INTRODUCTION

In the mammalian brain, reciprocal connections between sensory nuclei of the dorsal thalamus and specific areas of the neocortex are essential for the relay and processing of visual, auditory, sensory and motor information (O’Leary et al., 1994; Levitt et al., 1997; Monuki and Walsh, 2001; Pallas, 2001; Ragsdale and Grove, 2001; Ruiz i Altaba et al., 2001; Sur and Leamey, 2001; O’Leary and Nakagawa, 2002). During development, thalamocortical and corticothalamic axons grow into the subcortical telencephalon, where they meet and continue on their paths to the cortex and thalamus, respectively (Miller et al., 1993; Metin and Godement, 1996; Molnar et al., 1998a; Auladell et al., 2000). In this process, thalamic axons of a given nucleus grow through the ventral thalamus, telencephalic stalk and basal ganglia to the specific presumptive neocortical areas that they will invade (Crandall and Caviness, 1984; Catalano et al., 1991; Kageyama and Robertson, 1993; Miller et al., 1993; Metin and Godement, 1996; Molnar et al., 1998a; Auladell et al., 2000). This first level of topographic organization (an inter-areal map), in which a given thalamic nucleus projects to a specific neocortical region, is important to relay visual, auditory, somatosensory and motor information to different neocortical areas. Within each neocortical area, thalamic axons establish highly ordered projections with specific neocortical domains and the mechanisms controlling the initial topography of these projections remain to be characterized. To address this issue, we examined Ebf1−/− embryos in which a subset of thalamic axons does not reach the neocortex. We show that the projections that do form between thalamic nuclei and neocortical domains have a shifted topography, in the absence of regionalization defects in the thalamus or neocortex. This shift is first detected inside the basal ganglia, a structure on the path of thalamic axons, and which develops abnormally in Ebf1−/− embryos. A similar shift in the topography of thalamocortical axons inside the basal ganglia and neocortex was observed in Dlx1/2−/− embryos, which also have an abnormal basal ganglia development. Furthermore, Dlx1 and Dlx2 are not expressed in the dorsal thalamus or in cortical projections neurons. Thus, our study shows that: (1) different thalamic nuclei do not establish projections independently of each other; (2) a shift in thalamocortical topography can occur in the absence of major regionalization defects in the dorsal thalamus and neocortex; and (3) the basal ganglia may contain decision points for thalamic axons’ pathfinding and topographic organization. These observations suggest that the topography of thalamocortical projections is not strictly determined by cues located within the neocortex and may be regulated by the relative positioning of thalamic axons inside the basal ganglia.

Key words: Topography, Thalamocortical axons, Internal capsule, Neocortex, Basal ganglia, Dlx, Ebf1, Mouse
groups of cells in the basal ganglia have been proposed to be intermediate targets for cortical and thalamic axons, by producing attractants or forming transient projections to the thalamus (Mitrofanis and Guilbery, 1993; Metin and Godement, 1996; Tuttle et al., 1999). In addition, it was shown that preplate and subplate cortical axons, which pioneer the corticofugal pathway, form a scaffold that guides thalamic axons into the neocortex (McConnell et al., 1989; Ghosh et al., 1990; Ghosh and Shatz, 1992; Ghosh and Shatz, 1993; Molnar et al., 1998b; McQuillen et al., 2002). These observations provided the basis of the ‘handshake’ hypothesis, which proposes that thalamic and cortical axons require each other to reach to their targets (Molnar and Blakemore, 1995; Molnar et al., 1998a). Support for this model comes from analysis of the Tbr1 and COUP-TFI mutants, in which subplate defects impair the capacity of thalamocortical axons to reach the neocortex (Zhou et al., 1999; Hevner et al., 2001; Hevner et al., 2002). Similar results are also observed in Emx1/Emx2 mutants (K. M. Bishop, S. G., Y. Nakagawa, J. L. R. R. and D. M. M. O’Leary, unpublished). Conversely, in Gbx2 mutant mice, which have major thalamic defects, corticothalamic projections do not reach their target (Miyashita-Lin et al., 1999; Hevner et al., 2002). Taken together, these studies provide insights into how thalamic and cortical axons reach their respective target structure. However, very little is known about the mechanisms that control the targeting of thalamic axons to specific neocortical domains.

