European Journal of Neuroscience, Vol. 26, pp. 33–50, 2007 doi:10.1111/j.1460-9568.2007.05639.x

Novel genes differentially expressed in cortical regions during late neurogenesis

Sven Mühlfriedel,* Friederike Kirsch,* Peter Gruss, Kamal Chowdhury and Anastassia Stoykova
Department of Molecular Cell Biology, Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, 37077 Göttingen, Germany

Keywords: arealization, corticogenesis, expression, markers, microarray, mouse

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 a 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 neurones 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; Lukazewicz *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).

Correspondence: Dr Anastassia Stoykova, as above.
E-mail: astoyko@gwdg.de

*S.M. and F.K. contributed equally to this work.

Received 25 September 2006, revised 3 May 2007, accepted 21 May 2007

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*-TF1 (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/palleocortical 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 (Catalano *et al.*, 1991, 1996; Bicknese *et al.*, 1994). Thus, differential gene expression at E16 is likely to be intrinsic to the developing cortex and may be involved in the development of area-specific features, such as cytoarchitecture and layering as well as the set-up of the TCA map. E16 was therefore regarded as an interesting stage in mouse corticogenesis and hence chosen for this analysis. Moreover, comparison of the expression patterns at E16 (before TCA invasion) with those at E18 (after TCA invasion) allowed us to obtain insight into the expression changes during this critical period of cortical development.

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 mM 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, U74Bv1, 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 M [Na^+]; 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 diethylpyro–carbonate–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 7.5; 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 $MgCl_2$; 0.05% Tween-20)] 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 1A. Microarray expression data: genes expressed in the frontal cortex of the embryonic day 16 mouse cerebral cortex

Gene symbol and affymetrix ID	Fold change vs. Fr	Cg	Hi	Par	GenBank ID	Origin of *in-situ* probe (Image clone ID/reference)	Linearization/ probe synthesis	Gene name
Atp13a2 106964_at/v1	4.5	5.7	4.9	3.9	NM_029097	3167831	EcoRI/T3	ATPase type 13A2
Clone 3 105726_at/v1	6.4	3.6	6.1	2.6	NM_029007	518946	BamHI/T7	–
Clone 36 111310_at/v1	1.3	1.8	4.7	1.9	NM_198647	3465181	EcoRI/T3	–
Clone 43 (mScrt2) 107382_at/v1	2	2.3	3.2	2.1	AW060659	389580	EcoRI/T3	Scratch 2
Clone 67 108995_at/v1	1.8	1.7	3.5	1.8	NM_177618	1226693	EcoRI/T3	–
Nrip1 103288_at/v2	3.1	3.3	7.4	4	NM_173440 and AA959574	3376247	XhoI/T3	Nuclear receptor interacting protein 1
110650_at/v1	4.4	2.1	6	2.5				
110650_at/v2	2.9	2.7	5.9	2.9				
Ppp1r1b 108533_at/v1	18	3.8	16.9	7.9	NM_144828	921764	EcoRI/T3	Protein phosphatase 1, regulatory (inhibitor) subunit 1B
108533_at/v2	8.3	2.3	8.1	2				
Ppp2r5b 112902_at/v1	1.5	3	7.8	4	NM_198168	464888	XhoI/T3	Protein phosphatase 2, regulatory subunit B, beta isoform
Tox 112175_at/v1	5.7	2	5.9	2	NM_145711	573967	EcoRI/T3	Thymocyte selection-associated HMG box gene

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.

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

Gene symbol and affymetrix ID	Fold change vs. Fr	Cg	Hi	Par	GenBank ID	Origin of *in-situ* probe (Image clone ID/reference)	Linearization/ probe synthesis	Gene name
Clone 63								
134008_at/v2	2.8	4	2.9	3.1	AI536417	1181880	XhoI/T3	–
Clone 97								
135858_at/v2	2.9	3.2	2.4	2.2	AI481066	851221	EcoRI/T3	–
Flrt3								
110370_at/v1	5.4	6.1	2.8	2.2	NM_178382	3972772 and 1852927	EcoRI/T3 and XhoI/T3	Fibronectin leucine-rich transmembrane protein 3
110370_at/v2	7	9.7	3.2	2.7				
164233_at/v2	5	6.5	2.4	2.1				
167905_f_at/v2	6.3	8	3.3	2.2				
Nef3								
117269_at/v1	5.9	6.3	5.3	5.5	NM_008691	385063	XhoI/T3	Neurofilament 3, medium
117269_at/v2	6.2	6	4.8	5.4				
92346_at/v1	6	5.6	4.1	5.9				
92346_at/v2	9.5	7.8	6.7	8				
Nurr1								
163675_r_at/v2	5.6	7.1	2.7	5.9	NM_013613	1762441	EcoRI/T3	Nur-related protein 1
92248_at/v1	6.5	4.9	5.5	5.5				
92248_at/v2	6.1	17.7	4.8	15.4				
92249_g_at/v1	4	3.9	2.3	3.1				
92249_g_at/v2	3.2	5.3	4.8	5.1				
Odz3								
166915_at/v2	2.5	7.7	2.7	1.8	NM_011857	3513892	EcoRI/T3	Odd Oz/ten-m homologue 3 (*Drosophila*)
92500_at/v2	1.9	4	2.4	2.2				
PIPPIN								
116316_at/v1	1.9	4.5	3.5	9.9	AK081145	889232	EcoRI/T3	PIPPIN protein

See footnote to Table 1A.

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

Gene symbol and affymetrix ID	Fold change vs. Oc	Fr	Hi	Par	GenBank ID	Origin of *in-situ* probe (Image clone ID/reference)	Linearization/ probe synthesis	Gene name
Tyrp2								
103597_at/v1	33.9	3	23.2	27.2	NM_010024	Steel *et al.* (1992)	HindIII/T7	Tyrosinase-related protein 2
103597_at/v2	39.3	3.7	42.1	11.3				
164910_s_at/v2	33.6	4.8	17.6	46.9				
Hop								
96672_at/v1	3.6	3.4	1.9	2.8	NM_175606	1196783	BamHI/T3	Homeodomain only protein
96672_at/v2	3.7	2.6	1.9	3				
Zcchc12								
135902_at/v2	1.9	4.2	1.6	3.4	NM_028325	4010211	SalI/T7	Zinc finger, CCHC domain containing 12
Zic5								
116403_at/v1	6.7	7.7	1.8	10.4	NM_022987	633740	EcoRI/T7	Zinc finger protein of the cerebellum 5

See footnote to Table 1A.

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 1D. Microarray expression data: genes expressed in the hippocampus of the embryonic day 16 mouse cerebral cortex

Gene symbol and affymetrix ID	Fold change vs. Oc	Fr	Cg	Par	GenBank ID	Origin of *in-situ* probe (Image clone ID/reference)	Linearization/ probe synthesis	Gene name
Clone 19								
113738_at/v1	10.6	9	2.7	5.7	BC048953 and AI553462	1248381 and 1247849	EcoRI/T3 (both)	–
113738_at/v2	4.4	3.7	2.8	11.4				
115830_at/v1	4.8	4.8	1.9	4.2				
115830_at/v2	2.8	3.1	2.1	4				
Ebf3								
93913_at/v2	4	4.6	1.7	4	NM_010096	4316932	EcoRI/T3	Early B-cell factor 3
Grp								
165567_at/v2	15.5	25.3	23.5	17.8	NM_175012	440937	EcoRI/T3	Gastrin-releasing peptide
Nor1, Nr4a3								
105572_at/v1	3.3	2.8	1.8	5.5	NM_015743	3328870	EcoRI/T3	Nuclear receptor subfamily 4, group A, member 3
Nrp2								
116382_at/v1	3.4	12.5	2.6	14.2	NM_010939	3416226	EcoRI/T3	Neuropilin 2
116382_at/v2	3	13.4	2	13.3				
Oda8, Zbtb20								
116619_at/v1	5.2	5.2	2	5.6	NM_019778	2182632	EcoRI/T3	Zinc finger and BTB domain containing 20
116619_at/v2	2.9	3.5	2	5.6				
94780_at/v1	7.9	8.1	2.5	8.1				
94780_at/v2	3.3	4	2.5	7.1				

See footnote to Table 1A.

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 this screen was intended as a first filtering for the subsequent screening by RNA *in-situ* hybridization. Genes with mild expression 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 predicted enriched expression in one of the studied cortical regions were identified. Most of these genes or expressed sequence tags (ESTs) were found in the frontal cortex sample (72), followed by the occipital cortex (38), hippocampus (24), cingulate cortex (31) and parietal cortex (13). Expression data of these genes are given in Supplementary material, Table S1.

