Novel genes differentially expressed in cortical regions during late neurogenesis

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Abstract
Differential gene expression across the embryonic cerebral cortex is assumed to play a role in the subdivision of the cortex into distinct areas with specific morphology, physiology and function. In search for genes that may be involved in the cortical regionalization during late neurogenesis in mouse, we performed an extensive in-situ expression analysis at embryonic day (E)16 and E18. The examined candidate genes were selected beforehand by a microarray screen by virtue of their preferential expression in the anlagen of the motor, somatosensory, visual and cingulate cortices or hippocampus. We present new information about graded or regionally enriched expression of 25 genes (nine of which are novel genes) across the mouse embryonic cortex, in progenitor cells as well as in the cortical plate. The established differential expression of most of these genes is persistent at both stages studied, suggesting that their expression is regulated by an intrinsic programme. For some of the genes, the concept of intrinsic regulation is further substantiated by the high similarity of the reported expression patterns at E16 and E18 and published data from earlier stages. Few genes with robust expression in the E16 caudal cortex showed a more restricted pattern at E18, possibly because of their response to extrinsic cues. In addition, several genes appeared to be suitable novel markers for amygdalar and diencephalic nuclei. Taken together, our findings reveal novel molecular partitions of the late mouse cortex that are in accordance with the model of a leading role of intrinsic mechanisms in cortical arealization.

Introduction
In the adult cortex billions of neurons form a complex network that is subdivided in radially organized layers and tangentially arrayed functional areas. These cortical areas feature precise connectivity patterns and process distinct aspects of sensation, movement and cognition. Although it is generally accepted that the cortical layer identity is acquired during the last mitotic cycle of the progenitors in the germinative neuroepithelium, the ventricular zone (VZ) and subventricular zone (SVZ) (McConnell, 1988; McConnell & Kaznowski, 1991), little is known about the mechanisms of the cortical arealization process. According to the protomap model, molecular cues intrinsic to cortical germinal zones have a decisive role in the cortical arealization process (Rakic, 1988). Indeed, isolated cortical explants from early embryos are committed to express molecular markers specific to their region of origin when analysed in vitro or after heterotopical transplantations (Arimatsu et al., 1992; Ferri & Levitt, 1993; Cohen-Tanoudji et al., 1994; Tole et al., 1997; Gitton et al., 1999; Tole & Grove, 2001). Moreover, the analysis of Gbx2/−/− and Mash1/−/− mouse mutants, in which the thalamocortical projections (TCA) are distorted (Garel et al., 2002) or absent (Miyashita-Lin et al., 1999; Nakagawa et al., 1999), revealed that early regionalization of the cortex does not require afferent inputs but instead is controlled by intrinsic mechanisms. In contrast, the protocortex (or tabula rasa) model states that the cortical primordium is lacking any areal bias and requires information brought by ingrowing subcortical, mainly thalamocortical, projections (O’Leary, 1989). The discovered prolonged plasticity of the area identity in heterotopically transplanted explants supports this idea (Schlaggar & O’Leary, 1991). Accumulating evidence indicates, however, that both intrinsic and extrinsic mechanisms are responsible for cortical arealization (reviewed by O’Leary & Nakagawa, 2002; Sur & Rubenstein, 2005; Mallamaci & Stoykova, 2006). Furthermore, laminar and areal specification appear to be inter-related processes involving the control of cell cycle parameters (Caviness et al., 1995, 2003; Dehay et al., 1993; Polleux et al., 1997; Lukaszewicz et al., 2005) and signals from the VZ, cortical plate (CP) and mature cortex that progressively specify the final connectional phenotype of the cortical areas (Dehay et al., 2001; Polleux et al., 2001).

Cortical arealization starts with the patterning of the early cortical primordium by the regionalized expression of ligands belonging to the fibroblast growth factor (FGF), bone morphogenetic protein/wingless integrated and epidermal growth factor signalling pathways produced by forebrain signalling centres (Ferri & Levitt, 1995; Grove et al., 1998; Maruoka et al., 1998; Galceran et al., 2000; Bachler & Neubuser, 2001; Herbert et al., 2002; Assimacopoulos et al., 2003; Gimeno et al., 2003). In addition, Sonic Hedgehog from the prechordal mesendoderm contributes to the ventral patterning of the cortical primordium (Crossley et al., 2001; Shimamura et al., 1995; Shimamura & Rubenstein, 1997). Remarkably, altering or abolishing paracrine gradients such as the FGF8 gradient leads to predictable shifts in the size and location of the cortical areas (Fukuchi-Shimogori & Grove, 2001, 2003; Storm et al., 2003; Shimogori et al., 2004).
Secreted molecules from forebrain signalling centres are assumed to regulate the graded expression of transcription factors in a dose-dependent manner. The latter encode the positional information specific for distinct cortical fields. Up to now, only a few factors with gradual expression in cortical progenitors along the mediolateral (ML) axis [Lhx2 (Porter et al., 1997) and Foxg1 (Hanashima et al., 2004)] and along the anteroposterior (AP) axis [Pax6 (Walther & Gruss, 1991), COUP-TFI (Zhou et al., 2001) and Emx2 (Simeone et al., 1992; Mallamaci et al., 1998)] have been demonstrated to play a role in the cortical arealization. In mice lacking functional Pax6 or Emx2, the corresponding rostral or caudal cortical domains are shrunken, whereas opposite areas are enlarged (Bishop et al., 2000, 2002; Mallamaci et al., 2000; Muzio et al., 2002a,b; Hamasaki et al., 2004). Similarly, the LIM-box homeodomain gene Lhx2, expressed in a caudomedial-high to rostrolateral-low gradient in the cortical VZ, promotes hippocampal vs. neo/pallocortical specification programmes (Monuki et al., 2001; Vyas et al., 2003), whereas the winged helix transcription factor Foxg1 expressed in an opposite gradient in the progenitors is involved in the suppression of hippocampal fate (Muzio & Mallamaci, 2005). Late in development and after birth a number of genes show restricted expression across the cortex.