The chemoaffinity hypothesis stipulates that topographic projections are generated through the expression of molecules that mediate repulsion and/or attraction between the projecting axons and their target (Sperry, 1963; Goodhill and Richards, 1999). This mechanism was shown to play a key role in the establishment of retinotectal projections through Eph and ephrin protein interactions (Drescher et al., 1997; Goodhill and Richards, 1999; Feldheim et al., 2000) and is the prevailing model for the formation of topographic projections in the nervous system. It is implicated in the targeting of reticulogeniculate axons (Feldheim et al., 1998), of limbic thalamic axons to the limbic cortex (Barbe and Levitt, 1992; Mann et al., 1998) and hippocampal neurons to the septum (Gao et al., 1996), as well as in the formation of a topographic map within a neocortical area (Vanderhaegen et al., 2000). Recent studies show that several genes, including genes encoding Eph/Ephrines, are expressed locally or in gradients in the neocortex before and shortly after the arrival of thalamic inputs (Bulfone et al., 1995; Gao et al., 1998; Nothias et al., 1998; Donoghue and Rakic, 1999; Mackarehtschian et al., 1999; Miyashita-Lin et al., 1999; Nakagawa et al., 1999; Rubenstein et al., 1999; Liu et al., 2000; Sestan et al., 2001), suggesting that the expression of localized cues within the neocortex may control the targeting of thalamic axons. Consistent with this model, the inactivation of Emx2 and COUP-TFI transcription factor genes, that are expressed in high-caudal-low-rostral gradients in the cortical primordium, induces a change in neocortical molecular regionalization as well as a corresponding change in the topography of thalamocortical connections (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001; Muzio et al., 2002). However, there is evidence that mechanisms operating outside of the neocortex may participate in regulating the development of thalamocortical topography. Explant culture experiments indicate that thalamic axons can innervate any region of the neocortex in vitro, suggesting the absence of an instructive code within the cortex (Molnar and Blakemore, 1991; Molnar and Blakemore, 1995). Based on a variety of studies, it has been proposed that molecular and/or temporal interactions between cortical and thalamic axons inside the internal capsule may regulate the regional specificity of thalamocortical connections (Molnar and Blakemore, 1991; Ghosh and Shatz, 1992; Ghosh and Shatz, 1993; Molnar and Blakemore, 1995; Molnar et al., 1998a).

So far, little is known about the mechanisms that control the initial targeting of thalamic axons to specific neocortical domains and the proposed models remain to be tested. To address this issue specifically, we searched for mouse mutants that exhibited projection defects in subpopulations of thalamic axons. We chose to analyze Ebf1 mutant embryos because they have internal capsule pathfinding defects (Garel et al., 1999). Ebf1 (also known as Olf-1, O/E-1, Coe1) encodes a HLH transcription factor (Hagman et al., 1993; Wang and Reed, 1993; Dubois and Vincent, 2001), and its inactivation affects basal ganglia development (Garel et al., 1999). We show that, in Ebf1–/– mutant embryos, axons from the dLGN are misrouted inside the basal ganglia and the projections that do form between thalamic nuclei and neocortical domains have a shifted topography. This shift occurs in the absence of an apparent change in thalamic or neocortical regionalization and is preceded by a shift in the positions of thalamic axons in the basal ganglia. These results indicate that thalamic projections from different nuclei are not formed independently of one another and raise the possibility that defects in the basal ganglia of Ebf1–/– embryos participate in shifting the early topography of thalamocortical axons. To test whether defects in structures located along the path of thalamic axons might shift thalamocortical topography, we analyzed Dlx1/2 mutants that have defects in basal ganglia development (Anderson et al., 1997). Dlx1 and Dlx2 encode homeodomain transcription factors and are not expressed in cortical projection neurons or in the dorsal thalamus (Bulfone et al., 1993; Stühmer et al., 1997). In Dlx1/2–/– embryos, some thalamic axons fail to grow past the basal ganglia and, as in Ebf1 mutants, thalamic axons that do reach the neocortex have a shifted topographic organization in the neocortex and basal ganglia. Taken together, our study suggests that the early topography of thalamocortical projections is not strictly and solely governed by information located within the neocortex and dorsal thalamus and that the positioning of thalamic axons within the basal ganglia may have an important role in organizing these projections.

MATERIALS AND METHODS

Mouse lines and genotyping

Ebf1 heterozygous mice (Lin and Grosschedl, 1995) were maintained in a C57/B6 background and crossed to produce homozygous embryos. Dlx1/2 heterozygous mice (Qu et al., 1997) were maintained in mixed 129G and C57/B6 genetic background, and crossed to produce homozygous embryos. PCR genotyping of both lines was performed as described previously (Anderson et al., 1997; Qu et al., 1997; Garel et al., 1999). Heterozygous embryos did not show any phenotype and were used as controls. For staging of embryos, midday of the day of vaginal plug formation was considered as embryonic day 0.5 (E0.5).
In situ hybridization
Embryos were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. In situ hybridization were performed on 80-100 μm thick vibratome sections as described previously (Garel et al., 1999) with the following probes: cadherin 6 (*Cdh6*) (a gift of M. Takeichi); *Cdh8* (a gift of M. Takeichi); COUP-TFI (a gift of M. Tsai); *Ebf1* (a gift of R. Grosschedl); *Emx2* (a gift of A. Simeone); *Epha4* (a gift of A. Nieto); *Epha7* (a gift of A. Wanaka); ephrin A2 (Efna2 – Mouse Genome Informatics; a gift of J. Flanagan); ephrin A5 (Efna5 – Mouse Genome Informatics; a gift of U. Drescher); *Fgfr3* (a gift of D. Ornitz); *Gbx2* (a gift of G. Martin); *Id2* (*Idb2* – Mouse Genome Informatics; a gift of M. Israel); *Lhx9* (a gift of S. Retaux); netrin 1 (*Nnt1* – Mouse Genome Informatics; a gift of M. Tessier-Lavigne); *Sema6a* (a gift of W. Snider). Sections were mounted in glycerol and analyzed under a dissection microscope.

