Neuroscience 137 (2006) 401–412

ER81 IS EXPRESSED IN A SUBPOPULATION OF LAYER 5 NEURONS IN RODENT AND PRIMATE NEOCORTICES

H. YONESHIMA,[a] S. YAMASAKI,[b] C. C. J. VOELKER,[c] Z. MOLNÁR,[c] E. CHRISTOPHE,[d] E. AUDINAT,[d] M. TAKEMOTO,[b] M. NISHIWAKI,[b] S. TSUJI,[b] I. FUJITA[a] AND N. YAMAMOTO[b]*

[a]*Graduate School of Frontier Biosciences, Osaka University, 1-3 Machikaneyama, Toyonaka, Osaka 560-8531, Japan*

[b]*Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan*

[c]*Department of Human Anatomy and Genetics, University of Oxford, South Park Road, Oxford OX1 3QX, UK*

[d]*Laboratoire de Neurophysiologie, Institut National de la Santé et la Recherche Médicale U603, Centre National de la Recherche Scientifique FRE2500, Université Paris Descartes, 45 rue des StPères, 75006 Paris, France*

Abstract—Laminar organization is a fundamental cytoarchitecture in mammalian CNS and a striking feature of the neocortex. ER81, a transcription factor, has recently been utilized as a marker of cells in the layer 5 of the neocortex. We further pursued the distribution of ER81 to investigate the identity of the ER81-expressing cells in the brain. *Er81* transcript was expressed in a subset of pyramidal cells that were scattered throughout the entire width of layer 5. In the rat cortex, *Er81* transcripts were first detected in the ventricular zone at E15, remained expressed in putative prospective layer 5 neurons during infant and juvenile stages. The ER81-expressing subpopulation in adult layer 5 neurons did not segregate with the phenotypes of the projection targets. By retrograde labeling combined with immunohistochemistry or reverse transcription–polymerase chain reaction analysis, we found ER81 expression in nearly all of the layer 5 neurons projecting to the spinal cord or to the superior colliculus, while in only one-third of the layer 5 neurons projecting to the contralateral cortex. *Er81* was also detected in layer 5 neurons in a P2 Japanese macaque monkey but not in adult monkey cortices. These findings suggest that a neuron class defined by a molecular criterion does not necessarily segregate with that defined by an anatomical criterion, that ER81 is involved in cell differentiation of a subset of layer 5 projection neurons and that this mechanism is conserved among rodents and primates.

Key words: cortical development, gene expression, laminar structure, layer formation, Japanese monkey, macaque.

*Corresponding author. Tel: +81-6-6879-4636; fax: +81-6-6879-4637.
E-mail address: nobuhiko@fbs.osaka-u.ac.jp (N. Yamamoto).

Abbreviations: CPSC, contralateral primary somatosensory cortex; DIG, digoxigenin; IHC, immunohistochemistry; ISH, *in situ* hybridization; NGS, normal goat serum; P0, postnatal day 0; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RT-PCR, reverse transcription–polymerase chain reaction; SC, superior colliculus; SD, Sprague–Dawley; SpC, spinal cord; TBS, Tris-buffered saline; UTR, untranslated region.

The neocortex is composed of diverse classes of neurons, which can be defined according to morphological, connectional, neurochemical, electrophysiological and/or molecular characteristics (Peters and Jones, 1984; DeFelipe, 1993). The neocortex exhibits a laminar structure composed of six major layers that differ in their constituent neuron classes (Jones and Powell, 1973; Gilbert and Wiesel, 1985; Lund, 1988; McConnell, 1989). Thus laminar locations can sometimes be utilized as effective references of neuron classes; e.g. layer 4 and layer 5 neurons have distinct morphological and hodological characteristics. However, cell classes cannot be determined only from their laminar positions. For example, in layer 5, even adjacent pyramidal neurons project to different target areas, and have distinct dendritic morphology and electrophysiological properties (O'Leary et al., 1990; Kasper et al., 1994; Lewis and Olavarria, 1995).

How do cortical neurons differentiate into distinctive classes during development? It has been suggested that cortical cell types are determined in the ventricular zone just before the final proliferation (McConnell and Kaznowski, 1991). The neurons with the same 'birthday' in the ventricular zone are more or less distributed in similar laminar locations (Angevine and Sidman, 1961; Rakic, 1972). On the other hand, a precise 'birth hour' study of neurogenesis in rodent cortex has shown that cells born at the same time migrate to various radial positions spanning more than half of the cortical thickness (Takahashi et al., 1999), indicating that other factors than laminar constraints may also be involved in cell type specification. The cellular and molecular mechanisms of neuronal differentiation in the neocortex are still largely unknown.

