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
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 oriented 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 it is 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 anthem, 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 (Galeran et al., 1999; Lee et al., 2000). However, electroporation of a Wnt5a-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 Bmp5/7−/− 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 Fgfr1 results in olfactory bulb agenesy as well as in subtle patterning defects of the frontal cortex. Moreover, Garei 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 anthem 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.

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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\textsuperscript{high}–rostrolateral\textsuperscript{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., 1992). 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) \textit{in situ} detection of region-specific transcripts and area-specific transgene-driven activities; (ii) analysis of area-specific bromodeoxyuridine uptake profiles; (iii) \textit{labeled}–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 adenosiral transduction of an Emx2-expressing transgene into presumptive parietal cortex was followed by the invasion of this cortex by fibers 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 their 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 neurogenesis. Moreover, between E11 and E13 it expands less than normal, due to selective slowing down of DNA synthesis and exaggerated neurogenesis in this region. Remarkably, this is associated with up-regulation of cyclin-dependent kinase 2 inhibitor genes Kip1\textsuperscript{27} and Kip2\textsuperscript{28}, 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 (Frischer 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 neurogenetic 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\textsuperscript{high}–caudomedial\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{Sey/Sey} mutants are prefigured by severe malformation of the early Pax6\textsuperscript{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–dorsoventral low cortical gradient and the patterned distribution of Reelin 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 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 Reelin 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, contributing neuroblasts within the dorsal telencephalon to cortical development. 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). 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 orphan nuclear receptor gene Coup-tfl1 is specifically restricted to the caudal lateral 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-tfl1 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 Pax6Sey/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 Pax6Sey/Sey mutants, suggesting that the fully functional Pax6 protein may be necessary to achieve the Emx2-dependent confinement of Pax6 mRNA to ventral lateral 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-tfl1 expression domain is shifted caudallywards in Emx2−/− mutants and barely affected in Pax6Sey/Sey ones (A. Mallamaci and L. Muzio, unpublished observations), whereas no change in Emx2 and Pax6 expression patterns apparently takes place in Coup-tfl1−/− mutants. This suggests that Coup-tfl1 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...
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, 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.

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−/−Pax6Sey/Sey and of Emx1−/−Emx2−/−Pax6Sey/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 Trn+ 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, sFgf3c, 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 drastically 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<sup>Sey</sup>/Sey<sup>⁄</sup> 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 Bmp5 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 cross-talking 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<sup>−/−</sup> 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<sup>−/−</sup> mutants via in vivo electroporation of an sFgfr3c-encoding plasmid and verifying at E18.5 the reversal of the electroporated Emx2<sup>−/−</sup> brain to a quasi-normal rostrocaudal areal profile (Fukuchi-Shimogori & Grove, 2003). Consistently with this, when sFgfr3c was delivered to Emx2<sup>−/−</sup> 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 Ephp1 (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 Fgfs 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.

References


