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Molecular regionalization of the neocortex is disrupted in *Fgf8* hypomorphic mutants

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SUMMARY

The neocortex is divided into multiple areas with specific architecture, molecular identity and pattern of connectivity with the dorsal thalamus. Gradients of transcription factor expression in the cortical primordium regulate molecular regionalization and potentially the patterning of thalamic projections. We show that reduction of *Fgf8* levels in hypomorphic mouse mutants shifts early gradients of gene expression rostrally, thereby modifying the molecular identity of rostral cortical progenitors. This shift correlates with a reduction in the size of a molecularly defined rostral neocortical domain and a corresponding rostral expansion of more caudal regions. Despite these molecular changes, the topography of projections between the dorsal thalamus and rostral neocortex in mutant neonates appears the same as the topography of wild-type littermates. Overall, our study demonstrates the role of endogenous *Fgf8* in regulating early gradients of transcription factors in cortical progenitor cells and in molecular regionalization of the cortical plate

Key words: *Fgf8*, Neocortex, Regionalization, Topography, Thalamocortical axons

INTRODUCTION

The mammalian neocortex comprises multiple areas, each with unique architecture, connectivity and function. During embryogenesis, the neocortex forms in the caudodorsal region of the telencephalon or dorsal pallium (Rubenstein et al., 1998; Cobos et al., 2001; Ragsdale and Grove, 2001). An early characteristic of neocortical regionalization is the formation of region-specific projections from specific thalamic nuclei (Crandall and Caviness, 1984; O'Leary et al., 1994; Molnar and Blakemore, 1995; Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002). This process sets the framework for area-specific connectivity with thalamic nuclei, which relay different types of sensory and motor information (O'Leary et al., 1994; Pallas, 2001; Sur and Leamey, 2001; O'Leary and Nakagawa, 2002).

How is the regional organization of the neocortex established? This issue has been and remains controversial (O'Leary et al., 1994; Levitt et al., 1997; Rubenstein and Rakic, 1999; 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). It has been proposed that regionalization is induced by extrinsic cues, in particular by incoming thalamic axons, which convey positional and functional specification (the 'protocortex' model) (O'Leary, 1989). Conversely, it has been suggested that intrinsic regional differences are established within the neuroepithelium by molecular determinants that regulate neocortical areal specification, including the targeting of thalamic axons (the 'protomap' model) (Rakic, 1988). Although thalamic inputs have been implicated in regulating aspects of area-specific properties (O'Leary et al., 1994; Paysan et al., 1997; Gitton et al., 1999a; Dehay et al., 2001; Gurevich et al., 2001; Monuki and Walsh, 2001; Pallas, 2001; Ragsdale and Grove, 2001; Sur and Leamey, 2001; O'Leary and Nakagawa, 2002), several studies in mice have recently demonstrated that mechanisms intrinsic to the telencephalon play a major role in the regionalization of the neocortex.

Transplantation and in vitro experiments using the transgenic mouse line *H-2Z1*, which expresses β-galactosidase in the primary somatosensory area, have shown that positional information is already present at early stages of neocortical development (Cohen-Tannoudji et al., 1994; Gitton et al., 1999b). Furthermore, multiple genes whose expression prefigures neocortical areal domains or boundaries have been identified (Bulfone et al., 1995; Korematsu and Redies, 1997; Suzuki et al., 1997; Inoue et al., 1998; Nothias et al., 1998; Donoghue and Rakic, 1999a; Donoghue and Rakic, 1999b; 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). The expression of several of these genes is not perturbed by the lack of thalamic input in *Gbx2*$^{-/-}$ and *Mash1*$^{-/-}$ (*Ascl1* – Mouse Genome Informatics) mutant mice (Miyashita-Lin et al., 1999; Nakagawa et al., 1999). Similarly, cortical explant assays have demonstrated that thalamic inputs are not required for the induction of *H-2Z1* transgene expression (Gitton et al., 1999a). Thus, these experiments show that positional information is present within the neocortex at

early stages of development, and that later steps in neocortical molecular regionalization do not require thalamic innervation.

Several early positional information determinants have recently been identified: *Emx2* and *Pax6*, which encode homeobox transcription factors; and *COUP-TFI* (*Nr2f1* – Mouse Genome Informatics), which encodes an orphan nuclear receptor (Wang et al., 1991; Simeone et al., 1992; Stoykova and Gruss, 1994). *Emx2* is expressed in a high caudodorsal to low rostroventral gradient in the neuroepithelium of the cortical anlage, and *Pax6* is expressed in a complementary high rostroventral to low caudodorsal gradient (Simeone et al., 1992; Stoykova and Gruss, 1994; Gulisano et al., 1996; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001; Muzio et al., 2002b). Mice carrying null alleles of *Emx2* and *Pax6* have a rostral-to-caudal and caudal-to-rostral shift, respectively, in the expression of neocortical regionalization markers during embryogenesis (Muzio et al., 2002b) and at birth (Bishop et al., 2000; Mallamaci et al., 2000b; Bishop et al., 2003). *COUP-TFI* is expressed in a high caudoventral to low rostrodorsal gradient in the neocortex (Jonk et al., 1994; Qiu et al., 1994; Liu et al., 2000) and *COUP-TFI* homozygous mutant mice show molecular regionalization defects at birth, although *Emx2* and *Pax6* expression is not altered (Zhou et al., 2001). Furthermore, changes in the targeting of thalamic projections, which mirror the neocortical molecular defects, have been observed in *Emx2* and *COUP-TFI* mutants (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001). Thus, *Emx2*, *Pax6* and *COUP-TFI* control neocortical molecular patterning and may regulate the specific targeting of thalamic axons.

What are the molecules that control the early gradients of transcription factor expression within the telencephalon? Discrete sources of secreted signaling molecules that influence early telencephalic patterning include FGFs along the rostral and dorsal midline (Crossley et al., 1996; Shimamura and Rubenstein, 1997; Crossley et al., 2001; Ragsdale and Grove, 2001), BMPs and WNTs along the dorsal midline (Furuta et al., 1997; Grove et al., 1998; Lee et al., 2000; Monuki and Walsh, 2001), and SHH along the rostroventral margin (Kohtz et al., 1998; Crossley et al., 2001; Ruiz i Altaba et al., 2001). For example, BMP and WNT signaling pathways positively regulate *Emx2* expression in the telencephalon (Ohkubo et al., 2002; Theil et al., 2002), while ectopic FGF8 expression, which is generated either via bead implantation in chicken embryos or via electroporation in mouse explants, downregulates *Emx2* expression (Crossley et al., 2001; Storm et al., 2003). Furthermore, in utero electroporation experiments in mice have shown that early ectopic expression of *Fgf8*, or of a dominant-negative form of FGF receptor 3 (*Fgfr3*), shifts molecular, histological and functional aspects of regionalization in newborns and postnatal mice (Fukuchi-Shimogori and Grove, 2001). These results showed that modifying FGF signaling has a major impact on neocortical regionalization. However, the specific roles of endogenous levels of FGF8 in the formation of gradients of transcription factor expression, and in the establishment of thalamocortical connectivity, remain to be elucidated.

In the present study, we have undertaken a genetic approach to investigate the function of *Fgf8* in neocortical regionalization. Because a complete loss or severe reduction of *Fgf8* levels blocks embryonic development (Meyers et al., 1998; Sun et al., 1999) or severely perturbs telencephalic growth (Meyers et al., 1998; Reifers et al., 1998; Shanmugalingam et al., 2000; Storm et al., 2003), we took advantage of a hypomorphic allele that has been generated in mice by the intronic insertion of a *neo* cassette (*Fgf8neo*) (Meyers et al., 1998). In *Fgf8neo/neo* embryos, the insertion of the *neo* cassette causes aberrant splicing of *Fgf8* transcripts, reducing the amount of mRNA encoding functional FGF8 protein to ~40% of normal levels (G. Martin, unpublished) (Meyers et al., 1998). *Fgf8neo/neo* embryos survive until birth, have a hypoplastic midbrain and cerebellum, and appear to lack olfactory bulbs (Meyers et al., 1998). However, these embryos have a cerebral cortex of apparently normal size (Meyers et al., 1998), enabling the study of neocortical regionalization.

