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REVIEW ARTICLE
Gene networks controlling early cerebral cortex arealization

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Abstract

Early thalamus-independent steps in the process of cortical arealization take place on the basis of information intrinsic to the cortical primordium, as proposed by Rakic in his classical protomap hypothesis [Rakic, P. (1988) *Science*, **241**, 170–176]. These steps depend on a dense network of molecular interactions, involving genes encoding for diffusible ligands which are released around the borders of the cortical field, and transcription factor genes which are expressed in graded ways throughout this field. In recent years, several labs worldwide have put considerable effort into identifying members of this network and disentangling its topology. In this respect, a considerable amount of knowledge has accumulated and a first, provisional description of the network can be delineated. The aim of this review is to provide an organic synthesis of our current knowledge of molecular genetics of early cortical arealization, i.e. to summarise the mechanisms by which secreted ligands and graded transcription factor genes elaborate positional information and trigger the activation of distinctive area-specific morphogenetic programs.

Mechanisms controlling cortical arealization: protomap or protocortex?

From embryonic day 7.5 (E7.5) onward (in mice) the presumptive dorsal telencephalic field is progressively specified, thanks to a complex cascade of events involving secreted ligands released by the surrounding structures as well as transcription factor genes expressed by the field itself (Grove *et al*., 1998; Acampora *et al*., 1999; Gunhaga *et al*., 2000, 2003; Backman *et al*., 2005; Marklund *et al*., 2004; Tole *et al*., 2000; Suda *et al*., 2001; Muzio *et al*., 2002b; Kimura *et al*., 2005). The result of this specification, the cortical primordium of the E11 mouse embryo, looks like a thin neuroepithelial sheet and does not display any major region-specific morphological peculiarity. Subsequently, while developing throughout its extension according to common basic guidelines, it undertakes a complex and articulated process of regional diversification. This leads to the development of the mature cerebral cortex with its full repertoire of area-specific cytoarchitectural, myeloarchitectural and computational properties. This process of regional and areal differentiation of the cortical primordium is commonly termed 'cortical arealization'.

Two main models have been proposed for the cellular and molecular mechanisms controlling cortical arealization, the protomap model (Rakic, 1988) and the protocortex (or *tabula rasa*) model, originally put forward by Van der Loos & Woolsey (1973) and subsequently developed by O'Leary (1989). According to the former, cortical arealization would occur on the basis of molecular cues intrinsic to the cortical proliferative layer. These cues would be transferred by periventricular neural progenitors, lying in distinctive cortical regions, to their neuronal progenies, migrating along fibres of radial glia and sharing with them the same rostrocaudal and mediolateral locations. According to the latter, the cortical primordium would not have any areal bias at all. Arealization would take place on the basis of information transported to the developing cortex by subcortical afferents (mainly thalamocortical afferents). This information would be used to 'write' distinctive areal programs onto the cortical primordium, as if onto a clean table (hence '*tabula rasa*'). Both models are supported by very robust bodies of experimental data; this has resulted in a very heated scientific debate in this field. Two main lines of evidence support the protomap model. First, explants taken from different regions of the cortical anlage at E10.5–E12.5 (i.e. before the arrival of thalamocortical projections), grown *in vitro* or heterotopically transplanted, appear specifically committed to expressing molecular markers peculiar to their region of origin (Arimatsu *et al*., 1992; Ferri & Levitt, 1993; Tole *et al*., 1997; Gitton *et al*., 1999; Tole & Grove, 2001; Vyas *et al*., 2003). Second, the cortex of *Mash1* or *Gbx2* knock-out mice, constitutively lacking any thalamocortical projection, displays a normal molecular regionalization profile (Nakagawa *et al*., 1999; Miyashita-Lin *et al*., 1999). Two main lines of evidence also support the *tabula rasa* hypothesis. First, embryonal visual cortex transplanted to a parietal locale (and thus possibly exposed to information coming from the thalamic ventrobasal complex) acquires barrel features peculiar to the somatosensory cortex (Schlaggar & O'Leary, 1991). Second, surgical misrouting of visual information to adult somatosensory or auditory cortices (via the thalamic ventrobasal complex or the medial geniculate nucleus, respectively) makes these cortices acquire architectonic and high-order functional properties peculiar to the visual cortex (Schneider, 1973; Frost & Schneider, 1979; Sur *et al*., 1988).

A synthesis of these two models has recently been achieved and it is presently accepted that two main phases can be distinguished in the

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process of cortical arealization. During the earlier, prior to the arrival of thalamocortical projections, molecular regionalization of the cortical primordium would occur on the basis of information intrinsic to this primordium, as in the protomap model. During the latter, after the arrival of these projections (from E13.5 onward), cortical arealization would be refined based on information transported by thalamocortical fibres, as in the protocortex model. Special relevance to the whole process is ascribed to a particular developmental window, from E10.5 to E12.5, when cortical neuroblasts are areally committed or determined, i.e. their areal potencies become restricted in a progressively less reversible way.

At the moment, two main classes of molecules are supposed to be crucial for early regionalization of the cortical primordium: secreted ligands, released around the borders of the cortical field, and transcription factors, gradually expressed within primary proliferative layers of this field. Secreted ligands would diffuse through the cortical morphogenetic field where they would be degraded according to specific kinetics, so generating variously orientated concentration gradients. Secreted ligands would regulate the expression of cortical transcription factor genes, in dose-dependent manners, so accounting for the further generation of concentration gradients of these factors. Graded and transient expression of these factors would finally encode for positional values peculiar to distinctive regions of the cortical field. These values would be used 'on line' to properly regulate tangential expansion rates of distinct cortical regions and to size the final neuronal complement of their layers. They would be transferred, in a more stable format, to neurons generated in distinct cortical regions, thus eventually leading to selective activation of distinctive area-specific differentiation programs. (O'Leary & Nakagawa, 2002). Actually, differential area-specific regulation of key kinetic parameters controlling tangential expansion of the cortical primordium and sizing of its neuronal layers has been experimentally demonstrated in the anlagen of murine areas 3 and 6 (Polleux *et al*., 1997) as well as in those of primate areas 17 and 18 (Lukaszewicz *et al*., 2005). Remarkably, in the former case such differential regulation was documented at the time when deep-layer neurons are generated (Polleux *et al*., 1997), i.e. prior to the arrival of the thalamocortical radiation, which means it must rely on information intrinsic to the cortical primordium. On the other hand, none of the gradually expressed transcription factors identified so far is really restricted to a specific proto-area; rather, transcripts encoding for them are more abundant in specific regions than elsewhere. As such, they should be classified not as 'area-specific' but, more properly, as 'regionally enriched'. It is reasonable that the analogue positional information they bear might be subsequently digitized, via the combined activation of truly areally-restricted transcription factor genes, each of them able to trigger a specific areal morphogenetic program in its expression domain. However, none of these digital 'second level' effectors has as yet been identified (Funatsu *et al*., 2004; Sansom *et al*., 2005) and, at the moment, their existence is purely hypothetical.

