Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex

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Summary

Recent findings implicate embryonic signaling centers in patterning the mammalian cerebral cortex. We used mouse in utero electroporation and mutant analysis to test whether cortical signaling sources interact to regulate one another. We identified interactions between the cortical hem, rich in Wingless-Int (WNT) proteins and bone morphogenetic proteins (BMPs), and an anterior telencephalic source of fibroblast growth factors (FGFs).

Expanding the FGF8 domain suppressed Wnt2b, Wnt3a and Wnt5a expression in the hem. Next to the hem, the hippocampus was shrunken, consistent with its dependence for growth on a hem-derived WNT signal. Maintenance of hem WNT signaling and hippocampal development thus require a constraint on the FGF8 source, which is likely to be supplied by BMP activity. When endogenous BMP signaling is inhibited by noggin, robust Fgf8 expression appears ectopically in the cortical primordium.

Abnormal signaling centers were further investigated in mice lacking the transcription factor EMX2, in which FGF8 activity is increased, WNT expression reduced, and the hippocampus defective. Suggesting that these defects are causally related, sequestering FGF8 in Emx2 homozygous mutants substantially recovered WNT expression in the hem and partially rescued hippocampal development.

Because noggin can induce Fgf8 expression, we examined noggin and BMP signaling in the Emx2 mutant. As the telencephalic vesicle closed, Nog expression was expanded and BMP activity reduced, potentially leading to FGF8 upregulation. Our findings point to a cross-regulation of BMP, FGF, and WNT signaling in the early telencephalon, integrated by EMX2, and required for normal cortical development.

Key words: Cortical area map, In utero electroporation, Emx2 mutant mouse

Introduction

Normal functioning of the mammalian cerebral cortex depends on the partition of the cortical sheet into different types of cortex, and further subdivision into specialized areas (Nauta and Feirtag, 1986). These divisions form a map that is highly similar from one individual to another in the same species, and has common general features across species (Krubitzer, 1995). Considerable current research focuses on identifying the developmental mechanisms that generate this complex, reproducible pattern.

In a classic model of neocortical development (Rakic, 1988), a ‘protopmap’ of neocortical areas is set up in the cortical neuroepithelium. More recent findings suggest how such early cortical pattern might be achieved. It has been proposed that signaling centers in and around the cortical neuroepithelium secrete members of a select set of powerful embryonic signaling molecule families. These signaling molecules confer positional information and regulate regional growth (Assimacopoulos et al., 2003; Garel et al., 2003; Grove and Fukuchi-Shimogori, 2003; O’Leary and Nakagawa, 2002; Ohkubo et al., 2002; Ragsdale and Grove, 2001; Rubenstein et al., 1999; Shimamura and Rubenstein, 1997).

Two likely signaling centers for the mouse cortical primordium have been investigated in some depth. First, an anterior telencephalic source of FGF proteins, including FGF8 and FGF17 (Bachler and Neubuser, 2001; Crossley et al., 2001; Maruoka et al., 1998), imparts position along the anteroposterior (AP) axis of the developing area map (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003; Garel et al., 2003). If a new FGF8 source is introduced at the posterior pole of the cortical primordium, posterior cortex is induced to take on a more anterior fate (Fukuchi-Shimogori and Grove, 2001). As might be expected from a classic patterning theory (Wolpert, 1996), the two opposing AP signaling sources induce formation of apparently mirror-image partial area duplicates (Fukuchi-Shimogori and Grove, 2001). As might be expected from a classic patterning theory (Wolpert, 1996), the two opposing AP signaling sources induce formation of apparently mirror-image partial area duplicates (Fukuchi-Shimogori and Grove, 2001). As might be expected from a classic patterning theory (Wolpert, 1996), the two opposing AP signaling sources induce formation of apparently mirror-image partial area duplicates (Fukuchi-Shimogori and Grove, 2001). As might be expected from a classic patterning theory (Wolpert, 1996), the two opposing AP signaling sources induce formation of apparently mirror-image partial area duplicates (Fukuchi-Shimogori and Grove, 2001).
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(Panchision et al., 2001). These findings indicate a potential for medial/lateral (M/L) patterning of the cortical neuroepithelium by signals from the dorsomedial telencephalon.