Verification of the regionalized expression of selected candidate genes

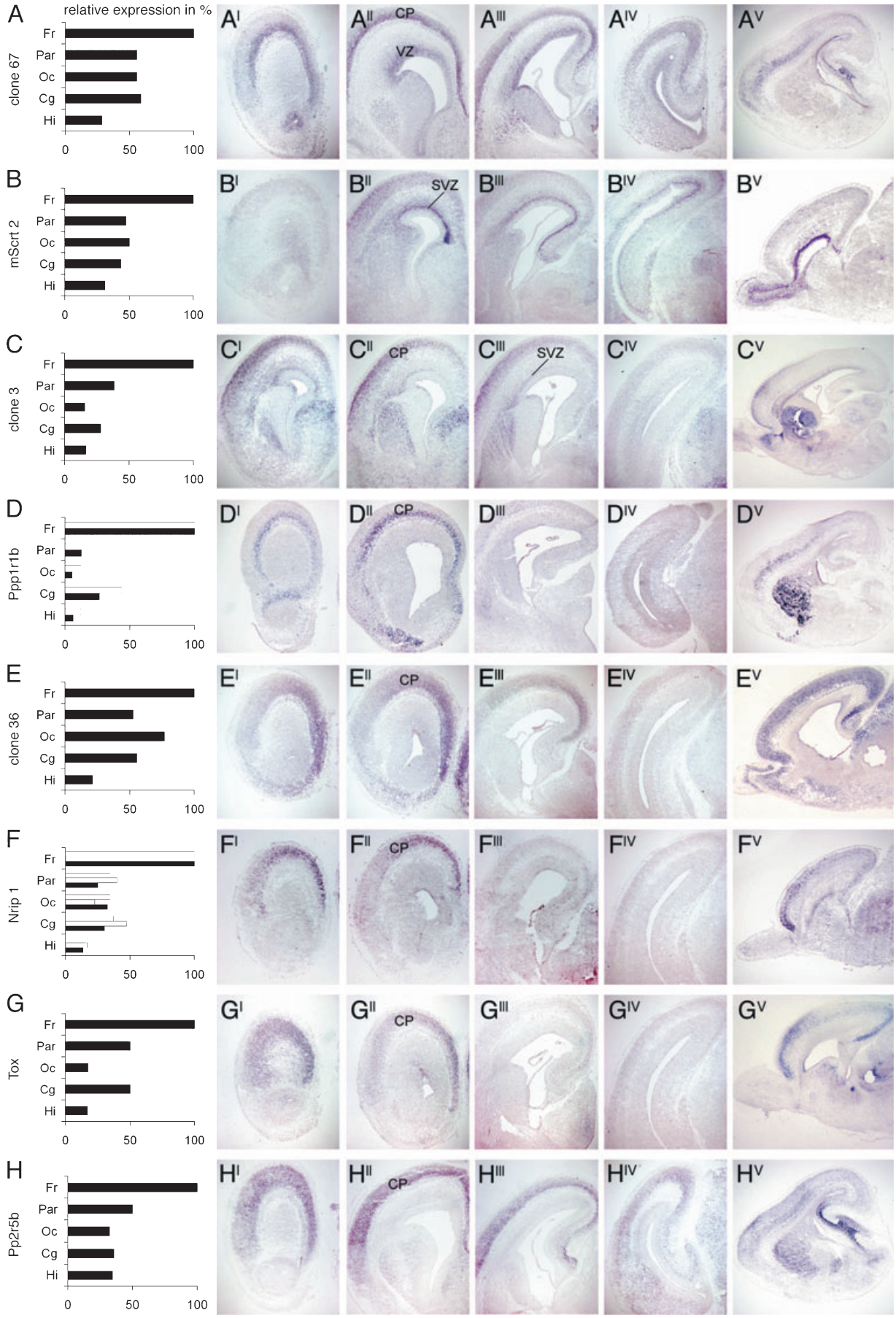
We selected 80 candidate genes and ESTs whose expression patterns and annotations were not present in public databases. An optimized *in-situ* hybridization protocol could be established for 42 clones, thus yielding a signal with the generated antisense RNA probes on sections of E16 and E18 mouse brains. 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 microarray data for these genes and the IMAGE clones 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, A^{I}–A^{IV}). The expression is



restricted to the lower part of the CP and is maintained at E18 (Fig. 1, A^V). Another EST, *Clone43/*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, B^I–B^{IV}) and E18 (Fig. 1, B^V). 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 rostrolateral-high to caudolateral-low expression in the SVZ and CP at E16 (Fig. 1, C^I–C^{IV}) and E18 (Fig. 1, C^V), whereas the occipital cortex is free of signal at both stages. A detailed view of the expression of *mScrt2* and *Clone 3* in the SVZ is provided in Fig. 4, J, J^I, K and K^I, respectively. The consistent graded expression of the three novel genes *Clone 3*, *Clone 67* and *mScrt2* in cortical progenitors and the CP at the two studied stages suggest that the expression patterns are the results of intrinsic regionalization of the cortical progenitors.

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. *Ppp1r1b* (also referred to as *DARPP-32*) encodes the regulatory inhibitory subunit 1B of the protein phosphatase 1 (Beckler *et al.*, 2003). The expression of *Ppp1r1b* is confined to the lower CP of the frontal, parietal and cingulate cortices at E16 (Fig. 1, D^I and D^{II}) and E18 (Fig. 1, D^V). At both stages no expression is detected in the occipital cortex. *Nrip1* (*Nuclear receptor interacting protein 1*; Fig. 1, F^I and F^{II}) and *Tox* (*Thymocyte selection-associated high mobility group box gene*; Fig. 1, G^I and G^{II}) show strong expression in the CP of the frontal and cingulate cortices at E16. This expression is maintained at E18 (Fig. 1, F^V and G^V). *Tox* reveals a complex spatiotemporal pattern; although initially expressed in the entire depth of the fronto-orbital and rostral cortex, at E18 *Tox* transcripts are detected only in the lower part of the parietal and occipital CP (Fig. 1, G^V). Consistent with these findings, graded expression along the AP axis of the CP was also reported for *Ppp1r1b* 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, rostrolateral-high to caudomedial-low, in the E16 CP (Fig. 1, H^I–H^{IV}). It is maintained at E18 in the lower frontal and parietal cortex (Fig. 1, H^V). 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, E^I–E^V). 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, D^{II}–D^{IV}), which is assumed to act as the forebrain inductive centre (Assimacopoulos *et al.*, 2003).

Graded expression in the cortical plate

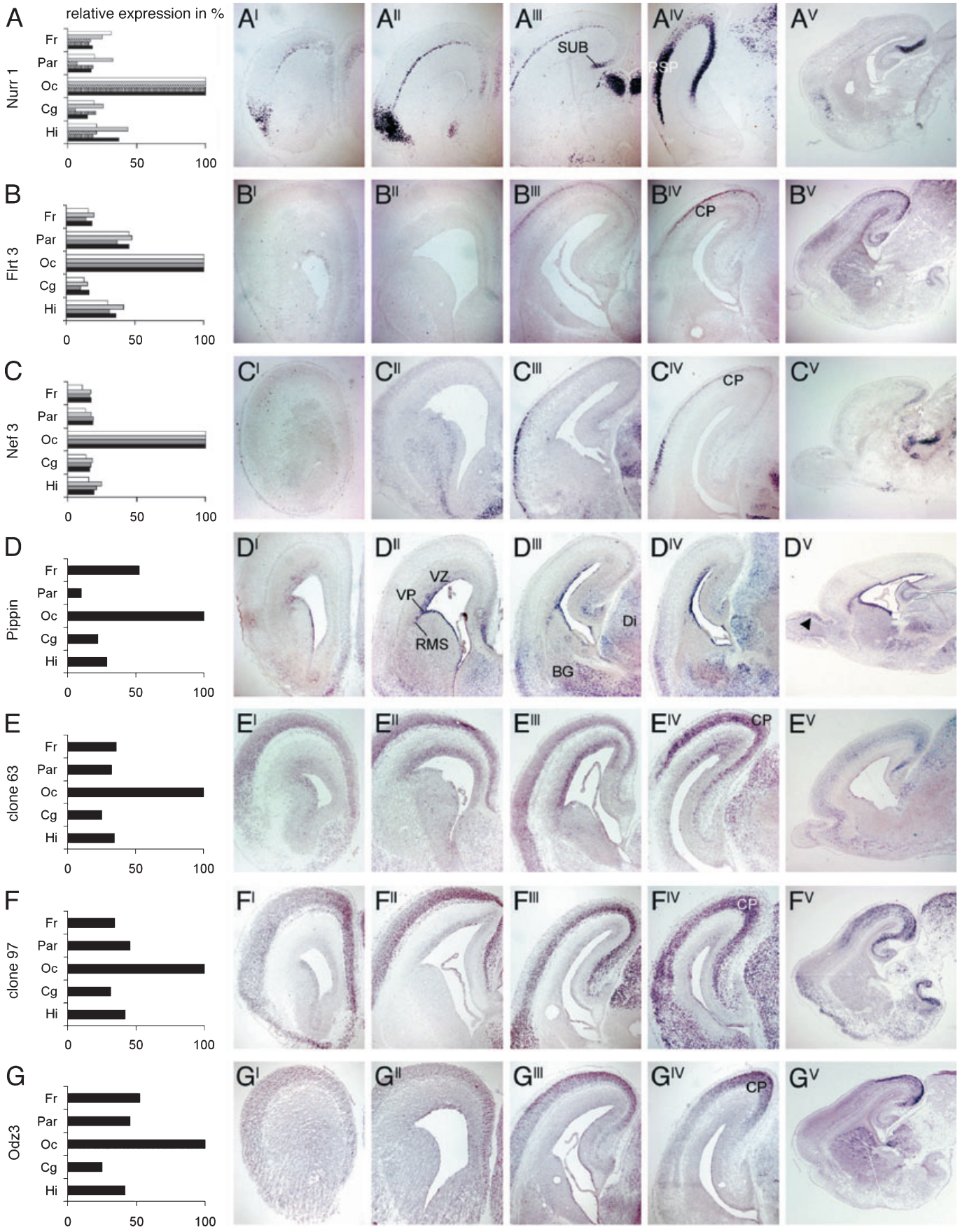
Nurr1 is expressed in the E16 caudal cortex (Fig. 2, A^{IV}) and in a deep layer (presumptive layer 6a) throughout the entire neocortex including the claustrum/endopiriform nucleus (Fig. 2, A^I–A^{III}). At E18 restricted expression was detected in the retrosplenial cortex and especially in the subiculum (Fig. 2, A^V).

