The ciliogenic transcription factor Rfx3 is required for the formation of the thalamocortical tract by regulating the patterning of prethalamus and ventral telencephalon

Dario Magnani1, Laurette Morlé2, Kerstin Hasenpusch-Theil1, Marie Paschaki2, Monique Jacoby3, Stéphane Schurmans4, Bénédicte Durand2 and Thomas Theil1,*

1Centre for Integrative Physiology, University of Edinburgh, Hugh Robson Building, Edinburgh EH8 9XD, UK, 2Centre de Génétique et de Physiologie Moléculaires et Cellulaires, CNRS UMR 5534, Université Claude Bernard Lyon 1, Villeurbanne, Lyon F69622, France, 3Institute of Immunology, Centre de Recherche Public de la Santé/ Laboratoire National de Santé, Luxembourg, Luxembourg and 4Laboratory of Functional Genetics, GIGA-Signal Transduction, GIGA B34, Université de Liège, Liège B-4000, Belgium

*To whom correspondence should be addressed at: Centre for Integrative Physiology, University of Edinburgh, Hugh Robson Building, Edinburgh EH8 9XD, UK. Tel: +44 131 650 3721; Fax: +44 131 650 6527; Email: thomas.theil@ed.ac.uk

Abstract

Primary cilia are complex subcellular structures that play key roles during embryogenesis by controlling the cellular response to several signaling pathways. Defects in the function and/or structure of primary cilia underlie a large number of human syndromes collectively referred to as ciliopathies. Often, ciliopathies are associated with mental retardation (MR) and malformation of the corpus callosum. However, the possibility of defects in other forebrain axon tracts, which could contribute to the cognitive disorders of these patients, has not been explored. Here, we investigate the formation of the corticothalamic/thalamocortical tracts in mice mutant for Rfx3, which regulates the expression of many genes involved in ciliogenesis and cilia function. Using Dil axon tracing and immunohistochemistry experiments, we show that some Rfx3−/− corticothalamic axons abnormally migrate toward the pial surface of the ventral telencephalon (VT). Some thalamocortical axons (TCAs) also fail to leave the diencephalon or abnormally project toward the amygdala. Moreover, the Rfx3−/− VT displays heterotopias containing attractive guidance cues and expressing the guidance molecules Slit1 and Netrin1. Finally, the abnormal projection of TCAs toward the amygdala is also present in mice carrying a mutation in the Inpp5e gene, which is mutated in Joubert Syndrome and which controls cilia signaling and stability. The presence of identical thalamocortical malformations in two independent ciliary mutants indicates a novel role for primary cilia in the formation of the corticothalamic/thalamocortical tracts by establishing the correct cellular environment necessary for its development.
Introduction

Primary cilia are complex subcellular organelles protruding from the cell surface, which mediate several sensory functions and are involved in regulating the cellular response to several signaling pathways and in particular to Sonic hedgehog (Shh) signaling (1). Moreover, primary cilia have important roles in human diseases, and defects in primary cilia formation and/or function underlie several human syndromes commonly referred to as ciliopathies (2,3). These disorders are associated with a large variety of manifestations that often include neurological features such as MR, but much less is known about the specific signaling pathways and the pathogenesis at the cellular and tissue level that ultimately result in neurological disease phenotypes (4,5).

A common feature of ciliopathies is malformations of the corpus callosum (CC) which as the major forebrain commissure provides the interhemispheric exchange of information between the two cortical hemispheres. While the significance of callosal abnormalities for the MR pathogenesis of ciliopathy patients remains unclear, this finding raises the possibility that other prominent axon tracts of the forebrain are affected in ciliopathies. To explore this, we here investigate the formation of the corticothalamic/thalamocortical tracts in two ciliary mouse mutants. The thalamocortical tract connects the dorsal thalamus with the cerebral cortex thereby conveying sensory information from the external environment to the cortex. The corticothalamic tract in turn sends processed sensory information back to the thalamus thereby providing the feedforward and feedback mechanisms essential in this processing unit (6). The formation of the thalamocortical tract requires a complex navigation of thalamocortical axons (TCAs) through the prethalamus and ventral telencephalon (VT), and involves several guidepost cues along the thalamocortical path that control the navigation of thalamic axons. Two populations of pioneer neurons are located in the prethalamus and the VT projecting their axons into the thalamus and providing scaffolds for TCAs growing into the prethalamus and across the diencephalic/telencephalic boundary, respectively (7–10). In addition, cells from the lateral ganglionic eminence (LGE) migrate into the medial ganglionic eminence (MGE) to form a permissive corridor and guide TCAs through the otherwise non-permissive MGE (11). These corridor cells also mediate the sorting of TCAs according to their rostral/caudal origin in the thalamus by expressing the Slit1 and Nestin1 guidance factors (12). Interestingly, the development of theVT and of the prethalamus, which provide these guidepost cues, is under the control of Shh signaling, raising the possibility that the formation of the thalamocortical tract might be affected in primary cilia mutants. However, this has not been explored yet.

