 mutant mice (Fig. 8). The number of thalamic axons growing into the subpallial telencephalon was severely reduced at E14.5 (Fig. 8B). At E16.5, a few thalamic axons grew into the hypothalamus and stalk of the telencephalon of Pax6 mutants, but no thalamic axons entered the cortex (Fig. 8D). The Pax6 mutant cortex also lacked retrogradely labeled neurons, which were present in the cortex of controls at E16.5 (arrowheads, Fig. 8C). Thalamic projections did not grow into the Pax6 mutant cortex by E18.5, nor did DiI injections into the thalamus label cortical neurons retrogradely at that age (Fig. 8F). These data indicated that corticothalamic as well as thalamocortical axons were deficient in Pax6 mutant mice.

DiI injections in the cortex revealed an abnormal cortical efferent pathway in Pax6 mutants. The early growth of cortical pioneer axons into the internal capsule appeared normal at E14.5 (Fig. 9A,B). However, at E16.5, it was evident that cortical axons followed an ectopic route through the lateral striatum and external capsule and did not enter medial portions of the internal capsule, nor did they exit the telencephalon (Fig. 9D). By E18.5, Pax6<sup>−/−</sup> cortical axons formed an ectopic bundle along the basal surface of the forebrain but did not grow into the thalamus or cerebral peduncle (Fig. 9G–J). Also, cortical axons did not cross the midline or enter the corpus callosum but formed a longitudinal (Probst) bundle (Fig. 9E,G). The lack of thalamocortical projections in Pax6 mutants was confirmed by the absence of retrograde neuronal labeling in the thalamus following DiI injections in the cortex at E16.5 and E18.5 (Fig. 9C,D,F,J).

**DISCUSSION**

Disturbance of either cortical or thalamic development (or both) causes a reciprocal reduction of thalamocortical and corticothalamic connections

We studied three different mutant mouse strains with primary developmental defects in the cortex (Tbr1), the thalamus (Gbx2), or both (Pax6). In all three lines, thalamocortical and corticothalamic projections usually were both missing. In one case, Gbx2 mutants produced a small thalamocortical pathway; in this instance, the corticothalamic pathway was also partially developed. In no case was there evidence that thalamocortical or corticothalamic projections developed independently of each other.
Previously, Kawano et al. (1999) and Pratt et al. (2000) showed that cortical axon projections in Pax6 (Sey\textsuperscript{mut}) mutant rats and mice grew normally as far as the internal capsule, but they did not evaluate cortical axon growth from the internal capsule to the thalamus. Thus, corticothalamic projections may be defective in Pax6 (Sey\textsuperscript{mut}) mutant rats as well as mice. In studies of other mouse mutant strains with thalamocortical defects, corticothalamic axon phenotypes were not reported (Tuttle et al., 1999; Zhou et al., 1999; Mallamaci et al., 2000; Leighton et al., 2001). Studies of the corticothalamic projections in those strains could pro-

Fig. 8. Absence of corticothalamic and thalamocortical projections in Pax6 (Sey\textsuperscript{mut}) mutant mice. All panels show DiI labeling from the internal capsule (ic) were almost entirely lacking in mutants. Asterisk indicates a tissue artifact. C,D: E16.5. Mutant thalamic axon bundles projected into the hypothalamus (hy; arrow; D,F) or through the ic toward the amygdala (amg; arrowheads; D,F). Mutants also lacked retrogradely labeled neurons in the cortex (ctx), which were numerous in controls (arrowheads; C,E). E,F: E18.5. Mutant thalamic axon bundles were concentrated in the amygdala and hypothalamus regions and did not enter cortex, nor were neurons labeled retrogradely in the ctx. dt, Dorsal thalamus. Scale bar = 0.5 mm.

Fig. 9. Pax6 (Sey\textsuperscript{mut}) mutants form an aberrant cortical efferent pathway. All panels show DiI labeling from the cortex (ctx). A,B: E14.5. Thalamic projections to the internal capsule (ic; arrowheads; A,B) developed normally in mutants. C,D: E16.5. Mutant cortical axons avoided the ic, and most grew along the edge of the striatum (str; arrowhead; D). E–J: E18.5. Controls (E,F) demonstrated projections to the corpus callosum (cc), the cerebral peduncle (cpd), and the ventral thalamus (vt) as well as retrogradely labeled neurons in the dorsal thalamus (dt; F, arrowhead). Mutant (G–J) cortical axons projected medially into a longitudinal Probst bundle (pb), or laterally through the lateral striatum and external capsule (ec) to form a dense bundle on the basal surface of the forebrain (arrowheads; G–J). Sections are progressively more caudal in G–J. amg, Amygdala; hy, hypothalamus. Scale bar = 0.5 mm.
provide further tests of the hypothesis that cortical and thalamic axons depend on each other for guidance.