An intriguing, but still unsolved, question is how the positional information encoded in the cortical primordium of the early embryo is translated into cortical areas with distinct and sharp boundaries at late developmental stages. Results from recent global expression analyses revealed that different domains in the adult brain have distinct transcriptional profiles (Evans et al., 2003) and contain an imprinted programme of embryonic gene expression (Zapala et al., 2005). However, only a few studies have so far attempted a large-scale examination of the regionalized gene expression in the developing cortex. By using a genomics-based strategy and in-situ hybridization expression analysis in cortical progenitor at embryonic day (E)12.5, Sansom et al. (2005) identified and confirmed the differential expression of 16 genes in the rostral cortex and 23 genes in the caudal cortex. The application of a similar approach revealed regionalized expression of 13 genes along the AP axis of the mouse cortex at E16.5 (Funatsu et al., 2004).

In the present study we performed DNA microarray analysis as a preliminary expression profiling of five microdissected neocortical regions of the frontal, parietal, occipital and cingulate cortex, and hippocampus isolated from the E16 mouse brain. At E16 the patterning events and neurogenesis are already advanced (Bayer & Altman, 1991) but the TCAs have mostly not yet invaded the CP regions of the frontal, parietal, occipital and cingulate cortex and hippocampus isolated from the E16 mouse brain. At E16 the patterning events and neurogenesis are already advanced (Bayer & Altman, 1991) but the TCAs have mostly not yet invaded the CP.

Candidate genes with predicted regionalized expression were selected from the microarray screen and subsequently validated by in-situ hybridization analysis at E16 and E18. The expression patterns of 25 genes, nine of which are novel, were confirmed and presented in this work. The majority of the identified genes show graded or more enriched expression along the AP or ML axis across the cortex in the VZ, SVZ, subplate (SP), CP or in the marginal zone (MZ). We present evidence for new candidate genes possibly involved in the regionalization of the cortical neuroepithelium during late neurogenesis.

Materials and methods

Animals

Embryos were derived from timed-mated wild type mice (strain HsdWin; NMRI, Harlan Winkelmann GmbH, Borchen, Germany). The plug date was considered as E0. Mice were killed by cervical dislocation. Animal care and procedures were in compliance with European Community Guidelines (86/609/EEC).

Dissection of the cortical tissue

In order to diminish the variability of the results inherent in the dissection technique, the same person carried out all of the cortex dissections. Small tissue pieces (2 × 2 mm) located in the central parts of the prospective cortical areas were isolated and tissue samples of 35 E16 embryos from six litters were pooled together. The tissue samples from five cortical regions were dissected on ice-cold diethylpyrocarbonate–phosphate-buffered saline (PBS) and kept at −20 °C in RNAlater™ (Ambion). The samples were representative of the frontal (presumptive motor area), parietal (presumptive somatosensory S1 area), occipital (presumptive visual area), cingulate and caudomedial cortex (hippocampus; see Supplementary material, Fig. S1). The remaining body without the spinal cord and genital ridge served as a control sample and allowed us to screen for brain and/or nervous system enriched genes.

RNA preparation, cDNA synthesis and target preparation

The entire procedure was carried out according to the Affymetrix microarray manual. Briefly, the total RNA was prepared with the Total RNA Isolation Kit (Qiagen). Thereafter, 5 μg of total RNA were reverse transcribed into double-stranded cDNA by using the Super Script Choice System kit (GibcoBRL). The cDNA was purified, precipitated and translated into biotin-labelled antisense cRNA targets with the BioArray™ High Yield™ RNA Transcript Labeling Kit (ENZO). cRNA (16 μg) was fragmented in fragmentation buffer (40 μM Tris-acetate, pH 8.1; 100 mM potassium acetate; 30 mM magnesium acetate) at 94 °C for 35 min and 1 μg of the fragmented cRNA was analysed for its size distribution on an agarose gel.

Array processing

The following steps were performed as described in the Expression Analysis Technical Manual (Affymetrix). (i) Hybridization in two independent experiments (v1 and v2) of the murine genome arrays U74Av1, U74Av2, U74Bv2 and U74Cv2 at 45 °C for 16 h with 15 μg fragmented cRNA in 300 μL hybridization solution (100 mM morpholinoethansulfonic acid; 1 mM NaCl; 20 mM EDTA; 0.01% Tween-20). (ii) Washing and staining in the GeneChip Fluidics Station 400 (Affymetrix). (iii) Scanning with the GeneArray Scanner (Agilent). For validating the quality of the cRNA targets, test chips (Microarray test 3, Affymetrix) were hybridized and analysed in advance.

Data analysis

The analysis of the microarray data was performed with the help of the Affymetrix MICROARRAY SUITE 4.0 software. For comparability the
chip data were scaled to a target intensity of 500. CHP-type files were created and the expression data for the cortical tissues and the control sample were compared pairwise with each other. Genes were selected for further work on the basis of their fold changes and difference calls fulfilling the following two criteria. (i) The difference call vs. the other cortical regions and the control was increased or marginal increased. (ii) The gene of interest showed a fold change > 3 towards at least one of the cortical regions.

**In-situ hybridization**

Embryonic day 16 brains were fixed in 4% formaldehyde in PBS for 2.5 h at 4 °C, washed twice in diethylpyrocarbonate–PBS and cryoprotected in 25% sucrose in PBS overnight. After embedding in Tissue-Tek (Jung), 16-µm-thick coronal cryosections were cut with a cryostat (Leica). Plasmids containing the partial or complete cDNAs of interest were obtained from the IMAGE Consortium (German Resource Center for Genome Research, Berlin, Germany). After confirmation by sequencing, the plasmids were linearized with the appropriate restriction enzymes (see Table 1). DNA (1 µg) was in-vitro transcribed into digoxigenin-labelled RNA probes with the RNA polymerase as indicated in Table 1. The non-radioactive in-situ hybridization was performed on sections as described in Moorman et al. (2001). In brief, the pretreatment of the sections included a proteolytic digestion for 4 min at 37 °C with 20 µg/mL proteinase K followed by a rinse in 0.2% glycine/PBS and refixation in 4% formaldehyde/0.2% glutaraldehyde/PBS. Sections were prehybridized in hybridization solution (50% formamide; 5× SSC (saline-sodium citrate), pH 4.5; 1 mg/mL yeast tRNA; 0.1% 3-[3-Cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS); 0.1% Tween 20; 5 mM EDTA, pH 8.0; 0.1 mg/mL heparin) for 1 h and hybridized overnight at 70 °C. After the hybridization three washes in 2× SSC and three washes in 2× SSC/50% formamide were carried out at 65 °C. For the immunohistochemical detection of the bound digoxigenin (DIG)-labelled riboprobes an alkaline phosphatase-conjugated anti-DIG antibody (ENZO) was used at 375 mU/mL in KTBT buffer (50 mM Tris-Cl, pH 9.5; 150 mM NaCl; 10 mM KCl; 1% Triton X-100). Nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, NBT/BCIP, (Roche) [1 : 50 diluted in NTMT buffer (100 mM Tris-Cl, pH 9.5; 100 mM NaCl; 50 mM MgCl2; 0.05% Tween-20)] was served as the substrate for the colourimetric detection. Photographs were taken on an Olympus BX60 microscope, processed and mounted with the SIS ANALYSIS 3.0 and Adobe PHOTOSHOP 6.0 software.

