FGF8 acts as a classic diffusible morphogen to pattern the neocortex

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SUMMARY
Gain- and loss-of-function experiments have demonstrated that a source of fibroblast growth factor (FGF) 8 regulates anterior to posterior (A/P) patterning in the neocortical area map. Whether FGF8 controls patterning as a classic diffusible morphogen has not been directly tested. We report evidence that FGF8 diffuses through the mouse neocortical primordium from a discrete source in the anterior telencephalon, forms a protein gradient across the entire A/P extent of the primordium, and acts directly at a distance from its source to determine area identity. FGF8 immunofluorescence revealed FGF8 protein distributed in an A/P gradient. Fate-mapping experiments showed that outside the most anterior telencephalon, neocortical progenitor cells did not express Fgf8, nor were they derived from Fgf8-expressing cells, suggesting that graded distribution of FGF8 results from protein diffusion from the anterior source. Supporting this conclusion, a dominant-negative high-affinity FGF8 receptor captured endogenous FGF8 at a distance from the FGF8 source. New FGF8 sources introduced by electroporation showed haloes of FGF8 immunofluorescence indicative of FGF8 diffusion, and surrounding cells reacted to a new source of FGF8 by upregulating different FGF8-responsive genes in concentric domains around the source. Reducing endogenous FGF8 with the dominant-negative receptor in the central neocortical primordium induced cells to adopt a more posterior area identity, demonstrating long-range area patterning by FGF8. These observations support FGF8 as a classic diffusible morphogen in neocortex, thereby guiding future studies of neocortical pattern formation.

KEY WORDS: Patterning, Neocortex, FGF8, FGF17, Area specification, Mouse

INTRODUCTION
Mammalian neocortex is divided into scores of functionally specialized and anatomically distinct areas that form a consistent area map in each species. Thus, the neocortical area map represents the fundamental way in which the perceptual, cognitive and behavioral functions of the neocortex are organized (Nauta and Feirtag, 1986). Furthermore, although aspects of an area map are species specific, the overall layout of primary sensory and motor areas is conserved across species (Krubitzer, 1995). How the neocortical area map is generated is therefore an important problem in neural patterning. A conceptually simple model (Wolpert, 1996), proposed for patterning diverse developing tissues, would be that classic diffusible morphogens establish initial positional values in the neocortical primordium, which are then read off as different area fates. The best current candidates for such morphogens in neocortex are secreted signaling molecules that belong to the fibroblast growth factor 8 (FGF8) subfamily of FGFs (Ornitz and Itoh, 2001), which have been implicated in patterning the area map along its anterior to posterior (A/P) axis (Dominguez and Rakic, 2008; Grove and Fukuchi-Shimogori, 2003; O’Leary et al., 2007; Sur and Rubenstein, 2005).

FGF8, Fgf17 and Fgf18 are expressed at the anterior pole of the telencephalon (Bachler and Neubuser, 2001; Cholfin and Rubenstein, 2008; Maruoka et al., 1998) at the embryonic stage at which area patterning is initiated (Shimogori and Grove, 2005). FGF17 is required to specify dorsal prefrontal areas but does not have clear effects outside prefrontal cortex (Cholfin and Rubenstein, 2007; Cholfin and Rubenstein, 2008). FGF8, by contrast, has more widespread effects on the neocortical area map. In mice hypomorphic for Fgf8, neocortical area boundaries shift anteriorly, towards the depleted source of FGF8 (Garel et al., 2003). Conversely, augmenting the FGF8 source shifts boundaries posteriorly, enlarging anterior areas at the expense of more posterior areas (Fukuchi-Shimogori and Grove, 2001). Most compelling, introducing a second source of FGF8 posteriorly induces mirror-image duplications in the area map (Fukuchi-Shimogori and Grove, 2001).

Several lines of evidence therefore indicate that FGF8 has organizer activity in neocortex. Nonetheless, there have been no direct tests of whether FGF8 meets the criteria for a classic morphogen in the neocortical primordium (Crick, 1970; Driever and Nusslein-Volhard, 1988; Green et al., 1992; Lander et al., 2002; Wolpert, 1969). In this case, FGF8 would form a diffusion gradient along the entire anterior to posterior (A/P) axis of the neocortical primordium, and act directly to impart positional identity, both close to the FGF8 source, and at a distance. An alternative possibility is that FGF8 acts locally to specify area identity and control growth, similar to FGF17. The more extensive effects of FGF8 on area identity would be indirect, mediated by a cascade of other secreted signaling molecules. The two models instigate highly divergent research programs on the cellular and molecular mechanisms that pattern the area map. We therefore sought to test the first model, and to determine whether FGF8 shows features of a classic, diffusible morphogen in the mouse neocortical primordium.