A devastated cerebral cortex is predicted if cortical signaling centers are too large, too small, or incorrectly positioned. In the present study, we therefore searched for mechanisms that could control the size and position of signaling sources. Classic embryonic signaling centers, studied longer and more fully, include those in the vertebrate limb bud, which organize the major axes of the limb and the size of limb components. These classic signaling centers regulate one another to coordinate limb patterning (Capdevila and Izpisua Belmonte, 2001; Dahn and Fallon, 2000; Niswander, 2002; Riddle et al., 1993; Sun et al., 2002; Wolpert, 1996), suggesting that the recently identified cortical patterning centers may also be controlled by a network of interactions.

More specific guidance comes from studies of dorsal telencephalic signaling centers in non-mammalian vertebrates (Crossley et al., 2001; Ohkubo et al., 2002; Shammugalingam et al., 2000). Major dorsal telencephalic circuitry is comparable among mammals, reptiles and birds (Karten, 1997; Medina and Reiner, 2000), suggesting homologies at the level of cell type, circuitry and, in at least some cases, function. Moreover, telencephalic signaling centers identified in mouse and chick are strikingly alike, suggesting a further consistency between the two species in the basic patterning of the telencephalic AP and ML axes.

The chick telencephalon, like that of the mouse, is exposed to an anterior source of FGF8 and a dorsomedial source of BMP proteins, both of which regulate morphological and gene expression patterning (Crossley et al., 2001; Golden et al., 1999; Ohkubo et al., 2002). In addition, Wnt7b and Wnt8b are expressed in the medial pallium, including a position comparable with that of the mouse cortical hem (Gard et al., 2002). Furthermore, interactions between signaling sources have been analyzed in the chick: Fgf8 expression is suppressed by BMP signaling, and enhanced by BMP inhibition (Crossley et al., 2001; Ohkubo et al., 2002). Thus, chick studies have already provided evidence of regulation between dorsal telencephalic signaling sources. We sought to extend this investigation to the mouse cerebral cortex.

Materials and methods

In utero micro- and macroelectroporation

At all ages, embryos were visualized by transillumination inside the intact uterus using a fiber optic light source. Expression plasmid DNA was mixed with fast green (Sigma) and injected into the cerebral ventricle via a glass capillary. Two alternative forms of electroporation were employed, depending on the age of the embryos, and differing in the type of electrode. At embryonic day (E) 10 and above, microelectroporation was used as previously described (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003), except that a CUY21 Electroporator (Nepagene) allowed the passage of current to be monitored. At E9.5, the fine electrodes needed for microelectroporation were difficult to introduce into the small embryo forebrain, and macroelectroporation was used instead. After DNA injection, platinum electrodes (Nepagene) were positioned outside the uterus and a series of three square-wave current pulses (30V, 100 msseconds×3) delivered, as before. After electroporation, the surgical incision in the mother was closed and embryos developed in utero with a survival rate of about 60%. As previously, 50% of surviving embryos showed effective electroporation, as indicated by strong expression of a transgene. The site and efficiency of transfection were monitored, whenever possible, by direct visualization of transgene expression. In some cases, this was aided by the co-electroporation of a marker gene encoding enhanced green fluorescent protein (EGFP).

Rescue of the Emx2 mutant cortex

For rescue experiments, the Emx2 mutant mouse line was maintained on a mixed CD1/C57BL/J6 background. In comparison with mice on a C57BL/J6 background, the addition of the CD1 outbred strain appeared to increase survival of electroporated embryos. Mice were genotyped as previously (Pellegrini et al., 1996). The neocortical and hippocampal phenotype, judged by morphology and gene expression, did not differ from the Emx2 mutant maintained on a C57BL/J6 background (Fukuchi-Shimogori and Grove, 2003). To test the hypothesis that excess FGF8/17 reduces WNT expression, leading in turn to hippocampal abnormalities, a construct encoding a truncated FGF8 receptor (see below) was electroporated into the anterior cortical primordium of Emx2 mutants at E9.5.

Constructs and histology

Expression plasmids carrying cDNA encoding human placental alkaline phosphatase (Ap), mouse FGF8 (isoform b) or the soluble, truncated FGFR receptor 3c (sFGFR3c) were used as previously (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003). EGFP-N1 (Clontech), chick Noggin and human BMP4 were cloned into the expression vector pEFX (Agarwala et al., 2001). Brains or whole embryos were fixed in 4% paraformaldehyde, and, where needed, sectioned in the coronal plane with a Leica sliding microtome. Section and whole-mount in situ hybridization followed described procedures (Agarwala et al., 2001; Grove et al., 1998).