RFX transcription factors have been shown to play fundamental roles in ciliogenesis by regulating the expression of genes involved in cilia assembly or function (13–15). Accordingly, Rfx3-deficient mouse mutants exhibit several hallmarks of ciliopathies, in particular left–right asymmetry defects and hydrocephalus (13,14), yet importantly they survive until birth, providing a rare opportunity to study the formation of forebrain axon connections in a cilia mouse mutant. Indeed, the Rfx3 mutation interferes with the formation of the CC (16). Here, we investigate the formation of the corticothalamic/thalamocortical tracts in Rfx3-deficient mice. In these animals, some corticothalamic axons (CTAs) abnormally migrate toward the amygdala. Moreover, only a small proportion of TCAs reach the dorsal telencephalon but many fail to migrate through the prethalamus, whereas others enter the VT but subsequently project ventrally toward the amygdala. These defects correlate with abnormal patterning of the prethalamus. In addition, the rostroventral telencephalon forms neural heterotopias at its pial surface containing a mixture of MGE- and LGE-derived cells expressing the Slit1 and Nestin1 guidance molecules. Finally, the abnormal ventral deflection of TCAs toward the amygdala is also found in a second ciliary mouse mutant, which is deficient for inpp5e, a Joubert syndrome disease gene. Taken together, these analyses indicate a novel role for primary cilia in the development of thalamocortical tract.

Results

Rfx3−/− mutant embryos display defects in the development of thalamocortical connections

Recently, we showed that the ciliogenic transcription factor Rfx3 is required for CC development (16). Here, we investigate whether an Rfx3 null mutation also affects the formation of the thalamocortical/corticothalamic tracts. TCAs progress in a multistep fashion over their intermediate territories, the diencephalon and the VT (17). Thalamic neurons send their axons ventrally through the prethalamus toward the diencephalic–telencephalic boundary. After entering the telencephalon at E13.5, thalamic axons migrate through the VT via the internal capsule (ic) to reach the pallial–subpallial boundary (PSPB) and finally enter the cortical intermediate zone at E14.5. CTAs leave the cortex at around E14.5 and follow the same trajectory through the VT as TCAs but in the opposite direction. This trajectory is revealed in E18.5 control embryos by placement of Dil crystals into the cortex. This analysis also reveals the path of TCAs by retrograde labeling (Fig. 1A–D). Rfx3−/− mutant brains show a similar labeling pattern; however, we noted a number of backlabeled nuclei in the rostroventral telencephalon close to a region of the VT containing a heterotopia. This analysis also revealed an abnormal ventral projection toward the amygdala at intermediate and caudal levels (Fig. 1E–H). To more specifically label TCAs, we placed Dil crystals into the thalamus. TCAs are highly fasciliated in the ic, are arranged in roughly parallel bundles in the stratum and reach the cortex (Fig. 1I and J). In contrast, in Rfx3−/− mutant brains, thalamic axons appear disorganized in the stratum with some axons projecting toward the PSPB in more ventral regions (Fig. 1M and N). At caudal levels, TCAs abnormally project into the VT toward the amygdala similar to our findings on the CTA trajectory.

Immunostaining for the panaxonal marker Neurofilament (NF) confirmed the pathfinding defects revealed by Dil labeling. In control embryos, this analysis showed parallel organized axons in the thalamus, prethalamus, ic, the stratium and in the intermediate zone of the cerebral cortex (Fig. 1K and L). In Rfx3−/− mutants, NF staining revealed several abnormal fiber tracts. Many axons are already disorganized in the thalamus, and the exit and entry zones of CTAs and TCAs into and out of the cortex appear broader (Fig. 1O, arrowheads). Many NF+ fibers ectopically project toward the amygdala forming a highly fasciliated axon bundle at more caudal levels (Fig. 1P, arrow).

