Genetic Control of Cortical Regionalization and Connectivity

John L.R. Rubenstein, Stewart Anderson, Limin Shi, Emily Miyashita-Lin, Alessandro Bulfone[1] and Robert Hevner

Nina Ireland Laboratory of Developmental Neurobiology, Center for Neurobiology and Psychiatry, Department of Psychiatry and Programs in Neuroscience, Developmental Biology and Biomedical Sciences, 401 Parnassus Avenue, University of California at San Francisco, CA 94143–0984, USA and [1]Tigem – Telethon Institute of Genetics and Medicine, Via Olgettina 58, 20132 Milan, Italy

Herein, the genetic control of regionalization and connectivity of the neocortex are reviewed. Evidence is accumulating which suggests that intrinsic mechanisms have a central role in controlling cortical regional specification and differentiation. Expression patterns of several genes (*Id-2*, *Tbr-1*, *cadherin-6*, *cadherin-8*, *neuropilin-2*, *Wnt-7b*, *Eph-A7* and *RZR-beta*) are described; the expressions of these genes have regional boundaries which demarcate distint functional areas of the cerebral cortex in neonatal mice.

Introduction

The vertebrate cerebral cortex, or pallium, is organized into several large functional subdivisions: the medial, dorsal, lateral and ventral pallium (L. Puelles *et al*., submitted for publication). In mammals, the medial pallium, or archicortex, includes the hippocampal and subicular regions; the dorsal pallium corresponds to the neocortex; the lateral pallium, or paleocortex, includes the primary olfactory cortex; and the ventral pallium includes the claustrum. Each of these regions has further subdivisions. For instance, within the neocortex, there are the prefrontal, motor and sensory regions, each with their own areal subdivisions. The extensive expansion of neocortical surface area and complexity in higher mammals (Northcutt and Kaas, 1995), especially in primates (Rakic, 1995), is probably causally related to the increased sophistication of higher cognitive processing of these species.

What are the genetic and developmental processes that produce the diversity of cortical regions? Two schools of thought have made important contributions to this subject. The Protomap model (Rakic, 1988) postulates that neocortical regionalization is primarily controlled by molecular determinants that are intrinsic to the proliferative zone of the neocortex. These determinants control local regional identity and proliferative capacity (Dehay *et al.*, 1993; Rakic, 1995; Polleux *et al.*, 1997); radial migration of newborn neurons from the proliferative zone to the overlying differentiating cortex keeps the regional specification properties of the proliferative zone in register with the overlying cortical mantle (see Tan *et al*., 1998; Anderson *et al*., 1999; Walsh *et al*., 1999).

In contrast to the Protomap model, the Protocortex model (O'Leary, 1989; Schlagger and O'Leary, 1991) suggests that neocortical regionalization is controlled in large part by extrinsic influences, such as thalamocortical axons or by the targets of cortical axons. Heterotopic transplantation experiments in rats provide evidence that ectopic immature cortical tissue can develop efferent projections and histology that are characteristics of the local cortical tissue (Stanfield and O'Leary, 1985; O'Leary and Stanfield, 1989; Schlagger and O'Leary, 1991). For instance, transplantation of late embryonic visual cortex into neonatal somatosensory cortex, results in the ectopic visual cortex to develop histological characteristics of the rat somatosensory cortex (barrels and associated boundary domains) (Schlagger and O'Leary, 1991). These results support the concept that neocortical sensory subdivisions in young animals have a high degree of functional plasticity. Furthermore, manipulations that alter the anatomy (ablation of whisker pads or ventrobasal thalamus) or that alter the function of the thalamic inputs to the rodent somatosensory cortex (e.g. mutations or pharmacological disruptions of monoamine oxidase, adenylate cyclase, serotonin levels, NMDA receptor), disrupt the development of the barrels (Killackey *et al*., 1995; Cases *et al*., 1996; Welker *et al*, 1996; Iwasato *et al*., 1997; Abdel-Majid *et al*., 1998). In addition, thalamic inputs are implicated in regulating the expression of the *H-2Z1* transgene in the somatosensory cortex (Gitton *et al*., 1999a). These experiments show that anatomical and/or functional changes in axonal inputs to the neocortex can play a role in modifying existing, and generating new, neocortical subdivisions (Innocenti, 1995; Krubitzer, 1995).

While the axonal inputs may regulate region-specific features of the postnatal rodent brain, we are unaware of evidence that afferent fibers regulate prenatal cortical development. Furthermore, several experiments suggest that some aspects of early cortical regionalization do not require extrinsic influences. For instance, transplantation and explant culture methods have provided evidence that the expression of regional molecular markers of the neocortex (Cohen-Tannoudji *et al*., 1994; Nothias *et al*., 1998; Gitton *et al*., 1999b) and lateral limbic cortex (Barbe *et al*., 1991; Levitt *et al*., 1997; Arimatsu *et al*., 1999) do not depend upon extrinsic factors. In addition, several genes are regionally expressed in the cerebral cortex before thalamic input has penetrated the cortical plate (Arimatsu *et al*., 1992; Bulfone *et al*., 1995), as exemplified by the discontinuous expression of *Tbr-1* in the mouse superficial cortical plate at embryonic day 16.5 (E16.5) (Bulfone *et al*., 1995).

A definite answer regarding the role of extrinsic factors on cortical regionalization will likely require experimental methods to prevent the cortex from communicating with other brain regions. Such an approach has already been used (Wise and Jones, 1978); these authors performed a thalamotomy in newborn rats and found that a histological characteristic of layer IV (dense granule cell aggregates) in the somatosensory cortex was maintained. More recently, we have studied neocortical regionalization in *Gbx-2* mutant mice (Wasserman *et al*., 1998) that lack thalamocortical fibers (Miyashita-Lin *et al*., unpublished data). *Gbx-2* is a homeobox gene that is expressed in the developing thalamus and is required for its proper differentiation (Bulfone *et al*., 1993) (E.M. Miyashita-Lin *et al*., unpublished data). Because these mice die on the day of birth, probably due to brainstem abnormalities (Wasserman *et al*., 1998), we have had to identify a panel of molecular markers that are regionally expressed in the cortex of neonatal mice. Herein, we describe several genes that are expressed in distinct subdivisions of the

mouse cerebral cortex. In the Discussion, we address potential mechanisms intrinsic to the telencephalon that may regulate regionalization of the cerebral cortex. We end by addressing some genetic mechanisms that regulate the control of cortical axonal connectivity.

Materials and Methods

Cd-1 mice aged E16.5 (2), E18.5 (3), P0 (3), P6 (3) were utilized. For mounted sections, brains were prepared as previously described (Anderson *et al.*, 1997). For floating sections, after perfusion and postfixation for 4 h in 4% paraformaldehyde/0.1 M phosphate-buffered saline (PBS), 100 μm thick sections were cut on a vibratome. Sections were stored for up to 2 weeks in PBS. In-situ hybridization was performed as described (Bulfone *et al.*, 1993; Henrique *et al.*, 1995), using riboprobes labeled with S^{35} or digoxigenin. Id-2 cDNA was kindly provided by M.C. Hernandez and M. Israel, NP-2 from M.Tessier-Lavigne, RZR-beta from M. Levin and S. McConnell, Eph-A7 from A. Wanaka. Immunohistochemistry was performed as described (Anderson *et al.*, 1997) using rabbit anti-5-HT polyclonal antibody (1:5000, Incstar). Cytochrome oxidase staining was performed as previously described (Vincent, 1992).