Axonal tracing
After overnight fixation in 4% PFA at 4°C, single crystals of the fluorescent carbocyanide dye DiI (1,1’-dioctadecyl 3,3’,3’-tetramethylindocarbocyanine perchlorate; Molecular Probes) or DiA (4-[4-(dihexadecyl amino)styryl]N-methyl-pyridinium iodide; Molecular Probes) were placed in single or multiple locations in the neocortex or dorsal thalamus (Godement et al., 1987; Metin and Godement, 1996). After 4-7 weeks at room temperature in 4% PFA to allow dye diffusion, the sample were embedded in 5% agarose and cut into 100 μm thick sections on a vibratome. Counterstaining was performed using Hoechst (Aldrich Chemicals) or SYTOX green (Molecular Probes) and digital images were taken using a Spot II camera on a fluorescent microscope or dissection microscope.

RESULTS

A specific subpopulation of thalamic axons is misrouted within the basal ganglia of *Ebf1*–/– mutant embryos
Previous analysis suggested that *Ebf1* inactivation may affect the navigation of specific populations of thalamic axons (Garel et al., 1999). To further characterize this phenotype, we performed axonal tracing experiments using DiI injections. Broad injections in the lateral part of the dorsal thalamus in E16 *Ebf1*+/– embryos showed that some thalamic axons are misrouted into the amygdalar region (Fig. 1A,B). These axons form a thick tract that ends in this region, except for a few axons that grow dorsally in the direction of the neocortex. On the contrary, at more rostral levels, thalamic axons appear normal within the internal capsule and grow into the neocortical intermediate zone (Fig. 1C,D). To identify the region of the thalamus that sends the misrouted axons, we placed a DiI crystal in the amygdalar region in E16.5 *Ebf1*+/– embryos. Retrogradely labeled cells were specifically found in a lateral thalamic area that has the characteristic shape and position of the presumptive dorsal lateral geniculate nucleus (dLGN) (Jones, 1985) (Fig. 1E,F). Thus, in the absence of *Ebf1*, dLGN axons are misrouted in the amygdalar region, whereas the rest of the thalamic axons normally grow into the internal capsule and the neocortex.

The topography of connections between the dorsal thalamus and the neocortex is shifted in *Ebf1*–/– embryos
Axons from specific thalamic nuclei normally project towards particular neocortical domains (Crandall and Caviness, 1984; O’Leary et al., 1994; Sur and Leamey, 2001). We thus investigated in *Ebf1* mutants if dLGN axons reach their final target, the occipital neocortex and whether the general topography of thalamocortical projections is normal. We placed DiI crystals in three locations of the neocortex at E16.5 (Crandall and Caviness, 1984; Molnar et al., 1998a) (Fig. 2). In wild-type embryos, a DiI injection in the occipital neocortex labeled thalamic cell bodies and cortical axons in the dLGN (Fig. 2A,B,D,E). A DiI injection in the parietal neocortex, however, labeled cells and axon terminals in a more medial thalamic domain, where the
The presumptive ventrobasal complex (VB) is located (Jones, 1985) (Fig. 2G,H). Finally, a DiI injection in the frontal neocortex labeled cells and axons in an even more medial domain, which includes the presumptive ventromedial nucleus (VM) (Jones, 1985) (Fig. 2K,L). In Ebf1−/− mutant embryos, DiI injections in these three neocortical zones systematically labeled cell bodies and axons in a thalamic domain located more medially than in controls (Fig. 2A-M). Thus, thalamocortical and corticothalamic projections are shifted. This medial shift was confirmed by a double injection in the parietal and occipital neocortex with DiA and DiI, respectively (Fig. 2N-P). Taken together, in Ebf1−/− embryos, axons of the dLGN do not reach the occipital neocortex and the topography of thalamocortical projections is shifted with a given thalamic nucleus projecting towards a more caudal neocortical domain (Figs 2, 10).
H, hippocampus; Ncx, neocortex; OB, olfactory bulb; Str, striatum.

Id2, development, at E17.5, the expression pattern of 2000; Mallamaci et al., 2000b; Zhou et al., 2001). Later in and participate in early cortical regionalization (Bishop et al., Liu et al., 2000; Ragsdale and Grove, 2001; Muzio et al., 2002) the anteroposterior axis of the neocortex (Simeone et al., 1992; Simeone et al., 1992; Garel et al., 1999) (Fig. 3). Thus, a straightforward explanation would be that, inactivation perturbs the inactivation does not severely perturb the molecular regionalization of the neocortex and dorsal thalamus. Thus, the shift in thalamocortical projections is unlikely to result from a general change in the positioning or molecular identity of the different thalamic nuclei.