Results

Excess FGF8 suppresses WNT gene expression in the cortical hem

At E11.5 (Fig. 1A), expression domains of Wnt3a and Fgf8 almost abut along the medial wall of the cortical hemisphere, suggesting a mutual antagonism between WNT3A and FGF8. To test this possibility, we overexpressed Ap, Fgf8 or Wnt3a at E11.5, directing the site of transfection to the anterior cerebral hemisphere. Brains were assessed at E13.5. In the electroporated hemisphere only, excess FGF8 regionally suppressed Wnt3a expression (Fig. 1B,C, n=4/4, brains selected from those that showed strong, correctly targeted Fgf8 expression), Fgf2b (Fig. 1E,F, n=6/9) and Fgf5a expression (Fig. 1I,J, n=5/7). Control electroporation of Ap had no effect (Fig. 1E,I, n=12/12).

Downregulation of WNT genes was not due to general tissue deterioration caused by excess FGF8, or by electroporation itself. The hem appeared morphologically normal as a characteristically translucent band of tissue. Foxg1 expression, which marks the boundary between the cortical hem and the rest of the cortical neuroepithelium, was unaltered after electroporation with Fgf8 (Fig. 1K,L), as was Zic3 expression, which appeared in its normal anterior telencephalic domain, near the standard anterior site of electroporation (n=4/4 for Ap and 5/5 for Fgf8).

Electroporated Fgf8 had no observable effect on expression of Bmp4 in the hem region (Fig. 1D), on expression of Bmp6 or Bmp7 (data not shown), or on BMP activity assessed by expression of Msx1, a direct reporter of BMP2 and BMP4.
activity (Fig. 1G,H, n=3/3 for Ap and 5/5 for Fgf8). Reciprocal effects of WNT3A on Fgf8 expression were also not detected. However, electroporation of Wnt3a at E10.5 or 11.5 promoted general growth of the cortical hemisphere (Fukuchi-Shimogori and Grove, 2001) (data not shown).

**Excess FGF8 produces an abnormal hippocampus**

Hippocampal development requires a WNT signal from the cortical hem (Galceran et al., 2000; Lee et al., 2000). We therefore examined the hippocampus at E15.5 in brains electroporated with Fgf8 (arrow). Fgf8 expression is robustly upregulated. In some cases, noggin overexpression induced patches of ectopic Fgf8 in the cortical primordium lateral to the midline (Fig. 3B, Fig. 4A, n=3). Sections through such a brain showed ectopic Fgf8 expression extending through the entire thickness of the cortical neuroepithelium (Fig. 4B,C). Normal midline expression of Fgf8 was also enhanced or extended posteriorly (Fig. 3B, Fig. 4A). Diminished BMP activity after Nog electroporation was inferred by downregulated expression of Msx2 (Fig. 3G,H, n=9 for Ap and n=5 for Fgf8) and Msx1 (data not shown), representing a functional readout of BMP signaling. Consistent with findings above, increased Fgf8 expression after Nog electroporation was accompanied by decreased Wnt3a expression in the cortical hem (Fig. 3E, n=6). Notably, the effects described were seen in all brains showing efficient transfection. No such effects were observed in brains in which EGFP fluorescence could not be detected, or appeared only in scattered cells; nor were effects seen in brains electroporated with EGFP alone (Fig. 3A,D,G).

Electroporation at E11.5 of either Bmp4 or Nog had little or no effect on Fgf8 expression. However, unlike WNT gene expression in the hem, which is not fully established until E11.5, an anterior FGF8 source is evident very early in telencephalic development, initially at the interface between ectoderm and neurectoderm, the ANR (Shimamura and Rubenstein, 1997). Thus, although we found that WNT expression in the hem is malleable as late as E11.5, anterior Fgf8 expression may not be. Consequently, mice were electroporated earlier, at E9.5, with EGFP as a control, or Nog together with EGFP. Embryos were collected 24 hours later and the site of transgene expression was detected by EGFP fluorescence in each brain (Fig. 3 insets). As in previous experiments, about half of the brains showed efficient transfection (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003), which is evident from dense patches of EGFP fluorescence. In co-electroporated brains with efficient transfection of EGFP, and presumably also Nog, Fgf8 expression was robustly upregulated. In some cases, noggin overexpression induced patches of ectopic Fgf8 in the cortical primordium lateral to the midline (Fig. 3B, Fig. 4A, n=3).