MATERIALS AND METHODS
Mice
Mice carrying null alleles of Fgf17, null or hypomorphic alleles of Fgf8 were obtained from David Ornitz (Washington University) and Anne Moon (University of Utah). InGenious Targeting Laboratory Incorporated generated an Fgf8-IRES-Cre mouse, inserting an IRES-Cre cassette into...
the 3’ end of the Fgf8 locus immediately downstream of Fgf8-coding sequence. Timed pregnant CD-1 mice were obtained from the University of Chicago Transgenic Facility. Noon of the day on which a vaginal plug was seen was termed embryonic day (E) 0.5. Animal use was in accordance with NIH guidelines, and was approved by the University of Chicago IACUC.

**Immunohistochemistry**

Embryos were fixed in 4% paraformaldehyde and brains sectioned at 10 μm on a Leica CM1830 cryostat. After citrate antigen retrieval, sections were incubated with primary antibody and appropriate HRP-conjugated secondary antibodies. To detect immunofluorescence (IFl), TSA Plus Fluorescence Systems (Perkin-Elmer) was used according to the manufacturer’s instructions. Sections were counterstained with 4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen) to label cell nuclei and co-stained with FGF8 and FGF17 antibodies with FGF17 and FGF8, respectively, or with FGF2, FGF3, FGF15 and FGF18, the lateral telencephalon of E10.5 CD-1 embryos was electroporated with mouse Fgf2 (IMAGE Consortium, clone AI158649), Fgf3 (Open Biosystems, subsidiary of Thermo Fisher Scientific), Fgf8 (David Ornitz, Washington University), Fgf15 (Suzanne Mansour, University of Utah), Fgf17 (Nobuyuki Itoh, Kyoto University) or human Fgf18 (Open Biosystems). Brains were collected at E11.5 and sectioned into three series. One series was processed with in situ hybridization to identify the Fgf8 electroporation site. The second was processed for FGF8 IFl, and the third for FGF17 IFl. Immunostaining of endogenous FGF8 and FGF17 provided an internal positive control. Neither the MAB323 antibody against FGF8 nor the MAB319 antibody against FGF17 crossreacted with any other FGF tested (n=3 or 4 for each FGF).

**Image capture and modification**

Images were captured using a Zeiss Axioscope, Axiocam and Axiosvision software, or a Leica TCS SP5 laser confocal microscope with LAS-AF software (Leica Microsystems). Deconvolution increased confocal image clarity (Huygens Professional software, Scientific Volume Imaging). For figures, digital images were adjusted for contrast, color and brightness using Adobe Photoshop CS4.

**Quantitative analysis of FGF8 immunofluorescence**

The gradient of FGF8 IFl intensity in the neocortical primordium at E9.5 was quantified by averaging from light microscopic images of 10 μm sagittal sections near the midline of E9.5 forebrains (one section from each of nine brains). FGF8 IFl is most intense at the midline. Anterior and posterior boundaries of the neocortical primordium were defined, respectively, by the anterior pole of the telencephalon and by an inflection in the neuroepithelium marking the border between neocortical and hippocampal primordia (Altman and Bayer, 1995; Ashwell and Paxinos, 2008; Theiler, 1989). The segment of neuroepithelium in which FGF8 IFl intensities were measured was further standardized for each brain, by setting the width at 25 μm, with the lower edge at the ventricular surface, thereby covering the region where FGF8 IFl is most evident (see Results).

The nine curved neuroepithelial segments were digitally straightened with an established method (Long et al., 2009). Digital straightening allowed samples with varying curvature to be aligned and stacked (ImageJ, series 1.4, NIH, average z-projection routine). This permitted an image of average FGF8 IFl intensities along the A/P length of the neocortical primordium to be generated from the nine samples, and FGF8 IFl intensity to be plotted in arbitrary units (AU, Image J) against A/P distance from the FGF8 source (Fig. 2D). For this plot, the mean IFl intensity through the ventricular to pial width of a segment was calculated at one-pixel A/P intervals. Measurements were obtained from the ‘average’ segment, or from individual segments, then averaged, with the same results. Similar procedures were used to quantify gradients of FGF8 IFl and pERK IFl in the neocortical primordium at E10.5. Because the neocortex is thicker at E10.5, the segment of neuroepithelium in which IFl intensity was measured was 60 μm in width.

