The mammalian neocortex develops layer organizations with regional differences represented by expression of multiple genes at embryonic stages. These genes could play important roles in the formation of areal cyto-architecture, yet, the number of genes identified so far is not sufficient to explain such intricate processes. Here we collected five regions — the medial, dorsal, lateral, rostral and occipital — from the dissected E16.5 mouse cerebral cortex and performed extensive gene expression analysis using the Affymetrix U74Av2 array with probes for 12,500 genes. After relative quantitative analysis, 34, 33 and 15 genes were selected as highly expressed genes in the medial, dorsal and lateral regions, respectively. The combination of GeneChip system, real-time quantitative reverse transcription polymerase chain reaction and in situ hybridization analyses allowed the successful identification of seven genes from the dorsal region (Neuropeptide Y, Wnt7b, Tgf-β, Rl, Nrf3, Bcl-6, MT4-MMP and Rptp), three genes from the medial region (Hoppending, HtrA and Crystallin), and three genes from the lateral region (Somatostatin, Ngf and Fxyd7). Particularly, all seven genes identified in the dorsal region demarcated the future somatosensory and auditory areas in the cortical plate with high rostro-lateral–low caudomedial gradation. Their expression patterns were not uniform, but delineated either the superficial or the deep layer in the cortical plate. Furthermore, the regional expression pattern of Neuropeptide Y was shifted rostrally and the layer specificity was disorganized in the Pax6-deficient mice. Our results provide new information about a subclass of regionally expressed genes in the cortical plate at the late embryonic stage, which may help understand the molecular mechanisms of neocortical arealization.

Keywords: cadherin 6, development, neocortical area, Pax6, regionalization.

Introduction

The mammalian neocortex develops in the dorsal pallium of embryonic telencephalic vesicle, later parcellating many areas with distinct functions and cytoarchitectures. According to the radial unit hypothesis, postmitotic neurons generated in the telencephalic ventricular zone (VZ), migrate marginally along the radial glial fibers and populate between the layers of Cajal–Retzius cells and subplate neurons in an inside-out manner, forming the cortical plate (CP) with laminated organizations (Rakic, 1988). The cortical ‘regions’ with unclear borders and/or the immature laminar structures are then converted at postnatal stages to area-specific cytoarchitectures with sharp limits. It has been assumed that cortical area formation is controlled by interplay between intrinsic and extrinsic mechanisms (Rakic, 1988; O’Leary, 1989). For instance, the thalamocortical axons (TCAs) are thought to exert major extrinsic influences to specify the cortical primordium into cytoarchitecturally and functionally different areas (O’Leary et al., 1994). It has indeed been demonstrated that electric activities of the TCAs play a significant role in shaping intracortical neural networks at the postnatal stages (Sur and Leamy, 2001). On the other hand, several recent studies have confirmed the intrinsic effects of various secreted molecules and transcription factors on neocortical patterning (Crossley et al., 2001; Monuki and Walsh, 2001; Ragsdale and Grove, 2001; Ruiz i Altaba et al., 2001; Hebert et al., 2002; O’Leary and Nakagawa, 2002; Ohkubo et al., 2002; Garel et al., 2003). For instance, when FGF8 was ectopically expressed in the early neocortex primordium, the duplicate mirror image ‘barrels’, the cortical topographic representative of the rodent somatosensory pathway, was induced in the caudal neocortex (Wooley and Van der Loos, 1970; Fukuchi-Shimogori and Grove, 2001, 2003). In addition, the homeodomain transcription factor EMX2 and the paired-box domain transcription factor PAX6 are expressed in the cortical VZ at the early stage with gradual and opposing fashions; the EMX2 is most abundant in the caudomedial cortex whereas the PAX6 in the rostral-lateral cortex. Mice deficient in EMX2 and PAX6 show shifts in the gene expression of some regional markers and abnormal thalamocortical connectivity, indicating that these factors are probably essential to areal specification under the regulation of downstream gene expressions (Bishop et al., 2000, 2002; Mallamaci et al., 2000; Muzio and Mallamaci, 2003). For the PAX6, a role in patterning of the lateral cortex has also been reported (Toresson et al., 2000; Yun et al., 2001; Hirata et al., 2002). Region and layer specifications in the later embryonic cortex are further known to be represented by expression of multiple genes including cell–cell recognition molecules such as cadherins (Suzuki et al., 1997; Inoue et al., 1998; Nakagawa et al., 1999; Rubenstein et al., 1999), immunoglobulin superfamily members (Pimenta et al., 1996) and Ephs/Ephrins (Donoghue and Rakic, 1999; O’Leary and Wilkinson, 1999; Prakash et al., 2000; Vanderhaeghen et al., 2000; Yun et al., 2003), Wnt receptors and inhibitors (Kim et al., 2001b), transcription factors and some other classes of genes (Nakagawa et al., 1999; Rubenstein et al., 1999). Such genes are probably involved in the conversion of cortical regions into functional areas together with extrinsic mechanisms. However, the numbers of regionally expressed genes are not well defined, and thus the molecular machinery involved in neocortical regionalization is poorly understood.