Results

Intracortical Limits of Id-2 Expression Correspond to Areal Boundaries in the Developing Cerebral Cortex

Id-2 is a member of a family of genes that regulate cellular differentiation by the negative control of basic helix–loop–helix genes (see Kadesch, 1993, for review). It has been shown (Bulfone *et al.*, 1995) that in neonatal mice that *Id-2* and *Tbr-1* have complementary patterns of expression in neocortical layer V, with *Tbr-1* expressed more highly in rostral neocortex and Id-2 expressed more highly in caudal neocortex. To determine when the layer V boundary forms, we examined *Id-2* expression in mice aged from embryonic day 16.5 (E16.5) to postnatal day 6 (P6).

Figure 1*A–C* shows *Id-2* expression in sagittal sections at E16.5. At this age, when there is little histological evidence of regional differentiation within the neocortex, expression is limited to postmitotic cells of the subplate and cortical plate and appears fairly uniform along the rostro–caudal axis. By E18.5, expression is detectable in the subplate and layers VI and V. While expression in the subplate and layer VI appears fairly uniform, there is a distinct rostral boundary in layer V (arrows in Fig. 1*D–F*). Caudal to this boundary expression of *Id-2* extends through the visual cortex, while rostral to this point the expression diminishes abruptly. The boundary appears at a more rostral level in more lateral positions. At postnatal day 6, when neocortical neuronogenesis and migration are complete, the boundary in layer V remains distinct (arrows in Fig. 1*G–I*). After P6, the strength of *Id-2* expression decreases throughout the cerebral cortex (data not shown).

Examination of *Id-2* expression in coronal sections at P0 (Fig. 1*J–M*) and P6 (Fig. 1*N–Q*) revealed a number of interesting details. First, strong expression in neocortical layer V is present laterally and decreases abruptly at a boundary that is progressively more medial in more caudal regions (see arrows in Fig. 1*J–Q*). Based on several properties, this boundary appears to correspond to the transition between sensory and motor areas (see Fig. 2). Second, the layer of strongest *Id-2* expression changes from neocortical layer V to piriform cortex layer II (arrowhead in Fig. 1*K* and *O*). Interestingly, cells in these layers have similar birthdates (Bayer and Altman, 1995). Third, in caudal regions there is a clear reduction of *Id-2* expression in the entorhinal cortex (arrowhead in Fig. 1*Q*). Finally, expression of *Id-2* is increased in the superficial layers of the medial neocortex (cingulate and retrosplenial areas; Fig. 1*O* and *P*) which extend to different lateral positions along the rostro-caudal dimension of the cortex (asterisks in Fig. 1*J–L*).

To assess whether the boundary of *Id-2* expression in layer V corresponds to a known neocortical areal boundary, sections were examined in P6 animals for *Id-2* expression, histological appearance (Nissl stain), immunoreactivity for the neurotransmitter serotonin (5-HT), and cytochrome oxidase staining (CO) (Fig. 2*A–H*). The regions of dense 5-HT immunoreactivity and CO staining in layer IV of somatosensory and mixed motor/sensory regions have a rostral boundary at approximately the same position as the rostral boundary of *Id-2* expression in layer V (Wong-Riley and Welt, 1980; Fujimiya *et al.*, 1986; D'Amato *et al.*, 1987, Zilles and Wree, 1995). This relationship indicates that *Id-2* expression in layer V demarcates the transition from sensory to motor regions. In addition, expression of the T-box containing putative transcription factor *Tbr-1* is much stronger in layer V rostral to the same boundary (Fig. 2*C* and see also Bulfone *et al.*, 1995).

Close examination of *Id-2* expression in neocortical layer V at P6 suggests that expressing cells may be organized in column-like clusters (Fig. 2*I* and *J*) separated by non-expressing cells. This columnar appearance is present at E18.5 (data not shown), and may indicate that *Id-2* is expressed in a subset of functionally distinct pyramidal neurons that are organized into columns. These columns appear to be preserved in the absence of thalamic input (Miyashita-Lin and Rubenstein, unpublished data). We suggest that these columns of *Id-2* positive cells could prefigure types of columnar organization that are present in the occipital neocortex in higher mammals (e.g. ocular dominance columns).

Other gene markers of cortical regionalization

In addition to *Id-2* and *Tbr-1*, we have collected a set of genes whose expression distinguishes different regions and layers in the developing cerebral cortex. The expression patterns of most of these genes have previously been reported by other groups. We felt that it would be useful to show the P0 expression patterns of these genes as an ensemble. We chose P0, because many mouse mutants die on, or around, the day of birth. We have been using these markers to analyze the cortical phenotypes of mice with mutations in transcription factor genes that have developmental defects in cortical or thalamic development (e.g. *Emx-1*, *Tbr-1*, *Lef-1*, and *Gbx-2*) (Qiu *et al.*, 1996; Bulfone *et al.*, 1998) (E.M. Miyashita-Lin *et al.*, unpublished data; R. Hevner *et al.*, unpublished data). Here we show in-situ hybridization patterns that illustrate the regional expression of genes encoding cell surface and secreted proteins (cadherin-6, cadherin-8, Eph-A7, neuropilin-2, and Wnt-7b) and transcription factors (*Id-2*, *RZR-beta* and *Tbr-1*) (Fig. 3). The regional patterns of the cadherin expression were discovered by Suzuki (Suzuki *et al.*, 1997). The cortical expression of the Eph-A7 (MDK1) receptor tyrosine kinase was reported by Mori (Mori *et al.*, 1995). Neuropilin-2 (NP-2), the receptor for semaphorins E and IV, was shown to be highly expressed in the hippocampus and olfactory cortex (Chen *et al.*, 1997). Wnt-7b expression in the cortex was first described by Parr (Parr *et al.*, 1993). The RZR-beta orphan nuclear receptor was reported to be expressed in layer 4 of the neocortex (Schaeren-Wiemers *et al.*, 1997). Finally, *Id-2* and *Tbr-1* cortical expression is discussed above (Bulfone *et al.*, 1995). Figure 3 shows the expression patterns of these genes at P0. As described above for P6 mice (see Fig. 2), 5HT staining at P0 also defines the border between the somatosensory and