### Ebf1 inactivation affects early thalamic axon pathfinding in the basal ganglia

To better understand this phenotype, we examined how it develops, by performing DiI injections in the neocortex and dorsal thalamus in early embryos, before thalamic and cortical axons meet in the internal capsule. Using DiI injections in the neocortex at E13.5 and E14.5, we did not detect any defects in the growth and trajectory of cortical axons into the mantle zone of the ganglionic eminences, the basal ganglia primordium, in Ebf1–/– embryos (Miller et al., 1993; Metin and Godement, 1996, Molnar et al., 1998a; Auladell et al., 2000). However, DiI injections in the dorsal thalamus at E13.5 showed that, in Ebf1–/– embryos, the thalamic axons growing into the ganglionic eminences do not make a sharp turn in direction of the neocortex (Fig. 6E,F). Instead, they are misrouted towards the pial surface (Fig. 6E,F). At E14.5 and E15.5, the misrouted thalamic axons begin to navigate towards the amygdala (Fig. 6G,H, Fig. 1A,B). Thus, whereas early cortical axons show normal navigation before encountering thalamic axons, thalamic axons are already misrouted when they enter the basal ganglia (Miller et al., 1993; Metin and Godement, 1996; Molnar et al., 1998a; Auladell et al., 2000).

We examined the different cell populations that have been proposed to guide thalamic axons in the ganglionic eminences. In wild-type embryos, thalamic DiI injections retrogradely label cells of the perireticular nucleus, which are located in the internal capsule and form transient projections with the thalamus (Mitrafis and Guillery, 1993) (data not shown). In Ebf1–/–, thalamic DiI injections label a group of basal ganglia cells that is located where the internal capsule axonal bundle would normally be in E13.5 embryos (arrow in Fig. 6F), indicating that at least some perireticular nucleus cells are normally positioned. Next, we examined the expression of axonal guidance molecules, such as Semaphorin 6A and netrin 1, which are implicated in regulating the growth of thalamic axons into and through the basal ganglia. Semaphorin 6A encodes a laminar patterns (Suzuki et al., 1997; Donoghue and Rakic, 1999; Mackarehtschian et al., 1999; Miyashita-Lin et al., 1999; Nakagawa et al., 1999; Rubenstein et al., 1999; Liu et al., 2000). At both ages, we did not observe any changes in the cortical expression patterns of these genes in Ebf1–/– mutant embryos (Fig. 4; data not shown).

We next studied the morphology of the dorsal thalamus as well as the expression patterns of Cdhh6, Cdhh8, COUP-TFI, EphA4, EphA7, ephrin A2, ephrin A5, Gbx2, Lhx9 and Sema6a. The expression of these genes is restricted to specific domains or nuclei in the dorsal thalamus, allowing us to establish the position and molecular properties of the developing thalamic nuclei (Suzuki et al., 1997; Zhou et al., 1997; Feldheim et al., 1998; Miyashita-Lin et al., 1999; Retaux et al., 1999; Liu et al., 2000; Nakagawa and O’Leary, 2001) (Fig. 5). In Ebf1–/– mutant embryos, we did not observe changes in the expression patterns of these genes between E14.5 and E16.5 (Fig. 5; data not shown).

Overall, our gene expression analysis shows that Ebf1 inactivation does not severely perturb the molecular regionalization of the neocortex and dorsal thalamus. Thus, the shift in thalamocortical projections is unlikely to result from a general change in the positioning or molecular identity of the different thalamic nuclei.

### No apparent defects in the dorsal thalamus and neocortex of Ebf1–/– embryos

How could such a shift in the topographic organization of projections occur? Ebf1 is expressed in layer I of the neocortex between E10.5 and E12.5, in the mantle of the dorsal thalamus between E12.5 and E14.5, and in several nuclei in the embryonic basal ganglia (Wang and Reed, 1993; Garel et al., 1997; Garel et al., 1999) (Fig. 3). Thus, a straightforward explanation would be that Ebf1 inactivation perturbs the positioning or specification of the various thalamic nuclei and/or affects regionalization within the neocortex.

Although the expression pattern of Ebf1 does not suggest a role in neocortical regionalization, we nevertheless checked whether its inactivation affected patterning or lamination of the neocortex, based on gene expression patterns. At E12.5, COUP-TFI, Enx2 and Fgfr3 are expressed in gradients along the anteroposterior axis of the neocortex (Simeone et al., 1992; Liu et al., 2000; Ragsdale and Grove, 2001; Muzio et al., 2002) and participate in early cortical regionalization (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001). Later in development, at E17.5, the expression pattern of COUP-TFI, Id2, Cdhh6, Cdhh8, EphA5 and EphA7 are in gradients or restricted to presumptive cortical areas and show specific