DNA microarray is currently accepted as a tool for gene expression analysis and has many advantages with respect to extensiveness, quantity, reproducibility and possibilities, compared with conventional methods such as differential display or subtraction technique (Lockhart and Winzeler, 2000). In the present study, we undertook an extensive screening approach to identify regionally expressed genes in

Gene Expression Analysis of the Late Embryonic Mouse Cerebral Cortex Using DNA Microarray: Identification of Several Region- and Layer-specific Genes

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the mouse cortex at E16.5 when the TCAs are vertically projecting to the CP with topography (Killackey et al., 1995). Using the GeneChip system with U74Av2 mouse arrays (Affymetrix), combined with a real-time quantitative reverse transcription polymerase chain reaction (QRT-PCR) and in situ hybridization analyses, we identified seven genes from the dorsal region (Neuropeptide Y, Wnt7b, TGFβRI, Nrfl3, Bcl-6, MT4-MMP and Rplt6), three genes from the medial region (Hop-pending, HtrA and Crystallin), and three genes from the lateral region (Somatostatin, Ngef and Fxyd7). In particular, the genes identified in the dorsal region were characterized by their regional distribution with high rostro-lateral–low caudo-medial gradation and the layer specificities were classified in two types (delineated either the superficial or deep layer). Furthermore, the region- and layer-specific expression pattern of Neuropeptide Y (Npy) was disorganized in Pax6-deficient mice. These results suggest that genes identified in this study play important roles in the formation of neocortical area-specific cytoarchitectures.

Materials and Methods

Animals

Time pregnant ICR mice were purchased from Clea JAPAN and used for all analyses. Pax6-deficient mice (Sey/+ CBA background), in which a point mutation in the Pax6 results in the generation of truncated nonfunctional protein (Hill et al., 1991), were provided by Drs Veronica van Heyningen and Pen Rashbass at the Medical Research Council Human Genetics Unit. For experiments, we isolated brains from embryonic day (E) 18.5 homoygous and wild-type embryos, which were phenotypically distinguishable. In stage the embryos, the midday of the vaginal plug was considered as E0.5. The experimental protocol was approved by the Ethics review Committee for Animal Experimentation of National Institute of Neuroscience (NCNP).

Dissection of E16.5 Cortex for RNA Extraction

After administration of sodium pentobarbital to timed-pregnant female, E16.5 embryos were removed by cesarean section in ice-cold phosphate-buffered saline (−)(Nissui, Japan). Whole brains were dissected out from the decapitated embryos and their hemispheres were isolated. The diencephalon and mesencephalon were excluded from the hemispheres. They were equally divided into three regions along the dorsal–ventral (D–V) axis: the anterior third and the posterior third were named the rostral and the occipital region, respectively (Fig. 2A). The middle third tissues were then separated into four pieces along the anterior–posterior (A–P) axis; the dorsal-most, dorsal, and ventral-most were named the medial, dorsal and lateral regions, respectively (Fig. 2A). After separation, the latter two tissue areas were removed from the ganglionic eminences. The above five dissected tissues were homogenized in ice-cold Sepazol RNA solution (Nacalai tesque, Japan), followed by immediate freezing on a block of dry-ice, and were then kept at −80°C until used for RNA preparation. For subsequent RNA extraction, we used frozen samples that could be prepared within one hour from cesarean section to homogenization in Sepazol RNA solution.

Preparation of RNA and Synthesis of cDNA

Total RNA was prepared using the protocol provided by the manufacturer of Sepazol RNA (Nacalai tesque). Poly A+ RNA was obtained through twice column procedures with oligo dT cellulose column (New England Biolabs, Beverly, MA) and was used for synthesis of cDNA. One microgram of polyA+ RNA was converted into double-stranded cDNA by reverse transcription using ReverTra Ace (ToYoBo, Japan) with oligo dT(24) primers containing the T7 RNA polymerase promoter sequence (Amersham Pharmacia Biotech, Japan). The integrity of RNA was confirmed by agarose electrophoresis and RNA contents were analyzed by measuring the optical density at 260 nm.

Target Preparation and Array Processing

The following procedures including preparation of cRNA targets, hybridization, washing, staining and scanning were performed as described in the Expression Analysis Technical Manual (Affymetrix, CA). Briefly, biotin-labeled antisense cRNA targets were synthesized in in vitro transcription reaction system (IVT) using cDNA as template (ENZO BioArray’s HighYield™ RNA Transcription Labeling kit with T7 RNA polymerase, Enzo, NY). The cRNA targets were size-fragmented to 51–200 bp in a buffer containing potassium and magnesium acetate at 94°C for 35 min and were used for hybridization as cRNA targets. The hybridization cocktail [containing 10 µg of target cRNA, control oligo BS (Affymetrix), and Eukaryotic Hybridization controls (Affymetrix)] was hybridized with a mouse U74Av2 array at 42°C for 16 h in GeneChip Hybridization Oven 640. Washing and staining were performed after hybridization under the fluids station protocol. EuGeneWS2 on GeneChip Fluidics Station 400. Then, we scanned the array twice on HP GeneArray™ scanner. Experiments were repeated twice with independent dissection.

Data Analysis

To quantify the expression level of a transcript, the average difference value (Adv) was calculated from the intensities against the probe cells using the global methods of normalization by Microarray Suite v. 4.0 (MAS4) (Affymetrix). Percent Present (%P) value and the ratio of 5′/5′′ probes of actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe sets were analyzed to assess the quality of the RNA sample and assay performance. The reproducibility between duplicate experiments was analyzed using a parameter designated as scaling intensity of 500 (Sandberg et al., 2000). The subsequent criteria were that (i) an expression change between the two experiments was considered to be ‘increased’, ‘marginally increased’, ‘decreased’ or ‘marginally decreased’, and (ii) the fold change value was up to 1.8. The raw data analyzed by MAS4 were available at http://www.ncbi.nlm.nih.gov/geo/ (accession numbers: GSM41591 ~ 1602; GSE9229).