To gain insights into the origin of these axonal defects, we performed NF immunofluorescence analyses and Dil labeling on E14.5 control and Rfx3−/− mutant brains. At this stage, NF labels axons in the developing thalamus, prethalamus and VT of control embryos (Supplementary Material, Fig. S1A and B). The ic contains highly fasciliated NF+ axons, whereas axons are more loosely organized in the stratum (Supplementary Material, Fig. S1C and D). In Rfx3−/− embryos, NF+ axons are disorganized in the VT projecting in several directions within the LGE and MGE,
Figure 1. Axon guidance defects in the E18.5 Rfx3−/− brains. Coronal sections through the brain of control (A–C and I–L) and Rfx3−/− (E–G and M–P) E18.5 embryos. (A–C and E–G) DiI placements in the cortex (asterisks) reveal the anterior commissure (ac) (A and E) and the corticothalamic and thalamocortical tract through anterograde and retrograde labeling, respectively. In Rfx3−/− mutants, both tracts are formed but note the backlabeled neurons close to a heterotopia at the ventral telencephalic surface (inset and white asterisks in E) and the abnormal projections toward the amygdala at intermediate and caudal levels (arrowheads in F and G). (I, J, M and N) Thalamic DiI labeling (asterisks) revealed the TCA trajectory in control embryos (I and J). In Rfx3−/− mutants, TCAs cross the FSPB in a broader entry zone (arrowheads in M and N). Several axons leave the ic prematurely and form an ectopic axon bundle running ventrally toward the pial surface (arrows in M and N). (K, L, O and P) Immunostainings for NF showed axonal pathfinding defects in the diencephalon (arrowheads in O) and an ectopic axon bundle running ventrally toward the amygdala of Rfx3−/− embryos (arrows in O and P). (D and H) Schematic drawings summarizing the CTA and TCA pathfinding defects in Rfx3−/− mutants. Scale bar: 100 µm.
and a thick axon bundle runs ectopically toward the pial surface (Supplementary Material, Fig. S1F and G, arrow), whereas caudally no axons reached the PSPB (Supplementary Material, Fig. S1H and I). In addition, several NF axons mis-project in the prethalamus resulting in the formation of abnormal bundles (Supplementary Material, Fig. S1I). These findings were confirmed by Dil labeling experiments of TCAs. In contrast to control embryos, very few TCAs traverse the VT with no axons reaching the PSPB in Rfx3−/− mutants (Supplementary Material, Fig. S1K, L and Q), but a large fiber bundle abnormally projects ventrally toward the telencephalic pia (Supplementary Material, Fig. S1R). To further dissect the path of thalamic axons, we performed calretinin (CR) immunofluorescence analysis. In control brains, CR selectively labels the first thalamic axons entering the VT and projecting through the ic (11) (Supplementary Material, Fig. S1O and P). In Rfx3−/− mutants, CR axons were detected in the ic at rostral levels (Supplementary Material, Fig. S1U) but consistent with the NF immunostaining and Dil labeling experiments, many CR axons migrated ventrally toward the pial surface after entering the VT at caudal levels (Supplementary Material, Fig. S1V). Also, some CR axons form abnormal clusters in the thalamus. Finally, placing Dil crystals into the cortex revealed CTAs in control embryos having penetrated into the VT and retrogradely labeled neurons in the thalamus (Supplementary Material, Fig. S1M and N). In Rfx3−/− mutants, backlabeled neurons were not observed, instead a large axon bundle projects from the cortex toward the pia of the VT (Supplementary Material, Fig. S1S and T). Collectively, these analyses indicate severe disruptions in the prethalamus but no significant expression pattern shown by qRT-PCR (Fig. 2B, C, E, F, H and I). Next, we investigated prethalamic patterning. In E14.5 control embryos, the prethalamic progenitor domain is characterized by Nkx2.2 expression (25) (Fig. 3A and B). This expression domain is expanded in Rfx3−/− mutants (Fig. 3C and D). Olig2, another marker for diencephalic progenitor cells, displays a more complex expression pattern. Immunofluorescence analysis showed a high-level Olig2 expression domain in prethalamic progenitors, and lower-level expression in thalamic progenitors, with a high level of expression in the diencephalon (Fig. 3E, F, I and J). In the Rfx3−/− mutant diencephalon, the dorsal thalamic expression of Olig2 and its expression gradient is maintained (Fig. 3G, H, K and L). However, Olig2 expression is expanded in the prethalamus of Rfx3−/− mutants (Fig. 3H and L). Finally, the expression domain of the Pax6 transcription factor normally extends ventrally from the zli encompassing progenitors from the prethalamus and from the eminencia thalami, thereby delineating the boundary between the thalamus and prethalamus (Fig. 3E, F, I and J). In the Rfx3−/− mutants, Pax6 expression is maintained in prethalamic progenitors, but its expression in the diencephalic mantle is reduced (Fig. 3G, H, K and L). Taken together, these results suggest regionalization defects in the prethalamus of the Rfx3−/− mutants.