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* (Fig. 2, B^V and C^V) and *Clone 97* (Fig. 2, G^V) become even more restricted to the presumptive anlage of the visual cortex (Fig. 2, B^V and C^V), 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

FIG. 1. Genes preferentially expressed in the frontal cortex. (A–H) Cortical gene expression as assessed by the microarray expression analysis. The highest expression is set to 100%, whereas the expression in the other cortex regions is calculated as 100% divided by the respective fold change. (A^I–A^V) *Clone 67* is expressed with a rostrolateral-high to caudomedial-low gradient in the ventricular zone (VZ) and lower cortical plate (CP) at embryonic day (E)16 (A^I–A^{IV}) and E18 (A^V). Sections designated by superscript I or IV are at rostral-most or caudal-most brain level, respectively. (B^I–B^V) *Clone 43* (*mScrt2*) shows a rostromedial-high to caudolateral-low expression gradient in the subventricular zone (SVZ) at E16 (B^I–B^{IV}) and E18 (B^V). (C^I–C^V) *Clone 3* is expressed with a rostrolateral-high to caudomedial-low gradient in the SVZ and the superficial part of the CP at E16 (C^I–C^{IV}) and E18 (C^V). Additional expression is detected in the striatum and dorsal thalamus (C^I–C^V). (D^I–D^V) The expression of *Ppp1r1b* is confined to the lower CP of the frontal, parietal and cingulate cortices at E16 (D^{II}) and E18 (Fig. 2, D^V). Additional gene expression is present in the basal ganglia (D^{III} and D^V). (E^I–E^V) *Clone 36* encodes a novel gene with a rostromedial-high to caudolateral-low expression gradient in the E16 CP (E^I–E^{IV}). (F^I–F^V) *Nrip1* shows strong expression in the CP of the frontal and cingulate cortices at E16 (F^I and F^{II}). The regionalized expression is also maintained in the E18 cortex (F^V). (G^I–G^V) *Tox* shows abundant expression in the CP of the frontal and cingulate cortices at E16 (G^I–G^{IV}) and E18 (G^V). (H^I–H^V) *Pp2r5b* exhibits a rostromedial-high to caudolateral-low expression gradient in the E16 cortex (H^I–H^{IV}). At E18 *Pp2r5b* is expressed throughout the entire depth of the frontal cortex but only in the lower cortical layers in the parietal domain (H^V). Cg, cingulate; Fr, frontal; Hi, hippocampus; Oc, occipital; Par, parietal.



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*, *Nrp*2, *Oda7* 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, G^{I} and G^{II}), whose detailed expression was recently published (Funatsu *et al.*, 2004; Muhlfriedel *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, G^{I}–G^{V}). An even more regionalized and robust expression in progenitors of these two cortical domains was found for *Tyrp2* (*Tyrosinase-related protein* or *Dct*) at both studied stages (Fig. 3, J^{I}–J^{V}). At E16 *Oda8* (*Zinc finger and BTB domain containing 20*, *Zbtb20*; Fig. 3, B^{III}–B^{IV}) and the novel gene *Clone 19* (Fig. 3, D^{III}–D^{IV}) exert abundant expression in the caudal progenitors and in the differentiating hippocampus. This pattern also remains consistent at E18 (Fig. 3, B^{V} and D^{V}).

Expression in the medial cortical plate

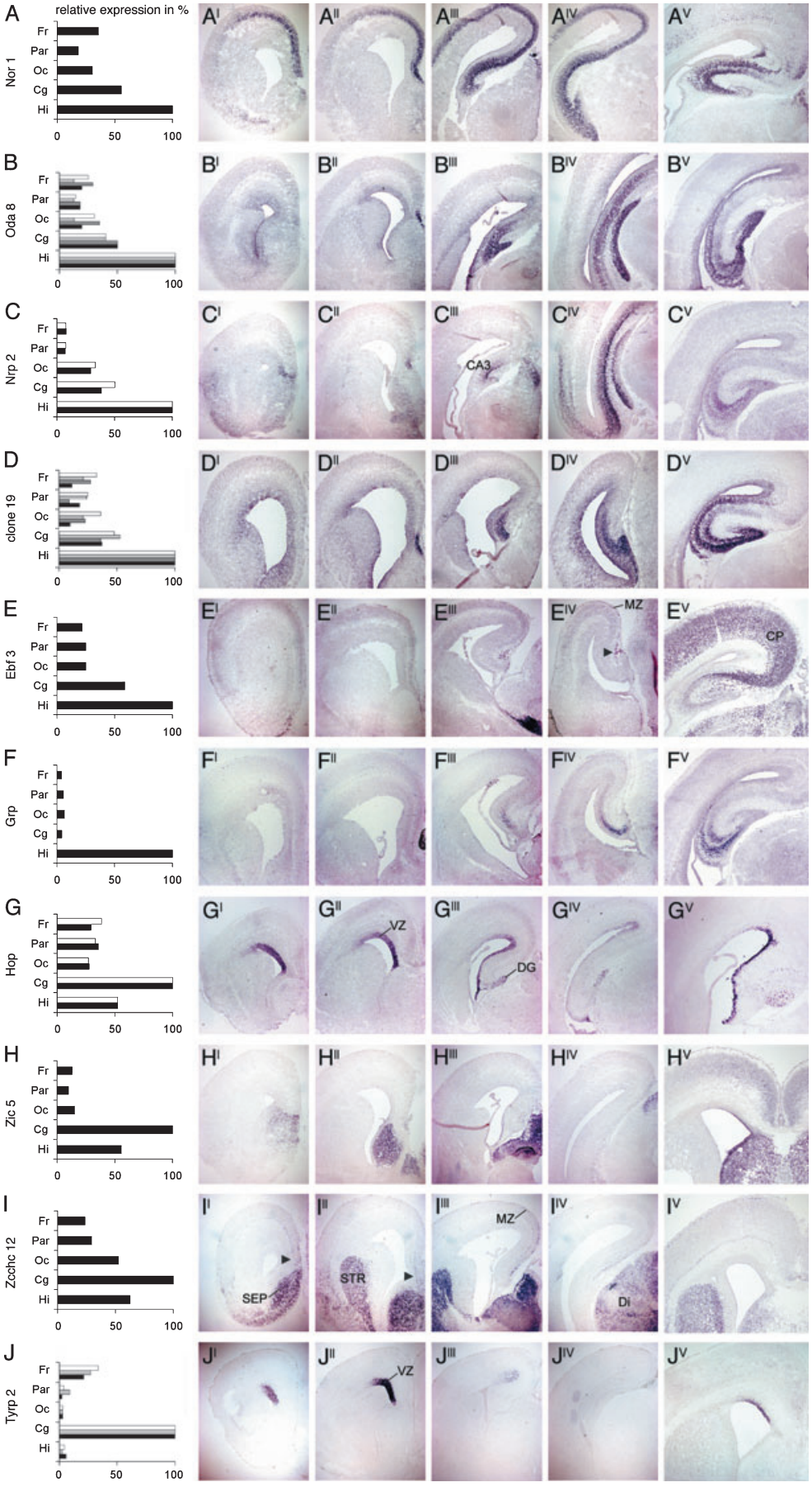
Nr4a3/Nor1 (*Nuclear receptor subfamily 4, group A member 3*) demonstrates a robust caudomedial-high to rostrolateral-low expression gradient in the E16 cortex that becomes more restricted to the region of the whole hippocampus at E18 (Fig. 3, A^{I}–A^{V}). By means of the microarray analysis, *Zic5* (*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, H^{I}–H^{V}). *Nrp2* (*Neuropilin 2*; Fig. 3, C^{I}–C^{V}) and *Grp* (*Gastrin-releasing peptide*, Fig. 3, F^{I}–F^{V}) transcripts are detected within the CA2/CA3 domains of the hippocampus. It is noteworthy that the restricted expression of *Oda8* 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 CCHC domain 12* (*Zcchc12*) in the databases, with an as yet unknown gene expression pattern. *Clone 86/*Zcchc12 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, I^{I}–I^{III}, and 4, G^{I}). Additional expression is seen in the MZ (Figs 3, I^{I}–I^{III}, and 4, G and G^{I}), 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, E^{I}–E^{IV}) 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 *Ppp1r1b*), 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, B^{I}, C, C^{I}, D, D^{I}, E and E^{I}). 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 A^{I}) and *ATPase13A2* (a novel protein with ATPase activity, *Atp13A2*; Fig. 4, F and F^{I}) 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 F^{I}), *Clone 86/*Zcchc12 (Fig. 4, G and G^{I}), *Flrt3* (Fig. 4, H and H^{I}) and *Ebf3* (Fig. 4, I and I^{I}), showed expression in the MZ of the E16 cortex.