The level of gene expression was compared among the five regions by GeneSpring 4.2 software (Silicon Genetics, CA). Adv was normalized by the per-chip normalization method with the default percentile (50th) to the distribution of all genes (nAdv). The average value of nAdv in duplicate experiments was calculated and used for the subsequent cluster analysis. Genes meeting the following conditions were selected as differentially abundant genes in each region: (i) Genes analyzed by MAS4 were ‘present’ in both experiments, (ii) nAdv was up to 0.5, and (iii) the fold change (FC) between baseline and experimental data sets was up to 1.5. With regard to selection in the lateral region, the FC value of 3.0 was used. To display these levels in a similar scale on the same graph in Figure 3, the nAdv was normalized to ‘Median’ for each gene among the five regions by the GeneSpring software.

In Situ Hybridization

Preparation of digoxigenin (Dig)-UTP-labeled RNA probes and in situ hybridization were performed as described previously with slight modifications (Inoue et al., 1998). Briefly, whole brains were removed after cesarean section and post-fixed (4°C, overnight), followed by immersion in a graded series of sucrose solutions (10–15–20%), embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Japan), and were then frozen on a block of dry-ice. Sagittal or coronal sections (10–20 µm) were prepared by cryostat microtome (Leica, Germany) and collected on slide glasses coated by Vectabond Reagent (Vector Laboratories, Burlingame, CA). For screening, two sets of nonconsecutive sagittal sections corresponding to the dorsal and medial cortex and three sets of nonconsecutive coronal sections corresponding to the frontal, parietal and occipital cortex were analyzed. The following steps were similar to those described by Inoue et al. (1998), except for adding 1 µg/ml proteinase K digestion (37°C, 5 min) after treatment with 0.2 N HCl. Stained sections were photographed under bright field on a Carl Zeiss Axiosplath 2 microscope.
Real-time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Each PCR reaction contained 5 ng of cDNA, 10 µl of 2X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and 50–900 nM of optimized primer pair. Primer sequences were designed using Primer Express™ v. 1.5 (Applied Biosystems). Primer sequences were all searched by BLAST to exclude other homologous genes to be amplified by them. Real-time quantitative PCR was performed in ABI PRISM 7700 Sequence Detection System by using the default thermal cycle condition (2 min at 50°C plus 10 min at 95°C for the hot start, and then 40 cycles of 15 s at 95°C plus 1 min at 60°C for amplification). Relative quantitation was performed using the comparative cycle threshold (Ct) method, as recommended by the manufacturer. The Ct value of each region was compared to that of E16.5 hemispheres using the GAPDH gene as an endogenous control and the resulting values were defined as ratio value. Duplicate experiments were repeated more than three times. Specific amplification of the PCR product was confirmed by ethidium bromide staining after 2% agarose gel electrophoresis.

Results

Regional Gene Expression in the Late Embryonic Cortex

First we examined the expression pattern of Cadherin-6 (Cdh6) in the developing mouse cortex by in situ hybridization to design a dissecting procedure in preparing target samples for GeneChip analyses. Cdh6 is a dorsal marker gene for the embryonic cortical plate (CP) and the expression domain postnatally demarcates the somatosensory and auditory cortex (Suzuki et al., 1997; Inoue et al., 1998). Consistently, Cdh6 expression was already detected in the dorsal CP at around E14.5, while it was weak and thin (Fig. 1A). As the CP development proceeded, the staining signal significantly increased and thickened at around E15.5 and the expression level peaked at E16.5 (Fig. 1B,C). This expression pattern was maintained until E18.5 (Fig. 1D) and gradually decreased in the postnatal ages (data not shown). Notably, Cdh6 expression pattern at E16.5 came to set a clear boundary between the neocortex and paleocortex (Fig. 1E). Gradual expression patterns at this stage also became clear along both D-V and A-P axes of the Cdh6 expression domain: the D-V gradient was higher in the ventral, while the A-P gradient was higher in the anterior (Fig. 1E; Inoue et al., 1998). Then we further examined if other regional markers such as Cadherin-8 (Cdh8), Klon and Lamp show restricted patterns of expressions in the E16.5 CP. In contrast to the expression pattern of Cdh6, the staining signal of Cdh8 at E16.5 was substantially found in the medial cortex; the future area for the hippocampus, cingulate cortex, and neocortical motor and visual areas (Fig. 1F). Expression of Cdh8 decreased gradually from the medial to the dorsal cortex along the D-V axis. Meanwhile, the future somatosensory and auditory cortex (CP) expressed Cdh8 at E16.5 (Fig. 1G). Expression of another member of IgLONs family, Lamp, was detected in the dorsal, lateral and medial cortex of E16.5 in our system (data not shown: Pimenta et al., 1996). Based on the above results, we concluded that the mouse CP at the E16.5 onward could molecularly and/or genetically be separated into at least three parts along the AP axis as well as along the D-V axis. Hence, we chose the E16.5 mouse cerebral cortex as a good source for subsequent analyses and divided the tissue into six regions to collect five of them: the medial (M), dorsal (D), lateral (L), rostral (R) and occipital region (O) (Fig. 2A).