Abnormal MGE development and formation of heterotopias in the Rfx3−/− VT

Since Rfx3−/− mutants show several corticothalamic/thalamocortical pathfinding defects in the VT, we next investigated its development. Recently, we reported a slight down-regulation of Shh signaling in the MGE of Rfx3−/− mutants using in situ hybridization (16), but qRT-PCR showed no effect on Shh and Gli1 expression, whereas Ptci expression was mildly up-regulated (P = 0.0384; n = 6; Supplementary Material, Fig. S4). Correct levels of Shh signaling are crucial for establishing the PSPB separating the dorsal telencephalon and VT. However, expression analyses for Dlx2, Gsh2, Pax6 and Dbx1 did not reveal abnormalities at this boundary (Fig. 4A and E, and Supplementary Material, Fig. S5). We next analyzed the subdivisions of the VT into the MGE and LGE. Dlx2 is expressed throughout the proliferative zone of the VT, whereas Nkx2.1 expression is restricted to the MGE (Fig. 4A and B). Moreover, Nkx6.2 expression is confined to progenitors on either side of the interganglionic sulcus (Fig. 4C). In Rfx3−/− embryos, these expression patterns are maintained except for a slight Nkx6.2 down-regulation (Fig. 4E–G). We also noted an ectopic accumulation of Dlx2 and Nkx2.1 expressing cells outside the VT at its ventral surface (Fig. 4E–G) reminiscent of the heterotopia we observed at E18.5 (Fig. 1E). These heterotopias were detected on both sides of the rostral telencephalon in all Rfx3 mutants but not in more caudal regions. Also, they varied in size with some extending toward the midline, whereas others were confined to the surface of the ventrolateral telencephalon. In addition, the heterotopias contain a mixture of progenitor cells and neurons. Expression of the MGE-specific genes Lhx6 and Lhx7, which regulate Shh expression in the MGE and which are in turn Shh target genes (26), was detected in the heterotopias (Fig. 4H and M) as well as that of TuJ1 characteristic of differentiating neurons (Fig. 4N). TuJ1 immunofluorescence also showed a size reduction of the MGE mantle. Finally, we investigated whether disruption of the basal lamina, which normally surrounds the telencephalon, could account for heterotopia formation. Laminin immunofluorescence showed that the basal lamina is disrupted in several positions at the ventral pial surface of E12.5 and E14.5 Rfx3−/− mutants (Fig. 4O and P). Taken together, these analyses show a size reduction of the MGE mantle and a disruption of the basal
lamina concomitant with the formation of neural heterotopias in $Rfx3^{-/-}$ embryos.

**Cellular and molecular guidance cues are affected in the VT of $Rfx3^{-/-}$ embryos**

Given these defects in the formation of the VT, we next analyzed whether ventral telencephalic guidance cues essential for TCA projection are also affected. Pioneer neurons located in the rostral MGE extend their axons into the thalamus and serve as scaffolds to guide TCAs across the diencephalic–telencephalic boundary (7–9). Since no markers are available to selectively label these MGE pioneer neurons, we investigated their formation by Dil placement into the E12.5 thalamus. In control embryos, these placements retrogradely labeled the pioneer axons and their cell bodies located in the rostral MGE (Fig. 5A and B).

While this analysis also revealed pioneer neurons cell bodies and axons in the MGE mantle of $Rfx3^{-/-}$ mutants, their distribution appeared less dense (Fig. 5C and D). In addition, some Dil-labeled cell bodies were located in the heterotopic tissue outside the MGE mantle.

In addition to the MGE pioneer neurons, neurons derived from the LGE migrate into the MGE and form a corridor along which TCAs project through the otherwise non-permissive environment of the MGE. In control E12.5 and E14.5 brains, these guidepost cells are marked by both $Ebf1$ and $Islt1$ expression from their origin in the LGE and during their migration into the MGE, where they form the ic (Fig. 6A–C). In E12.5 and E14.5 $Rfx3^{-/-}$ embryos, $Ebf1$-expressing cells originate normally in the LGE and migrate toward the MGE forming the corridor at caudal levels (Fig. 6F). However, at rostral levels, $Ebf1$-expressing cells migrate toward the heterotopias thereby abnormally connecting the

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**Figure 2.** Shh signaling in $Rfx3^{-/-}$ mutant diencephalon. Coronal sections through the diencephalon of E12.5 control (A–C) and $Rfx3^{-/-}$ mutant embryos (D–F) hybridized with the indicated probes. (A and D) No major differences are observed in Shh expression in the $Rfx3^{-/-}$ zli compared with control embryos. Note the enlargement of the third ventricle in mutant embryos (asterisks in D, E and F). (B and C) Ptc1 and Gli1 are expressed in the diencephalon of control brains, but not in the zli. (E and F) Expanded expression of both Ptc1 and Gli1 is found in the prethalamus of $Rfx3^{-/-}$ mutants (arrowheads in E and F). (G–I) Quantification of Shh (G), Ptc1 (H) and Gli1 (I) expression in the diencephalon using qRT-PCR revealed no significant changes in the expression levels of these markers ($n = 6$). Scale bar: 100 µm.
surface of the MGE mantel with the LGE (Fig. 6D and E). These abnormalities were con-

firm ed by immunostainings for Isl1/2. As in control embryos, Isl1/2+ cells normally populate the corridor (Fig. 6G, H, J and K), but were also found ectopically in the hetero-
topic tissue in Rfx3−/− embryos (Fig. 6J and K). Finally, Nkx2.1 expression labels the MGE ventricular zone and the globus pallidus of E14.5 control embryos, whereas the ic remains Nkx2.1 negative (Fig. 6I). The Nkx2.1 expression pattern in the Rfx3−/− MGE mantle appears unaltered in Rfx3 mutants but as with the corridor markers, Nkx2.1-expressing cells were also detected in the ectic tissue (Fig. 6L). These findings suggest that the MGE corridor forms at caudal levels, but that corridor cells abnormally migrate toward the heterotopias at rostral levels.