No errors were introduced into IFl intensity measures by the straightening process, which consisted of fitting, by hand, a line following the curve of the neocortical primordium. The ImageJ straightening algorithm fitted a spline to the curved line, with the lower border at the ventricular edge, selected points spaced 1 pixel apart, generated perpendicular lines at each point, and rigidly rotated the perpendicular lines to create the straightened version (Wayne Rasband, ImageJ, NIH). Plotting intensity values at one-pixel intervals along a line down the center of a sample, following its original curved contours, or after straightening, produced essentially identical results (see Fig. S1 in the supplementary material).

**RESULTS**

If FGF8 is a classic morphogen for the neocortex, FGF8 should form a gradient over the neocortical primordium during the period in which area patterning occurs. Based on previous electroporation experiments in which FGF8 levels were manipulated at different
Fig. 1. FGF8 is distributed throughout the neocortical primordium at E9.5. (A) E9.5 mouse embryo processed for FGF8-immuno/fluorescence (IFl). A hole visible in the telencephalon allowed reagent access. FGF8 IFl appears in the tail bud (tb), isthmus (iso) and anterior telencephalon (tel). (B-E,G) Sagittal brain sections, anterior towards the left, processed for FGF8 IFl. (B) FGF8 IFl extends throughout the neocortical primordium (ncxp). The meninges (mng) also show FGF8 IFl. (E-G) Sagittal sections from medial to lateral (M/L), one brain. FGF8 IFl intensity decreases along A/P and M/L axes. vtel, ventral telencephalon. (C,D) E9.5 forebrains, frontal view. Fgf8 expression marks an FGF8 source 200 μm in diameter (C, white double-headed arrow; black arrows indicate ncxp). (D) Gray lines outline the FGF8 source, based on gene expression in C, and indicate FGF8 IFl extending posterior and lateral to the source. Scale bar in A: 0.5 mm for A; 0.03 mm for B; 0.1 mm for C,D; 0.15 mm for E-G.

FGF8 is distributed throughout the neocortical primordium

Consistent with previous studies (Aoto et al., 2002; Bachler and Neubuser, 2001; Cholfin and Rubenstein, 2008; Crossley and Martin, 1995; Fukuchi-Shimogori and Grove, 2001; Maruoka et al., 1998), strong Fgf8 gene expression was localized to the anteromedial telencephalon at E9.5, marking the potential source of diffusible FGF8 (Fig. 1C). FGF8 immunofluorescence (IFl) was most intense at this site, and further revealed FGF8 protein throughout the neocortical primordium in a high to low A/P gradient (Fig. 1B,D-G). FGF8 IFl remained widespread in the neocortical primordium, at lower intensity levels, until at least E11.5 (see Fig. S2B in the supplementary material).

An exponentially declining gradient of FGF8

The FGF8 IFl intensity gradient along the A/P axis was quantified from standardized segments of neocortical neuroepithelium from nine brains (Fig. 2A, see Materials and methods for details). Curved segments of neuroepithelium were digitally straightened (Fig. 2B) and stacked to generate an ‘averaged’ image of the FGF8 IFl gradient, which showed an A/P gradient with an anterior plateau of high intensity (Fig. 2C). Mean IFl values were plotted against A/P distance with the plateau region excluded, and a declining exponential curve (Fig. 2D, red) was fit to the data (Fig. 2D, blue) with ImageJ regression analysis. IFl intensity declined by half over about 45 μm (Fig. 2D, representing about 10 cell widths (Fig. 2E). Given that cells can adopt different fates in response to as little as twofold differences in morphogen concentration (Green and Smith, 1990), the half-decline of FGF8 IFl, compared with the total length of the neocortical primordium at E9.5 (75-80 cell widths, n=9 brains), suggests that several different area fates for a neocortical ‘protomap’ (Rakic, 1988) could be obtained from the FGF8 gradient we estimate.