Tissue Preparation for GeneChip Analysis

In the process of embryonic sample preparations, we used >50 embryos for each GeneChip experiment to reduce potential inaccuracy in dissection. Starting from 50 embryonic brains, 4–9 µg of poly A+ RNA could be normally isolated from each region. After preparation of poly A+ RNAs, QRT-PCR analysis was performed for the regional markers to examine the adequacy of tissue dissection and integrity of RNAs using ABI 7700 system with the SYBR Green. Figure 2B-D shows the relative expression levels of marker genes among the five regions. Expression level of Cdh6 in the dorsal region was significantly the highest among the five regions (P < 0.01) (Fig. 2B). Expression levels of Cdh8 in the medial and lateral region were higher than those in the other three regions (Fig. 2C). Expression level of Klon was the highest in the lateral region (Fig. 2D) (P < 0.0005), followed by the occipital region, which may represent the expression in the entorhinal cortex (Fig. 1G). These expression patterns analyzed by QRT-PCR closely matched those examined by in situ hybridization (Figs 1 and 2), indicating that each dissected sample contained transcripts of high integrities and well kept endogenous levels of gene expressions through the experimental procedures. Thus, we used these samples for subsequent GeneChip analyses.

GenecChip Analyses

We next performed GeneChip analyses in duplicate by using the Affymetrix mouse U74Av2 array and target cRNAs prepared from each of the five regions. The qualities of target cRNA samples were considered good enough because the 5′/3′ signal ratio for GAPDH and beta-actin ranged from 0.86 to 1.02 in all assays. Reproducibility, which was represented by the number of genes having different expression levels between duplicate experiments (see Materials and Methods), was only 56 probes (0.4%) of 12 473 on average: 64 probes in the medial (0.5%); 24 probes in the dorsal (0.2%); 33 probes in the lateral (0.3%); 80 probes in the rostral (0.6%); and 78 probes in the occipital regions (0.6%). The average of present probe numbers was 53% in the medial, 53% in the dorsal, 52% in the lateral, 55% in the rostral, and 50% in the occipital region. Taken together, the quality of samples, arrays and assay performance was considered to be high.

We then examined whether our results from GeneChip analyses could reflect the expression levels of the known genes in the late embryonic CP. As summarized in Table 1, we found that relatively higher expressions of Wnt7b, SCIP, Sfpi2 and Cdh6 in the dorsal CP were clearly detected in our GeneChip analysis (see under ‘dorsal markers’). Likewise, our gene profiling completely recapitulated the medially higher expression of Preslomin, Mixx-I and EphA4, as well as the laterally higher expression of Latexin (see under ‘medial markers’ and ‘lateral markers’). On the other hand, Cdh8, other Ephs/ Ephrins, COUP-TFI, CHL1 and TNG36/Occ-1, whose expression patterns were reported to form a gradient along the A-P axis, displayed subtle regional differences with our expression indices (see under ‘other markers’). Taken together, the differential expression patterns along the D-V axis would likely be detected, whereas those along the A-P axis might be masked in our dissection strategy and/or GeneChip analyses. Unfortunately, any sequences encoding Klon, Lamp, Emx1, Fgf8,
Lhx2, p75 and RZR-beta were not included on the array, preventing us from further evaluation of this point.

It should also be noted that the expression level of a transcript in one sample can be correctly estimated in this system. For instance, the total level of Cdh6 among samples was very low (0.1–0.4) while that of Id2, Tbr-1 and BF-1 was very high (Id2: 6.1–12.0; Tbr-1: 5.3–8.1; BF-1: 3.4–7.4) (see under ‘other markers’). Moreover, we noticed that the expression of other genes including Bmp7, EphrinA5, Fgfr3, GABAa, Lef-1, Mnx2, Neuropillin2, Wnt2b, Wnt3a, Wnt5a and Wnt7a were estimated as ‘absent’ in relevant cortical regions by Microarray Suite (MAS) software. These results were due to low copies of transcripts and/or high ratio of cross-hybridization of target cRNAs with unspecific probes (data not shown).

We then screened ~12 500 genes on the mouse U74Av2 array to identify regionally expressed genes in the late embryonic cortex according to the following criteria: (i) genes analyzed by MAS4 were ‘present’ in an essential region; (ii) the ‘signal’ representing the expression level in an essential region was up to 0.5; and (iii) the fold change (FC) in an essential region was up to 3.0 for the lateral region and 1.5 for the other regions (see Materials and Methods). We identified the differential expression of 34 genes in the medial (0.56% of all ‘present’ genes); 33 genes in the dorsal (0.55% of all ‘present’ genes); and 15 in the lateral (0.25% of all ‘present’ genes) (Table 2). On the other hand, four genes were detected in the rostral (0.06% of all ‘present’ genes) and none in the occipital region (data not shown). Tables 3–5 (see supplementary material) provide lists

Figure 1. Combinatorial gene expression subdivides the embryonic cerebral cortex into several regions. In situ hybridization for known region-specific marker genes was performed on coronal sections including the parietal cortex of embryonic mouse brain. (A–D) Time course of Cdh6 expression pattern in the dorsal CP (*), at E14.5 (A), E15.5 (B), E16.5 (C) and E18.5 (D). Note that Cdh6 signal was already found at E14.5 and the level peaked at E16.5. (E) Cdh6 signal at E16.5 was detected in the dorsal CP (*) as well as VZ with a high lateral–low medial gradation. (F) Cdh8 expression at E16.5 demarcated the medial and dorsal cortex (***) with a high medial–low lateral gradation. (G) KilomRNA was strongly detected in the E16.5 lateral cortex including the piriform cortex (***)]. Arrowheads indicate the sharp boundary between the neocortex and paleocortex (E, G). Note that combinatorial gene expression subdivided the E16.5 cerebral cortex into several regions. Scale bars. 0.2 mm in (A)–(F), 0.1 mm in (G).
of the above genes with expression levels (see the supplementary data). The raw data analyzed by MAS4 were available at http://www.ncbi.nlm.nih.gov/geo/ (accession numbers: GSM14591 – 14602; GSE929).