The aim of this review is to summarise how positional information flows through the gene network encoding for secreted ligands and graded transcription factors expressed in the developing cortex, and how is it used to master regionalization and arealization of the cortical primordium.

Secreted ligands and cortical arealization

Ligands are released around three structures lying at the borders of the cortical field and relevant for its arealization: (i) the 'cortical hem', which forms between the cortical and the choroidal fields, at the caudomedial edge of the cortical neuroepithelial sheet; (ii) the commissural plate, at the rostromedial pole of telencephalon; (iii) the cortical antihem, a recently discovered signalling structure, which forms on the lateral side of the cortical field, at the pallial–subpallial boundary (Fig. 1A).

From E10, the cortical hem is a source of Wnts (Wnt2b, 3a, 5a, 7b, 8b) and bone morphogenetic proteins (Bmps; Bmp2, 4, 5, 6, 7), expressed in nested domains which also span the adjacent dorsomedial cortical field (Furuta *et al*., 1997; Lee *et al*., 2000). Wnt signalling apparently promotes archicortical morphogenesis, as suggested by disrupted hippocampal development peculiar to mice lacking *Wnt3a* or the β-catenin nuclear cofactor gene *Lef1* (Galceran *et al*., 1999; Lee *et al*., 2000). However, electroporation of a *Wnt3a*-expressing transgene into the wild-type E11.5 rostral cortex, while causing it to bulge possibly because of exaggerated neuroblast proliferation, did not up-regulate archicortical markers in this region, suggesting that Wnt signalling may normally promote the expansion of the archicortical progenitor pool without conferring on it any areal hippocampal determination (Fukuchi-Shimogori & Grove, 2001). Concerning Bmps, the analysis of $Bmp5^{-/-}Bmp7^{-/-}$ mutants revealed little about the role of Bmp ligands in telencephalic patterning because the resulting phenotype was confounded by early defects in neural tube closure (Solloway & Robertson, 1999). However, the electroporation of a transgene encoding for a constitutively active Bmp receptor 1a into the telencephalon as well as the conditional inactivation of *Bmpr1a* in this structure showed that *Bmpr1a* promotes choroidal vs. cortical specification without exerting any apparent influence on the subsequent regionalization of the cortical field (Panchision *et al*., 2001; Hebert *et al*., 2002).

From earlier than E10 to ∼E12.5, the commissural plate and the regions surrounding it release Fgf3, 8, 17 and 18 which, it has been predicted, would promote rostral vs. caudal areal programs (Bachler & Neubuser, 2001). In agreement with this prediction, Hebert *et al*. (2003) showed that telencephalon-restricted inactivation of the Fgf receptor gene *Fgfr1a* results in olfactory bulb agenesy as well as in subtle patterning defects of the frontal cortex. Moreover, Garel *et al*. (2003) showed that homozygosity for a hypomorphic *Fgf8* loss-of-function allele elicits a sensible caudalization of the rostrocaudal cortical molecular profile, even in the absence of any apparent anomaly in the distribution of thalamocortical afferents. However, the most spectacular demonstration of the relevance of Fgf signalling to neocortical arealization came from Fukuchi-Shimogori & Grove, (2001). These authors electroporated an *Fgf8*-expressing plasmid into rostral telencephalon and found that this lead to a caudal shift of the parietal cortex. A rostral shift of the somatosensory cortex was conversely obtained when a plasmid encoding for a truncated form of the Fgf receptor 3, able to chelate Fgfs and to counteract them, was electroporated. Remarkably, when *Fgf8* was delivered into caudal cortex this resulted in a partial mirror duplication of the somatosensory cortex, consistent with the idea that *Fgf8*, beyond any possible effects on neuroblast proliferation, may impart specific areal determinations to the various parts of the cortical field in a dose-dependent manner (Fukuchi-Shimogori & Grove, 2001).

Around E12.5 and afterwards, neural progenitors within the antihem specifically express five secreted signalling molecules: Fgf7, the Wnt-secreted inhibitor Sfrp2 and three Egf-related ligands, Tgf-α, Nrg1 and Nrg3 (Assimacopoulos *et al*., 2003). Even though their patterning activities on the cortex have not yet been characterized, however, Egf family members seem to be involved in the regional specification of cortical areas associated with the limbic system. This is suggested by the up-regulation of the limbic system-associated membrane protein LAMP occurring *in vitro*, in nonlimbic

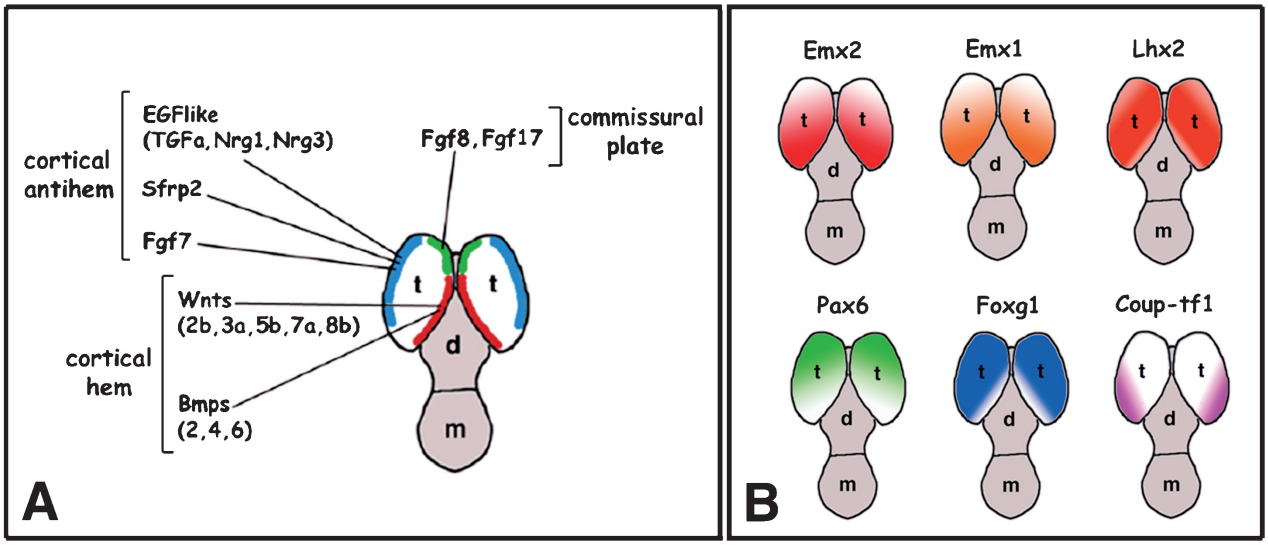

FIG. 1. Expression patterns of (A) secreted ligands and (B) graded transcription factor genes in the early cortical primordium. E12.5 brains, dorsal views: t, telencephalon; d, diencephalon; m, mesencephalon