In subsequent experiments, FGF8 levels were manipulated with in utero microelectroporation at E10.5; we therefore also assessed the FGF8 gradient at this age. Consistent with observations at E9.5, FGF8 IFl was distributed through the neocortical primordium with an A/P gradient that declined exponentially, following an anterior...
plateau of high intensity (see Fig. S3 in the supplementary material). The half decline of the gradient was roughly 100 μm at E10.5, consonant with the A/P growth of the neocortex between E9.5 and E10.5.

Given that growth factors mediated by receptor tyrosine kinases, including FGFs, activate the Ras-extracellular signal-regulated (Ras/ERK) pathway (Schlessinger, 2000), we hypothesized that a gradient of FGF8 would generate a gradient of activated Ras/ERK. Supporting the possibility, phospho-ERK (pERK) IFl at E10.5 showed an A/P gradient in the neocortical primordium (Fig. 3A-D, n=6 brains). A caveat in interpreting this finding is that other FGFs in the embryonic telencephalon (Bachler and Neubuser, 2001; Borello et al., 2008) could contribute to – or obscure – a pERK gradient induced by FGF8.

FGF dispersion through the neocortical primordium

Demonstrating a gradient of FGF8 raises the question of how FGF8 distributes across the neocortical primordium. FGF8 may diffuse from a source, as demonstrated in zebrafish (Scholpp and Brand, 2004; Yu et al., 2009), or an Fgf8 mRNA gradient established as the primordium develops may be translated into an FGF8 protein gradient, as in vertebrate head-to-tail patterning (Dubrulle and Pourquier, 2004). Alternatively, neocortical progenitor cells could inherit FGF8 from their founder cells, a possibility supported by the potential overlap between dorsal telencephalic founder cells and an Fgf8-expressing region in the anterior neural fold (Cobos et al., 2001; Sanchez-Arrones et al., 2009).

To evaluate the second two alternatives, we used Cre recombinase mapping to determine the fate of Fgf8-expressing cells from the onset of Fgf8 expression in the embryo, focusing on the specific contribution of the Fgf8-lineage to the neocortical primordium. In mice carrying both Fgf8-IRES-Cre and R26R reporter alleles, cells expressing Fgf8, or derived from Fgf8-expressing cells, were permanently labeled with lacZ. X-gal staining of embryos at E10.5 (n=4), accurately reflected the pattern of expression of Fgf8 in the anterior telencephalon, dorsal diencephalon and isthmic organizer (ISO), and showed expected staining in the body and tail (Fig. 4A). Very little X-gal staining was seen in the neocortical primordium, except in the most anteromedial regions, confirming that Fgf8 expressing cells and their progeny are confined to the anterior telencephalon at E10.5 (Fig. 4B,C). Coronal sections from E14.5 brains (n=4) showed dense X-gal cell labeling in the septal nuclei, and medial prefrontal cortex, but not elsewhere in the neocortical primordium (Fig. 4D). These observations indicate that most neocortical progenitor cells do not express Fgf8, nor are they descended from cells that do.

FGF8 dispersion by diffusion from a source

To determine the competency of FGF8 to diffuse through the neocortical primordium, ectopic sources of Myc-tagged FGF8 were introduced at E10.5 by in utero microelectroporation. Myc-Fgf8 electroporation was aimed towards lateral and posterior cortical primordium, away from the endogenous FGF8 source. Twenty hours after electroporation, Myc-tagged FGF8 was detected by Myc IFl, thereby distinguishing electroporated from endogenous FGF8. Borders of the electroporation site were determined in the same tissue section by tdTomato fluorescence (Fig. 5A), and by ectopic Fgf8 gene expression in a neighboring section. Brains were selected for a dense electroporation site, about 100-200 μm wide. Myc IFl indicated tagged FGF8 diffusion from each ectopic source reaching more than 30 cell widths (150-200 μm) from the edge of the electroporation site (Fig. 5B,C; n=5/5 brains).

A false appearance of FGF8 diffusion could result if Fgf8 upregulated its own expression during this experiment, generating FGF8 at a distance from the electroporation site. Extensive tangential cell movement from the electroporation site could give the same impression. Fgf8 and tdTomato were co-electroporated at E10.5, and expression of tdTomato and Fgf8 detected at E11.5 in single sections with double FISH. In no case did Fgf8 expression extend beyond the tdTomato-expressing electroporation site (see Fig. S4 in the supplementary material, n=6/6 brains, three sections/brain). Furthermore, few labeled cells appeared at a distance from the body of the electroporation site, suggesting minimal tangential cell movement.