![Fig. 4. Neocortical regionalization is not affected by Ebf1 inactivation. Sagittal sections of E17.5 heterozygous (left) and homozygous (right) Ebf1 mutants processed for in situ hybridization with the following probes: Id2 (A,B), COUP-TFI (C,D), Cdhh6 (E,F), EphA7 (G,H) and Cdhh8 (IJ). Arrowheads indicate rostrocaudal boundaries or changes in gene expression. These boundaries or gradients of expression are not changed in Ebf1+/– mutant embryos. H, hippocampus; Ncx, neocortex; OB, olfactory bulb; Str, striatum.](image-url)
transmembrane semaphorin (Zhou et al., 1997) and its inactivation affects the pathfinding of a subset of thalamic axons in the caudal region of the basal ganglia (Leighton et al., 2001). Sema6a is expressed in the basal ganglia and in the dorsal thalamus (Zhou et al., 1997; Leighton et al., 2001) (Fig. 6I,K). In Ebf1–/– mutant embryos, Sema6a expression in the thalamus was unaffected at E13.5 and E16.5 (Fig. 6I-L). By contrast, Sema6a expression was greatly reduced in a group of cells located close to the entrance point of thalamic axons in the basal ganglia (Fig. 3A–C), where thalamic axons start to show pathfinding defects in Ebf1 mutants (compare Fig. 6E-H with Fig. 6I-L). On the contrary, expression domains of the gene for netrin 1, which encodes a secreted molecule involved in the guidance of thalamic and cortical axons (Metin et al., 1997; Richards et al., 1997; Tuttle et al., 1999; Braisted et al., 2000), were not severely modified by Ebf1 inactivation (Fig. 6M-P).

Overall, in Ebf1 mutants, early thalamic axons fail to normally turn towards the cerebral cortex as they enter the basal ganglia, creating an abnormal tract in the amygdalar region. This defect may be due to a change in molecular properties in specific subsets of basal ganglia cells.

### Thalamic axons are caudally shifted in the internal capsule of Ebf1–/– embryos

Thalamic and cortical axons are topographically ordered along their trajectory in the internal capsule within the basal ganglia (Molnar et al., 1998a). We thus examined if the abnormal turn of thalamic axons inside the basal ganglia of Ebf1–/– embryos affected the topographic organization of axons inside the internal capsule.

In E16.5 wild-type embryos, double DiI and DiA injections in the frontal and parietal neocortex (Fig. 7A,B), or in the occipital and parietal neocortex (Fig. 7D,E), show that the axons originating from and projecting to a given neocortical domain form ordered bundles in the internal capsule (Fig. 7B,E). Ebf1 mutants showed a normal organization of these axon bundles (Fig. 7A-F).

We next examined the position of axons inside the internal capsule by double DiI and DiA injections into the putative dLGN nucleus and VB complex, respectively. In wild-type embryos, the bundle of axons labeled by dLGN injections runs through the caudal part of the striatum and caudal regions of the neocortex (Fig. 7G,H,J). Conversely, axons labeled by VB injections grow at an intermediate anteroposterior position and invade the parietal neocortex (Fig. 7G,H,J). In Ebf1–/– embryos, dLGN axons form the abnormal tract that travels towards the amygdalar region (Fig. 7I). VB injections label a bundle of axons located inside the internal capsule; however, these are located more caudally, where dLGN-labeled axons would normally be in wild-type embryos (Fig. 7J,K).

Thus, altogether, these results indicate cortical axons originating from a given domain have a normal navigation and topography in the internal capsule in Ebf1–/– embryos. Conversely, thalamic axons show a shift in their position inside the internal capsule, and, consistent with their new trajectory
Thalamocortical axons in *Ebf1* and *Dlx1/2* mutants

invade a more caudal region of the neocortex. Thus, we observe a global shift in the position of thalamic axons just after their turn into the basal ganglia.

**Dlx1/2 inactivation affects the topography of thalamocortical projections**

Our analysis of the *Ebf1* phenotype indicates that a misguidance of specific thalamic axons and a general shift in the position of the others can occur in the absence of apparent defects in the neocortex and dorsal thalamus. As *Ebf1* is expressed in the dorsal thalamus, the possibility that its inactivation may affect thalamic neurons cannot be excluded. Nevertheless, our results raise the possibility that developmental defects in the basal ganglia of *Ebf1*–/– embryos may shift the topography of thalamocortical connections. To further examine if affecting structures on the path of thalamic axons can deviate the topography of thalamocortical projections, we examined *Dlx1/2*–/– mutant embryos (Qiu et al., 1997). *Dlx1* and *Dlx2* encode homeodomain transcription factors and are expressed in the basal ganglia and in the ventral thalamus, but not in neocortical projection neurons or in the dorsal thalamus (Bulfone et al., 1993). *Dlx1/2*–/– mutant embryos have a block in differentiation of the basal ganglia (Anderson et al., 1997). DiI injections in the neocortex and dorsal thalamus of E14 *Dlx1/2*–/– embryos shows that both cortical and thalamic axons fail to make a sharp turn into ganglionic eminences and are displaced towards the pial surface (Fig. 8A-F). This defect is probably due to the expansion of the subventricular zone (SVZ*) in the basal ganglia (Anderson et al., 1997) and results in the formation of a displaced and highly disorganized internal capsule, as shown by DiI injections in the dorsal thalamus of E16.5 embryos (Fig. 8G-J). Although some thalamic axons reach the neocortex, a large number of the misrouted axons remain in the basal ganglia (Fig. 8G-J). The identity of the thalamic nuclei generating the axons that fail to reach to cortex could not be unequivocally determined because of the disorganization of the internal capsule.
The topography of thalamocortical projections was examined by introducing DiI and DiA crystals in the occipital or parietal neocortex of E16.5 heterozygous and homozygous embryos (Fig. 9A-L). These experiments retrogradely labeled fewer cells and axons in homozygous embryos than in controls (Fig. 9A-L), confirming that numerous thalamic axons do not reach the cortex. However, DiI injections (Fig. 9A-I) or double DiI and DiA injections (Fig. 9J-L) in the neocortex of \textit{Dlx1/2}–/– embryos systematically labeled cells located in a more medial domain (Fig. 9A-F) or in a wider domain that extends more medially (Fig. 9G-L) than in controls. Thus, the topography of thalamic projections into the neocortex is systematically shifted in \textit{Dlx1/2}–/– mutants.