**Results**

**Sample preparation and analysis of the microarray data**

Given the large cellular heterogeneity of the developing cerebral cortex, a lot of attention is required during the procedures of tissue dissection, sample isolation and subsequent expression analysis (see Materials and methods; Barlow & Lockhart, 2002). The cortex samples, taken from the dorsal domains of the frontal, parietal and occipital neocortex,

<table>
<thead>
<tr>
<th>Gene symbol and affymetrix ID</th>
<th>Fold change vs.</th>
<th>Origin of in-situ probe (Image clone ID/reference)</th>
<th>Linearization/probe synthesis</th>
<th>Gene name</th>
</tr>
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<td>Ap1l3a2</td>
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<td>106964 at/v1</td>
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<td>NM_029007</td>
<td>EcoRI/T3</td>
<td>ATPase type 13A2</td>
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<tr>
<td>Clone 3</td>
<td></td>
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<tr>
<td>105726 at/v1</td>
<td>6.4 3.6 6.1 2.6</td>
<td>NM_029007</td>
<td>BamHI/T7</td>
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<td>Clone 36</td>
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<tr>
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<td>NM_198647</td>
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<tr>
<td>Clone 43 (mScr2)</td>
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<td>Scratch 2</td>
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<tr>
<td>Clone 67</td>
<td>1.8 1.7 3.5 1.8</td>
<td>NM_177618</td>
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<td>EcoRI/T3</td>
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<td>NM_198168</td>
<td>Xhol/T3</td>
<td>Protein phosphatase 2, regulatory subunit B, beta isoform</td>
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<td>NM_145711</td>
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<td>Thymocyte selection-associated HMG box gene</td>
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Table 1A. Microarray expression data: genes expressed in the frontal cortex of the embryonic day 16 mouse cerebral cortex

BTB, BR-C, ttk and bab, CCHC, Zinc finger type with cysteine and histidine residues; Cg, cingulate cortex; Fr, frontal cortex; Hi, hippocampus; HMG, high mobility group box; Oc, occipital cortex; Par, parietal cortex.
mostly include the presumptive area of the motor, somatosensory (S1) and visual cortex, respectively. The samples isolated from the rostral and caudal medial telencephalic wall correspond to the presumptive region of the cingulate cortex and hippocampus.

The cRNA samples from the five regions were prepared and hybridized to the Affymetrix mouse arrays U74Av1/Bv1 and U74Av2/Bv2/Cv2 in two independent experiments. The quality of all cRNA samples was verified and found to be high, as in all hybridizations with the Affymetrix test array the 5’ : 3’ average difference ratio for β-actin and other housekeeping genes ranged from 0.9 to 2.1. This is in full agreement with the microarray quality control as suggested by the manufacturer. The average fraction of present

### Table 1B. Microarray expression data: genes expressed in the occipital cortex of the embryonic day 16 mouse cerebral cortex

<table>
<thead>
<tr>
<th>Gene symbol and affymetrix ID</th>
<th>Fold change vs.</th>
<th>GenBank ID</th>
<th>Origin of in-situ probe (Image clone ID/reference)</th>
<th>Linearization/probe synthesis</th>
<th>Gene name</th>
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<td>Fr</td>
<td>Cg</td>
<td>Hi</td>
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<tr>
<td>Fr</td>
<td>Cg</td>
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</tbody>
</table>

See footnote to Table 1A.

### Table 1C. Microarray expression data: genes expressed in the cingulate cortex of the embryonic day 16 mouse cerebral cortex

<table>
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<tr>
<th>Gene symbol and affymetrix ID</th>
<th>Fold change vs.</th>
<th>GenBank ID</th>
<th>Origin of in-situ probe (Image clone ID/reference)</th>
<th>Linearization/probe synthesis</th>
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<tbody>
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<td>Fr</td>
<td>Hi</td>
<td>Par</td>
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</tr>
<tr>
<td>Oc</td>
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See footnote to Table 1A.

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probes was 41.2% in the cingulate cortex, 40.2% in the hippocampus, 44.5% in the frontal cortex, 38.6% in the parietal cortex and 41.5% in the occipital cortex.

Genes were regarded as differentially expressed in a given cortical region and selected for further analysis when (i) the difference call indicated an increased or marginal increased expression in one tissue sample compared with all other samples and (ii) the fold change was greater than 3 in at least one of the comparisons. By combining fold change and difference call criteria the performed analysis was carried out in a rather stringent way, thus minimizing false-positive predictions. Priority was given to stringency vs. completeness, as out in a rather stringent way, thus minimizing false-positive changes and differences between the cortical regions may have been missed in this analysis. Therefore, it should be stressed that full genome coverage was not intended and statistical conclusions should not be drawn from any of the presented data.

By performing such an analysis 178 target sequences with hybridization events correlated well (differently shaded bars in the graphs of Figs 2D and F, 3B–D, G and J, and 4A–C). The result of these independent hybridization results of several of these candidate genes are described below.