Cortical cytoarchitecture has been investigated in various species to date. Although the mammalian neocortex shows some structural diversity among species (Krubitzer, 2000; Kaas and Collins, 2001; Preuss, 2001; DeFelipe et al., 2002), they share a similar six-layer structure (Cajal, 1911; Jacobson, 1991; Krubitzer, 1995) and developmental processes (Rakic, 2003). However, it still remains unknown to what extent neuron classes and their laminar distributions are conserved evolutionarily, which will provide a clue to an understanding of fundamental mechanisms of cortical cell differentiation.

Molecular profiling will help assign a cell of interest to a certain neuron class. By utilizing molecular tags, neurons can be visualized with single-cell resolution by immunohistochemistry (IHC) or *in situ* hybridization (ISH) method. These methods are technically easier and more efficient than tracer injections or electrophysiological methods. Several molecules with layer-specific expression patterns

0306-4522/06$30.00+0.00
doi:10.1016/j.neuroscience.2005.08.075

in the cerebral cortex have been identified to date (e.g. Arimatsu et al., 1999; Weimann et al., 1999; Liu et al., 2000; Hevner et al., 2001, 2003; Rice and Curran, 2001; Nakagawa and O'Leary, 2003; Zhong et al., 2004). However, the identified molecules are far from sufficient for us to explain all the complicated process of corticogenesis. Our knowledge about the relationship between neuron classes defined by molecular and other criteria such as morphology, connectivity and electrophysiology is still limited.

We have searched for the genes that are expressed with a specific pattern of layer distribution in the cerebral cortex by constructing a subtraction cDNA library (Zhong et al., 2004). We chose layer 5 for the present analysis, because the classes and projection patterns of layer 5 neurons are relatively well characterized (Larkman and Mason, 1990; Koester and O'Leary, 1992; Kasper et al., 1994). We found that ER81, a transcription factor of the ETS family (de Launoit et al., 1997), was expressed almost exclusively in layer 5 in the neocortex, as has been recently reported (Xu et al., 2000; Sugitani et al., 2002; Hevner et al., 2003; Beggs et al., 2003; Hasegawa et al., 2004; Gray et al.,2004). In the present study we further extended our analysis to the ontogenetic and phylogenetic development and the projection targets of *Er81*-expressing neurons.

EXPERIMENTAL PROCEDURES

Animals

Sprague–Dawley (SD) rats were used for cDNA library construction and ISH. P3 ICR mice were used for cloning by reverse transcription–polymerase chain reaction (RT-PCR). A P5 ICR mouse was used for ISH. Adult Wistar rats (P6–12 weeks) and adult C57BL/6 mice (P6–12 weeks) were used for a combined labeling of cortical neurons by a retrograde tracer and IHC. Three rats or three rats and one mouse were used for each injection paradigm: into the spinal cord (SpC), superior colliculus (SC) or the contralateral primary somatosensory cortex (CPSC). P20–P29 C3H mice were used for single cell RT-PCR analysis. Japanese monkeys (*Macaca fusucata,* a P2 infant and two adults) and an adult cynomolgus monkey (*Macaca fascicularis*) were also used for ISH. The day of vaginal plug detection in mother animals was designated as the embryonic day 0 (E0) and the day of birth as the postnatal day 0 (P0). We designed our experiments minimizing the number of animals used and their suffering. All experimental and animal care protocols were in accordance with the regulations and guidelines of Osaka University (Japan), University of Oxford (UK) and INSERM (France), and approved by the animal experiment committee of the respective institutes.

Construction and screening of layer 5-enriched cDNA library

A layer 5-enriched cDNA library was constructed from a P7 SD rat as described previously (Zhong et al., 2004). Layer 5 and layer 4 strips, approximately 500 μm in length, were dissected from the acute slices of primary somatosensory cortices with a pair of small scissors. The cortical barrel structures, which were visible under a trans-illuminating microscope, were used as a landmark for layer 4. The layer 5 strips were dissected from beneath the parts where the layer 4 strips were dissected (see Fig. 1 in Zhong et al., 2004). The cDNA from the layer 4 strips was subtracted from that from the layer 5 strips (SMART cDNA synthesis kit and PCR-Select cDNA subtraction kit, Clontech, Palo Alto, CA, USA). To enrich layer 5 specific clones, a differential screening and then ISH were performed (Zhong et al., 2004).