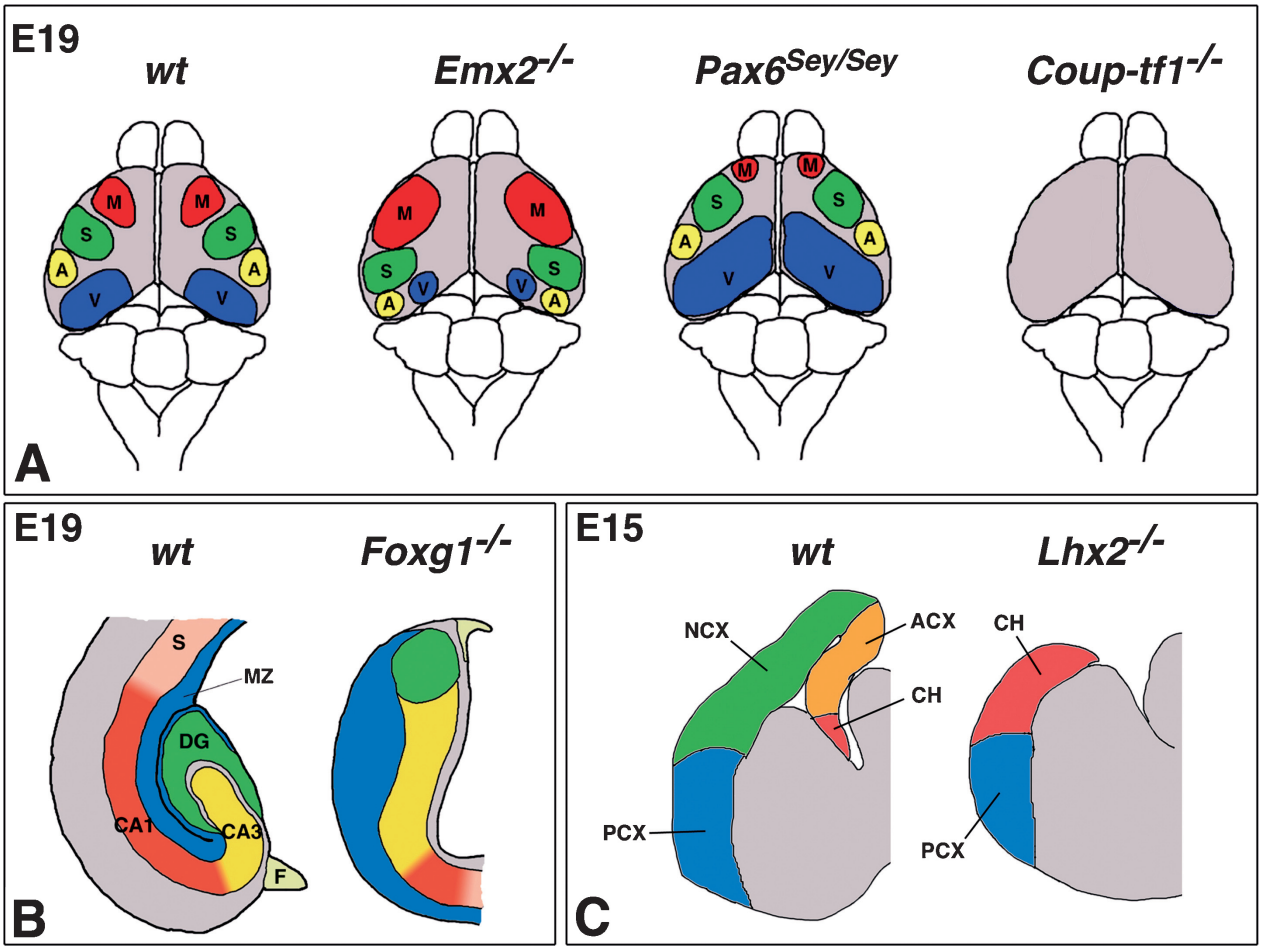

FIG. 2. Areal phenotypes of mice knock-out for the graded transcription factors (A) *Emx2*, *Pax6* and *Coup-tf1*, (B) *Foxg1* and (C) and *Lhx2*. (A) E19 brains, dorsal views: M, motor cortex; S, somatosensory cortex; A, auditory cortex; V, visual cortex. (B) E19 brains, mid-frontal sections: S, subiculum; CA1, cornu ammonis 1 field; CA3, cornu ammonis 3 field; DG, dentate gyrus; F, fimbria; MZ, marginal zone. (C) E15 brains, frontal sections: CH, cortical hem; ACX, archicortex; NCX, neocortex; PCX, palaeocortex.

cortical domains, in response to Egf family ligands (Ferri & Levitt, 1995; Levitt *et al*., 1997).

Gradually expressed transcription factor genes and regionalization of the cortical primordium

Several transcription factor genes, including *Emx2*, *Emx1*, *Lhx2*, *Pax6*, *Foxg1* and *Coup-tf1*, are expressed by neural progenitors within periventricular proliferative layers, in graded manners along the main tangential axes (Fig. 1B). As such, these genes were suspected of being crucial for imparting distinctive regional identities to neural progenitors. Remarkably, the analysis of mice mutant for each of them has to a large extent confirmed this suspicion (Fig. 2).

More than 10 years ago, it was suggested that the homeobox gene *Emx2*, expressed by the cortical primary proliferative matrix along a caudomedial^{high}–rostrolateral^{low} gradient (Simeone *et al*., 1992; Gulisano *et al*., 1996; Mallamaci *et al*., 1998), shapes the cortical areal profile as a promoter of caudomedial fates (O'Leary *et al*., 1994). Later, Bishop *et al*. (2000) and Mallamaci *et al*. (2000) tested this prediction on *Emx2*-knockout embryos, with success. A variety of experimental approaches were used, including: (i) *in situ* detection of region-specific transcripts and area-specific transgene-driven activities; (ii) analysis of area-specific bromodeoxyuridine uptake profiles; (iii) 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil)-based reconstruction of thalamocortical wiring profiles. The result was that, in the absence of *Emx2*, the full repertoire of areal identities was still preserved; however, as expected, caudomedial areas were shrunken and rostrolateral ones expanded. It was pointed out (López-Bendito *et al*., 2002) that abnormalities in cortical distribution of thalamic afferents taking place in $Emx2^{-/-}$ mutants might reflect subpallial misrouting of these afferents rather than problems in their final cortical sorting and targeting. However, the functional relevance of cortical *Emx2* mRNA dosage to cortical areal profiling was later confirmed by Leingartner *et al*. (2003). These authors showed that adenoviral transduction of an *Emx2*-expressing transgene into presumptive parietal cortex was followed by the invasion of this cortex by fibres coming from the lateral geniculate nucleus (normally directed to occipital cortex), even in the absence of any overt pathfinding abnormality in the basal telencephalon. More recently, it was shown that the overall areal profile is actually very finely tuned to the *Emx2* dosage. Relative and absolute sizes of occipital areas of $Emx2^{-/+}$ mutants are intermediate between null and wild-type mice and an expansion of caudal medial areas can be achieved by introducing one or, better, two alleles of a nestin-promoter-driven *Emx2*-expressing transgene into a wild-type genome (Hamasaki *et al*., 2004). Remarkably, areal profiling of $Emx2^{-/-}$ mutants was originally performed at late gestational ages (Bishop *et al*., 2000; Mallamaci *et al*., 2000). This left open the question whether areal dysmorphologies described in these mutants originated from an aberrant early regionalization of their cortical primordium, before and/or at the time of its areal commitment, or from selective impairment of tangential expansion rates of their occipitohippocampal anlage after this time. Muzio *et al*. (2002a) addressed this question and found that both explanations hold. The early occipitohippocampal anlage is already undersized at the beginning of neuronogenesis. Moreover, between E11 and E13 it expands less than normal, due to selective slowing down of DNA synthesis and exaggerated neuronogenesis in this region. Remarkably, this is associated with up-regulation of cyclin-dependent kinase 2 inhibitor genes $Kip1^{p27}$ and $Kip2^{p57}$, exaggerated proneural : antineural gene expression ratio and depression of the Delta–Notch–Hes axis in the same region (Muzio *et al*., 2005).