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. (A^{I}–A^{V}) *Nurr1* is expressed predominantly in the occipital cortex at embryonic day (E)16 and in the deep layer throughout the entire neocortex (A^{I}–A^{IV}). At E18 strong regionalized expression is seen in the retrosplenial cortex (RSP) and subiculum (SUB) (A^{IV}, E16; A^{V}, E18). (B^{I}–B^{V}) *Flrt3* is expressed in the uppermost cortical plate (CP) of the occipital cortex at E16 (B^{IV}) and E18 (B^{V}). (C^{I}–C^{V}) *Nef3* shows a strong signal in the uppermost part of the occipital CP (C^{IV}, E16; C^{V}, E18). (D^{I}–D^{V}) *mPippin* expression with a caudal (high) to rostral (low) gradient in the ventricular zone (VZ) of the E16 cortex. (D^{II}) 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 (D^{III}) and at E18 (D^{V}). (E^{I}–E^{V}) The identified novel gene encoded by *Clone 63* is expressed in the entire CP with highest expression in the occipital cortex (E^{I}–E^{IV}, E16; E^{V}, E18). (F^{I}–F^{V}) The novel gene encoded by *Clone 97* is expressed in the CP with a caudal (high) to rostral (low) expression gradient at E16 (F^{IV}) and E18 (F^{V}). (G^{I}–G^{V}) Strong expression of the Odd Oz/ten-m homologue (*Odz3*) in the upper CP of the occipital cortex at E16 (G^{IV}) and E18 (G^{V}). Cg, cingulate; Fr, frontal; Hi, hippocampus; Oc, occipital; Par, parietal.



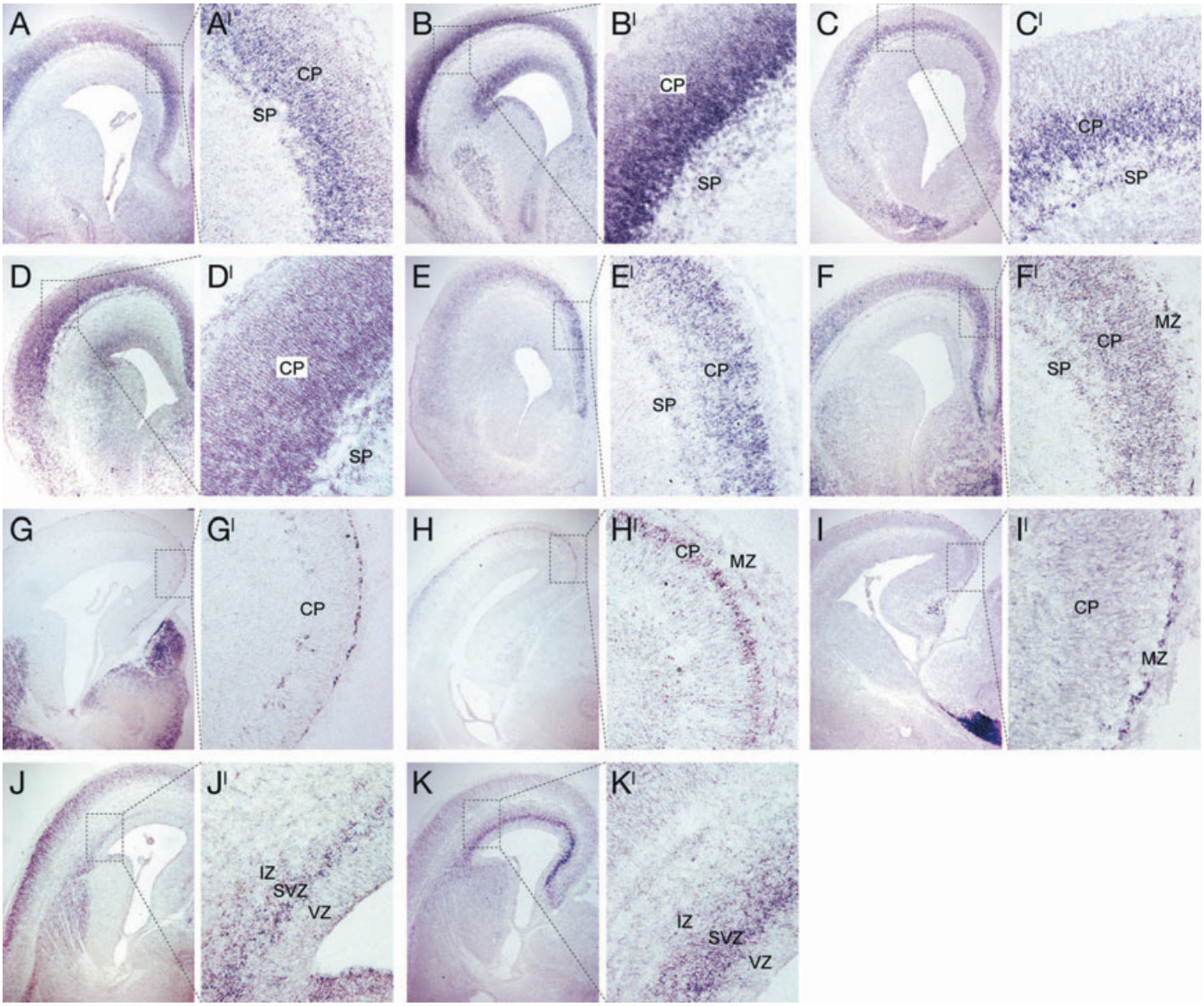


FIG. 4. Genes expressed in the subplate, marginal and subventricular zone (SVZ). *Clone 36* (A and A^I), *Clone 67* (B and B^I), *Ppp1r1b* (C and C^I), *Clone 63* (D and D^I), *Tox* (E and E^I) and the gene *ATpase 13A2* (*Atp13A2*; F and F^I) show expression in the cortical plate (CP) and subplate (SP). Expression in the marginal zone (MZ) is shown by *Atp13A2* (F and F^I), *Clone 86* (G and G^I), *Flrt3* (H and H^I) and *Ebf3* (I and I^I). The genes encoded by *Clone 3* (J and J^I) and *mScrt2* (K and K^I) show a restricted and strong expression in the SVZ. IZ, intermediate zone; VZ, ventricular zone.

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

FIG. 3. Genes preferentially expressed in the medial cortex. (A–J) Microarray expression data. Sections designated by superscript I or IV are at rostral-most or caudal-most brain level, respectively. (A^I–A^V) *Nr4a3* (or *Nor1*) shows a medial-high to lateral-low expression gradient in the cortical plate (CP) at embryonic day (E)16 (A^I–A^{IV}) and reveals more restricted expression in the entire hippocampus at E18 (A^V). (B^I–B^V) The expression of *Zbtb20* (or *Oda8*) is restricted to the hippocampus at E16 (B^I–B^{IV}) and E18 (B^V). (C^I–C^V) Restricted expression of *Neuropilin 2* (*Nrp2*) is present in the hippocampus, predominantly in the CA3 domain, at E16 (C^I–C^{IV}) and E18 (C^V). (D^I–D^V) The identified novel gene encoded by *Clone 19* is expressed in differentiating cells of the hippocampus at E16 (D^I–D^{IV}) and E18 (D^V). (E^I–E^V) The marginal zone (MZ) of the developing hippocampal fissure (arrowhead in E^{IV}) is positive for *Ebf3* at E16 (E^I–E^{IV}). At E18 (E^V) a wider *Eb3f* expression is detected throughout the cortical CP. (F^I–F^V) *Grp* shows restricted expression in the hippocampus (mostly CA3 region) at E16 (F^I–F^{IV}) and E18 (F^V). (G^I–G^V) *Hop* is expressed with a rostromedial-high to caudolateral-low gradient in the ventricular zone (VZ) and dentate gyrus (DG) of the E16 (G^I–G^{IV}) and E18 (G^V) cortex. (H^I–H^V) *Zic5* is expressed in the anlage of the cingulate cortex at E16 (H^I–H^{IV}). This regionalized expression pattern is preserved at E18 (H^V). (I^I–I^V) *Clone 86* (also *Zcchc12*) shows a complex expression pattern; positive are cell layers in the lower CP of the cingulate cortex (arrowheads in I^I and I^{II}) and MZ. Strong expression is seen in the septum (SEP), striatum (STR) and diencephalons (Di) (I^I–I^{IV}, E16; I^V, E18). (J^I–J^V) *Trp2* shows a highly restricted expression in progenitors of the presumptive domains of the cingulate and motor cortex at E16 (J^I–J^{IV}) and E18 (J^V). Cg, cingulate; Fr, frontal; Hi, hippocampus; Oc, occipital; Par, parietal.