We show that a reduction of *Fgf8* levels in *Fgf8neo/neo* embryos creates a rostral shift in the graded expression of transcription factors, including *Emx2* and *COUP-TFI*, in cortical progenitors. Furthermore, this early caudalization of neuroepithelial molecular properties correlates with a reduction in the size of a rostral neocortical molecular domain and a rostral expansion of more caudal regions. Surprisingly, we find that these molecular changes do not affect the targeting of thalamic axons to different neocortical domains. Taken together, our results show that *Fgf8* participates in regulating early gradients of cortical gene expression and in later neocortical molecular regionalization. Furthermore, our results raise the possibility that the initial targeting of thalamic axons may be partially independent of neocortical molecular regionalization.

MATERIALS AND METHODS

Mouse lines and genotyping

Fgf8neo heterozygous mice (Meyers et al., 1998) were maintained in a mixed 129G and CD1 genetic background and crossed to produce homozygous embryos. PCR genotyping was performed as described previously (Meyers et al., 1998). 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 was performed on 80-100 μm vibratome sections as described previously (Garel et al., 1999) with the following probes: cadherin 6 and cadherin 8 (*Cdh6* and *Cdh8*) (a gift from M. Takeichi); *COUP-TFI* (a gift from M. Tsai); *Dbx1* (a gift from F. Ruddle); *Emx2* (a gift from A. Simeone); *Epha7* (a gift from A. Wanaka); *Fgf8* (G. Martin); *Fgfr3* (a gift from D. Ornitz); *Id2* (a gift from M. Israel); *Lef1* (a gift from R. Grosschedl); *Otx2* (a gift from A. Simeone); *Pax6* (a gift from P. Gruss); *RZRβ* (a gift from M. Becker-Andre); and *Wnt3a* (a gift from A. McMahon). Sections were mounted in glycerol and analyzed on 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′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes) or DiA (4-4-dihexadecyl aminostyryl N-methyl-pyridinium iodide; Molecular Probes) were placed in single or multiple locations in the neocortex (Godement et al., 1987). After 3-7 weeks at room temperature in 4% PFA to allow dye diffusion, the samples were embedded in 5% agarose and cut at 100 μm on a vibratome.

Counterstaining was performed using Hoechst (Aldrich Chemicals) and digital images were taken using a Spot II camera on a fluorescent microscope or dissection microscope.

RESULTS

Reduced *Fgf8* expression in the rostral midline of *Fgf8neo/neo* embryos

We first examined how the *Fgf8neo/neo* mutation affects *Fgf8* expression in the rostral embryonic patterning centers (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997; Crossley et al., 2001). The use of a full-length *Fgf8* in situ hybridization probe allows the detection of both wild-type and *Fgf8neo* allele transcripts (Meyers et al., 1998). At E9.5, *Fgf8* expression domains in the commissural plate (rostral midline of the prosencephalon) and the olfactory placodes were reduced in *Fgf8neo/neo* embryos (Fig. 1A-B′). Aside from a slight reduction in the size of the telencephalic vesicles and a lateral displacement of the olfactory placodes, rostral head regions of *Fgf8neo/neo* embryos appeared normal (Fig. 1A-B′). At E12, the rostral midline of *Fgf8neo/neo* mutants was reduced in size and showed a smaller *Fgf8* expression domain (Fig. 1C,D). Otherwise, from E12-13, telencephalic size and morphology were grossly normal (Figs 1-3). Thus, in early *Fgf8neo/neo* embryos, rostral midline tissues are hypoplastic and express less *Fgf8*, whereas the rest of the telencephalon appears to develop normally.

A rostral shift in early neocortical gradients is present in *Fgf8neo/neo* embryos

We next investigated the effects of reduced *Fgf8* levels on the formation of cortical telencephalic structures. At E12.5, the cortex and its associated dorsal midline have several principal subdivisions that include the primordium of the neocortex (dorsal pallium), hippocampus (medial pallium), fimbria (or hem) and choroid plexus (Grove and Tole, 1999; Puelles et al., 2000; Wilson and Rubenstein, 2000). Components of the WNT signaling pathway, such as *Wnt3a*, *Lef1*, *Emx2* and *Pax6* are involved in dorsal and medial pallium formation (Pellegrini et al., 1996; Stoykova et al., 1996; Yoshida et al., 1997; Grove et al., 1998; Grove and Tole, 1999; Galceran et al., 2000; Lee et al., 2000; Mallamaci et al., 2000a; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001; Muzio et al., 2002a; Chenn and Walsh, 2002). In *Fgf8neo/neo* embryos, the major telencephalic subdivisions are present (Figs 1-8), as shown by the expression of *Otx2* in the choroid plexus, *Wnt3a* in the cortical hem, *Lef1* and *Emx2* in the medial pallium, and *Pax6* in the lateral pallium. However, subtle changes in the expression of *Emx2* and *Pax6* in the neocortical field of *Fgf8neo/neo* embryos (Fig. 2G-J) prompted us to examine further graded gene expression within the cortical neuroepithelium.

Emx2 expression normally shows a high caudodorsal to low rostroventral gradient (Simeone et al., 1992; Gulisano et al., 1996; Muzio et al., 2002b). In E11.5 and E12.5 *Fgf8neo/neo* embryos, the gradient of *Emx2* expression is shifted rostroventrally (Fig. 3A-B′). *COUP-TFI* expression, which normally forms a high caudoventral to low rostrodorsal gradient (Liu et al., 2000), was also affected (Fig. 3C-D′). Between E11.5 and E14.5, *COUP-TFI* expression is shifted

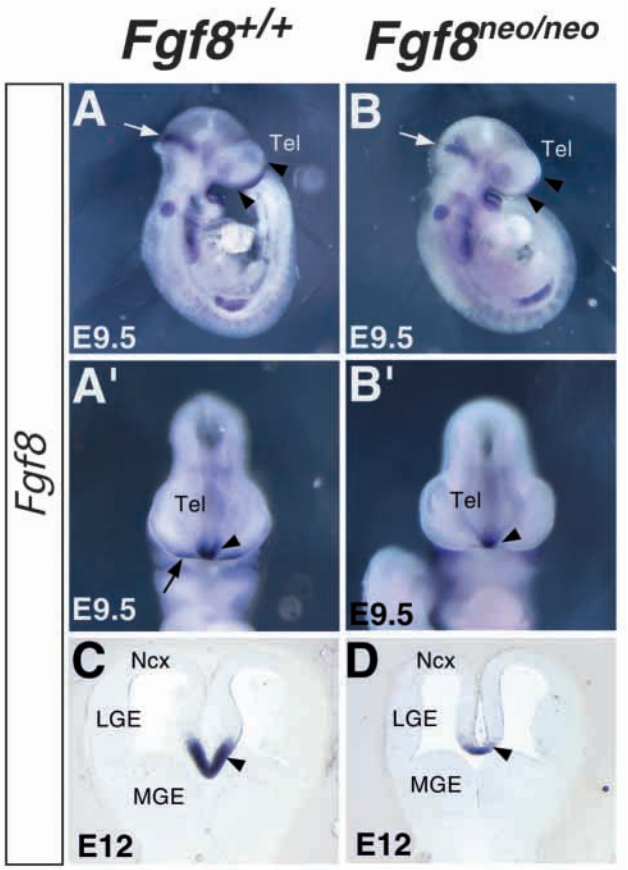

Fig. 1. Levels of *Fgf8* expression are reduced in *Fgf8neo/neo* embryos. (A-B′) Whole-mount *Fgf8* in situ hybridization on E9.5 wild-type (A,A′) and *Fgf8neo/neo* (B,B′) embryos. Lateral views (A,B) show that *Fgf8* expression domains in the midbrain-hindbrain boundary (white arrows) and in the rostral midline (black arrowheads) are present but reduced in size and intensity in *Fgf8neo/neo* embryos. Frontal views of the same embryos (A′,B′) show changes in both the commissural plate (black arrowheads) and the olfactory placodes (black arrow), the two sites of *Fgf8* expression in or in the vicinity of the telencephalic anlage. (C,D) *Fgf8* in situ hybridization on horizontal sections of wild-type (C) and *Fgf8neo/neo* (D) E12 embryos. In *Fgf8neo/neo* embryos, the neocortex primordium and the bulks of the ventral telencephalic ganglionic eminences are present. The rostral midline is hypoplastic and the width of *Fgf8* expression domain (black arrowheads) is reduced. LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Ncx, neocortex; Tel, telencephalon.