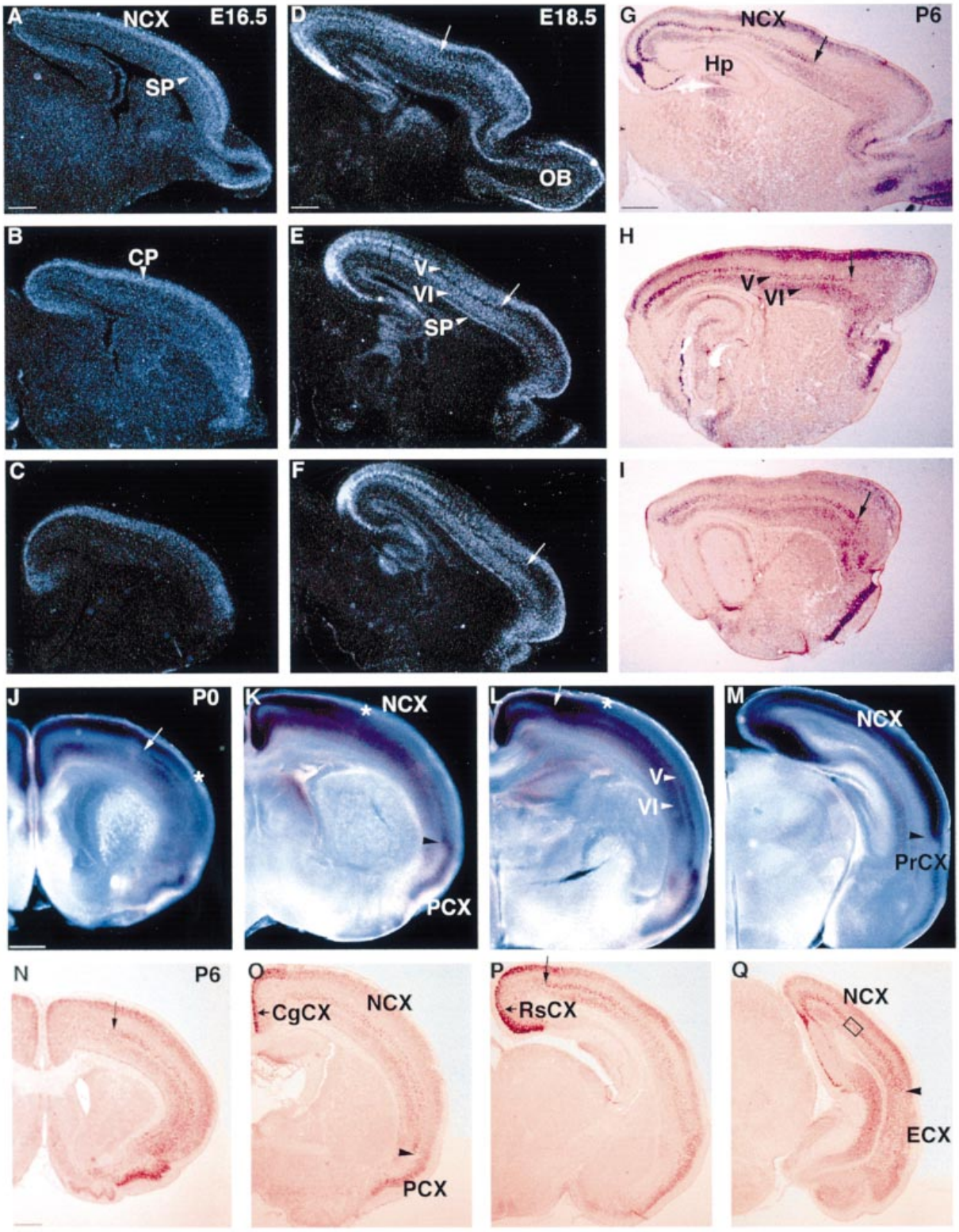

Figure 1. Expression of *Id-2* mRNA in the forebrain during late embryonic and early postnatal development. *A–I* Expression in sagittal sections from E16.5 (*A–C*), E18.5 (*D–F*) and P6 (*G–J*) mice. Arrows indicate the boundary of *Id-2* expression in neocortical layer V. This boundary appears to demarcate the transition from somatosensory to motor areas (see text and Fig. 2). *J–Q* Expression of *Id-2* in coronal hemisections from P0 (*J–M*) and P6 (*N–Q*) mice. Arrows indicate the medial boundary of expression in layer V. The arrowhead in panel K shows the transition of *Id-2* expression from layer V in the neocortex to layer II of the piriform cortex (PCX). The arrowheads in panels *M* and *Q* demarcate the abrupt decrease in *Id-2* expression at the transition of the caudal neocortex and the perirhinal cortex (PrCX; *M*) or the entorhinal cortex (ECX; *Q*). Note that there is also strong *Id-2* expression in layers II and III of the medial cortex, including the cingulate (CgCX; see *O*) and retrosplenial (RsCX, *P*) cortexes. The lateral extent of this *Id-2* expression in superficial neocortical layers is indicated by the asterisks in *J*, *K* and *L*. Scale bars, 500 μm. The boxed region in *Q* indicates the approximate region illustrated in panels *I* and *J* of Figure 2. Other abbreviations: CP, cortical plate; Hp, hippocampus; OB, olfactory bulb; NCX, neocortex; SP, subplate; *A–F*, dark-field photomicrographs of 12 μm sections showing *Id-2* signal from S-35 riboprobes. In *G–I* and *N–Q*, digoxigenin riboprobes were used. In *J–M*, digoxigenin riboprobes were used on floating 100 μm sections.