Verification of the regionalized expression of selected candidate genes

We selected 80 candidate genes and ESTs whose expression patterns and annotations were not present in public databases. An optimized in-situ hybridization protocol could be established for 42 clones, thus yielding a signal with the generated antisense RNA probes on sections of E16 and E18 mouse brains. Of these clones 25 showed a region-specific expression that strongly correlated with the microarray hybridization data. All cDNA clones corresponding to the specific target sequences were obtained from commercial sources (mostly IMAGE clones). Table 1 provides a detailed overview of the target sequences used for the in-situ probe synthesis. In order to facilitate the comparison of the microarray hybridization data and the corresponding expression patterns, both are shown next to each other in Figs 1–3. Hereby, the micrographs on the left-hand side indicate the relative expression in the cortical regions as assessed by the microarray. The strongest regional expression is set at 100%, whereas the other expression values are calculated as 100% divided by the fold change value. Some of the selected genes were represented several times on the microarrays. They were either present on both sets of chips (U74Av1/Bv1 and U74Av2/Bv2/Cv2) or applied by Affymetrix as different probe sequences. Noticeably, the results of these independent hybridization events correlated well (differently shaded bars in the graphs of Figs 2D and F, 3B–D, G and J, and 4A–C).

Genes with a rostral-high to caudal-low expression gradient

As a result of the first filtering 72 genes (20 of which were non-annotated ESTs, supplementary Table S1) were selected as putative candidates with abundant expression in the E16 frontal cortex. The in-situ hybridization results of several of these candidate genes are described below.

Graded expression in progenitors

Consistent with the microarray data (Table 1), Clone 67 shows a pronounced rostrolateral-high to caudomedial-low expression gradient in the VZ and lower CP at E16 (Fig. 1, AIII–AIV).
restricted to the lower part of the CP and is maintained at E18 (Fig. 1, A²). Another EST, Clone 43/mScrt2, shows a robust rostromedial-high to caudolateral-low expression in the progenitors of the SVZ of the frontal cortex and faint expression in the uppermost part of the CP at E16 (Fig. 1, B¹–B³) and E18 (Fig. 1, B⁴). The underlying EST sequence aligned significantly to the zinc finger transcription factors Scratch identified in *Drosophila melanogaster* and the mouse mScratch gene, without being identical to the latter (referred to as *mScrt1*, Nakakura et al., 2001). Thus, the identified gene encoded by Clone 43 represents a novel gene, further referred to as the mouse Scratch2 gene (mScrt2). This finding is consistent with the previous prediction of two Scratch genes in all vertebrate species (Manzanares et al., 2001). Clone 3 reveals rostromedial-high to caudolateral-low expression in the SVZ and CP at E16 (Fig. 1, C¹–C³) and E18 (Fig. 1, C⁴), whereas the occipital cortex is free of signal at both stages.

**Graded expression in the cortical plate**

Another group of genes shows consistent and predominant expression in the CP of the frontal cortex at both stages studied. PpIr1b (also referred to as DARPP-32) encodes the regulatory inhibitory subunit 1B of the protein phosphatase 1 (Becker et al., 2003). The expression of PpIr1b is confined to the lower CP of the frontal, parietal and cingulate cortices at E16 (Fig. 1, D¹ and D³) and E18 (Fig. 1, D⁴). At both stages no expression is detected in the occipital cortex. Nrip1 (Nuclear receptor interacting protein 1; Fig. 1, F¹ and F³) and Tox (Thymocyte selection-associated high mobility group box gene; Fig. 1, G¹ and G³) show strong expression in the CP of the frontal and cingulate cortices at E16. This expression is maintained at E18 (Fig. 1, F¹ and F³). Tox reveals a complex spatiotemporal pattern; although initially expressed in the entire depth of the frontal, parietal and cingulate cortices, at E18 Tox transcripts are detected only in the lower part of the parietal and occipital CP (Fig. 1, G³). Consistent with these findings, graded expression along the AP axis of the CP was also reported for PpIr1b and Nrip1 at E14.5 (http://www.Genepaint.org), indicating that the regionalized expression patterns of these genes in the CP could be caused by genetic imprints in the early cortical progenitors. The gene Pp2r5b (Protein phosphatase 2, regulatory subunit B) displayed an opposite robust expression gradient, rostromedial-high to caudolateral-low, in the E16 CP (Fig. 1, H¹–H³). It is maintained at E18 in the lower frontal and parietal cortex (Fig. 1, H⁴). In contrast, the enriched expression of Clone 36 in the anlage of the cingulate and motor cortex at E16 extends uniformly along the entire AP axis of the CP at E18 (Fig. 1, E¹–E⁵). This expression pattern probably reflects the gradient of normal CP differentiation.

**Genes expressed in caudal-high to rostral-low gradients**

After the microarray analysis, 38 genes (20 known and 18 non-annotated; supplementary Table S1) were selected as genes with predominant expression in the E16 occipital cortex. The expression patterns of seven of the selected genes were confirmed by the *in-situ* hybridization analysis and presented in this work (Fig. 2).

**Graded expression in progenitors**

Only one gene, Clone 12, exerted a caudal-high to rostral-low expression gradient in the cortical progenitors at E16. Sequence comparison revealed that the encoded gene is homologous to the rat *Pippin* gene (Nastasi et al., 1999) and therefore represents a new mouse gene (the mouse orthologue of the rat *Pippin* gene designated as *mPippin*). The *in-situ* expression analysis revealed that the highest expression of *mPippin* was detected in the region of the ventral pallium (Fig. 2, D¹–D³), which is assumed to act as the forebrain inductive centre (Assimacopoulos et al., 2003).

**Graded expression in the cortical plate**

*Nunr1* is expressed in the E16 caudal cortex (Fig. 2, A⁴) and in a deep layer (presumptive layer 6a) throughout the entire neocortex including the claustrum/endopiriform nucleus (Fig. 2, A¹–A³). At E18 restricted expression was detected in the retrosplenial cortex and especially in the subiculum (Fig. 2, A⁴).

Six genes showed a caudal-high to rostral-low expression gradient at E16, confined either exclusively to the upper portion of the CP (Flrt3, Nef3 and Odz3) or to the entire depth of the CP (Clone 63 and Clone 97). At E18 the expression of Flrt3, Odz3 (Clone 2, B¹ and C¹) and Clone 97 (Fig. 2, G¹) become even more restricted to the presumptive anlage of the visual cortex (Fig. 2, B¹ and C¹), suggesting a contribution of external factors to the refinement of these expression patterns. As the expression of Odz3 shows a consistent restricted expression in the caudal cortex at E15.5 (Zhou et al., 2003), E16 and E18 (this study), the highly regionalized expression of Odz3 in caudal cortex possibly involves an intrinsic patterning mechanism.