Quantitative RT-PCR Analysis

Following the GeneChip analyses, we performed QRT-PCR analyses for the 15 genes in order of expression level in the medial, 33 in the dorsal and 15 in the lateral among the above selected genes (Table 2). The number of regionally expressed genes was then narrowed down by comparative gene expression analysis with t-test ($P < 0.01$). We found 5 genes in the medial, 15 genes in the dorsal and 8 genes in the lateral to be significantly abundant in each region (the resultant genes are marked by '†', '‡' and '¶' in Table 2). Actual fold change values of the above genes were comparable between QRT-PCR analysis and GeneChip analysis (data not shown).

In Situ Hybridization Analysis

Using in situ hybridization, we finally examined the expression patterns of candidate genes selected by the QRT-PCR analyses. cDNA clones for 4 genes in the medial, 14 in the dorsal and 5 genes in the lateral region were successfully obtained by RT-PCR using polyA+ RNA of each region as template, but

Figure 2. Dissection of the E16.5 mouse cerebral cortex and relative expression levels of marker genes tested by real-time quantitative RT-PCR (QRT-PCR) analysis. (A) Orderly dissection of the E16.5 mouse cerebral cortex separates the regions demarcated by marker gene expressions. Briefly, the E16.5 mouse hemispheres were divided into three along the anterior-posterior axis and the middle part was cut into four along the dorso-ventral axis. Five of them were selected for subsequent QRT-PCR and/or GeneChip analyses and designated as the medial (M), dorsal (D), lateral (L), rostral (R) and occipital (O). (B–D) QRT-PCR analysis was performed using specific sets of primers for detecting Cdh6 (B), Cdh8 (C) and Kilon (D). cDNAs from the medial (M), dorsal (D), lateral (L), rostral (R), occipital regions (O) and E16.5 hemispheres were used as templates. The comparative cycle threshold value (Ct) of each region was compared to that of E16.5 hemispheres using the GAPDH gene as an endogenous control. Ratio values represented the mean of (expression levels of transcripts in each region/expression levels of transcripts in E16.5 hemispheres). Note that expression pattern of each gene was closely matched with that examined by in situ hybridization analysis in Figure 1. In all cases, data were plotted as mean ± SEM derived from duplicate experiments ($n \geq 3$).
Table 1
Marker gene expression levels were represented by normalized Avd in five regions: the medial (M), dorsal (D), lateral (L), rostral (R) and occipital region (O).

<table>
<thead>
<tr>
<th>gene name</th>
<th>M</th>
<th>D</th>
<th>L</th>
<th>R</th>
<th>O</th>
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<td>0.1</td>
<td>0.6</td>
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Gene Expression Patterns in the Dorsal Region

As shown in Figure 3, we confirmed the region-specific expression of Neurotrophin 7 (Npy), Wnt7b and TGF-β RI.

In situ hybridization and QRT-PCR data of finally identified genes are arranged in Figure 3 and their regional expression patterns are summarized in Table 2. In situ hybridization and QRT-PCR data of finally identified genes are arranged in Figure 3 and their regional expression patterns are summarized in Table 2.

The following is a summary of the expression profile of the identified genes.

Gene Expression Patterns in the Dorsal Region

As shown in Figure 3, we confirmed the region-specific expression of Neurotrophin 7 (Npy), Wnt7b and TGF-β RI. In contrast to MT4-MMP, the staining signals of Rptpα within the CP were weak in the deep layer and strong in the superficial layer (Figs 3H, 4B-3). Staining of the former was intense and broadly distributed in the neocortex with a high rostral–lateral, low caudomedial gradation, but the latter showed weak signals that were restricted to the more rostrodorsal neocortex. Rptpα, a member of type Ib receptor tyrosine phosphatases, is a cell adhesion molecule with homophilic adhesion property (Jiang et al., 1993; Sap et al., 1994). In contrast to MT4-MMP, the staining signals of Rptpα within the CP were weak in the deep layer and strong in the superficial layer (Figs 3H, 4B-3). Except for these seven genes, the expression pattern of Mef2c (L13177), unknown AI851348, unknown AI426400, Gpr1 (AI846077) and Neurotensin receptor type 1 (AB017027) showed less regional patterns of gene expressions in the neocortex (data not shown).
Gene Expression Patterns in the Medial/Lateral Region