We next examined the positioning of thalamic axons within the internal capsule at E16.5 by placing DiI and DiA crystals inside the presumptive dLGN and VB (Fig. 9M-Q). In \textit{Dlx1/2}–/– embryos, dLGN axons are present in ventral parts of the internal capsule (Fig. 9N,O) but most of these axons do not grow dorsally into the neocortex (Fig. 9P,Q). VB axons are shifted to a more caudal position within the internal capsule, particularly in its dorsal parts (Fig. 9R-S). Thus, in \textit{Dlx1/2}–/– mutants, a majority of dLGN axons fail to reach to neocortex and the topography of the remaining thalamocortical projections is shifted in the internal capsule and neocortex, as in the \textit{Ebf1}–/– mutants.

\section*{DISCUSSION}

During embryogenesis, different nuclei of the dorsal thalamus and cortical domains establish precise interconnections that are key to the relay and processing of sensory and motor information. We provide new information on the mechanisms regulating the initial targeting of thalamocortical axons to specific cortical domains. Our analysis of \textit{Ebf1} and \textit{Dlx1/2} mutant embryos shows that individual thalamic projections are not formed independently of each other, and suggests that altering the development of structures located on the pathway of thalamic axons shifts the early topography of thalamocortical projections inside the internal capsule and in the neocortex. These results indicate that cues regionally restricted within the neocortex and dorsal thalamus do not precisely dictate the initial topography of thalamocortical projections. Furthermore, our study suggests that the positioning of thalamic axons inside the basal ganglia is important for regulating this topography.

\textbf{Thalamic projections are shifted in the absence of apparent thalamic and neocortical defects in \textit{Ebf1} and \textit{Dlx1/2} mutant embryos}

We show that \textit{Ebf1} inactivation drastically affects the pathfinding of a subset of thalamic axons and creates a shift in the topography of the remaining thalamocortical projections (Fig. 10). This shift is observed in the absence of apparent thalamic or neocortical defects between E13.5 and E17 (Figs 4, 5) and is first detected within the basal ganglia primordium (Figs 10). Furthermore, during the period when thalamic axons are traveling through the basal ganglia primordium (E13.5-15.5) (Miller et al., 1993; Metin and Godement, 1996; Molnar et al., 1998a; Auladell et al., 2000), this structure exhibits
molecular defects (Garel et al., 1999), including an abnormal expression of *Semaphorin* (*Sema6a*) (Fig. 6I-L). Thus, although the loss of *Ebf1* expression in the dorsal thalamus may affect thalamic neurons, our molecular analysis shows that the shift in topography is not due to a general change in the molecular regionalization of the neocortex or dorsal thalamus. Furthermore, our results raise the possibility that this shift may be due to defects in the basal ganglia.

If defects in structures located on the path of thalamic axons can shift the topography of thalamocortical axons, we would expect to observe a similar phenotype in other mutant mice that have basal ganglia defects. We thus examined *Dlx1/2* mutants where differentiation of the basal ganglia and ventral thalamus is abnormal (Anderson et al., 1997; Marin et al., 2000; Yun et al., 2002). Furthermore, *Dlx1* and *Dlx2* are not expressed in the dorsal thalamus or in cortical projection neurons (Bulfone et al., 1993; Stuhmer et al., 2002). In *Dlx1* mutants, the formation of the internal capsule is perturbed, probably because of a block in basal ganglia differentiation (Anderson et al., 1997; Yun et al., 2002) and, as in *Ebf1* mutants, the topography of thalamocortical projections is shifted in the neocortex and internal capsule (Figs 9, 10). Thus, the phenotype of *Dlx1/2*–/– mice supports the possibility that affecting structures on the path of thalamic axons can shift the topography of thalamocortical projections. It could be argued that the reduction of cortical interneurons in *Dlx1/2* mutants (Anderson et al., 1997) might contribute to this phenotype. However, this is unlikely because *Nkx2.1* mutants, which also have a major deficit in neocortical interneurons, have normal thalamocortical projections (Marin et al., 2002).