The *Emx2* paralog *Emx1* is expressed in the primary proliferative layer of the cortex along a gradient similar to that of *Emx2*. Its expression, however, is not confined to intermitotic neuroblasts but extends into postmitotic glutamatergic neurons (Simeone *et al*., 1992; Briata *et al*., 1996; Gulisano *et al*., 1996; Chan *et al*., 2001). As such, it was suspected that *Emx1*, like *Emx2*, promoted cortical caudomedial fates. However, analysis of mutants lacking it did not confirm this suspicion (Yoshida *et al*., 1997).

Pax6 encodes for an evolutionarily conserved transcription factor (reviewed by Callaerts *et al*., 1997), including two DNA binding motifs, a paired domain (Bopp *et al*., 1986; Treisman *et al*., 1991) and a paired-like homeodomain (Frigerio *et al*., 1986). Its expression in the mouse begins at E8.0 and is restricted to the anterior surface ectoderm and the neuroepithelium of the closing neural tube in the regions of the spinal cord, forebrain and hindbrain (Walther & Gruss, 1991; Grindley *et al*., 1995). Within the telencephalon, *Pax6* is mainly expressed by the dorsal part and contributes to its pallial vs. subpallial specification (Stoykova *et al*., 1997; Toresson *et al*., 2000; Yun *et al*., 2001). In the absence of functional Pax6 protein, as seen in the *Pax6* mutant *Small eye* (*Sey*) (Hill *et al*., 1991), a progressive ventralisation of the molecular identity of the pallial progenitors occurs (Stoykova *et al*., 2000; Kroll & O'Leary, 2005) and, at birth, a significant proportion of cortical progenitors produce subpallial interneurons instead of generating cortical projection neurons (Kroll & O'Leary, 2005). Within the developing cortex, *Pax6* is expressed in a subpopulation of cortical progenitors, the radial glial cells (Götz *et al*., 1998), acting as pluripotent progenitors able to generate neuronal as well as glial cells (reviewed by Campbell & Götz, 2000). Here *Pax6* plays a potent neuronogenetic role as shown by both gain- and loss-of-function analysis (Heins *et al*., 2002; Haubst *et al*., 2004). Remarkably, within the cortical periventricular proliferative layer, *Pax6* expression shows a rostrolateral^{high}–caudomedial^{low} gradient (Stoykova *et al*., 1997; Muzio *et al*., 2002a). Thus it is highest rostrally, in the regions of the ventral and lateral pallium, including thereby the anlage of the motor cortex, while the medial pallium (the anlage of hippocampus) and the caudal cortex (the anlage of the visual cortex) express *Pax6* at much lower levels. Consistent with this gradient and based on the analysis of distribution of the area-specific adhesion molecules Cad6 and Cad8, a severe shrinkage of the rostral motor cortex area and enlargement of the posterior (visual) areas has been reported in $Pax6^{Sey/Sey}$ mutants. This suggested that *Pax6* plays a role complementary to that exerted by *Emx2* in the determination of cortical area sizes and of their distribution along the rostrocaudal axis of the cortex (Bishop *et al*., 2000). However, because of severe defects of the morphogenesis of the diencephalon (Stoykova *et al*., 1996; Warren & Price, 1997), the thalamocortical axons could not reach the cortex of *Pax6*-null ($Pax6^{lacZ/lacZ}$) mutants (Jones *et al*., 2002), thus precluding analysis of the hodological correlate of the molecular shifts characterising this structure. Unexpectedly, mapping of thalamocortical projections after cortex-restricted inactivation of *Pax6* indicated that the thalamocortical projections extend correctly between particular thalamic nuclei and the corresponding cortical areas, indicating that relevant, mature aspects of areal specification do not depend on *Pax6* (T. Tuoc and A. Stoykova, unpublished observations). More recently, consistent with the *Pax6* medial–lateral gradient, it has been reported that *Pax6* is crucial for the specification of subpopulations of ventral pallium progenitors, involved in morphogenesis of the lateral, basolateral and basomedial nuclei of the amygdalar complex as well as of the nucleus of the lateral olfactory tract (Tole *et al*., 2005). Finally, it is remarkable that the defects in cortical arealisation observed at perinatal stages in $Pax6^{Sey/Sey}$ mutants are prefigured by severe malformation of the early $Pax6^{Sey/Sey}$ cortical primordium, with reduced rostrolateral cortical domains and expanded

caudomedial ones. This suggests that the former defects may arise as a consequence of the latter. In this respect, it is also reasonable to hypothesize that over-expression of *Wnt8* and *Wnt3a* occurring in the caudomedial primordium of *Pax6* mutants might contribute to the genesis of their areal phenotype by over-stimulating the tangential expansion of the caudomedial pallium and thus contributing to relative shrinkage of the ventrolateral one Muzio *et al*., 2002a).

The winged helix transcription factor gene *Foxg1,* expressed in the early telencephalon along a caudomedial^{low}–rostrolateral^{high} gradient and relevant for basal ganglia morphogenesis as well as for cortical neuroblast differentiation (Xuan *et al*., 1995; Dou *et al*., 1999; Hebert & McConnell, 2000; Hanashima *et al*., 2002; Seoane *et al*., 2004; Martynoga *et al*., 2005), was recently reported as also being crucial for the proper laminar histogenetic progression of cortical progenitors. In its absence, neocortical neuroblasts would generate only preplate and not cortical plate, finally giving rise to an aberrant cerebral cortex where all neurons express the Cajal–Retzius cell marker *Reelin* (Hanashima *et al*., 2004). However, the complementarity between the *Foxg1* ventral^{high}–dorsal^{low} cortical gradient and the patterned distribution of *Reelin*^{on} neurons, generated to a large extent around the cortical hem (Meyer *et al*., 2002; Takiguchi-Hayashi *et al*., 2004) and, later, preferentially clustered in the archicortex, suggests that the overproduction of *Reelin*^{on} neurons occurring in *Foxg1*-null mutants might have a different origin. More than reflecting a blockage of histogenetic progression, such overproduction might indeed arise from large-scale dorsoventral mispatterning of the whole telencephalon and relative expansion of its dorsomedial fields. Accurate molecular profiling of *Foxg1*$^{-/-}$ brains confirmed this suspicion. In fact, in the absence of *Foxg1*, palaeo- and neocortex are undersized or absent, not all cortical neurons express *Reln* and the telencephalon develops as an enlarged and geometrically distorted hippocampus, where specific subdomains similar to CA1–3 and DG fields can be distinguished at topologically plausible locations (Muzio & Mallamaci, 2005). Remarkably, as in the case of *Emx2*$^{-/-}$ mutants, this phenotype seems to have a dual origin. It reflects a very early error in cortical regionalization (Muzio & Mallamaci, 2005) and it is exacerbated by a selective and progressive lengthening of neuroblast cell cycle in the rostral cortical field between E10.5 and E14.5 (Martynoga *et al*., 2005).