Antisense probes for Homeodomain only protein (Hop-pending) (AW123564), HtrA (AW125478) and Crystallin (AF039391) showed positive reaction in the medial cortex, the area of future the cingulate cortex and/or hippocampus (¶ and * in Table 2, Figs 3J–K, 4A). Hop-pending was expressed in the neuroepithelium of the cingulate cortex (cg) and hippocampus (hi) at E16.5 (Fig. 3J). Similarly, HtrA was expressed in the neuroepithelium of the hippocampus (hi), the subiculum (s), and the cingulate cortex (cg) (Fig. 3J). The Crystallin expression was found in the differentiating field of the hippocampus and the cingulate cortex (Cg) and QRT-PCR analysis showed that its expression level was high in the medial and occipital regions (* in Table 2, Fig. 3K). On the other

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**Table 2**

<table>
<thead>
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<th>Dorsal region (33)</th>
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<th>selection</th>
<th>Medial region (15)</th>
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**POU domain, class 3, transcription factor 1**

**Fibronectin 1**

**AB017026**

**EST, unknown**

**Neurotensin receptor type 1 (Ntsr)**

**Cenrin 3**

**B-cell leukaemia/lymphoma 6**

**Cytokine-receptor-like factor 1**

**Expressed sequence AW559717**

**Nif**

**Rglp k**

**MT4-MMP**

**ATPase, aminophospholipid transporter (APLT), class I, type 8A, member 1**

**RIKEN cDNA 5031419B10 gene**

**RIKEN cDNA 130001903 gene**

**Mek4**

**Calcium channel, voltage-dependent, alpha2/delta subunit 1**

**Mitogen activated protein kinase kinase 6**

**Nin**

**Expressed sequence AO041499**

**Nfr3**

**Ssd5**

**TbetaR1, TbetaR-1**

**Lateral region (15)**

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*Region-specific expression was confirmed by quantitative RT-PCR and in situ hybridization.

¶Region-specific expression was confirmed by quantitative RT-PCR, but not by in situ hybridization.

§Region-specific expression was confirmed by quantitative RT-PCR, but in situ hybridization analysis was impossible because partial cDNAs were not available.

*Region-specific expression was confirmed by in situ hybridization, but not by quantitative RT-PCR.

Blank column: region-specific expression was not confirmed by quantitative RT-PCR, and thus in situ hybridization was not performed.
Fig. 3

Dorsal cortex specific genes

A. Nissl

B. Npy

C. Wnt7b

D. TGF-β RI

E. Nrf3

F. Bcl-6

G. MT4-MMP

H. Rplo κ

<table>
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Intensity vs. region

Intensity vs. ratio

Significance levels:
- * p < 0.05
- ** p < 0.01
Figure 3. Expression patterns of genes identified in the E16.5 cortex. In situ hybridization analysis. (A–H) Sagittal sections corresponding to the dorsal cortex (sagittal); coronal sections corresponding to the parietal cortex (coronal); and the magnified views of the coronal sections (magnification) were arranged to show the Nissl staining (A) and the in situ hybridization results of Neuropeptide Y (B), Wnt7b (C), TGF-B RI (D), Nrf3 (E), Bcl-6 (F), MT4-MMP (G) and Rptp (H). The single asterisk represents positive signals in the deep CP layer. The double asterisks represent positive signals in the superficial CP layer. Note that expression of Neuropeptide Y, Wnt7b and Nrf3 was found in the subplate layer. (I–K) Sagittal sections corresponding to the dorsal cortex (left panel) and coronal sections corresponding to the parietal cortex (right panel) were used to examine Hop-pending (I), HtrA (J) and Crystallin (K) mRNA expression in the medial cortex. (L–N) Coronal sections including the central part of the parietal cortex (L, M) and the caudal part of the parietal cortex (N) were used to detect Somatostatin (L), Ngef (M) and Fxyd7 (N) expression in the lateral cortex by in situ hybridization. The clear boundaries between the piriform cortex and the perirhinal cortex were represented by arrows. Cg, cingulate cortex differential field; cg, cingulate cortex neuroepithelium; Hi, hippocampus differential field; hi, hippocampus neuroepithelium; IZ, intermediate zone; MZ, marginal zone; s, subiculum neuroepithelium; SP, subplate; St, striatum; pir, piriform cortex; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: 0.2 mm in sagittal and/or coronal sections (A–N), 50 µm in magnification views (A–H).

GeneChip analysis. The nAdv of GeneChip data in Tables 3–5 (see the Supplementary Data) were normalized to the median for each gene among five regions and the resulting intensity value was expressed as mean ± SD of duplicate experiments (GeneChip). QRT-PCR analysis. QRT-PCR analyses were performed as described in Figure 2 using five samples as template (QRT-PCR): M, medial; D, dorsal; L, lateral; R, rostral; O, occipital region. Note that the relative expression levels were comparable between GeneChip and QRT-PCR data and that these patterns closely matched those examined by in situ hybridization.
hand, the expression pattern of Atonal homologue 4 (Y07G21) and Cell division cycle 2 (M38724) showed a broader expression in the medial cortex (data not shown). Expressions of Somatostatin (X51468), Neuronal guanine nucleotide exchange factor (Ngef) (AW050346), and FXYD domain-containing ion transport regulator 7 (Fxyd7) (AI844246), were specifically detected in the lateral cortex where the olfactory area including the piriform cortex (pir) will be formed (¶ in Table 2, Fig. 3L–N). The boundary between the paleocortex and neocortex was clearly delineated by most genes identified in the dorsal and lateral cortex.