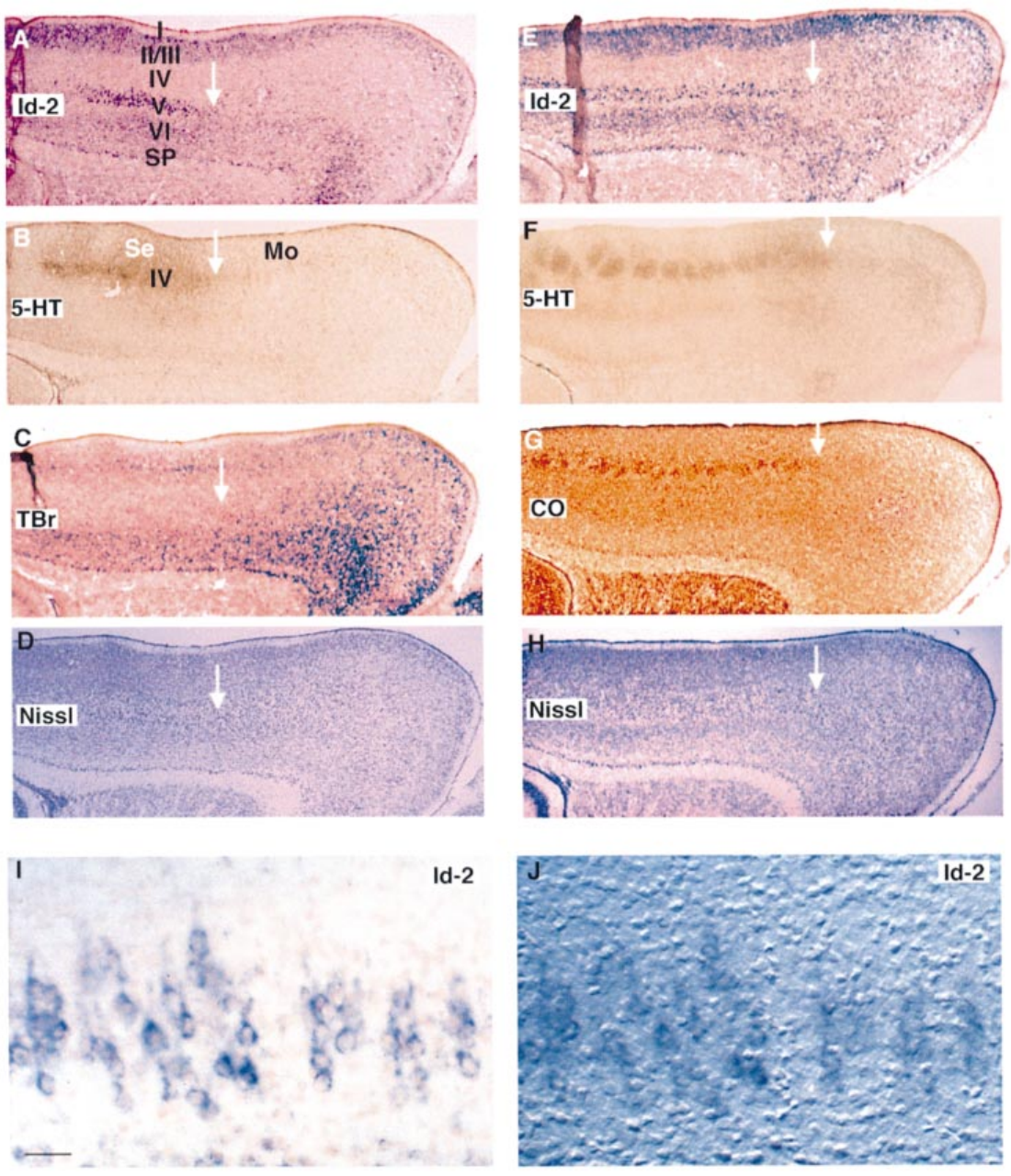

Figure 2. Expression of *Id-2* in layer V of the neocortex at P6 demarcates the sensory-motor boundary. *A–D* Adjacent parasagittal 12 μm sections; *E–H* adjacent lateral sagittal sections. *A* and *E Id-2* RNA expression using digoxigenin labeled riboprobe; *B* and *F* 5-HT immunoreactivity; *C Tbr-1* RNA expression using digoxigenin labeled riboprobe; *G* cytochrome oxidase (CO) expression; *D* and *H* Nissl stain. Arrows indicate that the rostral boundary of *Id-2* expression in layer V maps at the transition between sensory (including the mixed motor sensory hindlimb and forelimb areas) and motor regions, as defined by immunoreactivity for 5-HT in layer IV, CO activity in layer IV and histotypic characteristics seen in the Nissl stained sections. *Tbr-1* expression in layer V is more pronounced in the motor cortex rostral to the same boundary. *I* (brightfield optics) and *J* (same field as *I*, Nomarski optics) show *Id-2* expression in layer V of a coronal section through the primary visual cortex of a P6 animal. The approximate location of this panel is designated by the box in Figure 1*Q*. Note that the expression appears to occur in columns of cells. Se, sensory cortex; Mo, motor cortex; Sp. subplate. CO, cytochrome oxidase staining. I, II/III, IV, V, VI: neocortical layers. Scale bars. 500 μm in *A–H*, 50 μm in *I* and *J*.

motor regions (Fig. 3*L*) and matches the rostral border of Id-2 expression in layer 5 (Fig. 3*I*). Other than the somatosensory–motor boundary, a high resolution anatomical mapping of many of these genes remains to be described in part because at P0, it is difficult to make detailed assignments to functionally distinct cortical regions. Nonetheless, several of these genes have expression boundaries (or rapid changes in expression levels) within the neocortex (e.g. see arrows in Fig. 3*F*, *H*, *I* and *K*).

Discussion

Regionalization Mechanisms

Mounting evidence suggests that early cortical regionalization is

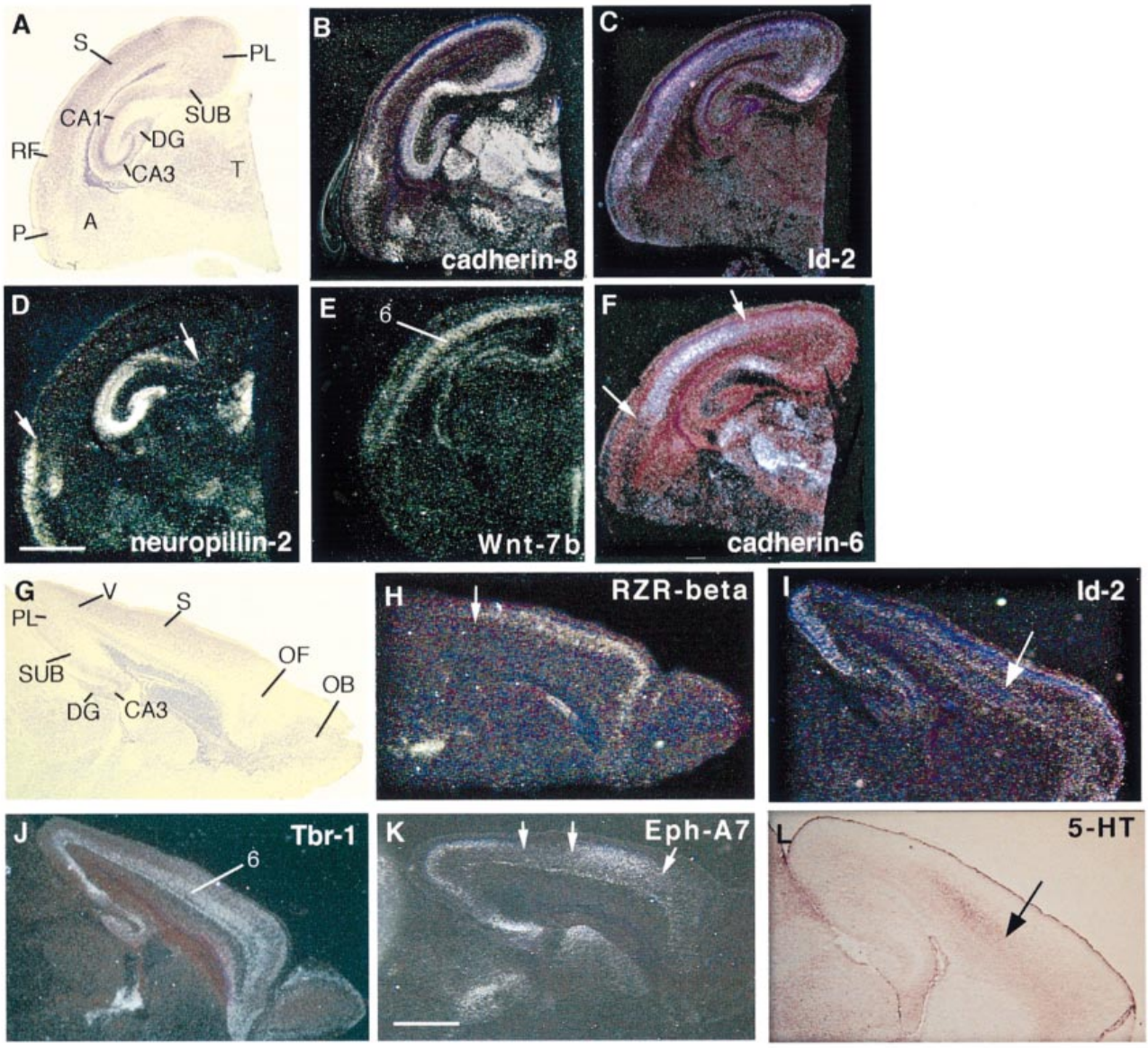

Figure 3. Regional expression of nine genes in the newborn mouse cortex as revealed by *in situ* RNA hybridization and immunohistochemistry. Coronal sections: *A* Nissl stain; *B* cadherin-8; *C* Id-2; *D* neuropillin-2; *E* Wnt-7b; *F* cadherin-6. Sagittal sections: *G* Nissl stain; *H* RZR-beta; *I* Id-2; *J* Tbr-1; *K* Eph-A7; *L* 5HT (serotonin). Note that Id-2 and 5HT expression analysis was performed in the same animal on sections 30 mm apart. The approximate location of various anatomical landmarks are indicated on the Nissl-stained pictures. Expression in layer 6 of the neocortex is indicated in panels *E* and *J*. The locations of intracortical boundaries of gene expression are indicated by arrows. Abbreviations: A: amygdalar region; CA1, CA3: pyramidal cell regions of the hippocampus; DG: dentate gyrus region of the hippocampus; OB: olfactory bulb; OF: orbitofrontal cortex; P: paleocortex (piriform or olfactory cortex); PL: posterior limbic cortex (probably including retrosplenial cortex); RF: rhinal fissure; S: somatosensory neocortex; SP: subplate; Sub: subiculum; T: thalamus; V: visual neocortex; 6: layer 6. Scale bars, 8 mm.