**Genes with abundant expression in the embryonic day 16 parietal cortex**

After the first filtering of the microarray data, only a few genes (13 genes, three known and 10 non-annotated, supplementary Table S1) were selected as candidate genes with a predominant...
expression in the parietal cortex. We tried to assess the expression of some of these candidates but the detected signals were at the limit of the background level. One explanation for this failure might be that, due to the central position of the parietal cortex along the AP axis, the identification of genes with differential expression in this domain is hampered. Along this line of evidence recent genome wide expression analysis at E12.5–E13.5 (Sansom et al., 2005) failed to identify genes with significant peaks of expression in the middle part of the neocortex. However, it is interesting to note that two of the selected genes, Nfe2l3 (Nuclear factor, erythroid-derived 2, like 3; NM_010903, supplementary Table S1) and Npy (Neuropeptide Y, supplementary Table S1), indeed have abundant expression in the parietal CP as shown for Nfe2l3 at E14.5 (http://www.Genepaint.org) and for Npy at E18.5 (Funatsu et al., 2004; Fig. 5).

**Genes with a medial-high to lateral-low expression gradient**

As a result of the microarray screen, 24 genes (16 known and eight non-annotated genes, supplementary Table S1) were predicted as candidates with predominant expression in the hippocampal anlage. Seven of these genes (Lhx9, Lhx5, Tac2, Lect1, Nr2p, Odad7 and Grp; supplementary Table S1) also exhibited restricted expression in the hippocampal anlage at E14.5/E15.5 (http://www.Genepaint.org), suggesting that an intrinsic patterning mechanism contributes to their regionalized pattern.

**Graded expression in progenitors**

At the rostral level, two genes showed well-defined expression in the E16 progenitors along the ML axis, most abundantly presented in the presumptive regions of the cingulate and motor cortex. Hop, encoding Homeodomain only protein (Fig. 3, G1 and G2), whose detailed expression was recently published (Funatsu et al., 2004; Mühlriedel et al., 2005), is expressed in a rostromedial-high to caudolateral-low gradient in the VZ of the cortex and in the dentate gyrus at E16 and E18 (Fig. 3, G3-G5). An even more regionalized and robust expression in progenitors of these two cortical domains was found for Tyrp2 (Tyrosinase-related protein or Oct) at both studied stages (Fig. 3, J1-J5). At E16 Odad8 (Zinc finger and BTB domain containing 20, Zbtb20; Fig. 3, B3-B4V) and the novel gene Clone 19 (Fig. 3, D3-D5) exert abundant expression in the caudal progenitors and in the differentiating hippocampus. This pattern also remains consistent at E18 (Fig. 3, B1 and D3).

**Expression in the medial cortical plate**

Nr4a3/Nor1 (Nuclear receptor subfamily 4, group A member 3) demonstrates a robust caudomedial-high to rostral-lateral-low expression gradient in the E16 cortex that becomes more restricted to the region of the whole hippocampus at E18 (Fig. 3, A1-A5). By means of the microarray analysis, Zinc5 (Zinc finger protein of the cerebellum) was predicted to be abundantly expressed in the E16 cingulate cortex. Utilizing in-situ hybridization this gene turned out to be expressed only in the MZ but extending into the CP of the cingulate and motor areas at E18 (Fig. 3, H1-H5). Nrp2 (Neuropilin 2; Fig. 3, C4-C4V) and Grp (Gastrin-releasing peptide, Fig. 3, F3-F5) transcripts are detected within the CA2/CA3 domains of the hippocampus. It is noteworthy that the restricted expression of Odad8 and Grp to the anlage of the whole hippocampus or to the presumptive CA2/CA3, respectively, can be detected as early as E14.5 (http://www.Genepaint.org).

Together with the results presented in this study, these findings indicate that these two genes might act as intrinsic determinants for the hippocampal differentiation. The novel gene encoded by Clone 86 shows a complex expression pattern. This gene corresponds to a full-length EST clone (GenBank entry NM_028325), which is referred to as Zinc finger containing CCHD domain 12 (Zcc12) in the databases, with an as yet unknown gene expression pattern. Clone 86/Zcc12 is strongly expressed in a thin layer of cells in the lower CP of the cingulate cortex, probably representing one of the prospective deep layers of the mature cortex (Figs 3, I1-I3, and G4). Additional expression is seen in the MZ (Figs 3, I1-I3, and G4 and G5), septum, striatum, diencephalon (Fig. 3) and amygdala (Fig. 6). The predicted higher expression of Ebf3 (Early B-cell factor 3) in the sample of the hippocampus compared with the other four regions was confirmed as expression in the hippocampal fissure (arrowhead in Fig. 3, E3-E4V) and MZ (see below).

**Genes expressed in the marginal zone and subplate**

At E16, TCAs reach the SP and extend tangentially below almost the entire CP. The SP is a transitory structure located below the CP that consists of the earliest-born neurones. It is assumed that the SP contains environmental cues with a role during the process of the TCAs finding their target regions before growing into the distinct areas of the CP (Allendorfer & Shatz, 1994). Interestingly, several genes that feature clear gradients in the CP, rostral-high to caudal-low (Clone 67 and Ppr1rb), caudal-high to rostral-low (Clone 63) or medial-high to lateral-low (Tox), also show expression in the SP (illustrated, respectively, in Fig. 4, B, C1, C1, D, D1, E and E1). It will be interesting to further study the function of these genes during the final steps of the TCA target finding. Clone 36 (Fig. 4, A and A3) and ATPase13A2 (a novel protein with ATPase activity, Atp13A2; Fig. 4, F and F1) showed wider expression in the SP along the AP axis.

The MZ consists of heterogeneous cellular components that are still not well characterized. Among the earliest-born neuronal types of MZ are the Cajal-Retzius cells. These cells secrete the extracellular matrix protein Reelin that has an important role for the proper layering of the cortex (Marin-Padilla, 1978; Ogawa et al., 1995). It is noteworthy that four of the described genes, Atp13A2 (Fig. 4, F and F1), Clone 86/Zcc12 (Fig. 4, G and G1), Flt3 (Fig. 4, H and H1) and Ebf3 (Fig. 4, I and I1), showed expression in the MZ of the E16 cortex.