The LIM-box-homeobox gene *Lhx2*, expressed in the whole telencephalic neuroepithelium except the cortical hem, along a caudomedial^{high}–rostrolateral^{low} gradient, plays two main roles in cortical development. First, it represses fimbriochoroidal programs, committing neuroblasts within the dorsal telencephalon to cortical fates (Bulchand *et al*., 2001; Monuki *et al*., 2001). Second, within the cortical field it promotes hippocampal vs. neo- and palaeocortical programs (Vyas *et al*., 2003). In the absence of *Lhx2*, the choroidal region and the cortical hem are considerably enlarged (Bulchand *et al*., 2001; Monuki *et al*., 2001), the residual pallium fails to activate the archicortical markers *Ephb1* and *KA1* and the same pallium conversely expresses specific sets of markers normally limited to ventral pallium, *NeuII*, *Sfrp2* and *Dbx1* at E12.5 and *Steel*, *Lmo3* and *ActRII* at E15.5 (Bulchand *et al*., 2001; Vyas *et al*., 2003).

The orphan nuclear receptor gene *Coup-tf1* is specifically restricted to the caudolateral cortex. Its inactivation leads to a complex areal phenotype, including deregulated widespread expression of a large panel of region- and area-specific markers and convergence of both somatosensory and visual thalamic afferents onto the parietal cortex. In view of this, *Coup-tf1* is supposed not to specifically promote a particular areal program but rather to be an integral part of the molecular machinery which allows cortical neuroblasts to appropriately read molecular cues encoded by other cortical patterning genes (Zhou *et al*., 2001).

Functional interactions among sources of secreted ligands

It was originally demonstrated by Ohkubo *et al*. (2002) that, within the chicken telencephalon, Bmp signalling represses the expression of *Fgf8*. More recently, the Grove group confirmed this interaction in the mouse and showed that, in the same model system, Fgf8 in turn down-regulates the expression of Wnt ligands (Shimogori *et al*., 2004), thus possibly limiting the expansion of the hippocampal progenitor pool (Fig. 3A). These two relevant interactions are the core of the functional network proposed by these authors as governing early steps of mammalian cortical arealization (see below; Shimogori *et al*., 2004).

Functional interactions among transcription factor genes

Valuable information about the topology of gene networks governing cortical arealization came from systematic inspection of expression patterns of gradually expressed transcription factor genes in mice knock-out for each of them (for a synopsis, see Fig. 3B).

Molecular analysis of *Emx2*$^{-/-}$ and *Pax6*$^{Sey/Sey}$ E11.5 embryos revealed that *Pax6* mRNA and *Emx2* mRNA, respectively, are up-regulated in regions which normally express them at lower levels, suggesting that *Emx2* and *Pax6* reciprocally inhibit the expression of each other. Paradoxically, *Pax6* is also up-regulated in the archicortical anlage of *Pax6*$^{Sey/Sey}$ mutants, suggesting that the fully functional Pax6 protein may be necessary to achieve the *Emx2*-dependent confinement of *Pax6* mRNA to ventraolateral pallium. Conversely, *Emx2* is selectively down-regulated in the archicortical anlage of *Emx2*$^{-/-}$ mutants, meaning that this gene is necessary to sustain its own expression in the medial cortical field (Muzio *et al*., 2002a). Moreover, the *Coup-tf1* expression domain is shifted caudalwards in *Emx2*$^{-/-}$ mutants and barely affected in *Pax6*$^{Sey/Sey}$ ones (A. Mallamaci and L. Muzio, unpublished observations), whereas no change in *Emx2* and *Pax6* expression patterns apparently takes place in *Coup-Tf1*$^{-/-}$ mutants. This suggests that *Coup-tf1* may act downstream of or in parallel with the other two (Zhou *et al*., 2001).

Several years ago it was found that inactivation of *Foxg1* leads to early up-regulation of *Emx2* (Dou *et al*., 1999). Recently, it has been shown that such up-regulation extends to later developmental stages and is associated with specification of the entire telencephalon as dorsomedial cortex (Muzio *et al*., 2005). Conversely, no up-regulation of *Foxg1* can be apparently detected in *Emx2*$^{-/-}$ mutants (A. Mallamaci and L. Muzio, unpublished observations). All this suggests that normal repression of dorsomedial programs exerted by *Foxg1* may occur through down-regulation of *Emx2*.

Additional information about mechanisms governing arealization came from phenotypic characterisation of embryos mutant for cortical transcription factor genes in various combinations.

Surprisingly, this analysis showed that, in addition to graded transcription factor genes listed above, *Otx* homeobox genes are also specifically required for the development of caudomedial cortical areas. This applies to *Otx1*, expressed by early cortical progenitors and deep-layer neurons derived from them, as well as to *Otx2*, withdrawing from the dorsal telencephalon at the time of its cortical specification (Simeone *et al*., 1993). This requirement might be due to implication of both *Otx* genes in early prosomeric subdivision of the anterior CNS and to the distinctive prosomeric origin of archicortex and neocortex (Puelles & Rubenstein, 1993). Aizawa and collaborators (Suda *et al*., 2001; Kimura *et al*., 2005), through accurate analysis of mice mutant for *Emx* and *Otx* genes, demonstrated that tight functional synergy among *Emx2*, *Otx1* and *Otx2* is crucial not only for primary large-scale patterning of the anterior neural plate and neural