under the control of patterning mechanisms intrinsic to the telencephalon (Barbe and Levitt, 1991; Ferri and Levitt, 1993; Cohen-Tannoudji *et al.*, 1994; Nothias *et al.*, 1998; Gitton *et al.*, 1999; Miyashita-Lin *et al.*, 1999). The elimination of thalamic input to the neocortex in the *Gbx-2* mouse mutants is perhaps the clearest example demonstrating that early neocortical regionalization does not require extrinsic information from the thalamus (Miyashita-Lin *et al.*, 1999). This result does not suggest that thalamic input is without importance in the formation of neocortical subdivisions at later developmental stages. Testing the role of thalamic input in later neocortical development will require analysis at postnatal stages which presently is not possible in the *Gbx-2* mutants due to the lethality of this mutation. Thus, conditional mutants in *Gbx-2* could circumvent this technical problem. In addition, there may be other genes that are required for development of the entire thalamus, or subsets of thalamic nuclei, that may prove to be useful in studying the role of thalamic input on cortical development. Finally, non-genetic experimental approaches, such as thalamotomy, can also be applied to this subject (Wise and Jones, 1978).

The intrinsic mechanisms that pattern the cortex are just beginning to be elucidated. It is likely that there several patterning centers (tissues that produce morphogens) which

control regionalization of the telencephalon (Rubenstein and Beachy, 1998; Rubenstein *et al.*, 1998). Ventral specification of the telencephalon is regulated by sonic hedgehog (*Shh*). Early ventral specification is regulated via a signaling center in the axial mesendoderm and later ventral specification may be under the control of a *Shh*-expression domain in the basal telencephalon. Thus, *Shh* mutants, which lack the function of both patterning centers, also lack basal telencephalic structures (Chiang *et al.*, 1996). An extensive analysis of the rudimentary cerebral cortex that forms in these animals has not been performed. Recently, analysis of mutants in the *Nkx-2.1* homeobox gene (Sussel *et al.*, 1999) shows that telencephalic expression of *Shh* is almost eliminated; despite this, the histogenesis of the major cortical subdivisions appears normal. Thus, available evidence suggests that *Shh*-expression within the forebrain is not essential for cortical regionalization.

A second putative telencephalic patterning center appears to be at the rostrodorsal midline in the region of the septum. This tissue may regulate anteroposterior patterning and growth of the telencephalon. The telencephalic rostrodorsal midline is derived from the anterior neural ridge (ANR) (Rubenstein *et al.*, 1998) which expresses *FGF8* (Crossley and Martin, 1995; Shimamura and Rubenstein, 1997). The ANR may be essential for development of the telencephalon through the regulation of the *BF-1* transcription factor (Shimamura and Rubenstein, 1997; Ye *et al.*, 1998). Ongoing studies are investigating the role of *FGF8* in the ANR and rostral midline of the forebrain in patterning the telencephalon (P.H. Crossley *et al.*, unpublished results).

A third putative patterning center is at the dorsal midline of the telencephalon. This tissue gives rise to roof plate-related structures where dorsal commissures cross, and where the choroid plexus and fimbria form. These tissues express genes that are implicated in dorsal patterning in the spinal cord, such as BMPs, and Wnts (Parr *et al.*, 1993; Furuta *et al.*, 1997; Tole *et al.*, 1997; Grove *et al.*, 1998; Lee *et al.*, 1998; Grove and Tole, 1999). Ectopic application of BMPs have been shown to dorsalize the chick telencephalon (Golden *et al.*, 1999) (Ohkubo and J.L.R. Rubenstein, unpublished data). The roles of Wnt proteins are suggested by hippocampal defects found in mice lacking the Lef-1 transcription factor, a protein that is implicated in Wnt signal transduction (J. Galceran *et al.*, unpublished data). The potential roles of the dorsal patterning center in regulating development of medial pallial tissues is discussed in greater detail by Grove and Tole (Grove and Tole, 1999).

There is evidence for other patterning centers flanking and within the telencephalon that control regionalization of the cerebral cortex. One includes the olfactory placode derived tissues and its associated mesenchyme. These tissues are implicated in regulating olfactory bulb growth through retinoid-mediated mechanisms (Anchan *et al.*, 1997). Additional tissues that might regulate regional specification of adjacent telencephalic structures may include the cortical/subcortical boundary region where there is an abrupt transition in gene expression patterns (Bulfone *et al.*, 1995) (L. Puelles *et al.*, unpublished data) and the caudal region of the telencephalic vesicle at its transition with the dorsal diencephalon.

Transduction of the signals from the patterning centers is mediated via transcription factors. The patterning signals regulate (induce or repress) the expression of transcription factors that then control regional identity of the neuroepithelium (e.g. *SHH* induces *Nkx-2.1*). Ventral patterning is mediated by transcription factors that are induced by *Shh*, such as Gli-2 (Matise *et al.*, 1998) and *Nkx-2.1* and *Nkx-2.2* (Briscoe *et al.*, 1999; Sussel *et al.*, 1999). For instance, loss of *Nkx-2.1* leads to a dorsalization of the pallidal parts of the basal ganglia (Sussel *et al.*, 1999).

Dorsal patterning is mediated by the *Gli-3*, *Emx-1*, *Emx-2* and *Lef-1* transcription factors; mutations of these genes result in loss of dorsal telencephalic structures such as parts of the hippocampus, corpus callosum and choroid plexus (Pellegrini *et al.*, 1996; Qiu *et al.*, 1996; Yoshida *et al.*, 1997; Grove *et al.*, 1998; Grove and Tole, 1999). Furthermore, BMPs can repress *BF-1*; this repression may explain why *BF-1* expression is excluded from dorsomedial regions of the telencephalon (Furuta *et al.*, 1997). In addition, transcription factors can regulate the expression of the secreted patterning molecules. For instance, loss of *BF-1* expression leads to increased expression of *Bmp-4* throughout the telencephalic vesicle (see Dou *et al.*, 1999).

Finally, anteroposterior patterning appears to be transduced in part via *Otx-1* and *Otx-2*. These homeobox genes are expressed in the midbrain and forebrain. As discussed (Acampora and Simeone, 1999), there is posteriorization of the forebrain in mice that are *Otx-1* –/– and *Otx-2* +/– (Acampora *et al.*, 1997). This is associated with ectopic expression of *Wnt-1* and *En-2* (markers of the midbrain and anterior hindbrain) in the dorsal telencephalon. It appears that reducing the dosage of OTX proteins leads to an anterior shift in the isthmic patterning center (the isthmic organizer regulates regionalization of the midbrain and anterior hindbrain). Furthermore, there is evidence that *FGF8*, in the isthmic organizer, functions in part through repression of *Otx* expression (Martinez *et al.*, 1999). As noted above, we are investigating whether *FGF8* expression in the anterior neural ridge (and commissural plate) may regulate forebrain patterning through modulation of the *Otx* genes.

Thus, we suggest that the patterning centers generate regional differences in the developmental potential of telencephalic ventricular zone cells. This regional specification process generates the primordia of the principal telencephalic anlage which include its dorsal (cortical) subdivisions. Radial migration of post-mitotic projection neurons transfers the ventricular zone regionalization map to the cortical mantle zone.

Regionalization and Cortical Connections

Different regions of cortex make characteristic sets of anatomical connections with other cortical and subcortical areas. These connections are area-specific and must be controlled in part by genetically encoded positional information. Herein, we describe the effects of specific mutations on the formation of distinct cortical connections.