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**Fig. 2.** Genes preferentially expressed in the occipital cortex. (A–G) Microarray expression data. Sections designated by superscript I or IV are at rostral-most or caudal-most brain level, respectively. (A1–A5) Nurr1 is expressed predominantly in the occipital cortex at embryonic day (E)16 and in the deep layer throughout the entire neocortex (A1-A5). At E18 strong regionalized expression is seen in the retrosplenial cortex (RSP) and subiculum (SUB) (A4, E16; A5, E18). (B1–B4) Fbrl3 is expressed in the uppermost cortical plate (CP) of the occipital cortex at E16 (B3V) and E18 (B5). (C1–C4) Nef3 shows a strong signal in the uppermost part of the occipital CP (C4V, E16; C4, E18). (D1–D5) mPippin expression with a caudal (high) to rostral (low) gradient in the ventricular zone (VZ) of the E16 cortex. (D5) Strong expression of Pippin in the ventral pallium (VP) and extending rostral migratory stream (RMS). In addition, mPippin transcripts are detectable in postmitotic cells in the differentiating basal ganglia (BG) and diencephalons (DC) at E16 (D3V) and at E18 (D5). (E1–E5) The identified novel gene encoded by Clone 63 is expressed in the entire CP with highest expression in the occipital cortex (E1–E3; E16; E4, E18). (F1–F5) The novel gene encoded by Clone 97 is expressed in the CP with a caudal (high) to rostral (low) expression gradient at E16 (F3V) and E18 (F5). (G1–G5) Strong expression of the Odd Oz/ten-m homologue (Odz3) in the upper CP of the occipital cortex at E16 (G1V) and E18 (G5). Cg, cingulate; Fr, frontal; Hi, hippocampus; Oc, occipital; Par, parietal.
In order to provide hints on the putative molecular function of the genes described in this study, we classified them with the help of the NetAffx internet portal (Liu et al., 2003). The classification is based on the Gene Ontology categories of molecular functions (Ashburner et al., 2000). For simplicity only the main categories were considered here (Fig. 5). Single genes can be mentioned multiple times in different categories. It is assumed that intermolecular and cellular interactions in late developmental stages contribute to the progressive

**Functional classification of the differentially expressed genes**

In order to provide hints on the putative molecular function of the genes described in this study, we classified them with the help of the NetAffx internet portal (Liu et al., 2003). The classification is based on the Gene Ontology categories of molecular functions (Ashburner et al., 2000). For simplicity only the main categories were considered here (Fig. 5). Single genes can be mentioned multiple times in different categories. It is assumed that intermolecular and cellular interactions in late developmental stages contribute to the progressive
delineation of interareal borders in the maturating CP. Indeed, most (20) of the genes presented in this study were assigned to the categories binding protein, receptor or membrane protein. These categories mainly stand for the processes of signal transduction or transcriptional regulation. Hence, these genes might have an essential function in the cortical arealization process at late developmental stages. Another six genes were predicted (or known) to exert enzyme or hormone activity or act as structural proteins. These genes probably represent marker genes for some already established features of the prospective cortical regions. For the putative gene products of three genes (Clone 3, Clone 36 and Clone 63) no similarity to existing protein domains, and hence no molecular function, could be established.

Novel marker genes for cortical, subcortical and diencephalic domains

Furthermore, we identified several genes with a restricted expression in nuclei of the amygdala. Recent evidence suggests a multiple origin of the amygdalar nuclei, being derived from either pallial or subpallial progenitors (Medina et al., 2004). The amygdala is a complex cortical structure located in the basal telencephalon that contains more than 40 distinct nuclei and the discovery of genes with a restricted expression in specific amygdalar nuclei will facilitate the study of its morphogenesis.

In order to assess the identity of the nuclei in our expression analysis, antisense probes for appropriate amygdalar markers (Medina et al., 2004) and the genes of interest were applied on adjacent sections of E16 and E18 brains. As shown in Fig. 6 the expression of Oda8 in the basolateral telencephalon is confined to the dorsal endopiriform nucleus of the claustroamygdalar complex (Fig. 6A and B). Similarly to Tbr1 (Medina et al., 2004), Oda8 is expressed in the intercalated, lateral and basomedial amygdalar nuclei (Fig. 6C–E). Clone 86/Zcchc12 shows restricted expression in the claustrum and in the dorsal endopiriform nucleus, basomedial amygdalar nuclei and lateral amygdalar nuclei, whereas the intercalated nucleus seems negative (Fig. 6F–I). The gene Nrp2 labels the dorsal endopiriform nucleus and piriform cortex, whereas at the caudal level it shows restricted expression in the medial amygdalar nucleus (Fig. 6K–N).

In addition, several genes showed restricted expression in differentiating diencephalic nuclei within the territory of the dorsal and/or ventral thalamus. The expression of Clone 87 (Fig. 7A and B) marks the dorsal thalamic nuclei (the paraventricular nucleus and medial habenular nucleus) and the several ventral thalamic nuclei. The expression of Clone 93 (Fig. 7C and D) is more restricted to differentiating dorsal thalamic nuclei and includes the ventrolateral part of the laterodorsal thalamic nucleus, the anteromedial and paracentral thalamic nuclei as well as the parataenial thalamic nucleus. Intriguingly, the expression of the transcription factor Zic5 (Fig. 7E–H) was restricted to several differentiating nuclei in the medial part of the telencephalon, encompassing at rostral levels the lateral and medial septal nuclei, medial preoptic area, septohypothalamic nucleus and bed nucleus stria terminalis. In more caudal levels the expression domain of Zic5 was confined to the dorsal and ventral geniculate nuclei, ventral posterior medial thalamic nucleus, anterior pretectal and precommissural nucleus.

Discussion

Identification of genes with regionalized expression in the developing cortex

Although it is expected that a large number of molecular determinants are involved in the normal cortical arealization, only a limited number...
of candidate genes playing a role in this process have been identified so far. The identification of new molecular determinants of the cortical area and layer formation is therefore a necessary step for understanding how the complexity of the mature cerebral cortex is achieved. In this work we describe for the first time the differential expression of nine novel and 16 known genes in the germinal or mantle zone of the E16 and E18 mouse embryonic cortex, thus implying a possible function of these genes in cortical arealization.