Cloning of *Pea3* subfamily members

The 3′ untranslated region (UTR) sequences of the mouse *Pea3* subfamily members (de Launoit et al., 1997; for details of *Pea3* subfamily, see "Layer 5-enriched expression of *Er81*" in Results section) were cloned by RT-PCR. Total RNA was extracted from a P3 ICR mouse brains, followed by first-strand cDNA synthesis. The cDNA fragment corresponding to bp 1782–2455 of the mouse *Er81* gene (GenBank accession no.: L10426) was amplified by PCR with a pair of specific primers (5′-TGT TGC ATT ATT CTA TGG TCT GCC-3′ and 5′-ATT CCG AAG GAT CAT GAC ATG TCA-3′). Likewise, the cDNA fragment corresponding to bp 1558–1970 of the mouse *Etv5* gene (GenBank accession no.: AY004174) was amplified (5′-AAC TGA TTT GGT ATT GGT GAA GGC-3′ and 5′-ATA AGA GGC AAA GGT GGA ATT AGG-3′) and bp 1843–2371 of the mouse *Pea3* gene (GenBank accession no.: X63190) was amplified (5′-AAC TGA TTT GGT ATT GGT GAA GGC-3′ and 5′-ATA AGA GGC AAA GGT GGA ATT AGG-3′). The PCR fragments were cloned into pGEM-T plasmid vector (Promega, Madison, WI, USA).

ISH

Digoxigenin (DIG)-labeled cRNA probes were used for ISH. The sequences of interest cloned into pGEM-T vectors were amplified by PCR using primers containing T7 and SP6 promoter sequences (5′-TTG TAA AAC GAC GGC CAG TG-3′ and 5′-TGA CCA TGA TTA CGC CAA GC-3′). The PCR products were purified (PCR purification kit, Qiagen, Hilden, Germany) and *in vitro* transcription was carried out (DIG-RNA Synthesis Kit, Roche Diagnostics, Mannheim, Germany). The product probes were purified (ethanol precipitation or Quick Spin Columns, Roche Diagnostics) and kept at −80 °C.

SD rats (one each of E15, E18, P0, P3, P14 and adult (200 g), and three P7), a P5 ICR mouse, Japanese monkeys (one P2 and two adults) and an adult cynomolgus monkey were deeply anesthetized by an i.m. injection of ketamine (6 mg/kg) followed by an i.p. injection of sodium pentobarbital (50 mg/kg) and perfused transcardially with cold phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). The brains were post-fixed in 4% PFA in 0.1 M PB (pH 7.4) for 1 h at room temperature and then 2 h at 4 °C. After overnight incubation in PBS containing 20% sucrose, the brains were frozen and then were sectioned at 7–10 μm using a cryostat (CM1850, Leica, Bensheim, Germany).

Sections were re-fixed in 4% PFA in 0.1 M PB (pH 7.4), washed with distilled water and 0.1 M triethanolamine, acetylated in 0.25% acetic acid in 0.1 M triethanolamine and rinsed in PBS. After pre-hybridization at 60 °C for 1 h in hybridization buffer (50% formamide, 5% SDS, 5× SSPE, 1 mg/ml tRNA), the sections were hybridized at 60 °C overnight in hybridization buffer containing 1 μg/ml DIG-labeled cRNA probe. After three washes in 50% formamide in 2× SSC at 60 °C, the sections were incubated in blocking buffer (Roche Diagnostics) for 1–4 h at room temperature and incubated overnight at 4 °C with alkaline phosphatase-conjugated anti-DIG antibody (diluted 1:2000, Roche Diagnostics). After washing, the color reaction was carried out at room temperature or 4 °C in BM Purple (Roche Diagnostics). The reaction was terminated by rinsing the sections in water and immersing the sections in 4% PFA in 0.1 M PB (pH 7.4). The sections were dehydrated and embedded permanently. The sections were observed with a microscope (E800, Nikon, Tokyo, Japan) and the images were captured with a CCD camera (DP70, Olympus, Tokyo, Japan).