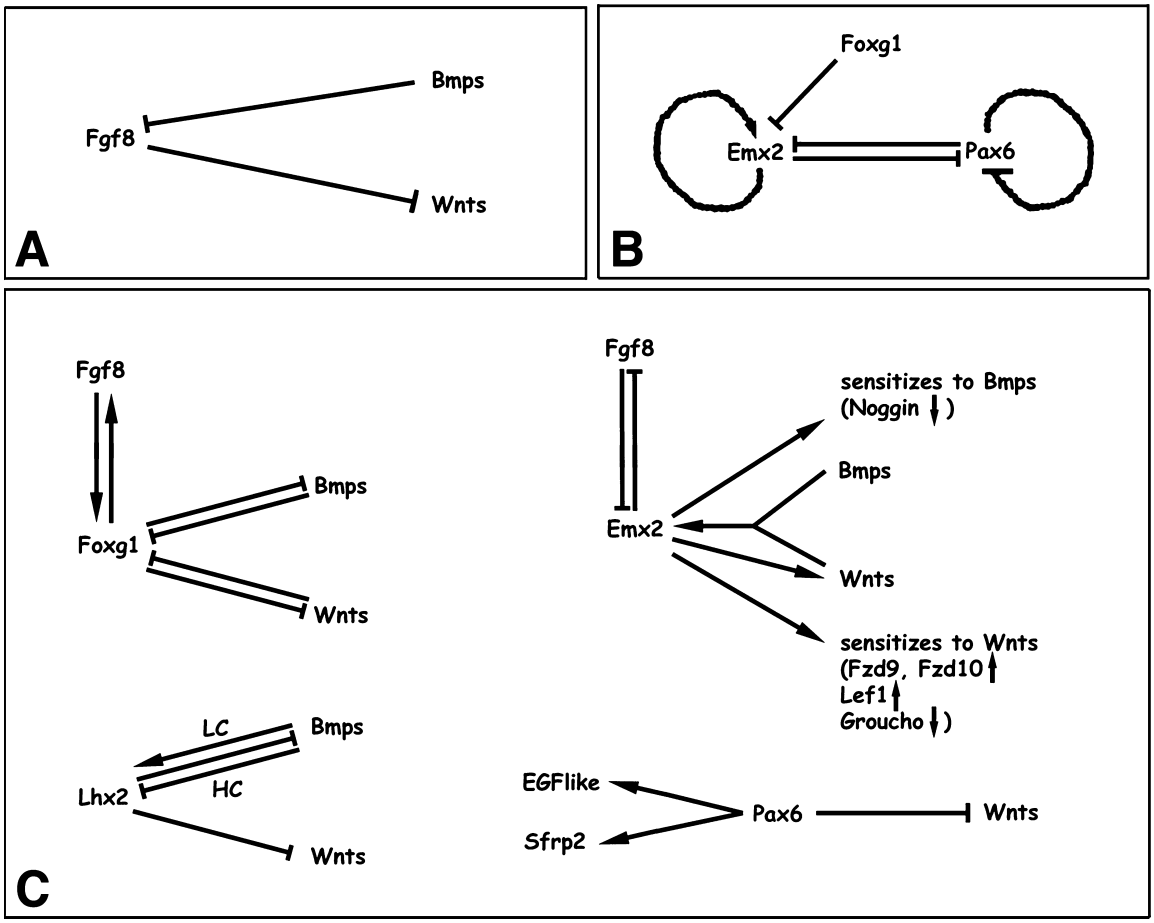

FIG. 3. Presumptive topology of gene networks governing early steps of cerebral cortex arealization: epistatic relationships (A) among secreted ligand genes, (B) among graded transcription factor genes and (C) among ligands and transcription factor genes.

tube but also for proper development of the hippocampus. In $Otx2^{+/-}Emx2^{-/-}$ mutants, not only is a large portion of the neural tube, from the pallium to the preotic sulcus, mispatterned (the rostral hindbrain is expanded, the midbrain is shifted rostrally, all of the thalamus except the posterior pretectum fails to develop, cortical development is impaired and the ganglionic eminence is enlarged) but, remarkably, distinct pallial regions are unequally affected. Lateral markers *Pax6* and *Ngn2* are easily detectable, neo-archicortical markers *Lef1* and *Wnt8b* are down-regulated, and medial markers, including archicortical markers *Ephb1* and *Prox1,* cortical hem markers *Wnt3a*, *Wnt5b* and *Wnt2a* and the choroidal plexus marker *Ttr*, are switched off. It has been proposed that selective impairment of cortical dorsomedial structures in $Otx2^{+/-}Emx2^{-/-}$ mutants might stem from their specific derivation from the fourth prosomere, tightly dependent on *Emx2* and *Otx2* for its proper development. This would not apply to neo- and palaeocortex, deriving from more rostral fifth and sixth prosomeres, apparently more tolerant to reduced *Emx2* and *Otx2* dosages (Suda *et al*., 2001; Kimura *et al*., 2005). However, it is possible that selective impairment of archicortical morphogenesis in $Otx2^{+/-}Emx2^{-/-}$ brains does not originate from their intrinsic inability to activate such a process but is due rather to disruption of Wnt signalling sustaining it. This point has to be carefully tested. Finally, an attenuated $Otx2^{+/-}Emx2^{-/-}$-like phenotype characterizes $Otx1^{-/-}Emx2^{-/-}$ mutants, but not $Emx1^{-/-}Otx2^{-/+}$ or $Emx1^{-/-}Otx1^{-/-}$ ones. This suggests that *Otx1* may be involved, like *Otx2*, in early allotment of a specific stripe of neural plate to hippocampal fates, but rules out any involvement of *Emx1* in such a process (Kimura *et al*., 2005).

Further suggestions about early molecular mechanisms shaping the cortical areal profile came from $Emx2^{-/-}Pax6^{Sey/Sey}$ mutants. Original analysis of these mice by Mallamaci and collaborators, aimed at testing the existence of *Emx2*- and *Pax6*-independent pathways controlling cortical arealization, did not hit its original target. This happened because the double-mutant dorsal telencephalon, already bearing hybrid pallial and subpallial features at E11.5, gets respecified into lateral ganglionic eminence between E11.5 and E14.5, thus precluding further characterization of its more mature cortical areal profile (Muzio *et al*., 2002b). However, further analysis of such brains by Aizawa and collaborators (Kimura *et al*., 2005) disclosed additional aspects of their phenotype, not previously addressed but nevertheless relevant to the problem of cortical arealization. These authors showed that dorsoventral telencephalic mispatterning of $Emx2^{-/-}Pax6^{Sey/Sey}$ mutants is paralleled by large-scale rostrocaudal mispatterning of their neural tube. The p1–p2 territory, caudal to the zona limitans intrathalamica (zli), is misspecified and, starting from E12.5, repatterned as a supranumerary mesencephalon, a mirror image of the original one. The p3 territory, delimited by zli and the telencephalic–diencephalic sulcus, collapses after E10.5. Prosomere P4 is also affected, as suggested by the absence of the eminentia thalami. Remarkably, the part of the dorsal telencephalon still bearing cortical specification at E12.5 displays molecular features peculiar to neocortex and lacks any hippocampal specification. It was suggested that failed development of the archicortex in these mutants might stem from its predicted derivation from this fourth prosomere, dependent on the availability of at least one functional *Emx2* or *Pax6* allele.

Remarkably, analysis of *Emx1*$^{-/-}$*Pax6*$^{Sey/Sey}$ and of *Emx1*$^{-/-}$ *Emx2*$^{-/-}$*Pax6*$^{Sey/Sey}$ mutants ruled out any *Emx2*-like involvement of *Emx1* in large-scale patterning of the early neural tube, including the proper development of the fourth prosomere (Kimura *et al*., 2005).