Sources of FGF8 induce expression of different target genes at different distances

FGF8 regulates the FGF8 synexpression gene group, which includes Spry genes, encoding negative regulators of FGF8 signaling, and Pea3/Etv4 and Erm/Etv5, encoding Ets transcription...
FGF8 is a classic morphogen in neocortex.

A dominant-negative FGF receptor sequesters FGF8 at a distance from its source

A dominant-negative (dn) FGF receptor, lacking the intracellular tyrosine-kinase signaling domain (Fig. 7A) (Amaya et al., 1991) was generated to determine whether FGF8 acts at a distance from its source to pattern the neocortex. Based on in vitro observations that FGF8 shows a high affinity for FGFR3 isofrom c (MacArthur et al., 1995; Ornitz and Itoh, 2001; Ornitz et al., 1996; Zhang et al., 2006) and on results obtained from electroporating a mutant FGFR3c in mouse (Fukuchi-Shimogori and Grove, 2001), the FGFR3c receptor isofrom was selected for these experiments. dnFGFR3c was predicted to sequester FGF8 from functional receptors at the electroporation site and thereby reduce FGF8 signaling.

Brains were electroporated with dnFGFR3c at E9.5 and processed for double IFI at E10.5 to detect endogenous FGF8 and Myc-tagged dnFGFR3c in the same tissue section. A prominent increase in FGF8-IFI intensity at the dnFGFR3c electroporation site showed an accumulation of endogenous FGF8 protein (Fig. 7B-D, n=7/7). Notably, dnFGFR3c sequestered FGF8 outside its anterior source, indicating diffusion of endogenous FGF8 (Fig. 7D,E, right arrows). Furthermore, the higher intensity of FGF8 IFI at the electroporation site compared with the FGF8 source itself (Fig. 7C), suggested that dnFGFR3c efficiently captures FGF8 as more protein diffuses from its source. Confocal microscopy confirmed colocalization of FGF8 and dnFGFR3c at the cell surface (Fig. 7J-M), and revealed FGF8 IFI puncta between electroporated cells (Fig. 7G,H, arrows), reflecting FGF8 that escaped sequestration.

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Consistent with FGF signal transduction (Cobb and Goldsmith, 1995; Tsang and Dawid, 2004), and previous observations described above, electroporation of Fgf8 at E9.5 activated the Ras-ERK pathway, indicated by increased pERK IFI at the Fgf8 electroporation site (see Fig. S5 in the supplementary material, n=9/9). Conversely, a large reduction of endogenous pERK IFI at sites of dnFgfr3c electroporation confirmed that dnFgfr3c reduces downstream FGF8 signaling (see Fig. S5 in the supplementary material, n=12/12). Based on these observations, dnFgfr3c was used to reduce FGF8 signaling at discrete sites in the neocortical primordium, testing the hypothesis that FGF8 acts directly at a distance to determine area identity.

**Evidence for long-range area patterning by FGF8**

The transcription factor gene Coup-TF1/Nr2f1 is expressed regionally in the posterior neocortical primordium, plays an important role in area specification (Armentano et al., 2007) and is downregulated by FGF8 (Sansom et al., 2005). Electroporation of dnFgfr3c at E10.5 in central/posterior neocortical primordium consistently caused an anterior expansion of the Nr2f1 expression domain at E13.5, evidently by removing FGF8-mediated inhibition. Anteromedial expression of Ets genes, far from the electroporation sites, was unaffected (see Fig. S6 in the supplementary material, n=10/11). These observations imply the ability of FGF8 to act at a distance from its source, in this case inhibiting a gene critical for neocortical patterning. We therefore examined the later effects of E10.5 dnFgfr3c electroporation on area gene expression at P0, when gene expression patterns indicate emerging neocortical area boundaries (Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003; Miyashita-Lin et al., 1999; Rubenstein et al., 1999) (n=10 brains, central/posterior electroporation verified with tdTomato fluorescence). Sections were processed with in situ hybridization for p75NTR/Ngfr, expressed posteriorly, for ephrinA5/Efna5, expressed strongly in presumptive primary somatosensory cortex (S1) (Mackarehtschian et al., 1999), and for Epha7, which shows a complementary pattern to Efna5 with both anterior and posterior expression domains (Yun et al., 2003).