**The specificity of thalamic axons targeting is not dictated by cues located within the neocortex**

Specific aspects of the phenotypes of *Ebf1* and *Dlx1/2* mutant embryos provide information on the mechanisms controlling the initial topography of thalamocortical projections. In particular, the systematic and coherent shift in the topography of thalamocortical axons indicates that projections of a given thalamic nucleus are not established independently of the projections of the other nuclei. In addition, this shift suggests that the mechanisms involved are likely to control the relative rather than absolute position of axons. This has been observed in the retinotectal system (Brown et al., 2000), suggesting that this feature may be a common characteristic for the formation of topographic projections.

Another aspect of the phenotypes of *Dlx1/2* and *Ebf1* mutants is that thalamic axons have a shifted position inside the basal ganglia before entering aberrant regions of the neocortex. If gradients of guidance cues within the neocortex were strictly governing the organization of thalamic inputs, we would have expected the thalamic axons to be redirected to
their appropriate destinations within the neocortex. Thus, positional information within the neocortex cannot correct the ectopic trajectory of the thalamic projections, at least at the early developmental stages we have studied. Therefore, either positional cues do not normally have a central role in regulating early thalamocortical projections, or perturbing axons on their way to the neocortex over-rides the activity of instructive gradients. These observations are in agreement with in vitro experiments in which thalamic axons can invade any region of the neocortex (Molnar and Blakemore, 1991). Furthermore, these data suggest that the initial organization of thalamocortical projections is not strictly regulated by a ‘classical’ chemoaffinity mechanism, where axons are guided by positional cues within the target structure (Drescher et al., 1997; Feldheim et al., 2000; Brown et al., 2000).

**The internal capsule: a decision point for the topography of thalamocortical projections?**

Our results suggest that the positioning of thalamic axons inside the internal capsule is important for the topography of the initial projection, as changes in this position correlate with changes in the neocortical target domains. What mechanism(s) could underlie this process? There is evidence that thalamic axons use cortical subplate axons as a scaffold to enter the cortex (McConnell et al., 1989; Ghosh et al., 1990; Ghosh and Shatz, 1992; Ghosh and Shatz, 1993; Molnar et al., 1998b). The handshake model proposed that thalamic and cortical axons interact inside the internal capsule and guide each other to their final target, potentially through specific molecular interactions or through positional alignment within the internal capsule (Molnar and Blakemore, 1995; Molnar et al., 1998a). Neocortical patterning is likely to regulate the molecular properties and navigation trajectories of cortical axons that grow towards the thalamus. For example, both *Emx2* and *COUP-TFI* mutant mice, which show caudal-to-rostral

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**Fig. 9.** The topography of thalamocortical projections is shifted in *Dlx1/2*⁺⁻ embryos. Coronal (A-L) or horizontal (M-Q) hemisections of E16.5 *Dlx1/2* heterozygous (left) and homozygous (right) mutant brains where DiI crystals (A-I) or DiI and DiA crystals (J-Q) were introduced in the occipital neocortex (A-C), the parietal neocortex (D-I), the occipital and parietal neocortex (J-L), or the putative dorsolateral geniculate nucleus (dLGN) and ventrobasal (VB) complex (M-Q). Schematic diagrams indicate the position of DiI and DiA crystals (A,D,G,J,M) and stars indicate their actual position in B,C. (A-L) Morphological landmarks, including the pial surface of the thalamus (broken line) and the retroflexus tract (white arrowhead), are used to position presumptive thalamic nuclei. In controls, injections in the occipital neocortex label cells in the putative dLGN (B,K) and injections in the parietal neocortex label cells in the VB complex (E,H,K). Open arrowheads indicate the medial boundary of the thalamic domain stained in wild-type embryos (B,E,H,K). This boundary is indicated in *Dlx1/2*⁺⁻ embryos and shows that thalamic domains labeled by occipital and parietal injections are shifted medially (C,F). Note that the number of cells labeled is reduced in homozygous mutant embryos (compare B with C and E with F). In some cases, the region containing labeled cells was broader in mutant embryos, partially including the domain labeled in controls as well as a more medial domain (H,I). Similarly, double occipital and parietal injections show that the labeled domains in mutant embryos are medially displaced (K,L). In this case there is some overlap in the regions labeled by each dye (arrow in L). (M-Q) Horizontal sections at ventral levels (N,O) and more dorsal levels (P,Q) of brains after a thalamic double injection in the presumptive dLGN and VB. Even though the tracer crystals were relatively small, a large number of axons were stained in our experiments because of the small size of the thalamus. In wild-type animals, putative dLGN axons (red) and VB axons (green) turn into the striatum and remain as two separate bundles in the caudal (open arrowheads in N and P) and intermediate regions of the internal capsule, respectively (N,P). In *Dlx1/2*⁺⁻ mutant embryos, dLGN axons are primarily detected ventrally (open arrowhead in O), and are mixed with VB axons. In more dorsal sections (Q), very few dLGN axons are visible (open arrowhead), indicating that they remain in ventral regions. On the contrary, a large number of VB axons are detected in a caudal region where normally dLGN axons travel (compare P with Q). dTH, dorsal thalamus; Fr, frontal neocortex; IC, internal capsule; Ncx, neocortex; Occ, occipital neocortex; Par, parietal neocortex; Str, striatum.
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changes in the molecular properties and connectivity of the occipital neocortex (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001), also have subplate defects (Zhou et al., 1999; Mallamaci et al., 2000a) that may contribute to the changes in thalamocortical connectivity.