**Size and position of the endogenous FGF8 source is constrained by time-limited BMP signaling**

In chick, Fgf8 expression in the anterior telencephalon is downregulated by BMP4 and expanded by the BMP inhibitor noggin (Ohkubo et al., 2002). In mice lacking chordin (Chrd) and with only a single copy of Nog, BMP activity is increased and Fgf8 expression is reduced in the anterior neural ridge (ANR) and anterior telencephalon (Anderson et al., 2002). We therefore tested whether inhibiting endogenous BMP signaling in living mouse embryos leads to ectopic expression of Fgf8 in the cerebral cortical primordium.

**Fig. 1.** Excess anterior FGF8 downregulates WNT gene expression in the hem. (A) E11.5 cerebral hemisphere viewed from the medial face, anterior towards the left, processed for two color in situ hybridization. The cortical hem is marked by Wnt3a expression (blue); Fgf8 expression (brown) is separated from the hem by a short gap (arrow). (B-L) Brains electroporated anteriorly at E11.5 with Ap or Fgf8 and analyzed at E13.5. B-D and K,L are coronal sections; E-J are hemispheres viewed from the medial face. (B,C) In adjacent sections from the same brain, ectopic expression of FGF8 (B) obliterates Wnt3a expression in the hem (C, black arrow indicates normal Wnt3a expression site). (Note endogenous Fg8 expression ventrally in B.) Wnt2b expression fills the cortical hem of an Ap electroporated brain (E) but is lost anteriorly in a brain electroporated with Fgf8 (arrow, F). Wnt5a shows a similar loss of expression (I, arrow in J). In contrast to WNT gene expression, Bmp4 expression is not downregulated at this age by excess FGF8 (D, white arrow indicates site of excess FGF8), nor is expression of Msx1, a indicator of BMP activity (G,H). Overexpression of FGF8 (L) has no apparent effect on Foxg1 expression at this age, indicating that the experimental protocol does not cause general damage. The boundary between the hem and the rest of the cortical neuroepithelium not obviously affected (K). Scale bar: 0.8 mm for A,E-J; 0.17 mm for B-D; 0.12 mm for K,L.
Early inhibition of BMP activity in the EMX2 homozygote mutant telencephalon

The patterns of Fgf8 and WNT gene expression following overexpression of noggin are reminiscent of those reported in mice that lack the transcription factor EMX2 (Fukuchi-Shimogori and Grove, 2003; Muzio et al., 2002). We therefore compared Nog-electroporated brains directly with Emx2 homozygote mutant brains at the same age. At E10.5, the Emx2 mutant and Nog-electroporated wild-type brains showed similarities. The Fgf8 expression domain was expanded in the Emx2 mutant, as previously reported (Fukuchi-Shimogori and Grove, 2003), and WNT gene expression in the cortical hem was reduced (Muzio et al., 2002) (Fig. 3C,F; n=9 for each of Fgf8 and Wnt3a). Expression of Msx2 appeared slightly reduced (Fig. 3I, n=3), which suggests lower levels of BMP activity. These findings suggest that the Emx2 mutant brain can profitably be studied, in part, as an illustration of abnormal cortical signaling centers.

At E8.75, in wild-type embryos, Nog was expressed at the anterodorsal telencephalic midline, close to the Fgf8-expressing anterior pole (Fig. 5A,I). In Emx2 homozygote mutants, matched to controls for overall development, we observed consistently increased expression of Nog (Fig. 5B,J,K,M, n=4/4). The anterior to posterior extent of Nog expression was roughly three times greater than in wild-type controls (compare Fig. 5B,J,K with A and I), and expression levels appeared increased (Fig. 5L,M). Bmp4 and Bmp7 were expressed along the telencephalic dorsal midline without a detectable difference between wild type and mutant (Fig. 5C-F, n=3 for each gene in wild type and mutant). However, indicating a difference in BMP activity, Msx2 expression appeared at the dorsal midline in wild type mice (Fig. 5G) but was depleted in the mutant (Fig. 5H, n=3).
Sequestering FGF8 in Emx2 mutants partially rescued hem WNT expression and hippocampal development