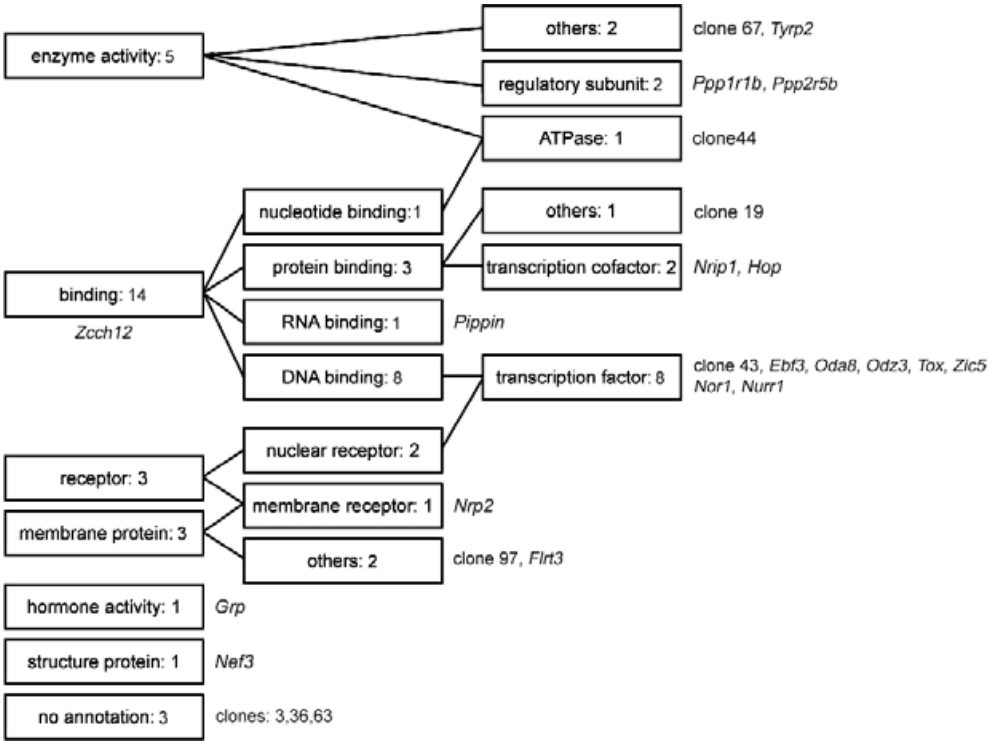


FIG. 5. Functional classification. The genes with proven expression in this study are classified according to their molecular function. The connecting lines indicate the relationships between these functional classes. Genes can be mentioned multiple times in different but related categories.

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 posteriomedial 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

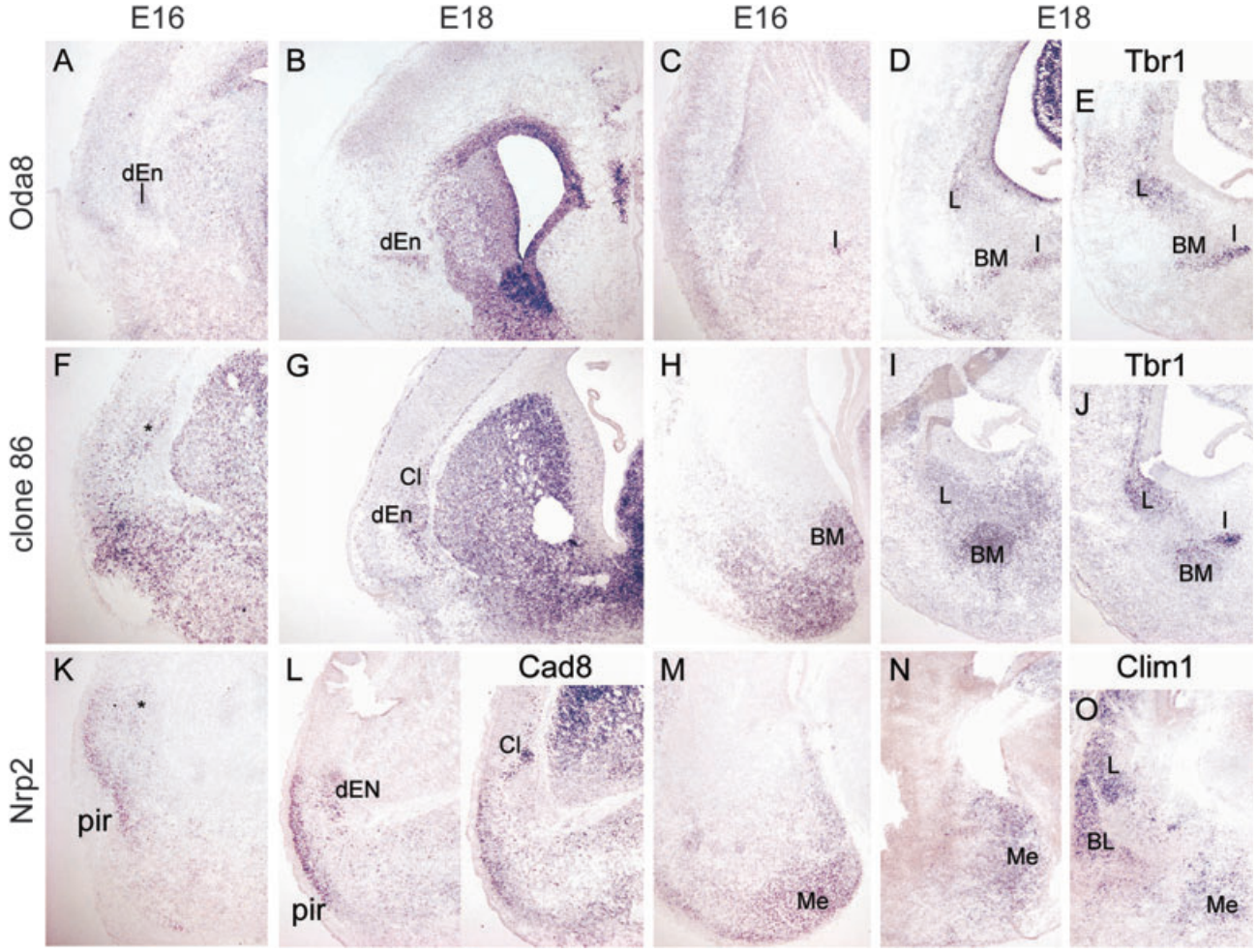


FIG. 6. New marker genes for amygdalar nuclei. The micrographs show the detected *in-situ* hybridization patterns of the studied genes at embryonic day (E)16 and E18 at two levels. The applied marker genes are *Tbr1* (E and J), *Cadherin 8* (M) and *Clim1* (O). (A–D) The expression of *Oda8* marks the dorsal endopiriform nucleus (dEn) and the lateral (L), basomedial (BM) and intercalated (I) amygdalar nuclei as confirmed by the *Tbr1* expression on adjacent section (E). (F–I) The expression of *Clone 86* outlines the claustrum (Cl), dEN, BM and L nucleus of the amygdala. (K–O) *Nrp2* is expressed in the dEn, medial (Me) and BL but not in the L amygdalar nuclei, all three of which are positive for *Clim1a* (see Remedios *et al.*, 2004). BL, basolateral amygdalar nuclei; Pir, piriform cortex.

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*, *Ppp1r1b*, *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.