Next, we investigated whether the defects in forebrain patterning and in the formation of cellular guidance cues correlate with altered expression patterns of axon guidance molecules. Interestingly, Sema6a−/− mice show a very similar thalamocortical pathfinding defect as Rfx3−/− mutants with TCAs being de-

flected toward the amygdala after entering the VT (27). Sema6a has a highly complex expression pattern in the developing fore-

brain. Rostrally, Sema6a expression was mainly confined to the ventricular zone of the VT, but weak expression was also found in the heterotopias of Rfx3−/− embryos (Fig. 7A and E). Caudally, Sema6a transcripts were detected in thalamic neurons and in groups of cells along the path of TCAs in the prethalamic and dorsally and ventrally to the permissive corridor. This expression pattern is not affected by the Rfx3 mutation (Fig. 7B and F). PlexinA4 encodes a semaphorin receptor, and groups of PlexinA4-expressing cells delineate the corridor ventrally and dorsally and also reside in the caudal VT where TCAs normally not project (Fig. 7C and D). In Rfx3−/− embryos, PlexinA4 expression largely persists in cells surrounding a smaller ic at rostral levels and in cells in the caudal VT where it is, however, adjacent to an ectopic axon bundle likely containing TCAs which mis-project toward the amygdala (Fig. 7G and H). Sema5b encodes a chemorepellent for corticofugal axons, and is expressed in telencephalic progeni-
tor cells and in neurons of the piriform cortex. This expression pattern is maintained in Rfx3−/− embryos with no Sema5b expres-
sion in the heterotopias (Fig. 7I, J, M and N). We also investigated expression of Netrin1 and Slit1 which interact in the rostral/caudal sorting of TCAs in the corridor (12). In addition, Slit1 regulates the migration of corridor cells in the MGE (28). Both genes are
expressed in ventral telencephalic progenitor cells, and Netrin1 transcripts were also found in the MGE mantle (Fig. 7K and L). These expression patterns remain unaltered in Rfx3−/− embryos, but both genes are ectopically expressed in cells located in the heterotopias (Fig. 7O and P). Taken together, these expression analyses did not reveal changes in the overall Sema5b, Sema6a...
and PlexinA4 expression patterns, but show differences in the organization of TCAs with respect to the expression domains of these guidance molecules and also indicate ectopic expression of axon guidance factors in the heterotopias.

The Inpp5e ciliary mouse mutant phenocopies the TCA pathfinding defects of Rfx3−/− mutants

The Rfx3 transcription factor has a prominent role in ciliogenesis by regulating the expression of many genes involved in cilia assembly and function, suggesting that the thalamocortical abnormalities of Rfx3−/− embryos are likely caused by structural and/or functional ciliary defects. However, scanning electron microscopy revealed that cilia are present in the diencephalon and telencephalon of E10.5 Rfx3−/− embryos without obvious morphological abnormalities (Supplementary Material, Fig. S6 and data not shown). To gain support for the involvement of cilia in the pathogenesis of these Rfx3−/− phenotypes, we characterized thalamocortical development in an additional ciliary mouse mutant that carries a mutation in the Inpp5e gene (29). Inpp5e encodes inositol polyphosphate-5-phosphatase E that hydrolyzes the 5-phosphate of the second messengers PI(4,5)P2 and PI (3,4,5)P3. The Inpp5e protein is localized at the axoneme of the primary cilium and has a role in cilia-mediated signaling and in regulating cilia stability (29,30). Moreover, its human homolog INPP5E is mutated in MORM and in Joubert syndrome (29,30). Inpp5e mouse mutants show defects in limb and kidney development typical for cilia dysfunction (29), but thalamocortical development has not been investigated yet. Similar to Rfx3−/− embryos, labeling of the thalamocortical tract in E18.5 Inpp5e−/− embryos by inserting Dil crystals into the thalamus revealed an abnormal broad trajectory of TCAs into the rostral cortex and a ventral delection of TCAs toward the amygdala (Fig. 8A, B, E and F). This phenotype was confirmed by NF immunohistochemistry staining, which also showed the absence of heterotopias in the VT of Inpp5e−/− embryos (Fig. 8C, D, G and H). Next, we investigated the origin of this axonal phenotype. In the diencephalon of Inpp5e mutant embryos, Shh expression in the zli was more diffuse, and the Gli1 expression domain is broader but thalamic patterning and neuronal specification were not obviously affected (Supplementary Material, Fig. S7). Similarly, a slight expansion in Gli1 and Ptc1 expression in the MGE had no obvious effect on dorsal/ventral patterning of the telencephalon (Supplementary Material, Fig. S8). Cortical lamination and the expression of