Tracer injection, IHC and quantification of labeled cells

Retrograde labeling with fluorescent microbeads and subsequent immunostaining were performed as described previously (Voelker et al., 2004). Adult rats were anesthetized with 2.7 mg/kg Hypnovel (Roche, Basel, Switzerland), Hypnorm (Janssen, Titusville, NJ, USA) and distilled H_2O (1:1:2 volume ratio), which was delivered i.p. and placed in a stereotaxic frame. After the skin was disinfected and incised, a microdrill was used to perform a craniotomy. Glass micropipettes (Clark Electromedical Instruments, Reading, UK) and a binocular stereomicroscope (Zeiss, Oberkochen, Germany) were used to inject 0.3–1.0 μl of microspheres (Lumafluor, Naples, FL, USA) into one of three pyramidal cell targets: SpC (rats, $n=3$; between thoracic 1 and thoracic 6), SC (rats, $n=3$; mouse, $n=1$; 6.5 mm posterior to bregma, 1.5 mm lateral of sagittal suture, 3.5 mm deep; Paxinos et al., 1985) or CPSC (rats, $n=3$; mouse, $n=1$; 3.0 mm posterior to bregma, 3.5 mm lateral of sagittal suture, 1 mm deep; Welker et al., 1996). Each target received two or three injections approximately 100 μm from each other. The micropipette was kept in place for 1–2 min before retraction. During the postoperative period, animals were kept under a heating lamp before returning them to their cages. All animals recovered quickly and resumed normal behavior following the procedure. Animals were allowed to survive for 24–48 h to permit adequate retrograde transport of the microspheres to the soma. They were then deeply anesthetized by an i.p. injection of sodium pentobarbital (50 mg/kg) and perfused transcardially with cold saline followed by 4% PFA in 0.1 M PB (pH 7.4). Brains were sectioned at 60 μm in the coronal plane using a vibroslicer (VT1000S; Leica) and serial sections through the primary somatosensory cortex or the primary motor cortex were collected. Free-floating sections were washed in 0.05 M Tris-buffered saline (TBS; pH 7.4) and incubated for 2 h in TBS containing 10% normal goat serum (NGS) and 0.1% Triton X-100 to mask non-specific binding sites. Sections were then incubated overnight in the primary polyclonal antibody against ER81 (a gift from Dr. T. Jessell, Columbia University) in TBS containing 1% NGS and 0.1% Triton X-100 (diluted 1:32,000). After rinsing in TBS, sections were incubated in biotinylated-conjugated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) in TBS with 1% NGS (diluted 1:100) for 2 h, rinsed in TBS and incubated in Cy3-conjugated to streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 2 h (diluted 1:500). After a final rinse in TBS, sections were counterstained with bisbenzimide Hoechst trihydrochloride (2.5 μg/ml in PBS; Sigma-Aldrich, St. Louis, MO, USA), mounted on gelatin-coated slides, air-dried and coverslipped with PBS. Slides were stored in 4 °C and protected from light. The IHC controls were negative. For ER81 and GAD67 double-labeling, the sections from a P21 and an adult SD rat were prepared according to the same protocol as that for ISH and were subjected to a similar procedure as that for ER81-IHC: A mixture of the anti-ER81 antibody and a monoclonal antibody against GAD-67 (diluted 1:1000, MAB5406, Chemicon, Temecula, CA, USA) were used as the primary antibodies. A mixture of a Cy3-conjugated anti-rabbit IgG (diluted 1:100, AP182C Chemicon) and an Alexa Fluor 488-conjugated anti-mouse IgG (diluted 1:100, A11029, Molecular Probes, Eugene, OR, USA) was used as the secondary antibody. After extensive washes, the sections were mounted with glycerol containing TBS.

The immuno-labeled sections from the tracer-injected animals were analyzed with a epifluorescence microscope (Diaplan, Leitz, Wetzlar, Germany) equipped with appropriate barrier filters for the various fluorophores in the same section. Quantification of double-labeled cells was restricted to the primary somatosensory cortex for SC and CPSC projecting cells and to the primary motor cortex for the SpC projecting cells. Two to three cortical sections for each injection paradigm were selected that contained numerous fluorescent back-labeled pyramidal cells within the primary somatosensory cortex and the primary motor cortex. All layer 5 pyramidal neurons per section that contained fluorescent microbeads were examined for immunostaining by changing the fluorescence filter under 100× oil immersion objective. True color images were captured using a digital camera (DC 500, Leica). The percentage of double-labeled cells identified (cells that contained beads and expressed ER81) per total beads-labeled cells was calculated for each animal. Approximately 130 layer 5 neurons containing microbeads per animal were analyzed for ER81 expression.

Laser-scanning confocal microscopy (TCS NT, Leica) was used to confirm the co-labeling of some of the cells that was counted in the quantification conducted under the fluorescence microscope and to capture the images in Fig. 6. Excitation was obtained with an argon-krypton laser, with lines set at 488 nm for fluorescein isothiocyanate (FITC) and 568 nm for tetramethyl rhodamine isothiocyanate (TRITC). Several optical sections (between six and eight sections, 0.2–0.5 μm of Z distance between each) were scanned through a single pyramidal cell. Images were taken in a 1024×1024 pixel format using a 100×/1.4 N.A. oil immersion objective. Individual optical sections and the *z* axis reconstructions were examined before images were compiled into a single image. The single images were then processed using Adobe Photoshop 6.0 or CS2.

To determine the proportion of ER81-expressing cells among layer 5 neurons, the immuno-labeled sections for both ER81 and GAD67 from the P21 and adult rats were analyzed with the epifluorescence microscope (Diaplan, Leitz). The number of nuclei stained with DAPI and that of ER81-positive cells were counted within layer 5 of the primary somatosensory cortex. Three sections each from the P21 and the adult cortices were used for this analysis. A defined area (0.5 mm×0.3 mm) of layer 5 was randomly selected from each section. In total, 968 and 660 cells were counted each.