Cortical–thalamic connections

Axonal connections with the thalamus are among the most numerous of the cerebral cortex. To date, three strains of mice with defined single gene mutations have been identified, in which the cortex and thalamus fail to connect with each other. Each of these mutations affects a different transcription factor gene implicated in forebrain patterning. Interestingly, each gene is expressed in distinct regions. *Tbr-1* is expressed in the developing cortex (including subplate and layer 6 neurons, which normally project to the thalamus), but not in the thalamus (Bulfone *et al.*, 1995). *Gbx-2* is expressed in the dorsal thalamus, but not in the cortex (Bulfone *et al.*, 1993). In *Tbr-1* and *Gbx-2* homozygous mutants, connections between thalamus and cortex both fail to develop, indicating that abnormal gene expression in either region leads to failure of the connection in

both directions (R. Hevner *et al.*, unpublished data). Furthermore, axon outgrowth stops in the internal capsule, suggesting that thalamocortical and corticothalamic axons interact and help guide each other beyond the internal capsule. The reciprocal requirement of both the thalamocortical and the corticothalamic fibers is consistent with the 'handshake' hypothesis (Molnar and Blakemore, 1995). Mutations in *Pax-6*, which is expressed widely in thalamus, cortex, and other regions, also result in loss of connections between cortex and thalamus (Kawano *et al.*, 1999) (R. Hevner *et al.*, unpublished data), probably due to abnormalities in multiple forebrain regions.

Besides transcription factors, another major class of molecules implicated in the development of thalamocortical connections are secreted and cell surface axon guidance proteins. As yet, no knockout mutants in this class have been identified with defects in thalamic–cortical connectivity, but redundant mechanisms may mask the effects of single gene inactivation. The diffusible chemoattractant netrin-1, released from the ganglionic eminences, probably has a role in guiding cortical axons into the internal capsule (Métin *et al.*, 1997). The Eph-A5 receptor protein tyrosine kinase may mediate exclusion of limbic thalamic axons from non-limbic sensorimotor cortex, which, unlike limbic cortical regions, expresses the inhibitory ligand molecule ephrin-A5 (Gao *et al.*, 1998). The Ig superfamily protein LAMP, which acts as a homophilic cell adhesion molecule, may facilitate the formation of limbic thalamocortical connections (Barbe and Levitt, 1992). Perhaps these axon guidance molecules are regulated by some of the same transcription factors involved in forebrain patterning.

Cortical Projections to the Brainstem and Spinal Cord

The cerebral peduncle is the major cortical efferent pathway that carries fibers to the brainstem and spinal cord. Our preliminary studies indicate that targeted inactivation of the linked homeobox genes *Dlx-1* and *Dlx-2* results in misrouting of many cerebral peduncle axons into the thalamus and pretectal regions (R. Hevner *et al.*, unpublished data). The genes that control pathfinding of corticothalamic and cerebral peduncle pathways may be largely independent, since the connections between cortex and thalamus are normal in the *Dlx-1/Dlx-2* knockout, and cerebral peduncle fibers develop normally (at least as far as the midbrain) in the *Tbr-1* and *Gbx-2* knockouts. Mice mutant for the widely expressed *Pax-6* gene demonstrate abnormalities of both pathways.

Cortical Commissural Projections

The corpus callosum, anterior commissure and hippocampal commissure are major pathways that connect cortical areas on opposite sides of the brain. Various mouse mutants affect one or more of these pathways. Agenesis of the corpus callosum can be caused by inactivation of single genes such as the *Emx-1* homeobox gene (Qiu *et al.*, 1996), but also occurs with relatively high frequency (50–70%) in certain mouse strains such as 129/J and BALB/cWah1 (Livy and Wahlsten, 1997), and in association with environmental insults such as fetal alcohol syndrome (Johnson *et al.*, 1996). Callosal agenesis is frequently associated with formation of a longitudinal Probst bundle, containing axons that were presumably meant to cross through the absent corpus callosum. In most strains with callosal agenesis, some cortical axons cross the midline through the hippocampal commissure (Ozaki and Wahlsten, 1993; Orioli *et al.*, 1996; Qiu *et al.*, 1996; R. Hevner *et al.*, unpublished data). Likewise, adventitious fiber crossing probably occurs in humans with callosal agenesis, who sometimes exhibit an enlarged hippocampal commissure (Barkovich, 1990). The anterior commissure may be affected independently of the corpus callosum, as observed in mice with a mutation of the receptor tyrosine kinase Eph-B2 (Orioli *et al.*, 1996). In *Tbr-1* mutants, both fiber bundles fail to develop. All three major bundles (corpus callosum, anterior commissure, and hippocampal commissure) are affected in mice with disruption of the homeobox gene *Hesx1* (Dattani *et al.*, 1998), or of netrin-1 (Serafini *et al.*, 1996). The targeting of callosal and commissural connections to specific cortical regions may be regulated by proteins such as LAMP (Barbe and Levitt, 1995).

Other Cortical Connections

Primary olfactory neurons, that express the same olfactory receptor gene, make precise topographic projections onto two glomeruli on the surface of the olfactory bulb (Mombaerts *et al.*, 1996). The precision of these projections appears to depend upon the type of olfactory receptor protein expressed on the axons (Wang *et al.*, 1998), but not on neuronal activity (Belluscio *et al.*, 1998) or on the presence of their principal targets (mitral cells and periglomerular cells (Bulfone *et al.*, 1998). It is postulated that the topographic map of the olfactory bulb may be encoded by radial glia (Bulfone *et al.*, 1998).

Little information is available regarding genes that govern the formation of less prominent cortical connections, with structures such as the striatum, ipsilateral cortex (i.e. associational connections), and short collaterals. No mutations affecting these connections have yet been reported. However, it has been found (Castellani *et al.*, 1998) that short axon collaterals may be guided to their appropriate laminar termination by attractive and/or repulsive interactions mediated by Eph-A5 and ephrin-A5. Further progress in this field will depend upon the identification of additional genes controlling forebrain patterning and axon guidance; generation of single and compound knockout mice; and detailed anatomical analysis.

Notes

The authors thank Phil Crossley for advice on conducting *in situ* hybridization on floating sections, and the following individuals for providing cDNA clones: Marc Israel for Id-2; Marc Tessier-Lavigne for NP-2; Andy McMahon for Wnt-7b; Masatoshi Takeichi for Cad-6 and Cad-8; Sue McConnell for RZR-beta; and Akio Wanaka for Eph-A7. This work was supported by the research grants to S.A. from NARSAD and NIMH K08-MH01620-1, and to J.L.R.R. from Nina Ireland, NARSAD, NINDS Grant NS34661-01A1 and NIMH K02 MH01046-01.

Address correspondence to John L.R. Rubenstein, 401 Parnassus Avenue, UCSF, San Francisco, CA, 94143-0984, USA. Email: jlrr@cgl.ucsf.edu.

References

Abdel-Majid RM, Leong WL, Schalkwyk LC, Smallman DS, Wong ST, Storm DR, Fine A, Dobson MJ, Guernsey DL, Neumann PE (1998) Loss of adenylyl cyclase I activity disrupts patterning of mouse somatosensory cortex. Nature Genet 19:289–291.

Acampora D, Barone P, Simeone A (1999) *Otx* genes in corticogenesis and brain development. Cereb Cortex 9:533–542.

Acampora D, Avantaggiato V, Tuorto F, Simeone A (1997) Genetic control of brain morphogenesis through *Otx* gene dosage requirement. Development 124:3639–3650.