Figure 5. Abnormal formation of MGE pioneer neurons in Rfx3−/− mutants. (A) Dil placements into the E12.5 thalamus of control embryos retrogradely labeled cell bodies located in the MGE and their axons. (C) Dil placed in the E12.5 Rfx3−/− thalamus revealed pioneer neuron cell bodies and axons in the MGE mantle and in ectopic locations in the heterotopias (arrows in C label the cell bodies and the asterisk the heterotopia). (B and D) Schematic representation of a series of coronal sections showing the location and axonal projections of MGE pioneer neurons. Note that these neurons reside in the rostral MGE and project their axons caudally toward the diencephalon. Scale bar: 200 µm.
Tbr1 and Ctip2 characteristic of corticofugal projection neurons also appeared normal in Inpp5e\(^{-/-}\) embryos (Supplementary Material, Fig. S9). In contrast, the MGE corridor was broader and the groups of Sema6a- and PlexinA4-expressing cells delineating the corridor were not discernible in mutant embryos (Supplementary Material, Figs S10 and S11), suggesting that defective ventral telencephalic development underlies the TCA pathfinding defects. Moreover, the similar ventral deflection of TCAs in Rfx3\(^{-/-}\) and Inpp5e\(^{-/-}\) embryos strongly implicates cilia dysfunction as the major cause of this phenotype in Rfx3\(^{-/-}\) mutants.
Figure 7. The Rfx3−/− heterotopias express thalamocortical guidance molecules. Coronal sections through the brain of control (A–D, I–L) and Rfx3−/− mutants (E–H, M–P) hybridized with the indicated probes. (A, B, E and F) At rostral levels, Sema6a shows expression in the ventral telencephalic ventricular zone and weak expression in the heterotopia of Rfx3−/− embryos (arrow). Caudally, Sema6a is expressed along the path of TCAs in the prethalamus and VT, and its expression is not affected in Rfx3−/− mutants. (C) PlexinA4 is expressed in the thalamus and delineates the corridor ventrally and dorsally (arrows). (D) PlexinA4-expressing cells also reside in the caudal VT where TCAs normally do not project. (G and H) In Rfx3−/− embryos, PlexinA4 expression is largely maintained in the VT and thalamus; however, in the caudal VT, it is expressed adjacent to an ectopic axon bundle projecting toward the amygdala (arrow). (I, J, M and N) Sema5b expression in telencephalic progenitors and in neurons of the piriform cortex (pc) is maintained in Rfx3−/− embryos. (K, L, O and P) Netrin1 and Slit1 are expressed in ventral telencephalic progenitor cells, and Netrin1 transcripts were also found in the MGE mantle in both control and Rfx3−/− embryos, but both genes are ectopically expressed in the heterotopias (arrows in O and P). Scale bar: 100 µm.
The Gli3 repressor does not rescue TCA pathfinding and heterotopia formation in Rfx3−/− mutants

Finally, we addressed the cilia-regulated signaling pathway(s) which could underlie the TCA and heterotopia phenotype in Rfx3 mutants. During forebrain development, primary cilia are essential for the processing of the Gli3 protein thereby controlling the balance between Gli3 repressor (Gli3R) and activator forms (Gli3A). Recently, we showed that this balance is disturbed in the Rfx3−/− forebrain (16). Rfx3 mutants also phenocopy the thalamocortical abnormalities of the Gli3 hypomorphic mutant Gli3<small>3<sup>9<sub>dm</sub></sup></small> (10). In addition, the olfactory bulb phenotype of the Ftm cilia mutant is rescued in Ftm<sup>−/−</sup>;Gli3<sup>3<sub>9<sub>dm</sub></sub></small> embryos (31) in which the Gli3<sup>3<sub>9<sub>dm</sub></sub></small> allele exclusively produces a short Gli3 isoform that resembles Gli3R (32) in a cilia-independent manner. These findings raised the possibility that the TCA pathfinding defects in Rfx3−/− embryos are caused by disrupting the Gli3R/Gli3A ratio, and we therefore analyzed thalamocortical tract and heterotopia formation in Rfx3−/−;Gli3<sup>3<sub>9<sub>dm</sub></sub></small>−/− mutants. However, Rfx3−/−;Gli3<sup>3<sub>9<sub>dm</sub></sub></small>−/− mutants still showed an abnormal projection of TCAs toward the amygdala and formed heterotopias in the ventrostral telencephalon (Supplementary Material, Fig. S12). Therefore, re-introduction of Gli3R is not sufficient to rescue these defects unlike the previously described rescue of the Ftm cilia mutant and the diencephalon (31). This finding could be explained by a requirement for Gli activator and not Gli repressor function in VT pathfinding and indeed, Gli3 western blots showed that the levels of Gli3R are not altered in the VT of Rfx3−/− embryos (Supplementary Material, Fig. S13).