Single-cell RT-PCR

After *in vivo* injections of fluorescent beads in the SC or the cerebral cortex as described above, P21–30 day old CH3 mice were deeply anesthetized by i.p. injection of either a mixture of ketamine (65 mg/kg)/xylazine (14 mg/kg), or sodium pentobarbital (27.4 mg/kg). Parasagittal sections (300 μm thick) comprising the left parietal association cortex and the medial part of the visual cerebral cortex were prepared (Kasper et al., 1994) using a vibroslicer (Leica). Slices were incubated 10 min at 35 °C and then at room temperature (20–25 °C) in a recording solution containing (in mM): 126 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose, 26 $NaHCO_3$, 5 pyruvate and was saturated with a mixture of 95% O_2, 5% CO_2 (325 mOsm/l, pH 7.2). Fluorescent cortical pyramidal cells from layer 5 were selected under visual control using an upright microscope equipped with Nomarski differential interference contrast optics and epifluorescence illumination (Leica). Cytoplasm harvesting and reverse transcription were performed as previously described (Lambolez et al., 1992). Briefly, patch pipettes were filled with 8 μl of solution containing (in mM): 130 K-gluconate, 15 Na-gluconate, 3 $MgCl_2$, 10 HEPES, 0.2 EGTA, 5.4 biocytin (pH 7.2, 295 mOsm). The content of the cells was aspirated into the pipettes and expelled in a 0.2 ml test tube where reverse transcription was performed overnight at 35 °C in a final volume of 10 μl. A two step PCR was then performed essentially as in Cauli et al. (1997). The cDNAs present in the reverse transcription reaction were first amplified in a final volume of 100 μl with 0.2 μM of the *Er81* primers (5′-CTC ATG ATT CAG AAG AAC TCT T-3′ and 5′-TGG 5′-GGT AGG TGC TGT CTG GT-3′; size of the expected fragment 426 bp), 2 mM of each of the deoxyribonucleotides triphosphate, 2.5 U of *Taq* polymerase and the buffer containing (in mM): 50 Tris (pH 8.9), 50 KCl, 1.5 $MgCl_2$. Twenty PCR cycles (45 s at 94 °C, 1 min 30 s at 56 °C, 1 min at 72 °C) were then performed, with an initial elongation period of 5

min at 94 °C and a final one of 10 min at 72 °C. Of this reaction, 2 μl were then used as template for second 35 PCR cycles using the same primer pair. Each PCR reaction (13 μl of the 100 μl reaction) was run on a 1.5% agarose gel stained with ethidium bromide, using a 100 bp DNA ladder molecular weight marker. The identity of all PCR products was further confirmed by restriction analysis using the enzyme *Kpn*I which generates two fragments of 84 and 342 bp.

RESULTS

Layer 5-enriched expression of *Er81*

We searched for genes that are expressed specifically in layer 5, using a subtraction cDNA library constructed from the primary somatosensory cortex of P7 rats. More than a thousand clones were subjected to differential screening and ISH. A clone (clone 262) was found to be expressed predominantly in layer 5 of P7 rat cerebral cortex. At this developmental stage the laminar configuration has just been formed in the cortex. Most cells with clone 262 signal were restricted to layer 5 and a small number of cells were found also in upper layer 6 (Fig. 1A and 1C). Within layer 5, clone 262-expressing cells were sparsely distributed throughout the width of the layer (Fig. 1B and 1C). Many of them showed radially-oriented pyramidal shape with a thick apical process (Fig. 1D), which is characteristic for pyramidal neurons.

Clone 262 showed 92% homology to a part of the open reading frame of the mouse *Er81* (Brown and McKnight, 1992; de Launoit et al., 1997), a member of the ETS transcription factor family (Fig. 2; based on results of DNA sequencing and database searches). However, these results do not necessarily indicate that *Er81* is the layer 5-dominant gene shown in Fig. 1. Clone 262 includes the sequence for the ETS domain, which is well conserved among *Pea3* subfamily members, *Etv5* and *Pea3* (Sharrocks et al., 1997). Therefore, there is a possibility that the ISH results using the clone 262 probe (Fig. 1) may reflect expression patterns of the other *Pea3* subfamily members. To examine this possibility, we conducted ISH on P5 mouse brain sections using probes from *Er81* and the 3′ UTRs of the mouse *Pea3* subfamily members, *Etv5* and *Pea3* (Fig. 3). The expression patterns of the *Er81*, *Etv5* and *Pea3* (Fig. 3A–C respectively) were compared with the clone 262 expression pattern (Fig. 1A). The *Er81* transcript was expressed in cortical layer 5 as well as in the subiculum, habenular nucleus, ventromedial thalamic nucleus and amygdaloid body (Fig. 3A), which was similar to the expression pattern of the clone 262 in rats (Figs. 1 and 4, described later). On the contrary, *Etv5* was weakly expressed in the medial part of the cerebral cortex in lower layers 2/3, 5 and 6 (Fig. 3B). No significant expression of *Pea3* was detected either in the cerebral cortex or in other brain structures (Fig. 3C). Thus we identified the clone 262 as a partial sequence of rat ER81 and utilized the clone 262 to generate the cRNA probe for ER81. Our present observation is not inconsistent with the previous study by Hasegawa et al. (2004) that Etv5 was expressed prominently in VZ of E14.5 mouse. They also showed that the expression of Etx5 declined from E16.5 onward in mouse VZ and our observation was done with P5 mouse.