In eight out of ten brains, posterior gene expression domains, including p75NTR/Ngfr, were expanded anteriorly, with a corresponding reduction of adjacent central domains. These shifts occurred at the dnFgfr3c electroporation sites and precisely correlated with the position and size of the sites. For example, dnFgfr3c electroporation in ‘brain 2’ extended anterior to that in ‘brain 1’ (Fig. 8B,C,E,F). In both brains, sagittal sections that cut through presumptive S1 in the parietal (Pa) domain, and primary visual cortex (V1) in the occipital (Oc) domain revealed an expanded Epha7-expressing Oc domain and a reduced Efna5-expressing S1, compared with a control brain (Fig. 8G-L). Brain 2, however, showed a larger anterior expansion of the Epha7-expressing Oc domain, and a more shrunken S1 than brain 1, correlating with the anterior borders of the electroporation sites in the two brains. These observations support the hypothesis that reducing FGF8 at discrete locations in the neocortical primordium induces cells to alter their fate and adopt a more posterior area identity.

A formal possibility is that electroporating dnFgfr3c affects area patterning by binding to other telencephalic FGFs, such as FGF2, FGF3, FGF15, FGF17 and FGF18 (Mason, 2007). Only FGF8 and FGF17 are implicated in area specification, however (Cholfin and Rubenstein, 2007; Cholfin and Rubenstein, 2008; Fukuchi-Shimogori and Grove, 2001; Garel et al., 2003; Grove and Fukuchi-Shimogori, 2003), and because FGF17 has less influence on overall neocortical patterning than FGF8 (Cholfin and Rubenstein, 2007) (see below), most of the effect of dnFgfr3c on area identity is likely to be mediated by sequestering FGF8.

FGF8 and FGF2 promote telencephalic growth (Borello et al., 2008; Garel et al., 2003; Raballo et al., 2000; Storm et al., 2003); thus, dnFgfr3c electroporation might induce differential growth in the cortical primordium, leading to apparent area shifts. This seems an unlikely explanation of present results, given that inhibiting FGF2 and FGF8 would decrease growth at sites of dnFgfr3c, leading to effects on area domains opposite to those we observed. In brain 1, for example, the dnFgfr3c electroporation site incorporates part of the Oc domain, yet we saw expansion, not shrinkage, of this domain.

**FGF17 cooperates with FGF8 to regulate A/P patterning of the neocortex**

The chief consequences of Fgf17 loss are in prefrontal cortex (Cholfin and Rubenstein, 2007), whereas, in mice hypomorphic for Fgf8, patterning shifts appear throughout neocortex (Garel et al., 2003). Nonetheless, the entire A/P extent of the neocortical primordium from E9.5 to E11.5 displays FGF17 IFI (see Fig. S2 in the supplementary material), suggesting FGF8 and FGF17...
hydrogenation. (A) Non-electroporated brain with cortical domains indicated. Red and green asterisks indicate centers of the dnFgfr3c electroporation sites in brains 1 and 2 (A-C, E, F). Yellow lines in B, C indicate section position in H, J, K, L. (D-F) Schematic sagittal sections illustrating electroporation sites (yellow) in brains 1 and 2. The site in brain 2 (F) is anterior to that in brain 1 (E). (G-L) Sagittal sections from a control hemisphere (G, J), brain 1 (H, K) and brain 2 (I, L) processed for situ hybridization. (G-I) In the control (G), expression of Epha7 picks out the Oc and Fr domains. In the control, brain 1 and brain 2, the Epha7-expressing Oc domain reaches progressively more anteriorly (arrowheads, G-I). (J-L) Efnas5 expression in the control (J) outlines developing S1, which is progressively smaller in brain 1 and brain 2, as central tissue is respecified to a more posterior fate. (M, N) tdTomato fluorescence documents the extent of each electroporation site in sagittal sections. Close inspection of the anterior boundary of the dnFgfr3c electroporation (ep) site in brains 1 and 2 shows near identity with the anterior boundary of the Oc domain, defined by gene expression (compare E, F with M, N and H, J). Abbreviation: Hp, hippocampus. Scale bar: 0.8 mm for A-C; 1.0 mm for D-L; 0.5 mm for M, N.