Structure and expression profiles similarities between *Emx1* and *Emx2* lead to hypotheses that the former could synergise with and/or substitute for the latter as a promoter of cortical caudomedial fates. Given the apparently normal areal profile of *Emx1*$^{-/-}$ mice (Yoshida *et al*., 1997), this hypothesis was re-tested by different groups who investigated whether coinactivation of both *Emx* genes would exacerbate the *Emx2*$^{-/-}$ areal phenotype. After a first, negative, report (Bishop *et al*., 2002), Mallamaci and collaborators demonstrated that coinactivation of both *Emx* paralogs actually lead to such a consequence; this was evident at E11.5 as well as at E18.5, suggesting that areal abnormalities peculiar to these double mutants might originate from errors in setting up the early areal protomap (Muzio & Mallamaci, 2003). More recently, this problem was re-addressed by the Aizawa and O'Leary groups (Shinozaki *et al*., 2002, 2004; Bishop *et al*., 2003), with consistent results. These authors showed that the development of medial-most cortical derivatives (Cajal–Retzius cells, dentate gyrus and hippocampus), already impaired in *Emx2*$^{-/-}$ mutants, is fully suppressed in the absence of both *Emx* genes. Moreover, they reported that the medial Wnt/Bmp signalling centre and the choroid plexus are not established and the cortical hem gets respecified as telencephalic roof plate. Remarkably, these patterning anomalies are already evident at E10.5–E12.5, again suggesting that late areal abnormalities of *Emx1*$^{-/-}$*Emx2*$^{-/-}$ mutants may stem from very early regionalization errors (Shinozaki *et al*., 2002; Bishop *et al*., 2003; Shinozaki *et al*., 2004).

Recently, Muzio & Mallamaci (2005) showed that coinactivation of *Emx2* and *Foxg1* suppresses over-production of Cajal–Retzius cells peculiar to *Foxg1*-null mutants. This validates the hypothesis that repression of dorsomedial programs normally exerted by *Foxg1* may occur through down-regulation of *Emx2*. However, inactivation of *Foxg1* also leads to up-regulation of canonical Wnt signalling machinery (Muzio & Mallamaci, 2005) as well as to higher Wnt signalling (L. Muzio and A. Mallamaci, unpublished observation). This suggests that the morphogenesis of cortical hem, dentate gyrus and hippocampus, which requires early Wnt activity, might be confined to the wild-type dorsomedial cortex, through early, *Foxg1*-dependent down-regulation of this pathway in the lateral part of it. Of course, given the capability of *Emx2* and Wnt signalling to reciprocally sustain each other (Theil *et al*., 2002; Muzio *et al*., 2005), these two hypotheses have to be considered not mutually exclusive.

Finally, *Foxg1* and *Lhx2*, each of them able to confine cortical hem programs to the dorsomedial-most telencephalic vesicle (Dou *et al*., 1999; Bulchand *et al*., 2001; Monuki *et al*., 2001; Muzio & Mallamaci, 2005), seem to synergise in repressing choroidal programs, as shown by the enlargement of the *Ttr*^{on} choroid field occurring in double *Foxg1*$^{-/-}$*Lhx2*$^{-/-}$ mutants as compared to simple *Foxg1*$^{-/-}$ and *Lhx2*$^{-/-}$ mutants. Moreover, over-generation of Cajal–Retzius cells, peculiar to *Foxg1*$^{-/-}$ embryos, is not rescued in double *Foxg1*$^{-/-}$*Lhx2*$^{-/-}$ mutants, suggesting that the *Lhx2* function is not necessary for the production and/or survival of these neurons (L. Muzio and A. Mallamaci, unpublished observation).

Crosstalk among graded transcription factors and diffusible ligands

It has been suggested that diffusible ligands synthesized and released by the borders of the cortical morphogenetic field may spread a large distance through this field and be degraded in a uniform way, so generating concentration gradients. These gradients would promote pan-cortical graded expression of genes encoding for primary transcription factors and these ones, according to a complex combinatorial syntax, would cell-autonomously dictate differential activation of distinctive area-specific programs (O'Leary & Nakagawa, 2002). Genetic dissection of cortical arealization performed in a number of labs worldwide indicates that, even if this paradigm holds to some extent, the molecular logic underlying cortical arealization is much more complex.

A first additional factor of complexity is that recurrent regulatory loops exist through which the transcription factors feedback-regulate the expression or at least the activity of their regulators, i.e. the diffusible ligands (for a synopsis, see Fig. 3C).

This is the case with *Emx2*, regulated in a coordinated manner by Bmp, Wnt and Fgf ligands and able, in turn, to modulate the activity of the three corresponding canonical signalling pathways. Ohkubo *et al*., 2002) reported that, in the chicken telencephalon, Bmp4 promotes *Emx2* expression and the Bmp inhibitor Noggin inhibits it. Theil *et al*. (2002) demonstrated that, in the mouse, *Emx2* is synergistically up-regulated by Wnt and Bmp ligands released by the cortical hem, thanks to two modules located within its telencephalic enhancer which bind to Smad1,5 and Tcf/Lef cofactors. Fukuchi-Shimogori & Grove (2003) found that electroporation of *Fgf8* into the anterior pole of the E11.5 mouse telencephalon results in a caudal shift of regions expressing high levels of *Emx2* whereas sequestering Fgf8 via electroporation of a truncated, high-affinity soluble form of an Fgf receptor, *sFgfr3c*, elicits the opposite effect, consistent with the up-regulation of *Emx2* observed in *Fgf8*-hypomorphic mutants by Garel *et al*. (2003). Remarkably, all of the three signalling pathways, Bmp, Wnt and Fgf, are in turn feedback-regulated by *Emx2*. In the *Emx2*$^{-/-}$ prosencephalon, *Nog* is over-expressed at an early stage, leading at ~E8.75 to a transient depression of Bmp signalling (Shimogori *et al*., 2004). [As we will see, this effect seems to be crucial for later patterning of the cortex, as early (E9.5) *Nog* electroporation into the rostral wild-type telencephalon can later phenocopy the classical *Emx2*$^{-/-}$ areal profile (Shimogori *et al*., 2004)]. Moreover, canonical Wnt signalling collapses in E11.5–E13.5 *Emx2*$^{-/-}$ brains, possibly as a consequence of misregulation of genes encoding for four functional layers of this signalling machinery: ligands (Wnt3a, 2b, 5a and 8b), plasma membrane receptor (Fzd9 and -10), a nuclear β-catenin agonist (Lef1) and an antagonist (Groucho) (Muzio *et al*., 2005). Finally, the *Fgf8* and *Fgf17* expression domains are largely expanded in the *Emx2*$^{-/-}$ E10.5 telencephalon, whereas electroporation of *Emx2* into wild-type cortical explants dramatically reduces them if performed by E10.5 (Fukuchi-Shimogori & Grove, 2003).