rostrally (Fig. 3C-D′, Fig. 4A,B). Similarly, we observed in *Fgf8neo/neo* embryos a rostral expansion of *Dbx1*, a homeobox transcription factor expressed in ventral pallial progenitors in the caudal telencephalon (Fig. 3E,F) (Yun et al., 2001). Likewise, there was a rostral shift in the expression of *Fgfr3*, which in controls is largely restricted to caudodorsal cortical regions (Fig. 3G,H, Fig. 4E,F) (Ragsdale and Grove, 2001; Muzio et al., 2002b). *Pax6* expression normally forms a high rostroventral to low caudodorsal gradient of expression (Stoykova and Gruss, 1994; Stoykova et al., 2000; Toresson et al., 2000; Yun et al., 2001). By contrast, the pattern of *Pax6* expression did not appear to be affected in sagittal sections of embryos between E12.5 and E14.5 (Fig. 4C,D). Thus,

Fgf8neo/neo mutants show a rostral shift in gradients of regulatory gene expression that leads to a molecular caudalization of the rostral cortical neuroepithelium.

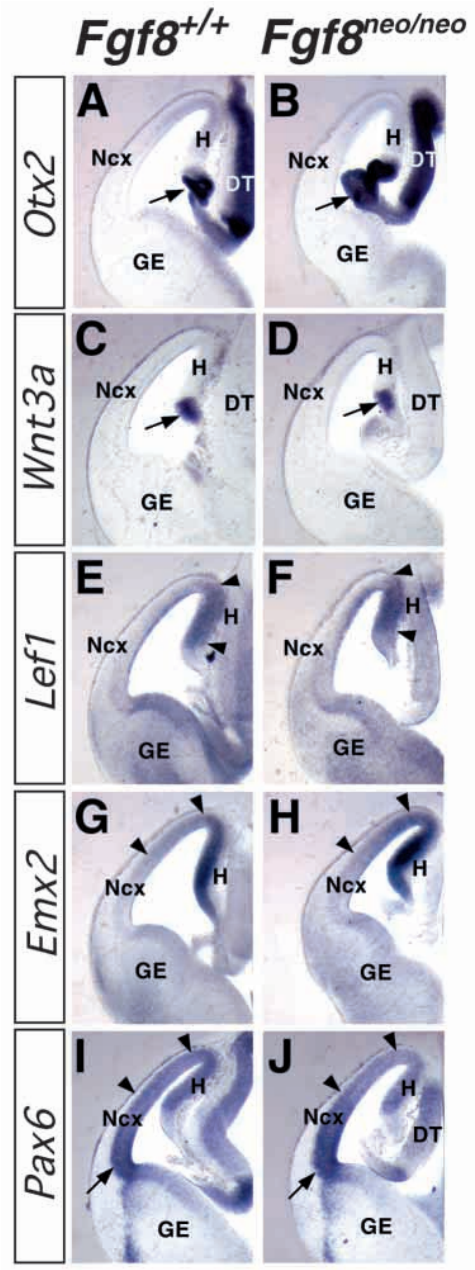

Fig. 2. The subdivisions of the dorsal telencephalon are present in *Fgf8neo/neo* embryos. In situ hybridization on coronal sections of wild-type (A,C,E,G,I) and *Fgf8neo/neo* (B,D,F,H,J) E12.5 embryos performed with the indicated riboprobes. *Otx2* expression in the choroids plexus (black arrows in A,B), *Wnt3a* expression in the cortical hem (black arrows in C,D), *Lef1* (between arrowheads in E,F) and *Emx2* (G,H) expression in the hippocampus primordium, and high levels of *Pax6* expression in the ventral-most region of the neocortex (black arrows in I,J) do not appear modified in *Fgf8neo/neo* embryos. *Emx2* in situ hybridization signal is slightly increased in the dorsal part of the neocortex (between arrowheads in G,H) in *Fgf8neo/neo* compared with wild-type embryos. The level of *Pax6* expression in this same region is slightly less intense (between arrowheads in I,J). DT, dorsal thalamus; GE, ganglionic eminences; H, hippocampus; Ncx, neocortex.

Molecular identity of the frontal neocortex is abnormal in *Fgf8neo/neo* late embryos and neonates

We examined if the early molecular modifications in the cortical neuroepithelium of *Fgf8* hypomorphic embryos changed the regional properties of the neonatal neocortex. Between E17.5 and birth, *Fgf8neo/neo* brains have a variable external phenotype (Meyers et al., 1998) that generally fit into two categories: (1) a 'mild' phenotype in which there is hypoplasia of the olfactory bulb, midbrain and cerebellum; (2) a 'severe' phenotype, in which the olfactory bulbs are not morphologically detectable and there is a large reduction of the midbrain and cerebellar structures. In both 'mild' and 'severe' cases the size and external morphology of the cerebral cortex appeared normal (Figs 5-8) (Meyers et al., 1998).

To study molecular regionalization of the neocortex at birth, we analyzed the distribution of genes that are expressed in a laminae- and region-specific pattern that correlates with functional subdivisions of the neocortex (Suzuki et al., 1997; Miyashita-Lin et al., 1999; Nakagawa et al., 1999; Rubenstein et al., 1999; Bishop et al., 2000; Bishop et al., 2003). *Id2* (*Idb2* – Mouse Genome Informatics) expression in layers 2/3 delimits a rostrodorsal domain in the orbitofrontal and frontal neocortices (*Id2* rostral-superficial, $Id2^{rs}$), which extends medially into the occipital neocortex (*Id2* caudal-superficial, $Id2^{cs}$), whereas *Id2* expression in layer 5 is restricted to a complementary parietal domain (*Id2* layer 5, $Id2^{5}$) (Fig. 5A,G) (Rubenstein et al., 1999). In 'mildly' affected *Fgf8neo/neo* embryos, we observed a pronounced reduction in the rostral $Id2^{rs}$ domain, a rostral expansion of $Id2^{5}$ and a slight rostral shift in $Id2^{cs}$ (compare Fig. 5A,G with 5B,H). Similarly, the *Cdh8* rostral superficial expression domain (*Cdh8* rostral-superficial, $Cdh8^{rs}$) is reduced (Fig. 5C,D), whereas the domain of high *Cdh6* expression, which is normally restricted to the parietal neocortex expands rostrally (Fig. 5E,F) (Nakagawa et al., 1999). In 'severe' *Fgf8neo/neo* embryos, these shifts are even more pronounced and in some cases $Id2^{rs}$ and $Cdh8^{rs}$ expression is not detected (see Fig. 8A,B; data not shown), whereas high *Cdh6* expression expands into the most rostrodorsal neocortex (Fig. 8C,D). By contrast, *Epha7* expression in the caudal and rostral neocortex and *RZRβ* (*Rorb* – Mouse Genome Informatics) expression in layer 4 (Fig. 5I,K) (Miyashita-Lin et al., 1999; Rubenstein et al., 1999) does not show a clear change (Fig. 5G,J and data not shown).

To better understand the relationship between the different shifts in expression that we observed in the rostral neocortex of *Fgf8neo/neo* neonates, we carefully compared the expression patterns of *Id2* and *Cdh6* in adjacent coronal sections (Fig. 6). In the wild-type rostral neocortex, $Id2^{rs}$, $Id2^{5}$ and *Cdh6* high expression define a boundary between two molecular domains: (1) a rostral and medial domain (including the presumptive prefrontal and motor neocortex) delimited by high $Id2^{rs}$, low $Id2^{5}$ and low *Cdh6* expression; (2) a complementary parietal domain (including the presumptive somatosensory cortex) delimited by low $Id2^{rs}$, high $Id2^{5}$ and high *Cdh6* (Fig. 6A-D′). In 'mildly' affected *Fgf8neo/neo* embryos, the boundary of expression between these two molecular domains was still present but was displaced to a more rostral position (Fig. 6E-H′).

