Molecular regionalization of the neocortex is disrupted in Fgf8 hypomorphic mutants

Sonia Garel, Kelly J. Huffman and John L. R. Rubenstein*

Nina Ireland Laboratory of Developmental Neurobiology, Department of Psychiatry, University of California, San Francisco, CA 94143-0984, USA

*Author for correspondence (e-mail: jlrr@cgl.ucsf.edu)

Accepted 31 January 2003

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; 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 (Nrtf1 – 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 gradient in the neuroepithelium of the cortical anlage, and Pax6 is expressed in a complementary high caudodorsal gradient 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 caudodorsal to low rostroventral 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 telencephalic 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; Shammugalingam 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 factory 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: caderhin 6 and caderhin 8 (Cd6 and Cd8) (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 (Gedement 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.
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; Puuelles 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; Chen 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 caudodorsal to low rostromedial gradient (Liu et al., 2000), was also affected (Fig. 3C-D’). Between E11.5 and E14.5, COUP-TFI expression is shifted rostrally (Fig. 3C-D’, Fig. 4A,B). Similarly, we observed in Fgf8neo/neo embryos a rostral expansion of Dlx1, 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. 3H, Fig. 4E,F) (Ragsdale and Grove, 2001; Muzio et al., 2002b). Pax6 expression normally forms a high rostroventral to low caudaldorsal 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,
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 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 laminar- 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 rostradorsal domain in the orbitofrontal and frontal neocortices (*Id2* rostral-superficial, *Id2⁎⁎), which extends medially into the occipital neocortex (*Id2* caudal-superficial, *Id2⁎⁎), whereas *Id2* expression in layer 5 is restricted to a complementary parietal domain (*Id2* layer 5, *Id2⁎⁎) (Fig. 5A,G) (Rubenstein et al., 1999). In ‘mildly’ affected Fgf8neo/neo embryos, we observed a pronounced reduction in the rostral *Id2⁎⁠⁎ domain, a rostral expansion of *Id2⁎ and a slight rostral shift in *Id2⁎ (compare Fig. 5A,G with 5B,H). Similarly, the *Cdhl6* rostral superficial expression domain (*Cdhl6* rostral-superficial, *Cdhl6⁎⁠⁎) is reduced (Fig. 5C,D), whereas the domain of high *Cdhl6* expression, which is normally restricted to the parietal neocortex expands rostrally (Fig. 5E,F) (Nakagawa et al., 1999). In ‘severely’ affected Fgf8neo/neo embryos, these shifts are even more pronounced and in some cases *Id2⁎⁠⁎ and *Cdhl6⁎⁠⁎ expression is not detected (see Fig. 8A,B; data not shown), whereas high *Cdhl6* expression expands into the most rostradorsal neocortex (Fig. 8C,D). By contrast, *Epha7* expression in the caudal and rostral neocortex and RZRB (*Rorb – Mouse Genome Informatics*) expression in layer 4 (Fig. 5L,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 *Cdhl6* in adjacent coronal sections (Fig. 6). In the wild-type rostral neocortex, *Id2⁎**, *Id2⁎⁠⁎** and *Cdhl6* 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⁎**, low *Id2⁎ and low *Cdhl6* expression; (2) a complementary parietal domain (including the presumptive somatosensory cortex) delimited by low *Id2⁎**, high *Id2⁎ and high *Cdhl6* (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. 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 choroid 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.
Fgf8 function in neocortical regionalization

The 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. Dil crystals alone, or
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 mediiodorsal (MD) nuclei, respectively (Fig. 7A,C,E,G,I,K) (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 (black arrowheads in A) coinciding 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 \( (Cdh6^{rs}) \) 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).

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 expression and show a massive...
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 Fgf8 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 (Maruoaka 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).

**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 rostral dorsal 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, *Fgf8* 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 Dil/DiA axonal tracing (E-L) were performed on the two hemispheres of one wild-type newborn (A,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 Cdh8 expression moves into the most rostral and medial neocortex (arrowheads in C and D). (E,F) Lateral views show that Dil 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 Dil 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 function in neocortical 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\(^{rs}\) 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 Pax6\(^{sey/sey}\) 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-TFI expression was observed (Figs 2-4). As Emx2 expression is also increased in Pax6\(^{sey/sey}\) mutant mice (Muzio et al., 2002b), our results suggest the possibility that the common molecular changes found in Pax6\(^{sey/sey}\) and Fgf8neo/neo embryos are both mediated by Emx2 expansion into the rostro-lateral 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-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-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 Dil 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 partial 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 local cortical cues and thalamic axons probably modify the initial pattern of thalamic projections and promote the formation of mature neocortical areas.

We thank Gail Martin for providing the Fgf8 mutant mice and for her generous support and advice. We thank Michael Depew, Oscar Marin, Elaine Storm and members of the Rubenstein laboratory for stimulating discussions and critical comments on the manuscript. We also thank M. Becker-Andre, R. Grosschedl, P. Gruss, M. Israel, A. McMahon, D. Ornitz, F. Ruddle, A. Simeone, M. Takeichi, M. Tsai and A. Wanaka for the gift of probes. This work was supported by the research grants to S.G. from Human Frontiers Science Program, to K.J.H. from NIMH and Giannini Family Foundation, and to J.L.R.R. from Nina Ireland. MIND Institute, NINDS #NS34661-05A1, NIMH RO1 MH94928-01, RO1 MH51561-01A1, K02 MH01046-01.

REFERENCES