In addition to the defects noted above, the Emx2 mutant shows dramatic anterior to posterior shifts in neocortical regional pattern (Bishop et al., 2000; Bishop et al., 2002; Mallamaci et al., 2000), similar to those seen after augmentation of the endogenous FGF8 source in wild-type mice (Fukuchi-Shimogori and Grove, 2001). In a previous study, we tested the effect on neocortical patterning of reducing excess FGF8 in the Emx2 mutant. We found that this reduction partially rescued neocortical patterning (Fukuchi-Shimogori and Grove, 2003). Based on the previous study, and on findings described above, we hypothesized that excess FGF8 also causes decreased WNT expression in the hem of Emx2 mutants and consequent abnormalities of the hippocampus.

To test this possibility, we reduced FGF8 activity, as before, by electroporation of a truncated FGF receptor. FGFR3c is a high-affinity receptor for FGF8 and for closely related FGF family members such as FGF17, which is also overexpressed in the anterior telencephalic domain of the Emx2 mutant (Fukuchi-Shimogori and Grove, 2003). A soluble, truncated form of the receptor, sFGFR3c, is presumed to sequester FGF8 and related FGFs, preventing them from binding to their endogenous receptors (Fukuchi-Shimogori and Grove, 2001; Ye et al., 1998).

The soluble receptor construct, sFGFR3c, and EGFP were co-electroporated into the anterior cortical primordium of Emx2 mutants at E9.5 – the age at which Wnt3a expression is initiated at the hem (Lee et al., 2000). Supporting the hypothesis, 4 days after electroporation, Emx2 mutant brains that were efficiently transfected showed increased expression of Wnt3a and Wnt2b in the cortical hem (Fig. 6C,F, n=8/18). Brains with sparse or no EGFP-fluorescence continued to show diminished expression of both WNT genes (Fig. 6B,E).

A prominent defect in the Emx2 mutant hippocampus is the absence of a morphologically distinct dentate gyrus (DG) (Pellegrini et al., 1996; Yoshida et al., 1997; Tole et al., 2000a). Given that reducing excess FGF8 in the mutant substantially restored Wnt3a in the cortical hem, we examined the DG in the ‘rescued’ mutant at E18.5. In wild-type mice at this age, Prox1-expressing cells were dense in the distal hippocampus, beginning to form the blades of the DG (Fig. 7A,D). By contrast, in homozygote Emx2 mutants, Prox1-expressing cells formed only tiny patches in the distal hippocampus – the presumptive region of the DG (Fig. 7B,E). In addition, abnormal Prox1 expression appeared along the ventricular surface of the hippocampus (Fig. 7E, asterisk). When homozygote Emx2 mutants were electroporated with sFGFR3c at E9.5, a dense domain of Prox1-expressing DG cells was restored, suggesting partial rescue of DG development (Fig. 7C,F, n=8/17). A similar partial recovery of expression (Fig. 7H,I,J) was observed.
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7I, n=8/17) was seen for the ephrin receptor gene EphB1, which fills the distal half of the hippocampus in wild-type mice (Fig. 7G), but is reduced in the Emx2 mutant (Fig. 7H). Efficiency of transfection could not be monitored directly in these brains because 9 days after electroporation the transgene was no longer expressed. Given that the rate of efficient transfection is about 50% in surviving mice (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003) (present study), the number of brains with a distinct DG (about 50%) suggests partial rescue in all efficiently electroporated Emx2 mutant brains.

The hippocampal DG appears exquisitely sensitive to the level of canonical WNT signaling available during development (Galceran et al., 2000; Zhou et al., 2004). This may be because the primordium of the DG is closest to the cortical hem and requires the highest level of WNT signaling for normal expansion. Both Wnt2b and Wnt3a appear in the hem (Grove et al., 1998), signal through the canonical WNT pathway (Kawakami et al., 2001; Takada et al., 1994), and are depleted in the Emx2 mutant.

Discussion

Regulation of BMP, FGF and WNT signals in the cortical primordium

Candidate patterning centers have been identified previously in the mouse cortical primordium, and existing evidence indicates that these regulate development of the primordium along both the AP and ML axes (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003; Hebert et al., 2002; Lee et al., 2000). The present study provides evidence that the cerebral cortical patterning centers interact to regulate one another, as do signaling centers in chick dorsal telencephalon (Crossley et al., 2001; Ohkubo et al., 2002) and classic signaling centers elsewhere in the embryo.