cooperate in patterning. Decreased Fgfa/Fgf17 gene dose reduces FGF8/FGF17 protein activity (Cholfin and Rubenstein, 2007; Cholfin and Rubenstein, 2008), therefore, if FGF8 and FGF17 act together to pattern the entire neocortex, a progressive decrease in Fgfa/Fgf17 gene dose should generate progressive posterior to anterior boundary shifts in the area map. An analysis of neocortical area patterning in mice carrying Fgf17-null alleles (Xu et al., 1999; Xu et al., 2000), alone or in combination with null or hypomorphic (hy) alleles of Fgfa (Moon and Cappecchi, 2000) supported this hypothesis, and confirmed that FGF8 is the primary agent of the two in overall neocortical patterning.

At P6, Cdh6, Cdh8 and Lmo4 expression demarcates a neocortical frontal domain (Fr), and primary visual cortex (V1) (Fig. 9C, D). In mice of various genotypes, ImageJ was used to measure the surface areas of Fr, V1 and the entire neocortex, with reference to Cdh6 and Lmo4 expression patterns. In Fgf8+/+;Fgf17+/− (n=6) or Fgf17+/− (n=12) mice, the Fr domain decreased, compared with controls (n=10), relative to total neocortical surface area (Fig. 9A). V1 appeared proportionally larger in Fgf17 nulls (Fig. 9B), but this could in principle result from shrinkage of the neocortex caused by reduction of the Fr domain. More compelling changes were seen when a functional Fgf8 allele was removed from the Fgf17 null, or an additional Fgf17 allele from the double heterozygote. The Fgf8+/+;Fgf17−/− genotype (n=7) displayed a greatly decreased Fr, and a significantly increased V1 in both proportional and absolute (P<0.001) size (Fig. 9A-D). The increase in absolute size of V1 in Fgf8+/+;Fgf17−/− mice is more impressive given that the total neocortical area in these mice is significantly decreased compared with controls (t-test, P<0.01, n=8, each group).
Very little loss of Fgf8 function is required to see combined effects of Fgf8 and Fgf17 depletion. Replacing a functional Fg8 allele in the Fgf17 null with a hypomorphic Fg8 allele (50% function) (Frank et al., 2002) expanded posterior neocortical domains. In sections through flattened cortex processed for 5HT-IR, the absolute length from the posterior edge of the neocortex to a common point in S1 (barrel A1) was greater in Fg8+/–;Fgf17−/− (n=6) than in Fg8+/– mice (n=5) (P=0.03) (Fig. 9E, red lines, arrows), despite a significantly smaller overall neocortical area in the former (P<0.01). Fgf17−/−, Fgf8−/−;Fgf17−/− and Fgf8−/− mice showed no significant differences with controls in overall neocortical area at this age (Cholfin and Rubenstein, 2007) (present study). These findings reveal functionally significant levels of both FGF8 and FGF17 in the posterior neocortical primordium that regulate regional specification and tissue growth.

**DISCUSSION**

**FGF8 as a neocortical morphogen**

Several lines of evidence indicate that FGF8 diffuses from its source in the anterior telencephalon, forms a gradient from anterior to posterior across the neocortical primordium, and acts at a distance from its source to regulate neocortical area identity. These findings support the hypothesis that FGF8 operates as a classic diffusible morphogen, establishing positional values along the A/P axis of the neocortical primordium. Additional findings indicate that the anterior source of FGF8 and FGF17 is an integral patterning center for the entire neocortex. Not supported are alternative models, in which, for example, FGF8 primarily controls development of anterior neocortex, patterning posterior neocortex indirectly through interactions with other secreted signaling molecules.