Discussion

Ciliopathies, syndromes caused by dysfunction of the primary cilium, are often associated with MR; however, the underlying pathogenesis remains largely unknown. Here, we investigated the development of the forebrain in mice mutant for the ciliogenic transcription factor Rfx3. We show that Rfx3 mutants display the formation of heterotopias in the VT and patterning defects in the prethalamus. Moreover, the development of guidepost neurons, which are provided by the VT and which guide TCAs from the thalamus into the cortex, is affected. In fact, many TCAs are unable to leave the thalamus or mis-project toward the amygdala after entry into the VT. As a similar ventral deflection of TCAs into the VT is also found in Inpp5e mutants, these findings provide a novel role for primary cilia in the development of the thalamocortical tract.

Rfx3 is required for patterning the VT and the diencephalon

Recent analyses have shown a prominent role of primary cilia in forebrain development, but only few cilia mouse mutants with forebrain phenotypes have been described yet. These analyses were also limited by severe patterning defects (33,34) or did not analyze diencephalic development (31). Therefore, roles of primary cilia in the VT and in the diencephalon remain largely unknown or have not been analyzed at all. Rfx3 mutant mice provide a useful tool for studying such functions. In contrast to previously analyzed cilia mutants, these animals show a relatively mild alteration in the Gli3 activator/Gli3 repressor ratio in the forebrain (16), an important determinant of Shh controlled patterning processes.

Interestingly, Rfx3−/− embryos show multiple and complex abnormalities in the VT. First, the size of the Rfx3<sup>3<sub>−/−</sub></small> MGE mantle is reduced. Secondly, Rfx3−/− embryos form heterotopias in which the basal lamina is disrupted and telencephalic cells are located outside the mantle zone. These heterotopias are a complex mixture of different cell types including progenitor cells (Dlx2<sup>+<sub></sub></small> and...
attributed to the in the ventricular zone of the VT. This discrepant PCR analysis using the whole VT revealed a mild up-regulation lead to an early overproduction of MGE neurons. While qRT-amiic patterning, production of post-mitotic neurons and initial others are either not able to exit the dorsal thalamus, or after hav-tering defects. Some thalamic axons reach the cortex whereas

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devlopment of the VT and in the patterning of the prethalamus. Regardless of the exact mechan-
ical and CTAs in Corticothalamic/thalamocortical tract defects

Our analyses also revealed pathfinding defects of thalamocorti-
cal and CTAs in Rx3−/− mutants occurring subsequently to pat-
tering defects. Some thalamic axons reach the cortex whereas others are either not able to exit the dorsal thalamus, or after hav-ing entered the VT mis-project toward the amygdala. Since thal-
amic patterning, production of post-mitotic neurons and initial growth of TCAs are not obviously affected, cell-autonomous de-
fects in thalamic neurons are unlikely to underlie these pathfind-
ding defects, but this requires further testing in transplantation experiments. In contrast, development of the prethalamus and the VT is compromised in Rx3−/− mutants. Both structures give rise to multiple neuronal cell types playing important roles in the guidance of thalamocortical/CTAs (17). Indeed, we show here an abnormal migration of corridor cells and MGE pioneer neurons into the ventral telencephalic heterotopias of Rx3−/− mutants. Similarly, mis-patterning of the prethalamus might have affected the development of prethalamic pioneer neurons, which normally project their axons into the thalamus thereby providing a scaffold for TCAs. Given the importance of pi-
oneur neurons in the development of forebrain connections (10,42-46), defects in the prethalamic pioneer neurons might be responsible for the clustering of TCAs in the diencephalon; however, we currently lack the molecular markers to test this hypothesis.

Moreover, our analysis provides first insights into the cellular and molecular mechanisms by which Rx3 controls thalamocor
tical tract formation. The Rfx transcription factors regulate the transcription of genes important for ciliogenesis and function, suggesting that ciliary defects may underlie the abnormal TCA pathfinding in Rx3−/− embryos. While cilia are present without obvious morphological abnormalities in the Rx3 mutant fore-
brain, their function might be affected as in J688 hypomorphic mutants which have severe telencephalic patterning defects (34). Taken together with our finding that inactivating two genes with essential but different roles in cilia, namely Rx3 or Inpp5e, which encode a transcription factor controlling cilia as-
sembly or an enzyme regulating cilia signaling and stability, res-
pectively, result in an highly similar TCA phenotype suggests that cilia dysfunction underlies this TCA pathfinding defect. The ectopic projection of TCAs toward the amygdala in Rx3−/− and Inpp5e−/− embryos also phenocopies the TCA pathfinding errors in the Gli3 hypomorphic mutant Gli3Pdn/Pdn (10), suggesting altered Gli3 processing as the key cilia controlled pathway responsible for this phenotype. However, while the Gli3R/Gli3FL ratio is altered in the whole forebrain (16), Gli3 western blotting did not reveal significant changes in this ratio in the VT of Rx3−/− embryos, similar to our previous finding in Gli3Pdn/Pdn mutants (10). Moreover, re-introducing a Gli3R allele in an Rx3−/− mutant background does not ameliorate the TCA abnormalities, unlike the rescue of olfactory bulb formation in Ftm−/− embryos (31) or of the callosal defects in Rx3−/− embryos (B. Durand, manuscript submitted). These findings rule out the involvement of altered Gli3 to Gli3R processing. Alternatively, these data sug-
ject that there could be different requirements for Gli3 repressor and activator functions in the dorsal telencephalon and VT. It will therefore be interesting to aim at rescuing the Rx3 phenotype using a Gli3 mutant that predominantly forms Gli3A (47).