Specifically, we find an antagonism between the WNT and BMP-rich cortical hem, and the anterior telencephalic source of FGF8. FGF8 suppresses WNT gene expression in the cortical hem, and may therefore normally set the anterior boundary of the WNT-rich hem. In turn, Fgf8 expression is suppressed by BMP signaling, which constrains the boundaries of the anterior telencephalic FGF8 source. A particularly striking finding is that a large part of the cortical primordium is competent to express Fgf8. That is, noggin can induce sites of Fgf8 expression far from the telencephalic midline, where Fgf8 is normally expressed. The implication is that BMP activity in the cortical primordium normally provides an extensive and crucial block of Fgf8 expression. The ability of BMP activity to regulate the FGF8 source appears to be time limited, perhaps because the lateral cortical primordium loses competence to express Fgf8.

Cross species comparisons

Our observations of mouse dorsal telencephalic signaling centers fit well with previous studies in the chick (Crossley et al., 2001; Ohkubo et al., 2002). As detailed above,
In the chick, as in other birds, basic pallial circuitry is comparable with that in mammals but its components are distributed as distinct cell types in different pallial nuclei (Karten, 1997). A particular functional specialization therefore co-opts parts or all of several nuclei. The avian Wulst is proposed to be most similar to neocortex (Medina and Reiner, 2000), containing somatosensory and visual representations. Its circuitry is organized in pseudolayers, which give a cortex-like appearance. However, the pseudolayers are in actuality radially adjacent slab-shaped nuclei (Medina and Reiner, 2000; Reiner et al., 2004), but not at the level of anatomical organization of circuitry.

Intense debate continues regarding homologies between specific avian and mammalian dorsal telencephalic structures (Reiner et al., 2004). A clearer comparison requires further conncetional and physiological investigation of the avian brain. Comparative studies of embryonic patterning should also be helpful, at both early (as in the present study) and later stages of development, as final morphology and connectivity emerge.

Effects of abnormal signaling sources; the Emx2 mutant mouse

Gene transfer experiments with wild-type mice uncover dramatic cortical patterning defects that occur when signaling centers are misregulated (Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003). Augmenting anterior FGF8 shifts the neocortical area map, suppresses WNT expression in the cortical hem and compromises hippocampal development. In humans, hippocampal and other cortical abnormalities accompany certain forms of thanatophoric dysplasia (TDs), devastating disorders caused by constitutive activation of FGFR3 (Ho et al., 1984; Neilson and Friesel, 1996; Wongmongkolrit et al., 1983). A human hem has been identified (Abu-Khalil et al., 2004); thus, a prediction to be tested is whether defects in hem WNT signaling appear in TDs – mouse expression and thereby influence BMP4/7 activity. BMP signaling is active early in choroid plexus development, perhaps specifying the choroid plexus epithelial cell fate. BMP4 signaling further regulates expression of Fgf8/17. FGF8/17 signaling is crucial for neocortical area patterning by regulating the AP axis of the cortical primordium, and at least indirectly, for hippocampal development. FGF8/17 inhibits WNT gene expression; canonical WNT signaling at the cortical hem is required for hippocampal development (Bonaccielli et al., 1993; Bulchand et al., 2001; Dou et al., 1999; Fukuchi-Shimogori and Grove, 2001; Fukuchi-Shimogori and Grove, 2003; Galceran et al., 2000; Garel et al., 2003; Hebert et al., 2002; Lee et al., 2000; Mallamaci et al., 1998; Monuki et al., 2001; Panchision et al., 2001; Shimamura et al., 1995; Shimamura and Rubenstein, 1997; Storm et al., 2003).

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The Emx2 mutant mouse line provides an informative illustration of the consequences of signaling center defects. Homozygous mutants display an expanded FGF8 domain, and predictably, given the present findings, a partial loss of WNT gene expression in the hem (Muzio et al., 2002). A previous study provided evidence that shifts in region-specific gene expression in the Emx2 mutant neocortex are in part caused by excess FGF8 (Fukuchi-Shimogori and Grove, 2003). Findings from the present study indicate that the expanded FGF8 source in the mutant reduces WNT signaling from the cortical hem, which in turn could contribute to defective development of the hippocampus.