To extend the correlation between our expression analysis performed at E16 and E18 with expression at earlier stages, we benefited from the availability of the gene expression database of the GenePaint organization (http://www.Genepaint.org). Of the 179 selected sequences revealing differential expression across the E16 cortex, 47 (indicated in light blue in supplementary Table S1) were also scrutinized at the earlier E14/E15 (Visel et al., 2004; http://www.Genepaint.org). Most of these 47 genes were not selected for expression analysis by in-situ hybridization in the present study. However, for 26 of them (indicated with a red star in supplementary Table S1), the cortical expression at E16 as assessed by the microarray hybridization was consistent with the differential expression at E14/E15 as reported by Visel et al. (2004). This consistency further supports not only the reliability of the performed microarray assay but also the assumption that these genes are intrinsically regulated in the embryonic cortex. In the mouse at E16 the TCAs from the dorsal thalamus have crossed the corticostriatal border and extended tangentially in the SP all along the entire dorsomedial surface of the telencephalon. However, invasion of thalamic collaterals is seen only in the more mature areas of the lateral-most cortex (Catalano et al., 1991, 1996; Bicknese et al., 1994), a region not included in our set of samples. Similarly, only very few thalamic axons have grown into the CP before E16–E17 in rat (corresponding to E14–E15 in mouse), mostly in the occipital cortex (Molnar et al., 1998). Therefore, except for the visual cortex, the consistent graded expression of genes detected in this study in cortical progenitors and the CP along the AP axis reflects predominantly the last stage of intrinsic cortical regionalization during the late neurogenesis. Indeed, six of the genes presented here (indicated in red in supplementary Table S1, Nrip1, Pppl1r1b, Nurr1, Odz3, Zbtb20/Oda8 and Grp) exert consistent expression gradients at E16 and E18 (this study) and at E14.5/E15.5 (http://www.Genepaint.org), suggesting that these genes could act as novel intrinsic molecular determinants of cortical arealization.
Novel genes with graded expression across the anteroposterior and mediolateral axis during late neurogenesis

The genes with a consistent expression at E16 and E18 fall into three main categories: (i) graded expression along the ML and AP axis confined exclusively to the proliferative neuroepithelium, VZ (Tyrp2, Hop and Pippin) and SVZ (mScrt2); (ii) graded expression in both the germinal zones and the CP (Clone 67, Clone 3, Clone 63, Clone 19 and Oda8) or only in the CP (Nor1 and Grp); and (iii) enriched expression in domains of the CP in rostral (Od23, Fr3, Nef3 and Nurr1) or medial (Nrp2, Nor1, Clone 36 and Pp2r2b) cortex.

It is important to note that we found a more pronounced graded expression in the germinative neuroepithelium of the E16 cortex along the ML as compared with the AP axis. At the rostral-most level Tyrp2 and Hop revealed strongly enriched expression in the VZ of the prospective cingulate and motor cortices at E16 and E18. Regionally restricted expression confined to progenitors of the frontal cortex at E12.5 was reported for Tyrp2 (see Fig. 4 in Steel et al., 1992; also Sansom et al., 2005) and Hop (Funatsu et al., 2004; Mühlfriedel et al., 2005), suggesting that the spatial expression of Tyrp2 and Hop might be an intrinsic molecular determinant for the fate commitment of progenitors of these two cortical areas. In addition, the identified novel mouse gene mScrt2, a member of the Snail family of zinc finger transcription factors, demonstrates strong graded expression rostrally in progenitors of the SVZ, suggesting a function in the generation of the supragranular cortical layers (reviewed by Guillemot et al., 2006).

Finally, mPippin, which encodes a protein containing an RNA and DNA binding cold shock domain (Castiglia et al., 1996), showed abundant expression along the AP axis in progenitors of the occipital cortex. The performed in-situ hybridization analysis revealed, however, that similarly to the expression of the transcription factor Pax6, the strongest expression of mPippin is confined to the rostral lateral cortical progenitors of the ventral pallium (or antihem). The antihem has recently been proposed to act as a signalling centre possibly depending on the function of Pax6 (Assimacopoulos et al., 2003). In support of this idea we found that the expression of mPippin is lost in the homozygous Pax6/Small eye mutant embryonic brain (data not shown).

Three novel genes were found to show graded expression in the germinative neuroepithelium and the lower CP. The enriched expression of Clone 3 and Clone 67 to the VZ and CP of the rostral cortex is consistent at both E16 and E18, indicating that the graded expression is not caused by the normal gradient of cortical

Fig. 7. Genes with restricted expression in diencephalon. (A and B) In two rostrocaudal levels the gene encoded by Clone 87 shows restricted expression in postmitotic cells of the paraventricular thalamic nucleus (PV) of the dorsal thalamus (DT), the medial habenular nucleus (MHb) of the epithalamus (ET) and the bed nucleus stria medular (BSM) and reticular thalamic nucleus (Rt), which are derivatives of the eminentia thalami (EMT) and ventral thalamus (VT), respectively. (C and D) The expression of the gene encoded by Clone 93 is restricted to the ventrolateral part of the laterodorsal thalamic nucleus (LDVL), the anteromedial (AM) and paracentral (PC) thalamic nuclei as well the parataenial thalamic nucleus (PT). Additional expression is detected in the subventricular zone (SVZ) of the basal ganglia (BG in D). (E–H) Micrographs from four different rostro-caudal levels illustrate the expression of Zic5 in distinct medial domains of the telencephalon. They include the lateral (LS) and medial (MS) septal nuclei, medial preoptic area (MPA in E), septohypothalamic nucleus (Shy), anterior part of the bed nucleus stria terminalis (BSTA in F), MHb/lateral (LHb) habenular nuclei, dorsal (DLG) and ventral (VLG) geniculate nuclei, LDVL, ventral posterior thalamic nucleus (VP in G), anterior pretectal nucleus (APT) and precommissural nucleus (PrC in H) (eml, external medullary lamina).
neurogenesis but rather seems to depend on an intrinsic patterning mechanism. Even more intriguingly, Clone 67 and Clone 63 are expressed in the SP, which is a transitory structure and, together with the deepest cortical layers, is assumed to play a role in TCA guidance (Kostovic & Rakic, 1990; De Carlos & O'Leary, 1992). An interesting characteristic of the novel gene Clone 3 is its graded expression confined to the SVZ and upper CP at E16 and E18 implying a function in the generation of the supragranular cortical layers. Restricted expression in both caudal progenitors and differentiating hippocampus was found for Oda8 and Clone 19. Oda8 and four other genes (Lhx9, Lhx5, Tac2 and Lect1, supplementary Table S1) that were scored as potentially expressed in the E16 hippocampus also show consistent expression at E14.5 (http://www.Genepaint.org). This suggests intrinsic expression properties of the caudomedial progenitors. It is noteworthy that the hypothetical protein encoded by Clone 19 shares similarities with the Prosaposin protein, a nervous system-associated protein, whose expression increases after injury in the peripheral and central nervous system (Gillen et al., 1995; Hiraïwa et al., 2003).