Thus, in *Fgf8neo/neo* newborns, there is a reduction of a molecularly defined rostral domain and a complementary expansion of the adjacent parietal domain. This change in the

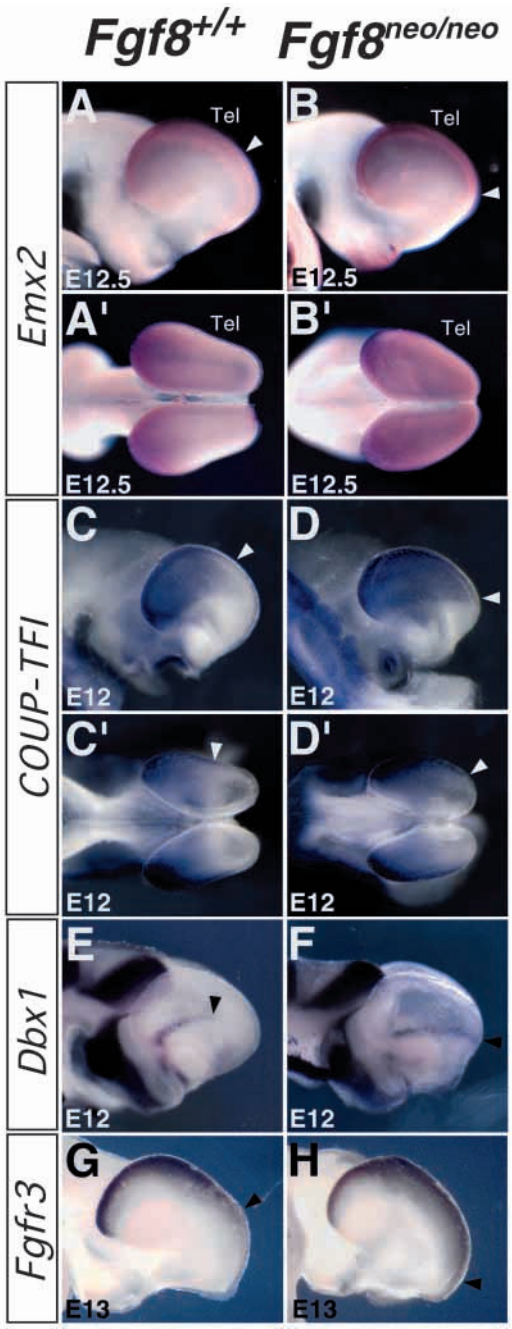

Fig. 3. Caudal-to-rostral gradients of expression are shifted rostrally in the dorsal telencephalon of E12/E13 *Fgf8neo/neo* embryos. Whole-mount in situ hybridization on dissected neural tubes of E12/E13 wild-type (A,A′,C,C′,E,G) and *Fgf8neo/neo* (B,B′,D,D′,F,H) embryos performed with the indicated probes. (A-B′) The high caudodorsal to low rostroventral gradient of *Emx2* expression in the dorsal telencephalon is detected in wild type on lateral (A) and dorsal (A′) viewing of the telencephalic vessicules. Lateral (B) and dorsal views (B′) of an *Fgf8neo/neo* embryo show the rostral shift in the anterior limit of this gradient (compare arrowheads in A and B). (C-D′) The high caudoventral-to-low rostrodorsal gradient of *COUP-TFI* expression is visible on lateral (C) and dorsal (C′) views of wild-type embryo. The anterior limit of high expression (white arrowheads) is shifted rostrally in *Fgf8neo/neo* embryos. (E,F) *Dbx1* expression is normally restricted to a stripe in the caudal part of the ventral pallium (black arrowhead in E). The anterior limit of *Dbx1* expression moves rostrally in *Fgf8neo/neo* embryos (black arrowhead in F). (G,H) *FgfR3* high-expression is excluded from the rostral-most part of the telencephalon in control embryos (black arrowhead in G). This expression domain expands into the rostral telencephalon of *Fgf8neo/neo* embryos (black arrowhead in H). Tel, telencephalon.

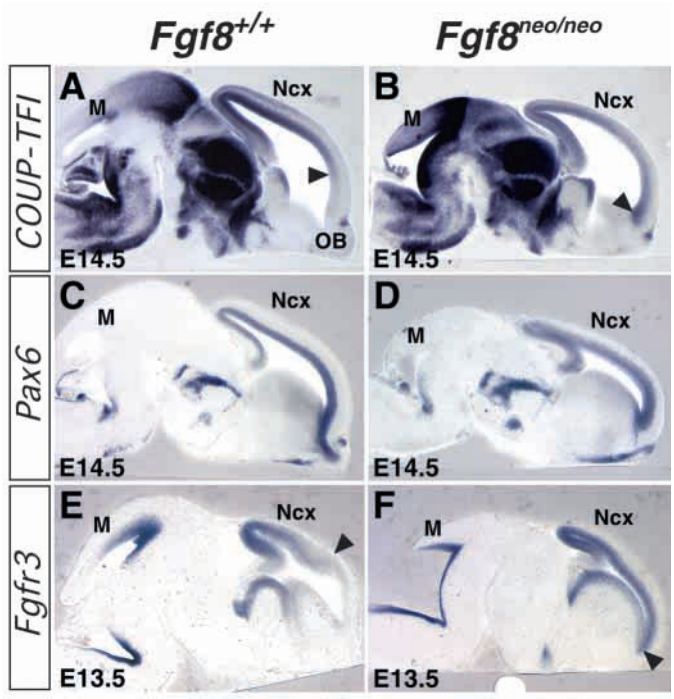

Fig. 4. Caudal gradients of gene expression are shifted rostrally in the dorsal telencephalon of E13.5 and E14.5 *Fgf8neo/neo* embryos. In situ hybridization on sagittal sections of wild-type (A,C,E) and *Fgf8neo/neo* embryos (B,D,F) with the indicated probes. The graded expression of *COUP-TFI* and *Fgfr3* (black arrowheads) expands into the rostral neocortex. *Pax6* expression appears normal in *Fgf8neo/neo* embryos (compare C with D). Note that the expression of these three genes outside of the neocortex is not affected in *Fgf8neo/neo* embryos except in the midbrain, which is dramatically reduced in size. M, midbrain; Ncx, neocortex; OB, olfactory bulb.

relative size of molecular domains implies that a substantial part of the frontal and orbitofrontal neocortex of *Fgf8neo/neo* neonates has molecular properties characteristic of the parietal neocortex.