Spatial and temporal distribution of *Er81*

Areal specificity of *Er81* mRNA expression was studied in P7 rat cerebral cortex. The signals were observed in all neocortical areas. Indeed, its expression was found throughout the brain from the frontal to the occipital

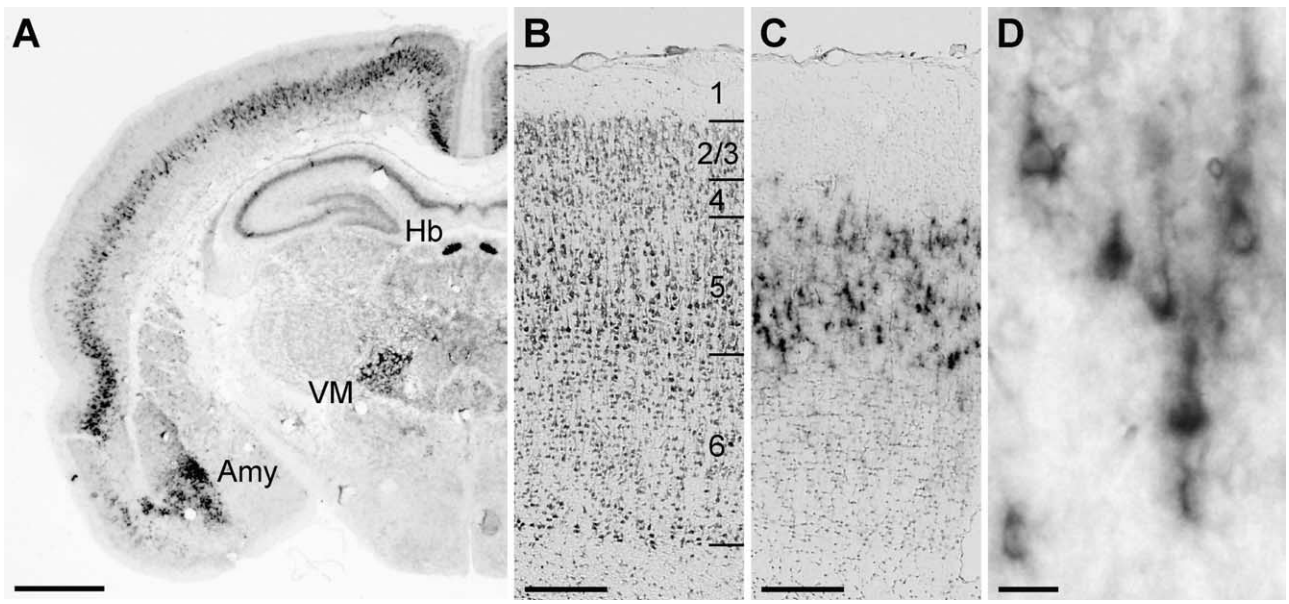

Fig. 1. Lamina-specific expression of clone 262 in rat cerebral cortex. The distribution of clone 262 in P7 rat brain was visualized by ISH (A, C, D). Nissl staining of an adjacent section to C is shown for layer assignment (B). Most cells with the signal were restricted to layer 5, but sparsely distributed throughout the width of this layer and a small number of labeled cells are scattered in layer 6 (A, C). Many of the labeled layer 5 cells showed radially-oriented pyramidal shape with a thick apical process (D). Amy, basal amygdaloid nuclei; Hb, medial habenular nucleus; VM, ventromedial thalamic nucleus. Scale bars = 1 mm (A); 200 μm (B, C); 20 μm (D).