Emx2 is expressed broadly in the cortical primordium, but its loss does not lead to a broad expansion of Fgf8 expression. Instead, the normally medial and anterior FGF8 domain is enlarged laterally and posteriorly, but retains clear boundaries. Findings from the present study suggest a partial explanation. A likely cause of the expanded FGF8 domain in the Emx2 mutant is early overexpression of noggin at the telencephalic midline, decreasing local BMP activity. BMP inhibition of Fgf8 expression is thereby relieved close to the midline, but
not at a distance. Remaining BMP activity may contain further lateral spread of Fgf8 expression.

We suggest that cortically expressed EMX2 influences signaling centers by direct gene regulation in the cortical primordium. However, an indirect influence by EMX2 function outside the cortical primordium remains a formal possibility. Emx2 expression appears at E8.5 in rostral brain, and continues in both the cortical and subcortical forebrain (Boncinelli et al., 1993), where EMX2 has diverse roles in development (Cecchi and Boncinelli, 2000). These complexities challenge easy interpretation of specific defects in the Emx2 mutant. For example, a misrouting of thalamocortical axons, first ascribed to the absence of EMX2 in the neocortex, may be partially due to loss of gene function in the ventral telencephalon where the thalamocortical pathway begins (Bishop et al., 2002; Lopez-Bendito et al., 2002).

Ultimately, the timing and sites of Emx2 expression that are crucial to particular aspects of development will be resolved by appropriate conditional deletions, or regional misexpression, of the gene. A recent advance has been the generation of a mouse that overexpresses Emx2 under the control of the nestin promotor (Hamasaki et al., 2004). FGF8 levels appear unaffected, perhaps because Emx2 is overexpressed too late, yet area boundaries are shifted. These findings, together with our own, indicate a primary effect of EMX2 on cortical patterning, and a secondary effect via two signaling sources.

Model of signaling interactions

We propose (Fig. 8) that early in telencephalic development, EMX2 acts directly or indirectly on noggin to derepress BMP activity. BMP activity constrains expansion of the anterior FGF8 source, and keeps the cortical hem clear of FGF8, protecting local WNT gene expression. Meanwhile, normal levels of midline noggin allow the FGF8 source to be established and maintained. Effectively completing a negative feedback loop, FGF8 downregulates Emx2 expression. These interactions help to ensure FGF and WNT/BMP sources of appropriate size, position and duration to regulate cortical patterning and growth.

Additional transcription factors have been implicated in the regulation of telencephalic signaling centers (Fig. 8). For example, the cortical hem, assessed by expression of BMP and WNT genes, is expanded in mice lacking the Lim homeobox domain transcription factor, LHX2 (Bulchand et al., 2001; Monuki et al., 2001). Restriction of Bmp4 expression to the hem region also requires the winged-helix transcription factor, FOXG1 (BF1) (Dou et al., 1999). Potential feedback pathways among signaling molecules and transcription factors add further complexity (see Fig. 8). For example, FGF8 and BMPs up- and downregulate Foxg1 expression, respectively (Furuta et al., 1997; Shimamura and Rubenstein, 1997; Storm et al., 2003); BMP signaling regulates Lhx2 expression (Monuki et al., 2001); FGF8/17 suppresses Emx2 expression (Fukuchi-Shimogori and Grove, 2003; Garel et al., 2003; Storm et al., 2003); and both BMP and WNT signaling pathways may control Emx2 transcription (Theil et al., 2002). A qualification is that in the different studies cited, the mouse dorsal telencephalon is analyzed at different embryonic ages, in different preparations, and in wild-type or engineered mutant mice. A systematic analysis will be needed to fit these observations into a comprehensive model of cortical patterning.

In conclusion, embryonic signaling centers and their interactions appear crucial for normal cerebral cortical patterning. Experiments using in utero electroporation and analysis of the Emx2 mutant mouse prompt a model in which two signaling centers, the anterior FGF source, and the WNT- and BMP-rich cortical hem interact antagonistically. Both the time dependency of gene misexpression effects and transient gene expression changes in the Emx2 mutant suggest there is a crucial period in early telencephalic development. During this period, the balance of signaling molecules governs the future functional organization of the cerebral cortex.

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