Regional Shift in Pax6-deficient Mice

It has been proposed that counter gradient expression of Pax6 and Emx2 in the early embryonic cortex is essential for patterning of the cortex (Bishop et al., 2000, 2002; Mallamaci et al., 2000; Muzio and Mallamaci, 2003). Pax6-deficient mice (Sey/Sey) showed abnormal regional expression of a few classes of genes. To examine whether the regional distribution of the present genes is controlled by Pax6, we determined the gene expression profiles in Pax6-deficient mice (Sey/Sey). Pax6-deficient mice exhibited morphological defects including a thin CP and absence of olfactory bulbs, in agreement with the findings of Schmahl et al. (1993). Npy staining signal in the dorsal deep CP layer at E18.5 was caudally distributed near the occipital pole in wild type mice (arrows in Fig. 5A), while it was shifted rostrally in Pax6-deficient mice (Fig. 5B). This was confirmed in the coronal sections of wild-type and Pax6-deficient mice by comparing Npy stainings in the rostral, central and caudal parts of the E18.5 mouse parietal cortex. Expression of Npy in the wild-type was highly detected in the central and caudal parts of the parietal cortex and was faint in the rostral part (Fig. 5C,E,G). On the other hand, expression of Npy in the mutant mice cortex was highest in the rostral CP (Fig. 5D,F,H). These results demonstrated that regional expression of Npy in the mutant mice was shifted rostrally in the dorsal CP along the rostro-caudal axis and suggest that Pax6 controls regional distribution of Npy in the CP. Interestingly, we found a broad distribution of Npy expressing cells in the deep CP in wild type mice (Fig. 5E,G), while most such cells in the Pax6-deficient mice formed an intensely labeled narrow band at the bottom of the CP and a few cells were abnormally scattered all over the thin CP (Fig. 5F,H), suggesting that Pax6 controls the layer-specific patterning of Npy expression or migration of Npy positive cells in the CP. Several previous studies advocated that Fgfr2, Emx2 and COUP-TFI independently regulate the regional identity of the rostral cortex (Bishop et al., 2000, 2002; Mallamaci et al., 2000; Fukushima-Shimogori and Grove, 2001, 2003; Zhou et al., 2001; Garel et al., 2003; Muzio and Mallamaci, 2003; Storm et al., 2005). How these genes regulate Npy expression pattern which is downstream of Pax6 should further be determined to understand the mechanism of cortical patterning.

Discussion

The combined use of fine evaluation tools of real-time QRT-PCR and in situ hybridization and the GeneChip system allowed us to identify several genes regionally expressed within the E16.5 mouse cerebral cortex. The genes identified in this study showed unique expression profiles with neocortical region and/or layer specificities during the late embryonic development, adding important information to our understanding of the intricate genetic mechanisms involved in neocortical area and layer formation.

Technical Considerations of DNA Microarray Analysis

The DNA microarray is being used in many laboratories to evaluate gene expression profiles in various tissues and/or cell populations at ease (Lockhart and Winzeler, 2000). Pitfalls in the present DNA microarray analysis include limitations in the
detection threshold level caused by the low copies of transcripts and/or high ratio of cross-hybridization. They produce a number of false-negative, such as ‘absent’ regional markers in the relevant regions, as well as a number of false-positive genes, which are selected by the GeneChip analysis but do not show any comparability with QRT-PCR and/or in situ hybridization.

**Figure 5.** Regional pattern of Npy expression is altered in the Pax6-deficient cortical plate. Npy expression was examined by in situ hybridization on sagittal section containing the parietal cortex (A, B) and on coronal sections containing the rostral (C, D), central (E, F) and caudal (G, H) parts of the parietal cortex from E18.5 wild-type (A, C, E, G) and Pax6-deficient brains (B, D, F, H). Note that the caudal boundary of the Npy expression in the deep CP was shifted rostrally in the mutant (arrows in A and B). In addition, Npy expression in the wild type parietal CP was peaked in the central part (E), while that in the mutant mice was shifted to the rostral part (D). (Bottom) Summary of the regional shift of Npy expression in the dorsal CP. Open diamonds represent the relative peak positions of wild type Npy expression along the rostro-caudal axis (left panel). A closed diamond indicates a shifted peak of Npy expression in the Pax6-deficient CP (right panel). Scale bars. 0.2 mm in (A) and (B), 0.1 mm in (C)-(H).
zation outcomes at all. Further improvement in probe sequences would make it possible to select candidate genes more effectively, which might help estimate expression levels of false-negative regional markers in our study.

In this study, we demonstrated that evaluating the quality of hybridization samples both in the GeneChip and QRT-PCR analyses allowed to establish a reliable and extensive system for profiling subtle differences in gene expression among heterogeneous tissue fragments. In addition, we noticed that optimization of the criteria used for signal intensity and/or fold change values enabled detection of many other genes with lower expression levels with careful comparison to the QRT-PCR and in situ hybridization data. For instance, while Cdh6 showed only 0.4 of the signal intensity in the dorsal region (the criterion for selection was up to 0.5), the expression level was double that in any other region, which was comparable to QRT-PCR and in situ hybridization results, thus providing a good dorsal marker. Combining GeneChip analyses with additional systems, such as QRT-PCR and in situ hybridization, is therefore essential to avoid false-negative and/or false-positive results in the present time.

In situ hybridization analyses of genes identified showed that their expression patterns had both regional and layer specificities in the developing CP. If a single cortical layer or dividing/differentiated population of cells were successfully isolated from the present heterogeneous cortical regions, a large number of layer and region specific genes would further be obtained effectively. For instance, usage of the laser capture microdissection technology or the FACS system with the PCR-based RNA amplification could help fine tissue isolation with subsequent DNA microarray analysis (our unpublished data; Luo et al., 1999).