Evidence about discrete domains of gene expression in the developing cortex is limited. During late neurogenesis graded or restricted expression patterns across the cortex were reported only for genes encoding cell interaction proteins such as cadherins (Suzuki et al., 1997; Donoghue & Rakic, 1999a), ephrins/Eph receptors (Donoghue & Rakic, 1999b; O'Leary & Wilkinson, 1999) and immunoglobulins (Pimenta et al., 1996; Mann et al., 1998). As demonstrated in this work, two genes (Tox and Nrip1) have strongly enriched expression in the area of the cingulate and motor cortex at E16 and E18. Tox is a member of a conserved family of high mobility group box proteins (O'Flaherty & Kaye, 2003) involved in the maturation of T-cells (Wilkinson et al., 2002). Its expression in the developing cortex has not been addressed before. Nrip1 acts as a retinoic acid-inducible corepressor of the TR2 nuclear receptor, assumed to be an interaction partner of histone deacetylases (Lee et al., 1998; Wei et al., 2000). This implies a possible involvement of the gene in chromatin modulation. Intriguingly, even more regionalized expression confined to the lower part of the E16 and E18 CP of the fronto-orbital and frontal cortex was detected for Ppp1r1b (also Perez & Lewis, 1992), a gene that is down-regulated in patients with schizophrenia (Foster et al., 1987). Furthermore, we found several genes with strongly enriched expression in the occipital cortex. One of them, Flrt3, belongs to a gene family that encodes membrane-integrated proteins with leucine-rich, fibronectin/collagen-like domains. FLRT3 protein was suggested to act as a receptor, signal transducer or participant in cell–cell contact (Lacy et al., 1999; Tsuji et al., 2004) and could regulate neurite outgrowth in sensory ganglia in vitro (Robinson et al., 2004). The Flrt3 expression in rat starts in a small region of the caudal dorsal telencephalon at E11.5 (mouse E9; see Fig. 4F in Robinson et al., 2004). Together with our present results demonstrating consistent expression in the occipital cortex until birth, these findings suggest an early role for Flrt3 in cortical arealization. Similarly, Odz3 and Nurr1 have restricted expression to the occipital cortex at E16, E18 (this study) and E15 (Odz3, Zhou et al., 2003) or E14.5 (Nurr1, http://www.Genepaint.org). This again implicates intrinsic properties of early progenitors as being responsible for the regionalized expression of these two genes. It is important to note that the enriched expression of Flrt3 and Odz3 in the E16 caudal cortex becomes more restricted and confined to the superficial layers of the presumptive visual cortex at E18, suggesting a contribution of the massive TCA ingrowth into occipital cortex at this stage to the pattern refinement.

Intrinsic mechanisms of cortical regionalization

In the spinal cord the nested expression of sets of transcription factors defines distinct neuronal progenitor domains along the dorsoventral axis, producing distinct interneuronal subtypes at a specific position (Briscoe et al., 2000). Such discrete expression of molecular determinants in cortical progenitors has not been shown so far. As mentioned above, our expression analysis revealed regionally enriched gene expression in the E16 cortical progenitors along the ML axis (the ventrodorsal axis of the neural plate before the neural tube closure). This expression follows either medial-high to lateral-low gradients (Tyrp2, Hop, mScr2, Clone 19 and Oda8) or opposite lateral-high to caudal-low gradients (Clone 67, Pippin, Clone 6 and Clone 3). Such accumulation of gene transcripts along the ML axis could be important in establishing a protomap for a particular cortical area at a distinct AP level (e.g. at rostral-most level, the cingulate, motor and perirhinal area; at caudal level, the hippocampus, visual and auditory area). Further experiments are required to study whether and how the expression of these genes at early developmental stages might be influenced by factors secreted from forebrain patterning centres. For one of these genes, Hop, we recently found that its strongly regionalized expression in cortical progenitors along the ML axis is indeed dependent on signalling influences from the roof plate, indicating that Hop might contribute to establishing elements of the early cortical protomap (Mühlriedel et al., 2005).

It is interesting to note that two of the identified novel genes (Clone 3 and mScratch2) show strong expression in progenitors of the SVZ. Few genes with restricted expression in the SVZ, namely Cux1, Cux2 (Nieto et al., 2004; Zimmer et al., 2004), Sver1 (Tarabykyn et al., 2001) and Satb2 (Britanova et al., 2005), have been implicated with the generation of the supragranular cortical neurons in a Pax6-controlled genetic pathway (reviewed by Guillenot et al., 2006). Further experiments are in progress to evaluate the genetic interplay between Pax6 and the newly identified genes during the corticogenesis.

In conclusion, our results provide further support for the model that, during late neurogenesis, regional expression of molecular determinants exists as a result of intrinsic assignment of positional identity at early stages, whereas afferent TCA input and possibly intermolecular and intercellular interactions between differentiating cells in the late CP might contribute to the progressive compartmentalization of the maturing cortex.

Supplementary material

The following supplementary material may be found on www.blackwell-synergy.com

Fig. S1. Dissection of the embryonic day 16.0 mouse cerebral cortex.

Table S1. Expression characteristics and annotations of the 179 genes scored from our microarray screen with predicted differential expression in distinct cortical domains in embryonic day (E)16 mouse brain.

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