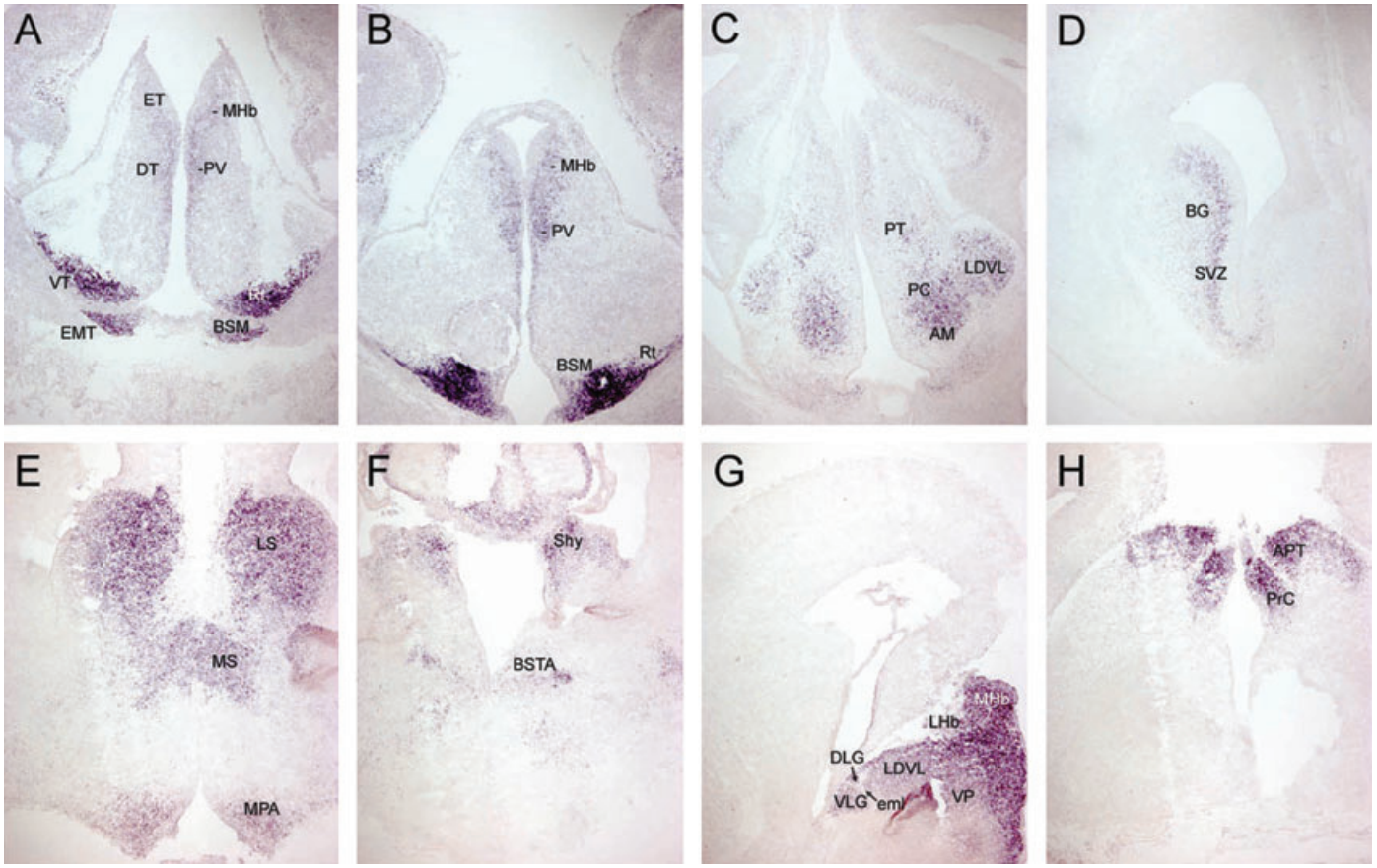


FIG. 7. Genes with restricted expression in diencephalon. (A and B) In two rostrocaudal levels the gene encoded by *Clone 87* shows restricted expression in postmitotic cells of the paraventricular thalamic nucleus (PV) of the dorsal thalamus (DT), the medial habenular nucleus (MHb) of the epithalamus (ET) and the bed nucleus stria medullaris (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).

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 (*Ppp1r1b*, *Nrip1* and *Tox*), caudal (*Odz3*, *Frt3*, *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; Muhlfriedel *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 rostrolateral 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

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; Hiraiwa *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*, *mScrt2*, *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 (Muhlfriedel *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), *Svet1* (Tarabykin *et al.*, 2001) and *Satb2* (Britanova *et al.*, 2005), have been implicated with the generation of the supragranular cortical neurones in a *Pax6*-controlled genetic pathway (reviewed by Guillemot *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.

Acknowledgements

We address our thanks to Dr K. Struwe for her help with bioinformatical questions. We thank M. Daniel and A. Conrad for their excellent technical assistance and S. Hille for DNA sequencing. We greatly appreciate the cooperation with Dr U. Teichman and the animal facility. Thanks are due to Mrs Wegener for the help with preparation of the illustration material. This work was financed and supported by the EU grant QLG3-CT-2000–00158 (A.S. and P.G.) and the Max Planck Society.

Abbreviations

AP, anteroposterior; CP, cortical plate; E, embryonic day; EST, expressed sequence tags; ML, mediolateral; MZ, marginal zone; PBS, phosphate-buffered saline; SP, subplate; SVZ, subventricular zone; TCA, thalamocortical projection; VZ, ventricular zone.

References

Allendorfer, K.L. & Shatz, C.J. (1994) The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex. *Annu. Rev. Neurosci.*, **17**, 185–218.

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

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T., Harris, M.A., Hill, D.P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J.C., Richardson, J.E., Ringwald, M., Rubin, G.M. & Sherlock, G. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.*, **25**, 25–29.

Assimacopoulos, S., Grove, E.A. & Ragsdale, C.W. (2003) Identification of a Pax6-dependent epidermal growth factor family signaling source at the lateral edge of the embryonic cerebral cortex. *J. Neurosci.*, **23**, 6399–6403.

Bachler, M. & Neubuser, A. (2001) Expression of members of the Fgf family and their receptors during midfacial development. *Mech. Dev.*, **100**, 313–316.

Barlow, C. & Lockhart, D.J. (2002) DNA arrays and neurobiology − what's new and what's next? *Curr. Opin. Neurobiol.*, **12**, 554–561.

Bayer, S.A. & Altman, J. (1991) *Neocortical Development*. Raven Press, New York.

Beckler, A., Moskaluk, C.A., Zaika, A., Hampton, G.M., Powell, S.M., Frierson, H.F. Jr & El-Rifai, W. (2003) Overexpression of the 32-kilodalton dopamine and cyclic adenosine 3′,5′-monophosphate-regulated phosphoprotein in common adenocarcinomas. *Cancer*, **98**, 1547–1551.

Bicknese, A.R., Sheppard, A.M., O'Leary, D.D. & Pearlman, A.L. (1994) Thalamocortical axons extend along a chondroitin sulfate proteoglycan-enriched pathway coincident with the neocortical subplate and distinct from the efferent path. *J. Neurosci.*, **14**, 3500–3510.

Bishop, K.M., Goudreau, G. & O'Leary, D.D.M. (2000) Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. *Science*, **288**, 344–349.

Bishop, K.M., Rubenstein, J.L. & O'Leary, D.D. (2002) Distinct actions of Emx1, Emx2, and Pax6 in regulating the specification of areas in the developing neocortex. *J. Neurosci.*, **22**, 7627–7638.

Briscoe, J., Pierani, A., Jessell, T.M. & Ericson, J. (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell*, **101**, 435–445.

Britanova, O., Akopov, S., Lukynov, S., Gruss, P. & Tarabykin, V. (2005) Novel transcription factor Satb2 interacts with matrix attachment region DNA elements in tissue-specific manner and demonstrates cell-type-dependent expression in the developing mouse CNS. *Eur. J. Neurosci.*, **21**, 658–668.

Castiglia, D., Scaturro, M., Nastasi, T., Cestelli, A. & Di Liegro, I. (1996) PIPPin, a putative RNA-binding protein specifically expressed in the rat brain. *Biochem. Biophys. Res. Commun.*, **218**, 390–394.

Catalano, S.M., Robertson, R.T. & Killackey, H.P. (1991) Early ingrowth of thalamocortical afferents to the neocortex of the prenatal rat. *Proc. Natl Acad. Sci. U.S.A.*, **88**, 2999–3003.

Catalano, S.M., Robertson, R.T. & Killackey, H.P. (1996) Individual axon morphology and thalamocortical topography in developing rat somatosensory cortex. *J. Comp. Neurol.*, **367**, 36–53.

Caviness, V.S. Jr, Takahashi, T. & Nowakowski, R.S. (1995) Numbers, time and neocortical neuronogenesis: a general development and evolutionary model. *Trends Neurosci.*, **23**, 379–383.

Caviness, V.S. Jr, Goto, T., Tarui, T., Takahashi, T., Bhide, P.G. & Nowakowski, R.S. (2003) Cell output, cell cycle duration and neuronal specification: a model of integrated mechanisms of the neocortical proliferative process. *Cereb. Cortex*, **13**, 592–608.

Cohen-Tanoudji, M., Babinet, C. & Wassef, M. (1994) Early determination of a mouse somatosensory cortex marker. *Nature*, **368**, 460–463.

Crossley, P.H., Martinez, S., Ohkubo, Y. & Rubenstein, J.L.R. (2001) Coordinate expression of Fgf8, Otx2, Bmp4, and Shh in the rostral prosencephalon during development of the telencephalic and optic vesicles. *Neuroscience*, **108**, 183–206.

De Carlos, J.A. & O'Leary, D.D. (1992) Growth and targeting of subplate axons in establishment of major cortical pathways. *J. Neurosci.*, **12**, 1194–1211.

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.

Dehay, C., Savatier, P., Cortay, V. & Kennedy, H. (2001) Cell-cycle kinetics of neocortical precursors are influenced by embryonic thalamic axons. *J. Neurosci.*, **21**, 201–214.

Donoghue, M.J. & Rakic, P. (1999a) Molecular evidence for the early specification of presumptive functional domains in the embryonic primate cerebral cortex. *J. Neurosci.*, **19**, 5967–5979.

Donoghue, M.J. & Rakic, P. (1999b) Molecular gradients and compartments in the embryonic primate cerebral cortex. *Cereb. Cortex*, **9**, 586–600.

Evans, S.J., Choudary, P.V., Vawter, M.P., Li, J., Meador-Woodruff, J.H., Lopez, J.F., Burke, S.M., Thompson, R.C., Myers, R.M., Jones, E.G., Bunney, W.E., Watson, S.J. & Akil, H. (2003) DNA microarray analysis of functionally discrete human brain regions reveals divergent transcriptional profiles. *Neurobiol. Dis.*, **14**, 240–250.