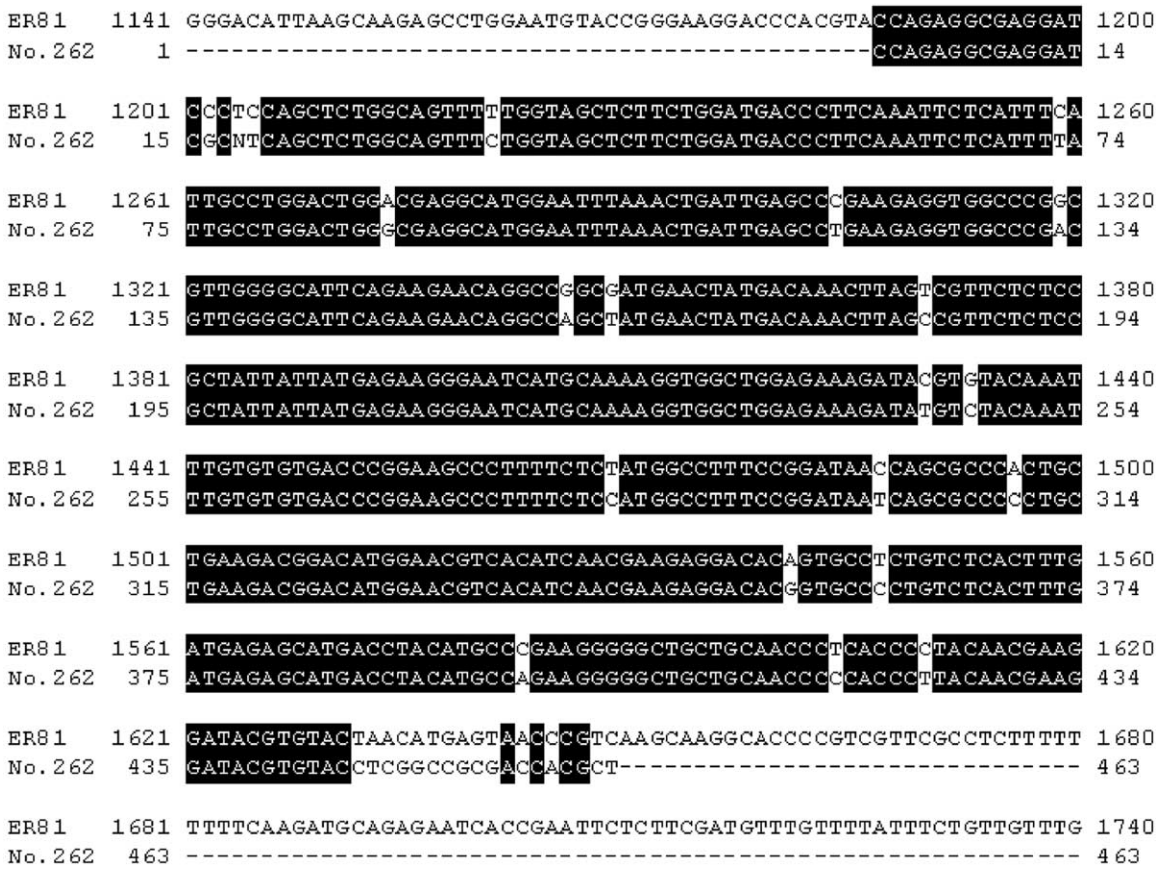

Fig. 2. DNA sequence of mouse *Er81* and clone 262. Identical residues are shaded in black. The two sequences showed 92% homology.

cortex (Fig. 4). The cingulate, retrosplenial and perirhinal cortices also expressed the transcript. The signal in these limbic areas was stronger than neocortical areas, which was attributed to the greater density of positive cells rather than the expression intensity within a single cell. In addition to the cerebral cortex, *Er81* was strongly expressed in the subiculum, medial habenular nucleus, basal amygdaloid nuclei, intermediate gray layer of SC, mesencephalic trigeminal nucleus and inferior olivary nuclei (Fig. 4) and ventromedial thalamic nucleus (Fig. 1A, Only the edge of the nucleus was included in Fig. 4B).

To study the relationship between *Er81* gene expression and the differentiation of layer 5 cells, developmental changes in *Er81* expression were examined by ISH in the embryonic and postnatal rat cortex. At E15, when layer 5 neurons are born in the ventricular zone (Miller, 1988), *Er81* was expressed exclusively in the ventricular zone (Fig. 5A). At E18, when most prospective layer 5 neurons have arrived to the cortical plate (Miller, 1988), *Er81*-expressing cells were located in the middle tier of cortical plate (Fig. 5B). At P0 and P3, *Er81*-expressing cells were located in the cortical position corresponding to immature layer 5 (Fig. 5C and 5D). At P7 and P14, *Er81*-expressing cells were located in layer 5 (Fig. 5E and 5F), and a small number of *Er81*-expressing cells were distributed in layer 6. No significant *Er81* expression was observed in the adult cortex (Fig. 5G).

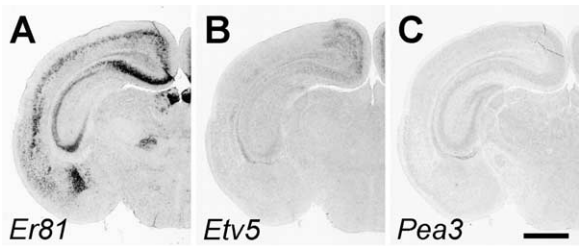

Fig. 3. Distribution of *Er81* and other *Pea3* subfamily members in brain. The distributions of *Pea3* subfamily members (A) *Er81*, (B) *Etv5*, (C) *Pea3* were specifically visualized by ISH in coronal sections of a P5 mouse brain. Only *Er81* (A) showed a similar distribution as clone 262. Scale bar=1 mm.