Anchan RM, Drake DP, Haines CF, Gerwe EA, LaMantia AS (1997) Disruption of local retinoid-mediated gene expression accompanies abnormal development in the mammalian olfactory pathway. J Comp Neurol 379:171–184.

Anderson S, Qiu M, Bulfone A, Eisenstat D, Meneses JJ, Pedersen RA, Rubenstein JLR (1997) Mutations of the homeobox genes Dlx-1 and Dlx-2 disrupt the striatal subventricular zone and differentiation of late-born striatal cells. Neuron 19:27–37.

Arimatsu Y, Miyamoto M, Nihonmatsu I, Hirata K, Uratani Y, Hatanaka Y, Takiguchi-Hayashi K (1992) Early regional specification for a molecular neuronal phenotype in the rat neocortex. Proc Natl Acad Sci USA 89:8879–8883.

Arimatsu Y, Ishida M, Takiguchi-Hatashi K, Uratani Y (1999) Cerebral cortical specification by early potential restriction of progenitor cells and later phenotype control of postmitotic neurons. Development 126:629–638.

Barbe MF, Levitt P (1991) The early commitment of fetal neurons to the limbic cortex. J Neurosci 11:519–533.

Barbe MF, Levitt P (1992) Attraction of specific thalamic input by cerebral grafts depends on the molecular identity of the implant. Proc Natl Acad Sci USA 89:3706–3710.

Barbe MF, Levitt P (1995) Age-dependent specification of the cortico-cortical connections of cerebral grafts. J Neurosci 15:1819–1834.

Barkovich AJ (1990) Apparent atypical callosal dysgenesis: analysis of MR findings in six cases and their relationship to holoprosencephaly. Am J Neuroradiol 11:333–339.

Bayer SA, Altman J (1995) Neurogenesis and neuronal migration. In: The rat nervous system (Paxinos G, ed), pp 1041–1078. New York: Academic Press.

Belluscio L, Gold GH, Nemes A, Axel R (1998) Mice deficient in G(olf) are anosmic. Neuron 20:69–81.

Briscoe J, Sussel L, Serup P, Hartigan-O'Connor D, Jessell T, Rubenstein JLR, Ericson J (1999) The Nkx2.2 Homeobox gene mediates graded sonic hedgehog signaling and controls ventral neuronal subtype identity. Nature (in press).

Bulfone A, Puelles L, Porteus MH, Frohman MA, Martin GR, Rubenstein JLR (1993) Spatially restricted expression of *Dlx-1*, *Dlx-2 (Tes-1)*, *Gbx-2*, and *Wnt-3* in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. J Neurosci 13:3155–3172.

Bulfone A, Smiga SM, Shimamura K, Peterson A, Puelles L, Rubenstein JLR (1995) *T-Brain-1:* a homolog of *Brachyury* whose expression defines molecularly distinct domains within the cerebral cortex. Neuron 15:63–78.

Bulfone A, Wang F, Hevner R, Anderson S, Cutforth T, Chen S, Meneses J, Pedersen R, Axel R, Rubenstein JLR (1998) An olfactory sensory map develops in the absence of normal projection neurons or gabaergic interneurons. Neuron 21:1273–1282.

Cases O, Vitalis T, Seif I, De Maeyer E, Sotelo C, Gaspar P (1996) Lack of barrels in the somatosensory cortex of monoamine oxidase-deficient mice: role of a serotonin excess during the critical period. Neuron 16:297–307.

Castellani V, Yue Y, Gao PP, Zhou R, Bolz J (1998) Dual action of a ligand for Eph receptor tyrosine kinases on specific populations of axons during the development of cortical circuits. J Neurosci 18:4663–4672.

Chen H, Chedotal A, He Z, Goodman CS, Tessier-Lavigne M (1997) Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron 19:547–559.

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA (1996) Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383:407–413.

Cohen-Tannoudji M, Babinet C, Wassef M (1994) Early determination of a mouse somatosensory cortex marker. Nature 31:460–463.

Crossley PH, Martin GR (1995) The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. Development 121:439–451.

D'Amato RJ, Blue ME, Largent BL, Lynch DR, Ledbetter DJ, Molliver ME, Snyder SH (1987) Ontogeny of the serotonergic projection to rat neocortex: transient expression of a dense innervation to primary sensory areas. Proc Natl Acad Sci 12:4322–4326.

Dattani MT, Martinez-Barbera JP, Thomas PQ, Brickman JM, Gupta R, Martensson IL, Toresson H, Fox M, Wales JKH, Hindmarsh PC, Krauss S, Beddington RSP, Robinson ICAF (1998) Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. Nature Genet 19:125–133.

Dehay C, Giroud P, Berland M, Smart I, Kennedy H (1993) Modulation of the cell cycle contributes to the parcellation of the primate visual cortex. Nature 366:464–466.

Dou CL, Li S, Lai E (1999) Dual role of brain factor-1 in regulating growth and patterning of the cerebral hemispheres. Cereb Cortex 9:543–550.

Ferri RT, Levitt P (1993) Cerebral cortical progenitors are fated to produce region-specific neuronal populations. Cereb Cortex 3:187–198.

Fujimiya M, Kimura H, Maeda T (1986) Postnatal development of serotonin nerve fibers in the somatosensory cortex of mice studied by immunohistochemistry. J Comp Neurol 2:191–201.

Furuta Y, Piston DW, Hogan BL. (1997) Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. Development 124:2203–2212.

Gao PP, Yue Y, Zhang JH, Cerretti DP, Levitt P, Zhou R (1998) Regulation of thalamic neurite outgrowth by the Eph ligand ephrin-A5: implications in the development of thalamocortical projections. Proc Natl Acad Sci USA 95:5329–5334.

Gitton Y, Cohen-Tannoudji M, Wassef M (1999a) Role of thalamic axons in the expression of H-2ZI, a mouse somatosensory cortex specific marker. Cereb Cortex 9:611–620.

Gitton Y, Cohen-Tannoudji M, Wassef M (1999b) Specification of somatosensory area identity in cortical explants. J Neurosci (in press).

Golden JA, Bracilovic A, McFadden KA, Beesely JS, Rubenstein JLR, Grinspan JB, Ectopic BMP5 and BMP4 in the chick forebrain leads to cyclopia and holoprosencephaly. Proc Natl Acad Sci USA (in press).

Grove E, Tole S (1999) Patterning events and specification signals in the developing hippocampus. Cereb Cortex 9:551–561.

Grove EA, Tole S, Limon J, Yip L, Ragsdale CW (1998) The hem of the embryonic cerebral cortex is defined by the expression of multiple Wnt genes and is compromised in Gli3-deficient mice. Development 125:2315–2325.

Henrique D, Adam J, Myat A, Chitnis A, Lewis J, Ish-Horowicz D (1995) Expression of a Delta homologue in prospective neurons in the chick. Nature 6534:787–790.

Innocenti G (1995) Exuberant development of connections, and its possible permissive role in cortical evolution. Trends Neurosci 18: 397–402.

Iwasato T, Erzurumlu RS, Huerta PT, Chen DF, Sasaoka T, Ulupinar E, Tonegawa S (1997) NMDA receptor-dependent refinement of somatotopic maps. Neuron 19:1201–1210.

Johnson VP, Swayze VW II, Sato Y, Andreasen NC (1996) Fetal alcohol syndrome: craniofacial and central nervous system manifestations. Am J Med Genet 61:329–339.

Kadesch T (1993) Consequences of heteromeric interactions among helix–loop–helix proteins. Cell Growth Diff 4:49–55.