The organization of thalamocortical projections to the rostral neocortex is not modified in *Fgf8neo/neo* embryos

In wild-type embryos, different thalamic nuclei form connections with specific neocortical domains (Crandall and Caviness, 1984; Miller et al., 1993; O'Leary et al., 1994; Molnar and Blakemore, 1995; Levitt et al., 1997; Monuki and Walsh, 2001; Pallas, 2001; Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002). As previous analysis of *Emx2*$^{-/-}$ and *COUP-TFI*$^{-/-}$ mice suggested that changes in patterning are linked with changes in the specificity of thalamic connections (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001), we examined the organization of thalamocortical projections in *Fgf8neo/neo* neonates. DiI crystals alone, or

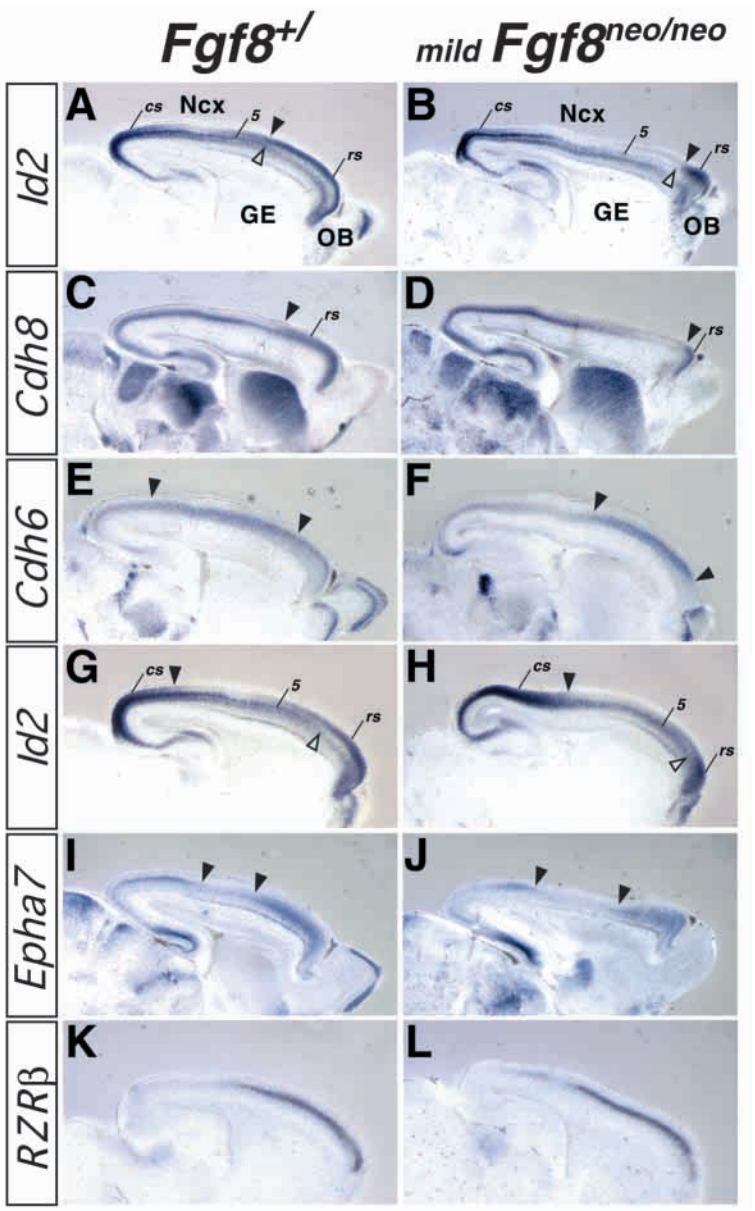

Fig. 5. Molecular regionalization of the neocortex is perturbed in 'mild' *Fgf8neo/neo* neonates. In situ hybridization performed on sagittal sections of wild-type or *Fgf8neo/+* (A,C,E,G,I,K) and *Fgf8neo/neo* (B,D,F,H,J,L) newborns (A-F) and E18.5 embryos (K-J) with the indicated probes. Note that medial sections (A-F,G,H) of *Fgf8neo/neo* neonates show the presence of olfactory bulbs. (A-F) Medial sagittal sections of a wild-type brain show *Id2* rostral superficial expression (*Id2*^{rs}), *Id2* parietal expression in layer 5 (*Id2*^{5}) and *Id2* occipital superficial expression (*Id2*^{cs}). The caudal limit of high *Id2*^{rs} (black arrowhead in A) and rostral limit of *Id2*^{5} (open arrowhead in A) coincide in wild type brains. In *Fgf8neo/neo* newborns, these two limits still coincide but both move rostrally towards the olfactory bulb (black and open arrowheads in B). The caudal limit of the superficial *Cdh8* expression domain (*Cdh8*^{rs}) in the frontal neocortex moves rostrally in *Fgf8neo/neo* newborns (compare black arrowheads in C and D). This modification correlates with a corresponding rostral shift in the high *Cdh6* expression domain (black arrowheads in E and F). (G,H) In more lateral sections, *Id2* strong caudal expression (*Id2*^{cs}) shows a rostral shift in *Fgf8neo/neo* embryos (black arrowheads). The shift in the rostral *Id2* domain is still observed (open arrowheads). (I-L) In medial (I,J) or lateral sections (K,L) there was no consistent changes in *Epha7* or *RZRβ* , although minor changes such as an increased spacing between the caudal and rostral *Epha7*-positive domains (black arrowheads in I,J) were observed in some *Fgf8neo/neo* embryos. *5*, layer 5 expression; *cs*, caudal superficial expression; GE, ganglionic eminences; Ncx, neocortex; *rs*, rostral superficial expression; OB, olfactory bulb.

paired with DiA crystals, were placed in occipital, parietal or frontal neocortices of control, 'mildly' and 'severely' affected *Fgf8neo/neo*neonatal brains to retrogradely label thalamic cells projecting towards these domains (Fig. 7). We assessed the severity of the rostral-to-caudal molecular shift in each of these brains by performing in situ hybridization with *Id2*, *Cdh6* and/or *Cdh8* on the opposite hemisphere (Fig. 8).

Dye injections in control occipital, parietal and frontal neocortex labeled cells in the dorsal lateral geniculate nucleus (dLGN), the ventroposterior nucleus (VP) and a more medial domain including the ventromedial (VM) and mediodorsal (MD) nuclei, respectively (Fig. 7A,A′,C,C′,E,E′,G,G′) (Jones, 1985; Molnar et al., 1998). Surprisingly, in both 'mild' and 'severe' *Fgf8neo/neo* embryos, dye placement within the occipital (*n*=5) and parietal neocortex (*n*=9) showed the same pattern of connectivity as in controls (Fig. 7). However, we noted two differences in caudal neocortical dye tracing experiments in *Fgf8neo/neo* mutants. First, dye placement in the occipital neocortex of four out of nine mutants, did not label axons even within the cortical plate. Second, large dye injections in the caudal parietal cortex of *Fgf8neo/neo* embryos sometimes labeled a few cells in the dLGN (Fig. 7C-D′) (two cases out of five), although the vast majority of labeled cells were detected in VP, as in controls (Fig. 7C-F′).

As molecular changes are more robust in the rostral neocortex of *Fgf8neo/neo* neonates, we focused on the pattern of connectivity of this neocortical region. Dye injections within the rostral parietal and frontal neocortex of 'mild' (*n*=5) and 'severe' (*n*=3) *Fgf8neo/neo* neonates labeled cells and axons in similar thalamic domains as in controls (data not shown and Fig. 7G-H′). To test if this observation was due to the persistence of a small rostral molecular domain, we examined thalamocortical projections in 'severe' *Fgf8neo/neo* newborns that lack detectable *Cdh8*^{rs} expression and show a massive