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

Ferri, R.T. & Levitt, P. (1995) Regulation of regional differences in the differentiation of cerebral cortical neurons by EGF family–matrix interactions. *Development*, **121**, 1151–1160.

Foster, G.A., Schultzberg, M., Hokfelt, T., Goldstein, M., Hemmings, H.C. Jr, Ouimet, C.C., Walaas, S.I. & Greengard, P. (1987) Development of a dopamine- and cyclic adenosine 3′:5′-monophosphate-regulated phosphoprotein (DARPP-32) in the prenatal rat central nervous system, and its relationship to the arrival of presumptive dopaminergic innervation. *J. Neurosci.*, **7**, 1994–2018.

Fukuchi-Shimogori, T. & Grove, E.A. (2001) Neocortex patterning by the secreted signaling molecule FGF8. *Science*, **294**, 1071–1074.

Fukuchi-Shimogori, T. & Grove, E.A. (2003) Emx2 patterns the neocortex by regulating FGF positional signaling. *Nat. Neurosci.*, **6**, 825–831.

Funatsu, N., Inoue, T. & Nakamura, S. (2004) Gene expression analysis of the late embryonic mouse cerebral cortex using DNA microarray: identification of several region- and layer-specific genes. *Cereb. Cortex*, **14**, 1031–1044.

Galceran, J., Miyashita-Lin, E.M., Devaney, E., Rubenstein, J.L. & Grosschedl, R. (2000) Hippocampus development and generation of dentate gyrus granule cells is regulated by LEF1. *Development*, **127**, 469–482.

Garel, S., Yun, K., Grosschedl, R. & Rubenstein, J.L. (2002) The early topography of thalamocortical projections is shifted in Ebf1 and Dlx1/2 mutant mice. *Development*, **129**, 5621–5634.

Gillen, C., Gleichmann, M., Spreyer, P. & Muller, H.W. (1995) Differentially expressed genes after peripheral nerve injury. *J. Neurosci. Res.*, **42**, 159–171.

Gimeno, L., Brulet, P. & Martinez, S. (2003) Study of Fgf15 gene expression in developing mouse brain. *Gene Expr. Patt.*, **3**, 473–481.

Gitton, Y., Cohen-Tannoudji, M. & Wassef, M. (1999) Specification of somatosensory area identity in cortical explants. *J. Neurosci.*, **19**, 4889–4898.

Grove, E.A., Tole, S., Limon, J., Yip, L. & Ragsdale, C.W. (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.

Guillemot, F., Molnár, Z., Tarabykin, V. & Stoykova, A. (2006) Molecular mechanisms of cortical differentiation. *Eur. J. Neurosci.*, **23**, 857–868.

Hamasaki, T., Leingartner, A., Ringstedt, T. & O'Leary, D.D. (2004) EMX2 regulates sizes and positioning of the primary sensory and motor areas in neocortex by direct specification of cortical progenitors. *Neuron*, **43**, 359–372.

Hanashima, C., Li, S.C., Shen, L., Lai, E. & Fishell, G. (2004) Foxg1 suppresses early cortical cell fate. *Science*, **303**, 56–59.

Herbert, J.M., Mishina, Y. & McConnell, S.K. (2002) BMP signalling is required locally to pattern the dorsal telencephalic midline. *Neuron*, **35**, 1029–1041.

Hiraiwa, M., Liu, J., Lu, A.G., Wang, C.Y., Misasi, R., Yamauchi, T., Hozumi, I., Inuzuka, T. & O'Brien, J.S. (2003) Regulation of gene expression in response to brain injury: enhanced expression and alternative splicing of rat prosaposin (SGP-1) mRNA in injured brain. *J. Neurotrauma*, **20**, 755–765.

Kostovic, I. & Rakic, P. (1990) Developmental history of the transient subplate zone in the visual and somatosensory cortex of the macaque monkey and human brain. *J. Comp. Neurol.*, **297**, 441–470.

Lacy, S.F., Bonnemann, C.G., Buzney, E.A. & Kunkel, L.M. (1999) Identification of FLRT1, FLRT2 and FLRT3: a novel family of transmembrane leucine-rich repeat proteins. *Genomics*, **62**, 417–426.

Lee, C.H., Chinpaisal, C. & Wei, L.N. (1998) Cloning and characterization of mouse RIP140, a corepressor for nuclear orphan receptor TR2. *Mol. Cell Biol.*, **18**, 6745–6755.

Liu, G., Loraine, A.E., Shigeta, R., Cline, M., Cheng, J., Valmeekam, V., Sun, S., Kulp, D. & Siani-Rose, M.A. (2003) NetAffx: Affymetrix probesets and annotations. *Nucl. Acids Res.*, **31**, 82–86.

Lukazewicz, A., Savatier, P., Cortay, V., Giroud, P., Huissoud, C., Berland, M., Kennedy, H. & Dehay, C. (2005) G1 phase regulation, area-specific cell cycle control and cytoarchitectonics in the primate cortex. *Neuron*, **47**, 323–325.

Mallamaci, A. & Stoykova, A. (2006) Gene networks controlling early cerebral cortex arealization. *Eur. J. Neurosci.*, **23**, 847–856.

Mallamaci, A., Iannone, R., Briata, P., Pintonello, L., Mercurio, S., Boncinelli, E. & Corte, G. (1998) EMX2 protein in the developing mouse brain and olfactory area. *Mech. Dev.*, **77**, 165–172.

Mallamaci, A., Muzio, L., Chan, C.H., Parnavelas, J. & Boncinelli, E. (2000) Area identity shifts in the early cerebral cortex of Emx2-/- mutant mice. *Nat. Neurosci.*, **3**, 679–686.

Mann, F., Zhukareva, V., Pimenta, A., Levitt, P. & Boltz, J. (1998) Membrane-associated molecules guide limbic and nonlimbic thalamocortical projections. *J. Neurosci.*, **18**, 9409–9419.

Manzanares, M., Locascio, A. & Nieto, M.A. (2001) The increasing complexity of the Snail gene superfamily in metazoan evolution. *Trends Genet.*, **17**, 178–181.

Marin-Padilla, M. (1978) Dual origin of the mammalian neocortex and evolution of the cortical plate. *Anat. Embryol. (Berl.)*, **152**, 109–126.

Maruoka, Y., Ohbayashi, N., Hoshikawa, M., Itoh, N., Hogan, B.L.M. & Furuta, Y. (1998) Comparison of the expression of three highly related genes, Fgf8, Fgf17 and Fgf18, in the mouse embryo. *Mech. Dev.*, **74**, 175–177.

McConnell, S.K. (1988) Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. *J. Neurosci.*, **8**, 945–974.

McConnell, S.K. & Kaznowski, C.E. (1991) Cell cycle dependence of laminar determination in developing neocortex. *Science*, **254**, 282–285.

Medina, L., Legaz, I., Gonzalez, G., De Castro, F., Rubenstein, J.L. & Puelles, L. (2004) Expression of Dbx1, Neurogenin 2, Semaphorin 5A, Cadherin 8, and Emx1 distinguish ventral and lateral pallial histogenetic divisions in the developing mouse claustroamygdaloid complex. *J. Comp. Neurol.*, **474**, 504–523.

Miyashita-Lin, E.M., Hevner, R., Wassarman, K.M., Martinez, S. & Rubenstein, J.L. (1999) Early neocortical regionalization in the absence of thalamic innervation. *Science*, **285**, 906–909.

Molnar, Z., Adams, R. & Blakemore, C. (1998) Mechanisms underlying the early establishment of thalamocortical connections in the rat. *J. Neurosci.*, **18**, 5723–5745.

Monuki, E.S., Porter, F.D. & Walsh, C.A. (2001) Patterning of the dorsal telencephalon and cerebral cortex by a roof plate-Lhx2 pathway. *Neuron*, **32**, 591–604.

Moorman, A.F., Houweling, A.C., de Boer, P.A. & Christoffels, V.M. (2001) Sensitive nonradioactive detection of mRNA in tissue sections: novel application of the whole-mount in situ hybridization protocol. *J. Histochem. Cytochem.*, **49**, 1–8.

Muhlfriedel, S., Kirsch, F., Gruss, P., Stoykova, A. & Chowdhury, K. (2005) A roof plate-dependent enhancer controls the expression of Homeodomain only protein in the developing cerebral cortex. *Dev. Biol.*, **283**, 522–534.

Muzio, L. & Mallamaci, A. (2005) Foxg1 confines Cajal-Retzius neurogenesis and hippocampal morphogenesis to the dorsomedial pallium. *J. Neurosci.*, **25**, 4435–4441.

Muzio, L., DiBenedetto, B., Stoykova, A., Boncinelli, E., Gruss, P. & Mallamaci, A. (2002a) Emx2 and Pax6 control regionalization of the pre-neurogenic cortical primordium. *J. Neurosci.*, **19**, 877–885.

Muzio, L., DiBenedetto, B., Stoykova, A., Boncinelli, E., Gruss, P. & Mallamaci, A. (2002b) Conversion of cerebral cortex into basal ganglia in *Emx2*$^{-/-}$ *Pax6*$^{Sey/Sey}$ double mutant mice. *Nat. Neurosci.*, **5**, 737–745.