In Ebf1 mutants, we did not observe defects in the pattern or timing of subplate or cortical plate axonal outgrowth into the internal capsule (Fig. 6A-D). However, thalamic axons show pathfinding defects as they enter the basal ganglia, suggesting that the phenotype we observe may be due to a mismatch between normally positioned cortical axons and shifted thalamic axons. In Dlx1/2 mutants, neocortical axons are displaced in the basal ganglia, and thus probably contribute to the disorganization of the internal capsule and to the reduction in the number of axons reaching their target structures (Fig. 8). However, the timing of their outgrowth, and their rostrocaudal distribution, does not seem affected. Thalamic axons, however, had a shifted position within the internal capsule (Fig. 9). Therefore, while defects in both thalamic and cortical axons could contribute to the shift in thalamocortical projections, our observations suggest that it may be primarily due to the displacement of the thalamic axons. Thus, if thalamic and cortical axons do directly interact (‘handshake’), our data suggest that a displacement in thalamic axons, and in the alignment of thalamic and cortical axons, can induce a shift in their final target zone in the neocortex as well as a reciprocal shift in cortical projections. Overall, our study suggests that the position of thalamic axons in the basal ganglia, an intermediate structure located between the thalamus and neocortex, is an important decision point for the initial topography of thalamocortical projections.

The role of basal ganglia in guidance and positioning of thalamic axons

Previous work has identified groups of cells in the basal ganglia that participate in the guidance of thalamic and cortical axons, by producing chemoattractants (Metin and Godement, 1996; Metin et al., 1997; Richards et al., 1997; Braisted et al., 1999; Braisted et al., 2000) and or by forming transient axonal scaffolds (Mitrofanis and Guillery, 1993). Defects in some of these cells, caused by the Mash1 mutation, are correlated with the inability of thalamocortical axons to reach the neocortex (Tuttle et al., 1999). These results support the idea that the basal ganglia form an intermediate target for axons interconnecting the neocortex and thalamus (Metin and Godement, 1996). Our results provide evidence that the ordering of thalamic axons within the basal ganglia may play an important role in the final topographic organization of thalamocortical projections.

How is this order established or maintained? Although our study does not provide definitive answers, it allows us to discuss different hypotheses. One possibility is that an array of guidance cues regulates the spatial organization of thalamic axons inside the basal ganglia, by a chemoaffinity mechanism within this structure. However, so far, there is no obvious candidate molecule for such function. An alternative mechanism involves both guidance cues and the timing of thalamic axon outgrowth (Molnar and Blakemore, 1995; Molnar et al., 1998a). Indeed, there is a gradient of differentiation in the dorsal thalamus (Jones, 1985) and thalamic axons of different zones grow into the internal capsule at different times (Molnar et al., 1998a). Localized guidance cues or groups of cells may determine the position of pioneering thalamic axons. Candidate molecules include Sema6a: a subpopulation of thalamic axons is misrouted in the amygdalar region of Sema6a mutants (Leighton et al., 2001). These pioneer axons would provide a landmark for the next set of thalamic axons to follow.
of incoming axons, which would navigate to an adjacent location. Thus, as new axons arrive, they would stack in a temporally determined array. In both models, the shift in topography we observe in Dlx1/2 and Eph/II mutant embryos could be a consequence of the axonal pathfinding defects of specific thalamic axons within the basal ganglia. This mechanism may also participate in the phenotype of Emx2 and COUP-TFI mutant embryos, where a subset of thalamic axons do not reach the neocortex (Zhou et al., 1999; Mallamaci et al., 2000b; Lopez-Bendito et al., 2002).

Conclusion
The favored model for the formation of topographic projections proposes that this process is regulated by cues located in the two interconnected structures (Sperri, 1963). This mechanism is implicated in the targeting of retinotectal (Drescher et al., 1997; Goodhill and Richards, 1999; Feldheim et al., 2000) and reticulogeniculate (Feldheim et al., 1998) axons, of limbic thalamic axons to the limbic cortex (Barbe and Levitt, 1992; Mann et al., 1998) and hippocampal neurons to the septum (Gao et al., 1996), as well as in the formation of a topographic map within a neocortical area (Vanderhaeghen et al., 2000). We have presented evidence that the initial topography of thalamocortical projections is not strictly determined by the information located inside the target structure and that the position of axons within intermediate structures may be important for the regulation of this topography. In particular, our results suggest a role for the basal ganglia in the organization of thalamic axons and the choice of their final target destination. More generally, these observations raise the possibility that intermediate structures, and/or the relative position of axons inside a fiber pathway, may regulate the formation of topographically organized long-range projections within the central nervous system.

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