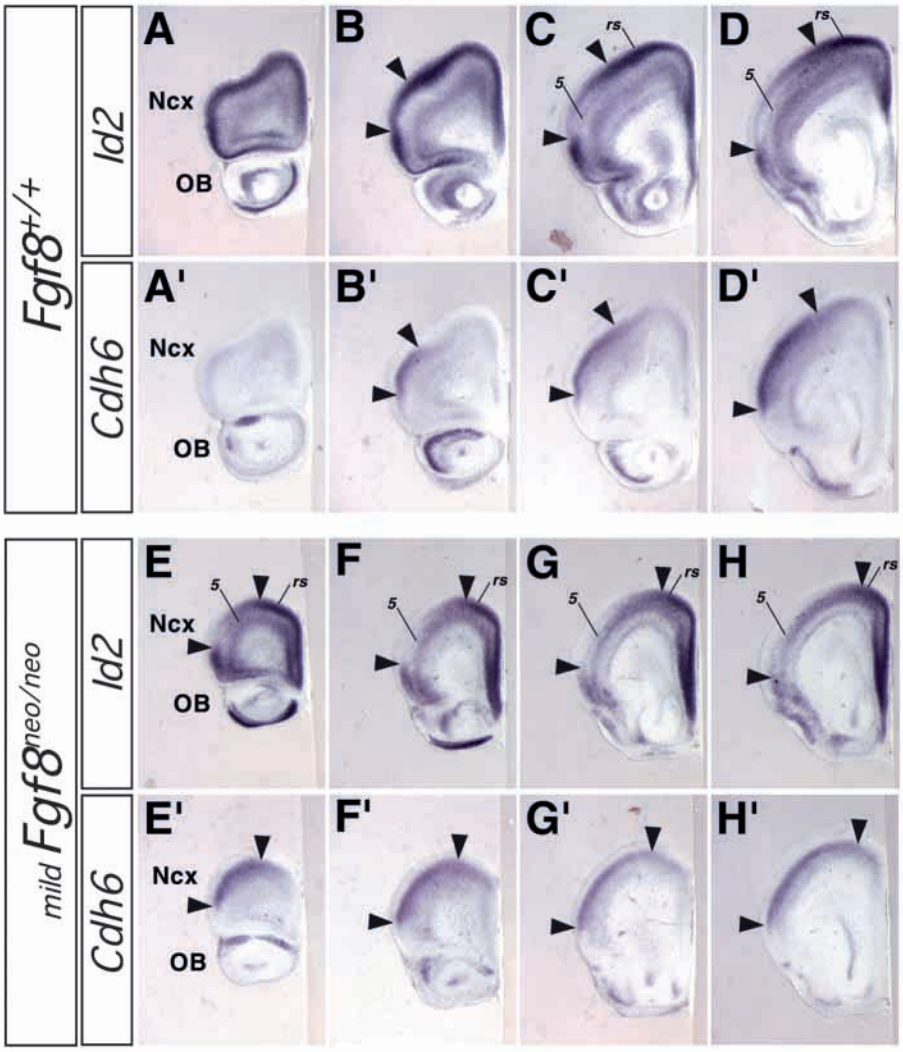

Fig. 6. Complementary *Id2* and *Cdh6* expression domains show a molecular shift in the orbitofrontal and frontal neocortex of *Fgf8neo/neo* newborns. *Id2* (A-H) and *Cdh6* (A′-H′) in situ hybridization were performed on adjacent 100 μm sections (A-H and A′-H′) from the rostral brain of a wild-type (A-D′) and a *Fgf8neo/neo* (E-H′) newborn. In wild type, *Id2* and *Cdh6* expression delimit two complementary zones (black arrowheads in A-D′): a rostral and medial domain of high $Id2^{rs}$, low $Id2^{5}$ and low *Cdh6* expression, and a lateral parietal domain of low $Id2^{rs}$, high $Id2^{5}$ and *Cdh6* expression. These two zones are still observed in *Fgf8neo/neo* newborns (black arrowheads in E-H′). However the rostral and medial domain is severely reduced and the lateral parietal domain expands both medially and rostrally (E-H′), even into the rostralmost neocortex (E,E′). *5*, layer 5 expression; Ncx, neocortex; *rs*, rostral superficial expression; OB, olfactory bulb.

rostral expansion of high *Cdh6* expression (Fig. 8A-D). Dye injections in the frontal and rostral parietal neocortex of such 'severe' mutants labeled a similar distribution of thalamic regions as in controls (Fig. 8E-L). Thus, in *Fgf8neo/neo* neonates, although the frontal neocortex has molecular characteristics of more caudal neocortical regions, it lacks a detectable defect in its pattern of connections with the thalamus.

DISCUSSION

We show that the reduction of *Fgf8* levels in *Fgf8neo/neo* embryos shifts gradients of gene expression in the embryonic cerebral cortex thereby modifying the molecular identity of rostral cortical progenitors. Despite affecting neocortical regionalization at birth, these modifications do not perturb the general pattern of connectivity with the dorsal thalamus. Thus, our study demonstrates the role of *Fgf8* in regulating early gradients of transcription factors in the rostral neocortex and suggests that this regulation may be partially independent of the mechanisms that control the initial targeting of thalamic axons.

Reduced *Fgf8* levels in *Fgf8neo/neo* embryos allow the study of neocortical regionalization

FGF8 has been implicated in a variety of processes including cell proliferation, cell death, cell migration and tissue regionalization (Crossley et al., 1996; Lewandoski et al., 1997; Shimamura and Rubenstein, 1997; Meyers et al., 1998; Reifers et al., 1998; Ye et al., 1998; Martinez et al., 1999; Sun et al., 1999; Trumpp et al., 1999; Lewandoski et al., 2000; Shanmugalingam et al., 2000; Wilson and Rubenstein, 2000; Crossley et al., 2001; Martin, 2001; Shinya et al., 2001; Kobayashi et al., 2002; Ornitz and Marie, 2002). In utero electroporation experiments driving the ectopic expression of *Ffg8* or blocking FGF signaling in the telencephalon of E11.5 mouse embryos have implicated FGF signaling in neocortical regionalization (Fukuchi-Shimogori and Grove, 2001). As multiple Fgf genes are expressed in the telencephalon (Maruoka et al., 1998; Bachler and Neubuser, 2001; Shinya et al., 2001; Gimeno et al., 2002), genetic analyses that eliminate the function of a single gene are required for the investigation of their specific function(s) in neocortical patterning. The use of hypomorphic *Fgf8neo/neo* mutants (Meyers et al., 1998) allowed us to circumvent the effects of a complete loss or severe reductions in *Fgf8* expression on

early telencephalic development (Meyers et al., 1998; Reifers et al., 1998; Sun et al., 1999; Shanmugalingam et al., 2000; Shinya et al., 2001; Storm et al., 2003) and to study the effects of reduced *Fgf8* levels on the regionalization of a roughly normally sized neocortex (Figs 1-4) (Meyers et al., 1998). Our results show that a reduction of endogenous *Fgf8* levels changes the molecular regional properties of neuroepithelial progenitors and postmitotic neurons in the rostral neocortex (Figs 3, 5 and 6).