**Regional Expression Patterns of Genes Identified in the Late Embryonic Neocortex**

Figure 4A summarizes the expression patterns of genes identified and/or confirmed in this study. Noticeable is the fact that genes specifically expressed in the dorsal neocortex showed a higher rostral-tolateral-lower caudomedial expression gradient (Fig. 4B). This type of gradation seemed to have been established since the appearance of CP, and was maintained until the neonatal stages, where the expression levels were noted to decrease after birth (data not shown). Interestingly, both intensive and extensive differences in gene expression appeared to attribute to this gradation: in the caudomedial region, both the expression level per cell and number of positive cell was decreased (N. Funatsu, unpublished observation). Importantly, we could not detect any gradual gene expressions within the VZ even at earlier stages, except for Npy and Cdh6. These observations suggest that the graded gene expression pattern in the CP was established not by a simple picture of gradual cortical maturation, but by regional interactions of gene products, which could either be affected by cellular properties already acquired in the VZ or be newly emerged within the CP.

Several types of regionally restricted gene expression patterns within the late embryonic neocortex have been previously reported. For example, the expression of RZR-β both in the VZ and CP is strong in the rostral cortex and gradually decreased in the caudal cortex at E17 (Schaeren-Wiemers et al., 1997). This pattern is similar to that of genes identified from the dorsal region in the present study, indicating that a common machinery could regulate the expression patterns. In contrast, others have shown that the expressions of COUP-TFI, CHL1, Lhx2 and P75 are lower in the rostral cortex and the level gradually increases in the caudal cortex (Mackarehtschian et al., 1999; Nakagawa et al., 1999; Liu et al., 2000). Expression of Cdh8 and EphA7 are markedly lost in the neocortex midsection containing the somatosensory and auditory areas (Korematsu and Redies, 1997; Rubenstein et al., 1999; Yun et al., 2003). In the present study, we found that Mef2c (L131771), Neurotensin receptor type 1 (AI017027), Grp-1 (AI846077), AI851348 and AI426400 were broadly expressed within the CP at late embryonic stages (data not shown). These patterns of gene expression, sometimes overlapped each other, might therefore represent a 'protomap' of future neocortical areas.

**Possible Roles of Genes Identified in the Neocortical Development**

The regional differences in gene expressions in the CP should eventually convert into functionally distinct cortical areas with anatomically distinguishable borders after birth. Whilst incoming TCAs could mediate the formation of area-specific cytoarchitectures (O’Leary et al., 1994), what mechanism(s) regulates the innervation of TCAs around E16 in a cortical region-specific manner remains elusive. Furthermore, how topologically innervated TCAs in the CP modulate the area-specific cellular organization is poorly understood at present. Here we selected the E16.5 neocortex as a good source for GeneChip analysis and identified several genes whose expression pattern could determine the topological innervation of TCAs. For instance, the pioneer guidance axons from the cortical subplate neurons are plausible candidates directing TCAs into the neocortical targets in a topological manner (Molnar and Blakemore, 1995), and the gradual expression patterns of Npy, Wnt7b and Nr3f3 in the dorsal subplate might induce gradual maturation of the subplate neurons thereby contributing to establish the topographic innervation pattern of TCAs.

TCAs then specifically project into the cortical layer IV granule cells. This step is thought to be controlled by various molecules including cell adhesion molecules, secreted factors and transcriptional factors, whose expression specifically demarcates the relevant cortical layers (Vanderhaegen et al., 2000; Mann et al., 2002; Takemoto et al., 2002; Yamamoto, 2002; Dufour et al., 2003; Lopez-Bendito and Molnar, 2003; Seibt et al., 2005). Considering the layer-specific expression of genes identified in the present study with their possible functions assessed in many contexts other than the neocortex (Fig. 4B), it is likely that they have also significant roles in regulating the TCAs projection into a specific layer.

The postmitotic neurons generated in the telencephalic VZ migrate along the radial fiber to settle in the CP (Rakic, 1988). Bayer and Altman (1991) further observed that the CP is primarily built in two separate tiers with little overlap in their birth date. The deep tier consists of the medium-sized pyramidal cells in layer VI and large pyramidal cells in layer V, while the superficial tier consists of the star pyramidal cells in layer IV and small pyramidal cells in layer II/III. Most of the deep tier is already finished before the superficial tier is started and the constituents of each tier have widely overlapped. Curiously, the dorsally-expressed genes identified in the present study can be classified into two groups by their layer-specific expression (Fig. 4B). These patterns may reflect the two populations of cells with different birth dates described in the Bayer and
Altman’s observation. If the cortical layers II/III–VI were laminated from the initial two layered structure (tiers) of the embryonic CP, the cortical cells might be rearranged according to their positional information within each CP layer. The genes identified in this study could play roles in the final determination of specific neuronal types and sorting these cells into specific areas and/or layers in the CP. Such tantalizing scenarios must further be tested in the next step as systematic loss of and/or gain of function analyses in utero could be applied to finally elucidate the intricate genetic mechanisms involved in neocortical patterning and area formation.

**Supplementary Material**

Supplementary material can be found at: [http://www.cercor.oupjournals.org/](http://www.cercor.oupjournals.org/).

**Notes**

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**References**


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