Kawano H, Fukuda T, Kubo K, Horie M, Takeuchi K, Uyemura K, Osumi N, Eto K, Kawamura K (1999) Pax-6 is required for the thalamocortical pathway formation in fetal rats. J Comp Neurol (in press).

Killackey HP, Rhoades RW, Bennett-Clarke CA (1995) The formation of a somatotopic map. Trends Neurosci 18:402–407.

Krubitzer L (1995) The organization of neocortex in mammals: are species differences really so different? Trends Neurosci 18:408–417.

Lee KJ, Mendelsohn M, Jessell TM. (1998) Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. Genes Dev 12:3394–3407.

Levitt P, Barbe MF, Eagleson KL (1997) Patterning and specification of the cerebral cortex. Annu Rev Neurosci 20:1–24.

Livy DJ, Wahlsten D (1997) Retarded formation of the hippocampal commissure in embryos from mouse strains lacking a corpus callosum. Hippocampus 7:2–14.

Martinez S, Crossley PH, Cobos I, Rubenstein JLR, Martin GR (1999) FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on *Otx2* expression. Development (in press).

Matise MP, Epstein DJ, Park HL, Platt KA, Joyner AL (1998) Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 125:2759–2770.

Métin C, Deléglise D, Serafini T, Kennedy TE, Tessier-Lavigne M (1997) A role for netrin-1 in the guidance of cortical efferents. Development 124:5063–5074.

Miyashita-Lin EM, Hevner R, Montzka Wassarman K, Martinez S, Rubenstein JLR (1999) Early neocortical regionalization is preserved in the absence of thalamic innervation. Science (in press).

Molnar Z, Blakemore C (1995) How do thalamic axons find their way to the cortex? Trends Neurosci 18:389–397.

Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M,

Edmondson J, Axel R (1996) Visualizing an olfactory sensory map. Cell 87:675–686.
Mori T, Wanaka A, Taguchi A, Kazumasa M, Tohyama M (1995) Localization of novel receptor tyrosine kinase genes of the eph family MDK1 and its splicing variant, in the developing mouse nervous system. Mol Brain Res 34:154–160.
Northcutt RG, Kaas JH (1995) The emergence and evolution of mammalian neocortex. Trends Neurosci 18:373–379.
Nothias F, Fishell G, Ruiz i Altaba A (1998) Cooperation of intrinsic and extrinsic signals in the elaboration of regional identity in the posterior cerebral cortex. Curr Biol 8:459–462.
O'Leary DDM (1989) Do cortical areas emerge from a protocortex? Trends Neurosci 12:400–406.
O'Leary DDM, Stanfield BB (1989) Selective elimination of axons extended by developing cortical neurons is dependent on regional locale: experiments utilizing fetal cortical transplants. J Neurosci 9:2230–2246.
Orioli D, Henkemeyer M, Lemke G, Klein R, Pawson T (1996) Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. EMBO J 15:6035–6049.
Ozaki HS, Wahlsten D (1993) Cortical axon trajectories and growth cone morphologies in fetuses of acallosal mouse strain. J Comp Neurol 336:595–604.
Parr BA, Shea MJ, Vassileva G, McMahon AP (1993) Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. Development 119:247–261.
Pellegrini M, Mansouri A, Simeone A, Boncinelli E, Gruss P (1996) Dentate gyrus formation requires Emx2. Development 122: 3893–3898.
Polleux F, Dehay C, Moraillon B, Kennedy H (1997) Regulation of neuroblast cell-cycle kinetics plays a crucial role in the generation of unique features of neocortical areas. J Neurosci 17:7763–7783.
Qiu M, Anderson S, Chen S, Meneses JJ, Hevner R, Kuwana E, Pedersen RA, Rubenstein JLR (1996) Mutation of the Emx-1 homeobox gene disrupts the corpus callosum. Dev Biol 178:174–178.
Rakic P (1988) Specification of cerebral cortical areas. Science 241: 170–176.
Rakic P (1995) A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. Trends Neurosci 18:383–388.
Rubenstein JLR, Beachy PA (1998) Patterning of the embryonic forebrain. Curr Opin Neurobiol 8:18–26.
Rubenstein JLR, Shimamura K, Martinez S, Puelles L (1998) Regionalization of the prosencephalic neural plate. Annu Rev Neurosci 21:445–478.
Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87: 1001–1014.
Schaeren-Wiemers N, Andre E, Kapfhammer JP, Becker-Andre M (1997) The expression pattern of the orphan nuclear receptor RORbeta in the developing and adult rat nervous system suggests a role in the processing of sensory information and in circadian rhythm. Eur J Neurosci 9:2687–2701.
Schlagger BL, O'Leary DDM (1991) Potential of visual cortex to develop an array of functional units unique to somatosensory cortex. Science 252:1556–1560.
Shimamura K, Rubenstein JLR (1997) Inductive interactions direct early regionalization of the mouse forebrain. Development 124:2709–2718.
Stanfield BB, O'Leary DDM (1985) Fetal occipital cortical neurons transplanted to the rostral cortex can extend and maintain a pyramidal tract axon. Nature 313:135–137.
Sussel L, Kimura S, Rubenstein JLR (1999) Loss of *Nkx2.1* homeobox gene function results in a ventral to dorsal respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. Development 126:3359–3370.
Suzuki SC, Inoue T, Kimura Y, Tanaka T, Takeichi M (1997) Neuronal circuits are subdivided by differential expression of type-II classic cadherins in postnatal mouse brains. Mol Cell Neurosci 9:433–447.
Tan SS, Kalloniatis M, Sturm K, Tam PP, Reese BE, Faulkner-Jones B(1998) Separate progenitors for radial and tangential cell dispersion during development of the cerebral neocortex. Neuron 21:295–304.
Tole S, Christian C, Grove EA (1997) Early specification and autonomous development of cortical fields in the mouse hippocampus. Development 124:4959–4970.
Vincent SR (1992) Histochemistry of endogenous enzymes. In: Experimental Neuroanatomy (Bolam JP, ed.), pp. 168–169. Oxford: Oxford University Press.
Wang F, Nemes A, Mendelsohn M, Axel R (1998) Odorant receptors govern the formation of a precise topographic map. Cell 93:47–60.
Wasserman KM, Lewandowski M, Campbell K, Joyner AL, Rubenstein JLR, Martinez S, Martin GR (1997) Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on Gbx2 gene function. Development 124:2923–2934.
Welker E, Armstrong-James M, Bronchti G, Ourednik W, Gheorghita-Baechler F, Dubois R, Guernsey DL, Van der Loos H, Neumann PE (1996) Altered sensory processing in the somatosensory cortex of the mouse mutant barrelless. Science 271:1864–1867.
Wise SP, Jones EG (1978) Developmental studies of thalamocortical and commissural connections in the rat somatic sensory cortex. J Comp Neurol 178:187–208.
Wong-Riley MTT, Welt C (1980) Histochemical changes in cytochrome oxidase of cortical barrels after vibrissal removal in neonatal and adult mice. Proc Natl Acad Sci USA 77:2333–2337.
Ye W, Shimamura K, Rubenstein JLR, Hynes M, Rosenthal A (1998) Intersections of the FGF8 and Shh signals create inductive centers for dopaminergic and serotonin neurons in the anterior neural plate. Cell 93:755–766.
Yoshida M, Suda Y, Matsuo I, Miyamoto N, Takeda N, Kuratani S, Aizawa S (1997) Emx1 and Emx2 functions in development of dorsal telencephalon. Development 124:101–111.
Zilles K, Wree A (1995) Cortex: areal and laminar structure. In: The rat nervous system (Paxinos G, ed.), pp. 649–678. New York: Academic Press.