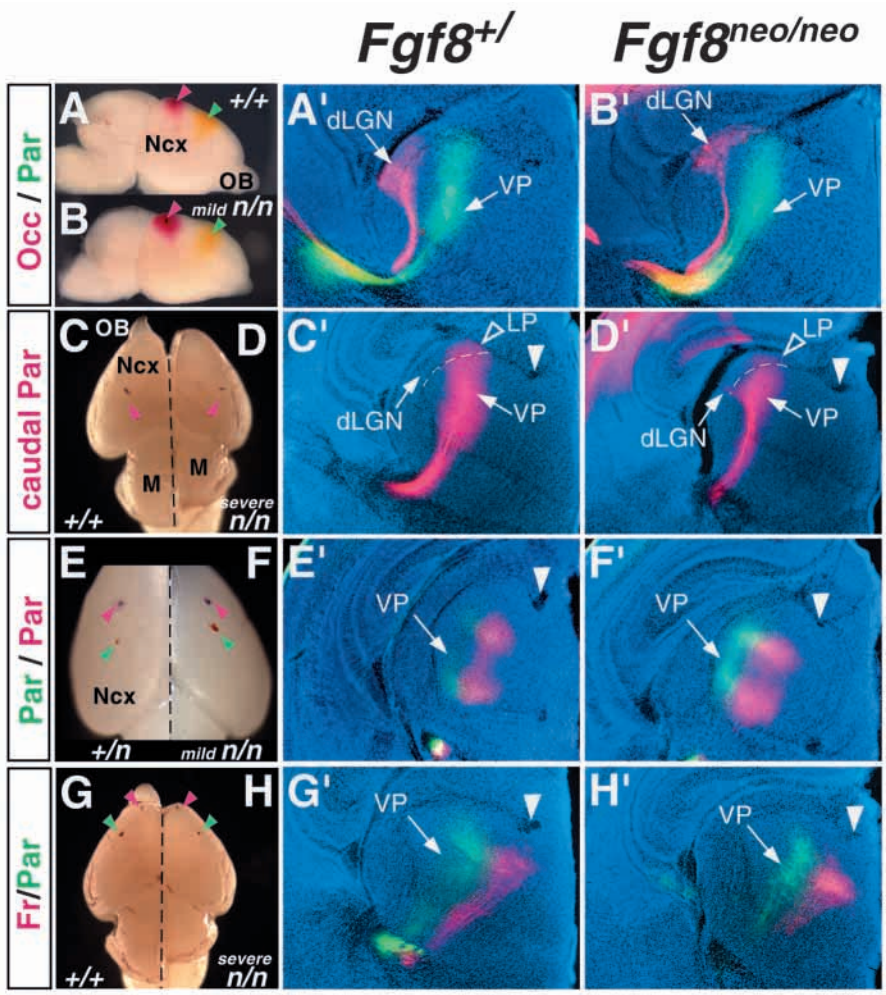

Fig. 7. Topographic organization of thalamocortical projections in *Fgf8neo/neo* neonates. DiI and DiA crystals or single DiI crystals were placed in the occipital (Occ) and parietal (Par) neocortex (A-B′), the caudal parietal neocortex (C-D′), the parietal neocortex (E-F′) and the rostral parietal and frontal (Fr) neocortex (G-H′). In A-H, crystal placements (red and green arrowheads) are shown in lateral (A,B) or dorsal views (C-H) of the brain hemispheres. These placements are shown before dye diffusion, except in A,B. (A′-H′) Dorsal thalamus coronal sections of the corresponding brains after dye diffusion. The retroflexus tract (white arrowhead) and the external medullary lamina (broken line) are morphological landmarks used to position and identify presumptive thalamic nuclei. Sections are counterstained with Hoechst. (A-B′) DiI injection in the occipital neocortex and parietal neocortex retrogradely stain the same presumptive thalamic nuclei in *Fgf8neo/neo* brains. The dorsal lateral geniculate nucleus (dLGN) is labeled in pink/red with DiI and the lateral part of the ventroposterior complex (VP) is labeled in green with DiA. (C-D′) Large DiI injections in the caudal parietal neocortex normally label cells in the lateral VP domain and spread into the presumptive lateral posterior nucleus (LP) (open arrowheads) in both wild type and mutant neonates. In this particular *Fgf8neo/neo* neonate, the injection also labeled a few cells located more lateroventrally in the dLGN (arrows). (E-F′) Small injections of DiI and DiA into the parietal neocortex label two overlapping domains within the VP complex in both control and *Fgf8neo/neo* newborns. (G-H′) Small injections of DiI into the orbitofrontal/frontal neocortex and of DiA in the rostral parietal neocortex label a medial VP domain as well as a more medial domain in both wild-type and *Fgf8neo/neo* newborns. White arrowheads in C′,D′,E′,F′,G′,H′ indicate the position of the fasciculus retroflexus. dLGN, dorsal lateral geniculate nucleus; LP, lateroposterior nucleus; M, midbrain; *n/n, Fgf8neo/neo*; Ncx, neocortex; OB, olfactory bulb; VP, ventroposterior nucleus.

Fgf8 levels regulate *Emx2* and *COUP-TFI* expression gradients

The inactivation of *Emx2*, *Pax6* and *COUP-TFI* has revealed the key roles of these transcription factors in the molecular regionalization of the neocortex (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001; Bishop et al., 2003; Muzio et al., 2002b). These genes have a graded expression within the cortical neuroepithelium, suggesting that gradients of diffusible molecules may regulate their expression patterns. Experiments in chicken embryos (Crossley et al., 2001) and in mouse telencephalic explants (Storm et al., 2003) have shown that ectopic FGF8 in the telencephalon downregulates *Emx2* expression, suggesting a link between FGF signaling and *Emx2* in neocortical regionalization. Our study shows that a hypomorphic *Fgf8* mutation shifts rostrally the graded expression of *Emx2* and *COUP-TFI*, indicating that reduced *Fgf8* levels caudalize the rostrodorsal telencephalon. Furthermore, it suggests a role for FGF8 in creating and/or maintaining a rostral neuroepithelial domain where caudally expressed genes such as *Emx2* and *COUP-TFI* are not present. This role may not be restricted to the neocortex, because we observe a similar rostral expansion of *Dbx1*expression (Fig. 3E,F), which is normally restricted to the caudal part of the non-neocortical ventral pallium (Puelles et al., 2000; Yun et al., 2001).

At this point, we are uncertain about how FGF8 regulates gradients of transcription factor expression in the embryonic cortex. Interestingly, *Fgfneo/neo* embryos have a rostral expansion of *FgfR3* expression. Thus, as at the midbrain/hindbrain boundary, FGF8 may regulate the expression of one of its own receptors in the forebrain (Sleptsova-Friedrich et al., 2001). In addition, *Emx2* inactivation has been shown to reduce the expression domain of *Fgfr3* (Muzio et al., 2002b), suggesting that *Emx2* may modulate FGF-signaling in the neocortex. Taken together, these observations raise the possibility of a negative feedback loop between *Fgf8*, *Emx2* and *Fgfr3*, which could contribute to patterning the rostral neocortical neuroepithelium.

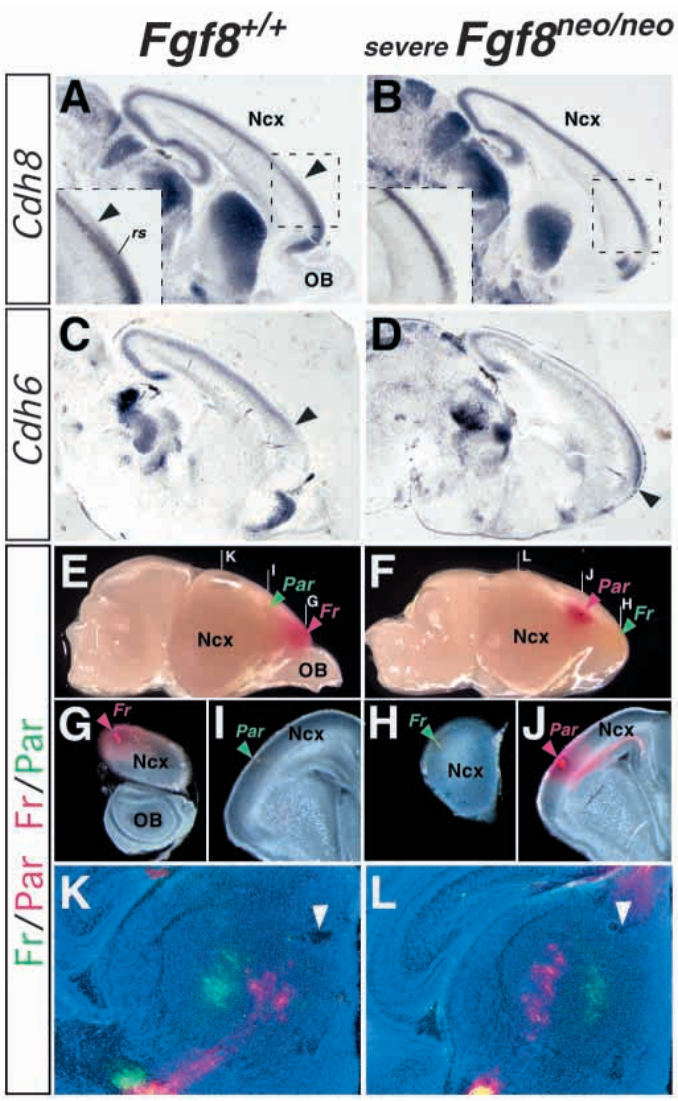

Fig. 8. Normal thalamocortical projections with the rostral neocortex of 'severe' *Fgf8neo/neo* newborns. In situ hybridization on sagittal sections (A-D) and DiI/DiA axonal tracing (E-L) were performed on the two hemispheres of one wild-type newborn (A,C,E,G,I,K) and one 'severe' *Fgf8neo/neo* newborn lacking olfactory bulbs (B,D,F,H,J,L). (A-D) The superficial *Cdh8* rostral expression (arrowhead in A) is absent in the rostral neocortex of the *Fgf8neo/neo*, as seen in a high-magnification view of the rostral neocortex (inset). In the same *Fgf8neo/neo* brain, the anterior limit of high *Cdh6* expression moves into the most rostral and medial neocortex (arrowheads in C and D). (E,F) Lateral views show that DiI and DiA crystals (red and green arrowheads) were placed in the frontal (Fr) and parietal (Par) neocortices and into the parietal and frontal neocortex of the wild-type and *Fgf8neo/neo* brain, respectively (E,F). Approximate levels of the coronal sections presented in G,I,K and H,J,L are indicated by white lines. (G-J) Dark field pictures of coronal sections showing the position of the dye crystals (red and green arrowheads) in the frontal (G,H) and parietal (I,J) neocortices. Note that DiI and DiA crystals have inverted positions in wild-type and *Fgf8neo/neo* newborns. (K,L) Coronal sections through the dorsal thalamus, counterstained with Hoechst, show very similar retrograde labeling in the wild type and *Fgf8neo/neo* brain (arrowheads indicate the fasciculus retroflexus. Ncx, neocortex; OB, olfactory bulb.