Similar phenomena were also described for *Foxg1*. Bmp2 and -4 (but not Bmp6 and -7) repress *Foxg1* in mouse E10.5 brain explants (Furuta *et al*., 1997). *Foxg1* inactivation leads to up-regulation of *Bmp4* throughout the mutant telencephalon (Dou *et al*., 1999). Down-regulation of canonical Wnt signalling occurring in *Lef1* loss-of-function mutants leads to over-expression of *Foxg1* (Galceran *et al*., 1999) [similar phenomena can be also detected upon conditional inactivation of the same pathway at E8.5 or E11.5 (Backman *et al*., 2005)]. Canonical Wnt signalling is strengthened in *Foxg1*$^{-/-}$ mutants (L. Muzio and A. Mallamaci, unpublished observations), possibly due to up-regulation of Wnt ligands (*Wnt3a, 5a* and *8b*), a plasma membrane receptor (*Fzd9*) and a nuclear β-catenin agonist (*Lef1*) (Muzio & Mallamaci, 2005). Early expression of *Foxg1* may be promoted by *Fgf8* (Shimamura & Rubenstein, 1997). *Fgf8* is, in turn, down-regulated in *Foxg1*$^{-/-}$ mutants (Martynoga *et al*., 2005).

Regulation of peripheral signalling centres by pallial transcription factors has also been shown in the case of *Pax6*. In the antihem of $Pax6^{Sey/Sey}$ mutants the expression of *Tgf-α* and *Nrg1* is missing, suggesting that *Pax6* might stimulate the generation of EGF-like ligands secreted by this patterning centre (Assimacopoulos *et al*., 2003). Moreover, in the same mutants the presumptive Wnt inhibitor gene *sFRP2*, normally expressed by the antihem, is absent and *Wnt3a* and *Wnt8b*, expressed around the cortical hem, are up-regulated (Ragsdale *et al*., 2000; Kim *et al*., 2001; Muzio *et al*., 2002a). This suggests that *Pax6* may antagonize Wnt signalling throughout the early cortical neuroepithelium by acting on different functional layers of its machinery.

Finally, an even more complex circuitry involves *Lhx2*. Monuki *et al*. (2001) showed that, in E11.5–E12.5 mouse cortical explants, high levels of Bmp2 and 4 (but not of Bmp6) shut *Lhx2* down; conversely, low levels promote its expression. (This is consistent with the restriction of *Bmps* to the cortical hem and with the expression profile of *Lhx2*, absent in the hem, high in the hippocampal anlage and lower in presumptive neocortex). Remarkably, in the absence of *Lhx2*, *Bmp4* as well as *Wnt3a*, *5b* and *5b* are up-regulated (Bulchand *et al*., 2001); *Fgf8* is not affected (Vyas *et al*., 2003).

Transcription factor-independent ligand-dependent arealization

A further divergence from the classical model 'diffusible ligands → graded transcription factors → arealization' comes from the fact that diffusible ligands may apparently dictate the cortical areal profile independently of the graded transcription factors crosstalking with them.

This has been specifically shown in the case of *Emx2* and *Wnts*. *Emx2* down-regulates neuronogenesis rates within the caudomedial cortical primordium, so normally allowing the proper expansion of the progenitor pool giving rise to the hippocampus. Remarkably, pharmacological reactivation of canonical Wnt signalling in $Emx2^{-/-}$ mutants rescues to a large extent the exaggerated neuronogenesis characterizing their brains, implying that the size of the hippocampal progenitor pool may be regulated by Wnts regardless of the available *Emx2* dosage (Muzio *et al*., 2005). Even more interestingly, similar phenomena have also been shown in the case of *Emx2* and *Fgfs*. Fukuchi-Shimogori and Grove (2003) noticed that early *in vivo Emx2* electroporation was followed by stable caudalization of the cortex, only provided that the expression plasmid was delivered into the anterior pole of the telencephalon. Strikingly, electroporation of *Emx2* into the somatosensory cortex anlage, a region in the very middle of the *Emx2* rostrocaudal gradient and, as such, very sensitive (according to the classical model) to changes in *Emx2* dosage, did not elicit any alteration. This suggested that *Emx2* might shape the areal profile not directly, as previously believed, but by modulating the expression of *Fgf8* and *Fgf17* in the rostral brain. This prediction was confirmed by buffering at E11.5 the Fgf excess peculiar to $Emx2^{-/-}$ mutants via *in vivo* electroporation of an *sFgfr3c*-encoding plasmid and verifying at E18.5 the reversion of the electroporated $Emx2^{-/-}$ brain to a quasi-normal rostrocaudal areal profile (Fukuchi-Shimogori & Grove, 2003). Consistently with this, when *sFgfr3c* was delivered to $Emx2^{-/-}$ brains earlier, at E9.5, inspection of the cortical hem at E13.5 did not reveal any collapse of *Wnts*, which was followed at E18.5 by partial rescue of dentate gyrus markers *Prox1* and *Ephb1* (Shimogori *et al*., 2004). On the basis of these findings as well as of the previous discovery that Bmp signalling down-regulates Fgf8 expression (Ohkubo *et al*., 2002), Shimogori *et al*. (2004) proposed that the true morphogen gene shaping the cortical areal profile would be not *Emx2* but *Fgf8*. The very function of *Emx2* would be to repress *Nog* and consequently to allow the early Bmp-dependent confinement of Fgf expression to the rostromedial pole of the telencephalon, so protecting the Wnt-expressing hem from inhibitory influences exerted by Fgf ligands. These conclusions were recently corroborated by the finding that artificial, layer-restricted overexpression of an *Fgf8* transgene in the early cortical primordium is sufficient to elicit a pronounced caudal shift of afferents coming from the ventrobasal thalamus, normally directed to the somatosensory area (Shimogori & Grove, 2005). However, hierarchical relationships between *Emx2* and *Fgf8* are still highly debated and controversial. In contrast with the above findings, O'Leary and collaborators (Leingartner *et al.*, 2003; Hamasaki *et al.*, 2004) recently reported new evidence supporting the idea that not *Fgf8* but *Emx2 per se* is the 'master' of cortical arealization. They showed that adenovirus-mediated transduction of *Emx2* into the rat cortical primordium is followed by misrouting of a substantial fraction of fibres coming from the dorsal geniculate nucleus towards areas rostral to their natural target, i.e. the occipital visual area. Remarkably, this also happens when viral transduction takes place as late in rat as E13.5 (Leingartner *et al*., 2003), corresponding to mouse E12.0, a developmental age too late to perturb *Fgf8* expression (Fukuchi-Shimogori & Grove, 2003). Moreover, Hamasaki *et al*. (2004) recently reported that transgenic mice expressing additional copies of *Emx2* under the control of the nestin promoter undergo a relevant expansion of caudomedial areas at the expense of rostromedial ones, in the absence of any detectable down-regulation of *Fgf8* in the rostromedial commissural plate. Discrepancies between these different reports concerning the capability of *Emx2* to repress *Fgf*s in the rostral brain, the very core of the problem, might be due to the different technologies the two groups used for overexpressing *Emx2*, by classical transgenesis and by somatic electroporation. Moreover, to explain these discrepancies, the diverse strengths of the promoters they chose for these manipulations, the nestin- and the CMV-promoter, should be taken into account as well. However, at the moment it is hard to reconcile such different conclusions and further experimental work is necessary to solve this problem.

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Abbreviations

Bmp, bone morphogenetic proteins; E, embryonic day; *Sey*, *Small eye*.

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