Finally, the formation of neural heteropias might also affect CTA pathfinding in the VT. The ventral TCA deflection and het-
erotopia formation occur at different rostral/caudal levels. In con-
trast, CTAs are deflected at rostral levels where heterotopias are present, and this deflection is absent in Inpp5e−/− and Gli3Pdn/Pdn embryos which lack heterotopias. Moreover, the heterotopias contain Ebf1− and Isl1/2− neurons. These cells normally migrate from the LGE in the MGE to form a corridor permissive for TCAs and CTAs, but are abnormally scattered throughout the MGE mantle and even form a stream of cells connecting the LGE with the heterotopias. These also contain cells expressing Netrin1 and Slit1, which individually act as chemoattractant or repellent of TCAs, respectively, but their combined action is required for the rostral/caudal sorting of TCAs (12). Therefore, the heteroto-
pias could provide alternative migration routes for CTAs toward the thalamus (27).
Conclusion

Our study highlights essential roles of Rfx3 for the correct development of the VT and the prethalamus and emphasizes how subtle defects in these tissues can lead to severe pathfinding defects in the thalamocortical tract. These findings are highly relevant to human ciliopathies, which are often associated with MR. The thalamocortical tract as a major forebrain axon tract conveys most of the sensory information from the environment to the cerebral cortex and its disruption is predicted to interfere with the normal functioning of the cortex. Indeed, development of the thalamus and cortex is tightly linked and abnormalities in the thalamocortical pathways correlate with sensory and motor defects in preterm born children (48,49). In addition, Bardet-Biedl syndrome patients have recently been reported with a reduced thalamic size (50,51). Moreover, we show here that an identical TCA pathfinding defect is present in mice mutant for the Inpp5e gene, whose human homolog is mutated in Joubert Syndrome (29,30). It will therefore be interesting to analyze thalamocortical tract formation in ciliopathy patients which could lead to a new understanding of the pathogenesis of MR in these patients.

Materials and Methods

Mice

All animal research has been conducted according to relevant national and international guidelines. Rfx3-deficient embryos were generated and genotyped as previously described (14). Gli3\(^{-/-}\) and Inpp5e null mutant mice used in this work have previously been described (29,32). For each marker and each stage, 3–6 mutant embryos were analyzed and compared with 3–6 controls. All reported phenotypes were fully penetrant.

In situ hybridization and immunohistochemistry

Antisense RNA probes for Dbx1 (52); Dbx2 (53); Ebf1 (11); Gbx2 (54); Gli1 (55); Ngn2 (56); Nkx2.1 (57); Lhx2 (EST2101448); Lhx6 and Lhx7 (58); Nkx2.2 (59); Ptc (60) and Shh (61) were labeled with digoxigenin.

In situ hybridization on 10 µm serial parasagittal sections of mouse brains was performed as described (62).

Immunohistochemical analysis was performed as described previously (62) using antibodies against the following molecules: β-III-tubulin (Tuj1 antibody; 1 : 1000, Sigma); CR (1 : 1000, CHEMICON); Gsh2 (1 : 2500, a gift from K. Campbell, Cincinnati Children’s Hospital Medical Center, OH); Isl1/2 (1 : 100; DSHB); laminin (1 : 100, Sigma); NF (1 : 5; DSHB); Nkx2.2 (1 : 50; DSHB); Olig2 (1 : 1000, Millipore) and Pax6 (1 : 200; DSHB). Primary antibodies for immunohistochemistry were detected with Alexa- or Cy3/2-conjugated fluorescent secondary antibodies, and sections were incubated the nuclear counterstain TOPRO3 (0.2 µM) overnight at 4°C.

Western blotting

Protein was extracted from the VT of E13.5 wild-type and Rfx3\(^{-/-}\) embryos as described previously (10). About 10 µg protein were subjected to gel electrophoresis on a 3–8% gradient Tris-acetate gel (Invitrogen), and protein was transferred to a nitrocellulose membrane, which was incubated with rabbit polyclonal anti-Gli3 antibody (1 : 500; Abcam). After incubating with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1 : 2000; Dako), signal was detected using an ECL Plus detection kit (Amersham GE healthcare). Band intensity was determined using the ImageJ software. The levels of Gli3R, Gli3A and the Gli3R/Gli3A ratio were compared between wild-type and mutant tissue using the Mann–Whitney test.

Supplementary Material

Supplementary Material is available at HMG online.
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