Fgf8 regulates rostral neocortical molecular regionalization

Our study shows that regionalization markers of the cortical plate are preferentially perturbed in the rostral neocortex in *Fgf8neo/neo* neonates. These modifications, which are reminiscent of the ones induced by reducing of FGF-signaling in the cortex (Fukuchi-Shimogori and Grove, 2001), include the reduction of frontal expression of $Cdh8^{rs}$ and $Id2^{rs}$, and the expansion of parietal $Id2^{5}$ and *Cdh6* expression. Overall, the changes in *Id2*, *Cdh8* and *Cdh6* expression in *Fgf8neo/neo* mice are opposite to the ones observed in $Emx2^{-/-}$ mice and share similarities with the ones observed in the *Pax6sey/sey* phenotype (Bishop et al., 2000; Mallamaci et al., 2000b; Bishop et al., 2003; Muzio et al., 2002b). However, in *Fgf8neo/neo* embryos, we have detected only subtle changes in *Pax6* expression, whereas a clear rostral shift in *Emx2* and *COUP-TF1* expression was observed (Figs 2-4). As *Emx2* expression is also increased in *Pax6sey/sey* mutant mice (Muzio et al., 2002b), our results suggest the possibility that the common molecular changes found in *Pax6sey/sey* and *Fgf8neo/neo* embryos are both mediated by *Emx2* expansion into the rostrolateral neocortical neuroepithelium.

Thalamic projections to the abnormally patterned frontal neocortex are apparently normal

Neocortical areas are defined by their architecture, molecular identity and connectivity and distinct neocortical areas are specifically interconnected with different thalamic nuclei. Thus, understanding the mechanisms regulating the early targeting of thalamic axons during embryogenesis and the establishment of these connections is a key step in the study of neocortical regionalization. In both $Emx2^{-/-}$ and $COUP\text{-}TFI^{-/-}$ mutant mice, the occipital neocortex forms aberrant embryonic thalamic connections with the ventroposterior nucleus, instead of the dLGN (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001). $Emx2^{-/-}$ and $COUP\text{-}TFI^{-/-}$ mice have reduced caudal molecular markers and abnormal molecular regionalization, respectively (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001; Bishop et al., 2003), suggesting that early gradients of *Emx2* and *COUP-TFI* expression may regulate the expression of guidance cues within the neocortex that direct the targeting of thalamic axons during embryonic development.

We show that in *Fgf8neo/neo* embryos, despite a rostral shift in *Emx2* and *COUP-TFI* expression and a change in the molecular identity of the rostral neocortex, this rostral neocortical domain has a pattern of axonal connections with the dorsal thalamus that is indistinguishable from wild-type embryos. It is important to note that DiI and DiA injections may not allow the detection of subtle changes in connectivity, owing to the difficulty in controlling the size of the cortical region where axons are labeled, as well as the lack of morphological boundaries delineating cortical areas and thalamic nuclei in neonates. Thus, in 'mildly' affected *Fgf8neo/neo* newborns, the potential rerouting of thalamic axons by the moderate shift in molecular cues might induce technically undetectable changes in connectivity. However, the pattern of thalamocortical projections appears normal even in 'severely' affected mutants that lack the frontal $Cdh8^{rs}$ domain and have the extensive rostral expansion of *Cdh6* expression (Fig. 8).

How could this apparent uncoupling between molecular regionalization and thalamocortical connectivity in *Fgf8neo/neo* mutants occur? One possibility is that the mutation might not extensively perturb the expression of the molecules that control the early targeting of thalamic axons such as *Pax6* (Fig. 4C,D) (Hevner et al., 2002; Jones et al., 2002). In such a case, it would imply that the changes in gradients of *Emx2* and *COUP-TFI* cortical expression might not be sufficient to modify the initial targeting of thalamic axons. This hypothesis is not supported by a straightforward interpretation of the phenotypes observed in *Emx2*$^{-/-}$ and *COUP-TFI*$^{-/-}$ mice (Bishop et al., 2000; Mallamaci et al., 2000b; Zhou et al., 2001; Bishop et al., 2003). However, it is possible that the thalamocortical topographic defects in *Emx2*$^{-/-}$ and *COUP-TFI*$^{-/-}$ mice may not be entirely due to neocortical defects. For example, in both mutants thalamic axons show pathfinding errors inside the basal ganglia (Mallamaci et al., 2000b; Zhou et al., 2001; Lopez-Bendito et al., 2002). As *COUP-TFI* is widely expressed in the dorsal thalamus and basal ganglia (Jonk et al., 1994; Qiu et al., 1994; Liu et al., 2000), and *Emx2* is expressed at the boundary between the basal ganglia and the diencephalon (Lopez-Bendito et al., 2002), these pathfinding defects may be due to abnormalities in the dorsal thalamus and/or basal ganglia. Thus, early molecular regionalization of the neocortex and the initial targeting of thalamic axons during embryogenesis might be regulated by partially independent mechanisms. Support for this hypothesis is provided by the study of *Ebf1*$^{-/-}$ and *Dlx1/2*$^{-/-}$ embryos. In these mice, which have basal ganglia defects, subsets of thalamic axons fail to reach the cortex. Remarkably, the axons that do reach the neocortex have a shifted topography in the absence of neocortical molecular defects (Garel et al., 2002). Thus, the analysis of *Fgf8neo/neo*, *Ebf1*$^{-/-}$ and *Dlx1/2*$^{-/-}$ mutants support the possibility that neocortical molecular regionalization and neocortical targeting of thalamic axons are partially independent during embryonic development.

Integration of molecular regionalization in neocortical arealization

How might these early embryonic events affect the formation of postnatal cortical areas? It is possible that if the *Fgf8neo/neo* mice lived beyond the day of birth, the distribution of thalamocortical projections would more closely match the molecular changes in the neocortex. This possibility is consistent with several experimental results, including those observed after ectopic expression of *Fgf8* in the caudal neocortical anlage. This treatment induces an astonishing mirror-image duplication of the somatosensory barrel field, which is tightly linked to thalamic innervation and peripheral sensory information (Fukuchi-Shimogori and Grove, 2001). These observations are made several days after birth, when thalamic axons have grown into the cortical plate and formed collaterals (Crandall and Caviness, 1984; Miller et al., 1993). In addition, several lines of evidence suggest that important regulatory processes in thalamocortical connectivity may take place shortly after birth in rodents. For example, cortical transplantation experiments at birth have shown that thalamic axons can later be redirected towards the ectopically grafted cortex (Levitt et al., 1997; Frappe et al., 1999; Gaillard and Roger, 2000; Ragsdale and Grove, 2001; O'Leary and Nakagawa, 2002). Thus, in response to local cues within the neocortex, early projections may be redirected or refined and collaterals specifically formed and targeted (Levitt et al., 1997; Gao et al., 1998; Mann et al., 2002). Therefore, our study supports a model in which the initial targeting of thalamic axons during embryogenesis is not strictly controlled by regionally expressed cues in the neocortex. However, once thalamic axons reach the neocortex, interactions between regional cortical cues and thalamic axons probably modify the initial pattern of thalamic projections and promote the formation of mature neocortical areas.

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