**Primary defects in the thalamus or cortex cause pathfinding errors of the reciprocal projection in the internal capsule**

In the Tbr1 and Gbx2 mutant strains, in which cortical and thalamic development were primarily affected, respectively, the reciprocal projections arose from neurons whose gene expression was presumably intact. For example, thalamic neurons do not express Tbr1 and therefore were probably not affected by loss of Tbr1 function at early stages of axon pathfinding to the cortex. Nevertheless, in both Tbr1 and Gbx2 mutants, pathfinding errors affected the reciprocal projection, and, in each case, the errors first occurred in the subpallium as the projections were forming the internal capsule. These results suggest that cortical and thalamic axons become dependent on each other for axon guidance in the internal capsule, after growing independently into the subpallium by other mechanisms. Alternatively, pathfinding defects in the internal capsule could be caused by abnormal development of subpallial cells. Because Tbr1 is not expressed in this region (Bulfone et al., 1995; Puelles et al., 2000; Hevner et al., 2001), and Gbx2 is expressed only in the ventral pallidum (Bulfone et al., 1993), a region that is not traversed by the internal capsule, it is highly unlikely that either Tbr1 or Gbx2 acts in subpallial cells to regulate the growth of cortical or thalamic fibers.

It is also possible that abnormal development of nearby regions caused pathfinding defects in the internal capsule. For example, the eminentia thalami, a diencephalic structure adjacent to the ventral thalamus and the internal capsule, expresses high levels of Tbr1 (Bulfone et al., 1995; Puelles et al., 2000; Hevner et al., 2001). This structure could be abnormal in Tbr1 mutants; however, we have not observed defects in the growth or pathfinding of thalamic axons in the diencephalon, so it seems unlikely that Tbr1 expression in the eminentia thalami contributes to the pathfinding of thalamic axons in the subpallium.

**Cortical and thalamic axons associate in the internal capsule**

Our results confirm previous studies showing that cortical and thalamic axons intimately associate in the same fiber bundles in the developing internal capsule of rats and mice (Molnár et al., 1998a, b). Their close apposition indicates that thalamic and cortical axons could guide each other using short-range signals or by contact mechanisms such as fasciculation. In Tbr1 mutant mice, cortical and thalamic axons still associated in the internal capsule (Fig. 7), indicating that each population of axons was correctly guided to the intermediate target. However, the subsequent pathfinding of Tbr1→ corticothalamic axons to the thalamus and cortex, respectively, was impaired. The simplest explanation for this phenotype in Tbr1 mutants is that the expression of bidirectional signaling molecules was defective in cortical axons, so that cortical and thalamic axons associated but did not signal each other. Further analysis of the molecular interactions between cortical and thalamic axons will be necessary to clarify this point.

**Corticothalmic and thalamocortical axon guidance after the intermediate target**

Much evidence has accumulated to indicate that cell populations in the subpallium act as intermediate targets for cortical and thalamic axons, in part through secretion of the chemoattractant netrin-1 (Métin and Godement, 1996; Métin et al., 1997; Richards et al., 1997; Braisted et al., 2000; S. Garel and J.L.R.R., unpublished). Intermediate targets are “choice points” at the end of each segment of axon growth, where axons typically change their response properties to navigate the next segment of the pathway (Tessier-Lavigne and Goodman, 1996). We hypothesize that the subpallium, by acting as an intermediate target for cortical and thalamic axons, promotes mingling between the two sets of axons. Recognition and bidirectional signaling between the cortical and the thalamic axons might then direct the axons to ignore netrin signaling and, instead, follow reciprocal axons or related cues for guidance to their final targets. This schema is not only consistent with experimental results but accounts for the precise reciprocal connections between cortical areas and specific thalamic nuclei. Alternatively, changes in long-range guidance signals could underlie the pathfinding defects that we have observed. For instance, the Tbr1 and Gbx2 mutations might block the production of secreted chemoattractants by the cortex (Molnár and Blakemore, 1999) and thalamus, respectively. According to such models, separate mechanisms would account for defects in direct and reciprocal projections. In future studies, we hope to identify the guidance molecules affected in these mutants.

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**LITERATURE CITED**


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