Nakagawa, Y., Johnson, J.E. & O'Leary, D.D. (1999) Graded and areal expression patterns of regulatory genes and cadherins in embryonic neocortex independent of thalamocortical input. *J. Neurosci.*, **19**, 10 877–10 885.

Nakakura, E.K., Watkins, D.N., Schuebel, K.E., Sriuranpong, V., Borges, M.W., Nelkin, B.D. & Ball, D.W. (2001) Mammalian Scratch: a neural-specific Snail family transcriptional repressor. *Proc. Natl Acad. Sci. U.S.A.*, **98**, 4010–4015.

Nastasi, T., Scaturro, M., Bellafiore, M., Raimondi, L., Beccari, S., Cestelli, A. & di Liegro, I. (1999) PIPPin is a brain-specific protein that contains a cold-shock domain and binds specifically to H1 degrees and H3.3 mRNAs. *J. Biol. Chem.*, **274**, 24 087–24 093.

Nieto, M., Monuki, E.S., Tang, H., Imitola, J., Haubst, N., Khoury, S.J., Cunningham, J., Gotz, M. & Walsh, C.A. (2004) Expression of Cux-1 and Cux-2 in the subventricular zone and upper layers II–IV of the cerebral cortex. *J. Comp. Neurol.*, **479**, 168–180.

O'Flaherty, E. & Kaye, J. (2003) TOX defines a conserved subfamily of HMG-box proteins. *BMC Genomics*, **4**, 13.

Ogawa, M., Miyata, T., Nakajima, K., Yagyu, K., Seike, M., Ikenaka, K., Yamamoto, H. & Mikoshiba, K. (1995) The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron*, **14**, 899–912.

O'Leary, D.D. (1989) Do cortical areas emerge from a protocortex? *Trends Neurosci.*, **12**, 400–406.

O'Leary, D.D. & Wilkinson, D.G. (1999) Eph receptors and ephrins in neural development. *Curr. Opin. Neurobiol.*, **9**, 65–73.

O'Leary, D.D.M. & Nakagawa, Y. (2002) Patterning centers, regulatory genes and extrinsic mechanisms controlling arealization of the neocortex. *Curr. Opin. Neurobiol.*, **12**, 14–25.

Perez, R.G. & Lewis, R.M. (1992) Regional distribution of DARPP-32 (dopamine- and adenosine 3′,5′-monophosphate-regulated phosphoprotein of Mr = 32,000) mRNA in mouse brain. *J. Comp. Neurol.*, **318**, 304–315.

Pimenta, A.F., Reinoso, B.S. & Levitt, P. (1996) Expression of the mRNAs encoding the limbic system-associated membrane protein (LAMP). II. Fetal rat brain. *J. Comp. Neurol.*, **375**, 289–302.

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.

Polleux, F., Dehay, C., Goffinet, A. & Kennedy, H. (2001) Pre- and post-mitotic events contribute to the progressive acquisition of area-specific connectional fate in the neocortex. *Cereb. Cortex*, **11**, 1027–1039.

Porter, F.D., Drago, J., Xu, Y., Cheema, S.S., Wassif, C., Huang, S.P., Lee, E., Grinberg, A., Massalas, J.S., Bodine, D., Alt, F. & Westphal, H. (1997) Lhx2, a LIM homeobox gene, is required for eye, forebrain, and definitive erythrocyte development. *Development*, **124**, 2935–2944.

Rakic, P. (1988) Specification of cerebral cortical areas. *Science*, **241**, 170–176.

Remedios, R., Subramanian, L. & Tole, S. (2004) LIM genes parcellate the embryonic amygdala and regulate its development. *J. Neurosci.*, **24**, 6986–6990.

Robinson, M., Parson Perez, M.C., Tebar, L., Palmer, J., Patel, A., Marks, D., Sheasby, A., De Felipe, C., Coffin, R., Livesey, F.J. & Hunt, S.P. (2004) FLRT3 is expressed in sensory neurons after peripheral nerve injury and regulate neurite outgrowth. *Mol. Cell Neurosci.*, **27**, 2001–2214.

Sansom, S.N., Hébert, J.M., Thammongkol, U., Smith, J., Nisbet, G., Surani, M.A., McConnell, S.K. & Livesey, F.J. (2005) Genomic characterisation of a Fgf-regulated gradient-based neocortical protomap. *Development*, **132**, 3947–3961.

Schlaggar, B.L. & O'Leary, D.D. (1991) Potential of visual cortex to develop an array of functional units unique to somatosensory cortex. *Science*, **252**, 1556–1560.

Shimamura, K. & Rubenstein, J.L. (1997) Inductive interactions direct early regionalization of the mouse forebrain. *Development*, **124**, 2709–2718.

Shimamura, K., Hartigan, D.J., Martinez, S., Puelles, L. & Rubenstein, J.L. (1995) Longitudinal organization of the anterior neural plate and neural tube. *Development*, **121**, 3923–3933.

Shimogori, T., Banuchi, V., Ng, H.Y., Strauss, J.B. & Grove, E.A. (2004) Embryonic signaling centers expressing BMP, WNT and FGF proteins interact to pattern the cerebral cortex. *Development*, **131**, 5639–5647.

Simeone, A., Gulisano, M., Acampora, D., Stornaiuolo, A., Rambaldi, M. & Boncinelli, E. (1992) Two vertebrate genes related to *Drosophila empty spiracles* gene are expressed in embryonic cerebral cortex. *EMBO J.*, **11**, 2541–2550.

Steel, K.P., Davidson, D.R. & Jackson, I.J. (1992) TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development*, **115**, 1111–1119.

Storm, E.E., Rubenstein, J.L. & Martin, G.R. (2003) Dosage of FGF8 determines whether cell survival is positively or negatively regulated in the developing forebrain. *Proc. Natl Acad. Sci. U.S.A.*, **100**, 1757–1762.

Sur, M. & Rubenstein, J.L. (2005) Patterning and plasticity of the cerebral cortex. *Science*, **310**, 805–810.

Suzuki, S.C., 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.

Tarabykin, V., Stoykova, A., Usman, N. & Gruss, P. (2001) Cortical upper layer neurons derive from the subventricular zone as indicated by Svet1 gene expression. *Development*, **128**, 1983–1993.

Tole, S. & Grove, E.A. (2001) Detailed field pattern is intrinsic to the embryonic mouse hippocampus early in neurogenesis. *J. Neurosci.*, **21**, 1580–1589.

Tole, S., Christian, C. & Grove, E.A. (1997) Early specification and autonomous development of cortical fields in the mouse hippocampus. *Development*, **124**, 4959–4970.

Tsuji, L., Yamashita, T., Kubo, T., Madura, T., Tanaka, H., Hosokawa, K. & Tohyama, M. (2004) FLRT3, a cell surface molecule containing LRR repeats and a FNIII domain, promotes neurite outgrowth. *Biochem. Biophys. Res. Commun.*, **313**, 1086–1091.

Visel, A., Thaller, C. & Eichele, G. (2004) GenePaint.org: An atlas of gene expression patterns in the mouse embryo. *Nucl. Acids Res.*, **32**, D552–D556.

Vyas, A., Saha, B., Lai, E. & Tole, S. (2003) Paleocortex is specified in mice in which dorsal telencephalic patterning is severely disrupted. *J. Comp. Neurol.*, **466**, 545–553.

Walther, C. & Gruss, P. (1991) Pax-6, a murine paired box gene, is expressed in the developing CNS. *Development*, **113**, 1435–1449.

Wei, L.N., Hu, X., Chandra, D., Seto, E. & Farooqui, M. (2000) Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing. *J. Biol. Chem.*, **275**, 40 782–40 787.

Wilkinson, B., Chen, J.Y., Han, P., Rufner, K.M., Goularte, O.D. & Kaye, J. (2002) TOX: an HMG box protein implicated in the regulation of thymocyte selection. *Nat. Immunol.*, **3**, 272–280.

Zapala, M.A., Hovatta, I., Ellison, J.A., Wodicka, L., Del Rio, J.A., Tennant, R., Tynan, W., Broide, R.S., Helton, R., Stoveken, B.S., Winrow, C., Lockhart, D.J., Reilly, J.F., Young, W.G., Bloom, F.E. & Barlow, C. (2005) Adult mouse brain gene expression patterns bear an embryologic imprint. *Proc. Natl Acad. Sci. U.S.A.*, **102**, 10 357–10 362.

Zhou, C., Tsai, S.Y. & Tsai, M.J. (2001) COUP-TFI: an intrinsic factor for early regionalization of the neocortex. *Genes Dev.*, **15**, 2054–2059.

Zhou, X.H., Brandau, O., Feng, K., Oohashi, T., Ninomiya, Y., Rauch, U. & Fässler, R. (2003) The murine Ten-m/Odz genes show distinct but overlapping expression patterns during development and in adult brain. *Gene Exp. Patt.*, **3**, 397–405.

Zimmer, C., Tiveron, M.C., Bodmer, R. & Cremer, H. (2004) Dynamics of Cux2 expression suggests that an early pool of SVZ precursors is fated to become upper cortical layer neurons. *Cereb. Cortex*, **14**, 1408–1420.