**Downstream of FGF8**

Transcription factors responsive to FGF8 are already implicated in neocortical area patterning (Mallamaci and Stoykova, 2006; O’Leary et al., 2007). Emx2 is downregulated by FGF8 and promotes the development of posterior neocortical areas (Bishop et al., 2000; Hamasaki et al., 2004). FGF8 also downregulates Nr2f1 (Sansom et al., 2005), and of the known mechanisms that mediate FGF8 signaling, this has the most striking consequences for area patterning. Nr2f1 expression defines a comparatively sharply bounded domain in the posterior neocortical primordium (Rash and Grove, 2006). Conditional deletion of Nr2f1 in cortex causes primary sensory areas to shrink, making way for a vastly expanded anterior cortex (Armentano et al., 2007; Faedo et al., 2008). Both Emx2 and Nr2f1 are upregulated by bone morphogenetic protein and Wnt signaling, originating in the mouse from the postcommissural cortical hem (Domínguez and Rakic, 2008; Ohkubo et al., 2002; Theil et al., 2002). Still missing from the picture, however, are FGF8-responsive, positive regulators of anterior and central area fates. The switch from a central to a more posterior fate when FGF8 signaling is inhibited by dnFgfr3c electroretropion implies the existence of such positive regulators, as does the ability of ectopic FGF8 to induce duplicate somatosensory barreifields, a central area fate, in posterior neocortex (Fukuchi-Shimogori and Grove, 2001). Genes expressed in gradients in the neocortical primordium have been found (Sansom et al., 2005), but FGF8-responsive genes that are regionally expressed at the particular time when area patterning is initiated have not yet been sought.

**Integrating FGF8 levels and time of exposure**

Fate-mapping experiments demonstrated that most neocortical progenitor cells do not belong to the Fgf8 lineage. Progenitors of ventromedial prefrontal cortex are exceptional, however, lying inside the anterior FGF8 source. These cells may be specified to generate ventromedial prefrontal cortex in response to both high levels of FGF8 and prolonged exposure to FGF8. The process of integrating morphogen concentration with time of morphogen exposure to arrive at a specific cell fate has been described previously in the limb (Harfe et al., 2004; Tabin and McMahon, 2008). Sonic hedgehog (Shh), produced by the zone of polarizing activity (ZPA) in the limb bud, induces the orderly A/P array of digits (Riddle et al., 1993). In mice, cells inside the ZPA generate digits 5 and 4, whereas digit 2 and part of 3 develop in response to a Shh gradient outside the ZPA. Both 5 and 4 are exposed to the maximum level of Shh, but are distinguished because cells that spend the longest time in the ZPA generate digit 5 (Harfe et al., 2004; Tabin and McMahon, 2008). Given that Fgf17 is expressed in a larger domain than Fgf8, a substantial region of prefrontal cortex may derive from progenitors inside the FGF8/FGF17 patterning source. Previous findings indicate that FGF17 is required for specification of dorsal, but not ventromedial, prefrontal cortex (Cholfin and Rubenstein, 2007). An appealing model is that area specification in prefrontal cortex is controlled by the cellular integration of FGF8 and FGF17 levels together with the time of exposure to each FGF. This model can be explored by fate-mapping experiments that follow the Fgf8 and Fgf17 lineages as the neocortex develops.

**Patterning human neocortex**

A working assumption is that mechanisms that pattern the cerebral cortex in the mouse will have a similar function in other mammals, including humans. For example, the cortical hem signaling center in the mouse generates Wnt proteins and bone morphogenetic proteins (BMPs) (Furuta et al., 1997; Grove et al., 1998), and is an organizer for the hippocampus (Mangale et al., 2008). At least by Wnt and BMP gene expression, and position next to the hippocampal primordium, the cortical hem has a human analog (Abu-Khalil et al., 2004). Moreover, human cortical abnormalities associated with faulty FGF8 or FGFR3 signaling (Frank et al., 2002; Hevner, 2005) suggest an FGF8 source regulates pattern and growth in the embryonic human telencephalon.

A key question is whether the same molecular mechanisms can pattern both small and large brains, given spatial constraints on the range of signaling molecule diffusion. This problem seems likely to be solved, not by adding mechanisms in large species to give morphogens vast ranges of action, but rather by keeping the primordia universally small. At 35 days of gestation, for example, the human neocortical primordium is at a developmental stage equivalent to E10 in the mouse, and is about 0.5 mm long (Coppen, 2005), close to the size of the E10 mouse neocortical primordium, and, according to the present study, within the diffusion range of FGF8. Thus, the neocortex of the mouse and human, which differ 1000-fold in final surface area, may be patterned by the same signaling mechanisms because, at the appropriate stage of development, their primordia are similar in size.

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References


Rabbino, D., Rhee, J., Lyon-Cook, R., Leckman, J. F., Schwartz, M. L. and Vaccarino, F. M. (2000). Basic fibroblast growth factor (Fgf2) is necessary for...
cell proliferation and neurogenesis in the developing cerebral cortex. J. Neurosci. 20, 5012-5023.