Projection types and identity of ER81-expressing cells

We analyzed the correlation between ER81 protein expression and projection phenotypes in rat and mouse at single cell resolution by combining retrograde tracer injections and IHC for ER81. Fluorescent microbeads served as retrograde tracers (Katz et al., 1984) and were injected into one of the three targets (SpC, SC, or CPSC; Fig. 2A, E and I in Voelker et al., 2004). Three rats for SpC injection and three rats and one mouse for SC and CPSC injection were used. The cells in the primary motor cortex retrogradely labeled from SpC were restricted to layer 5 (Fig. 2A,

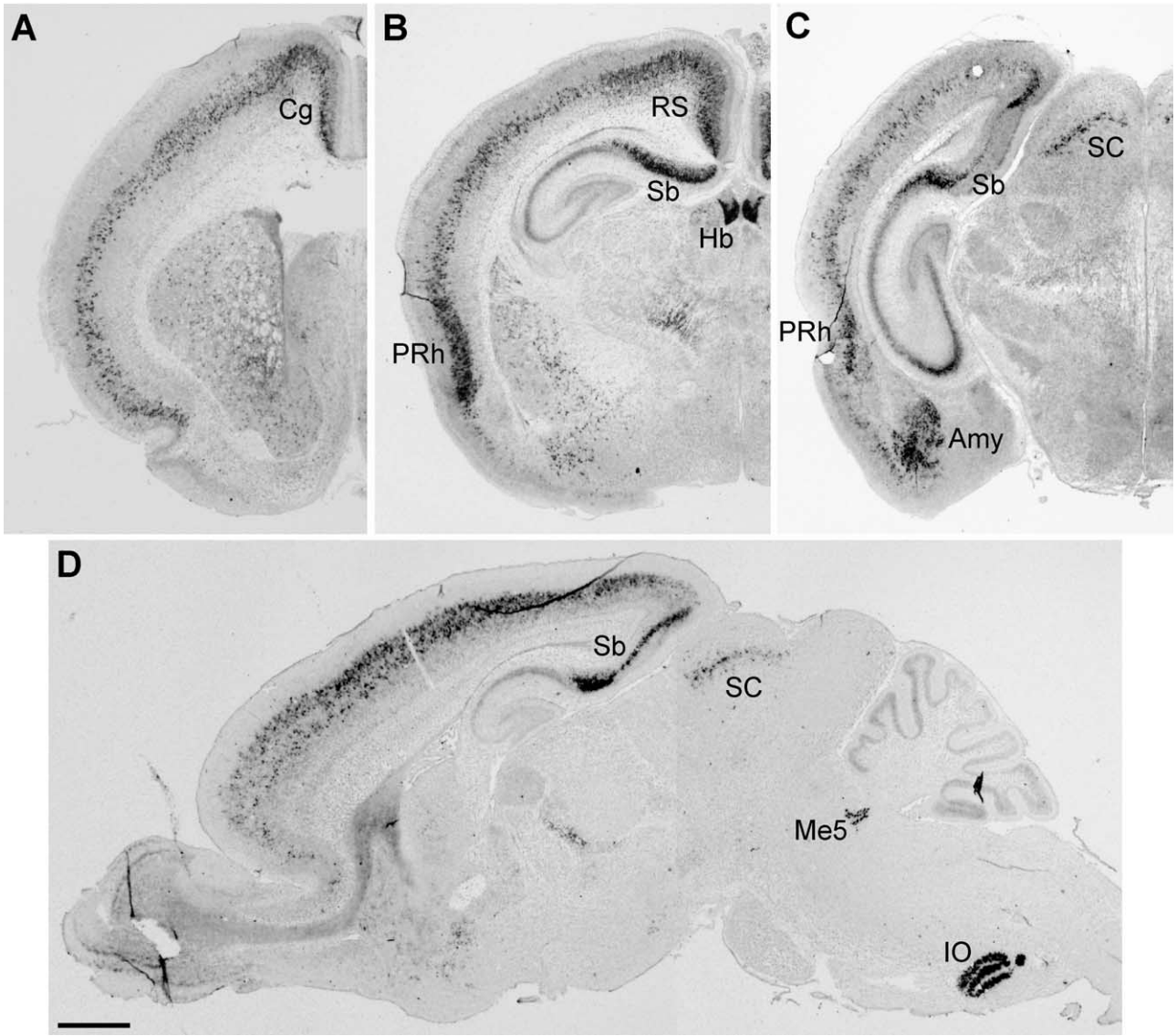

Fig. 4. Distribution of *Er81* in cerebral cortex and other brain structures. The distribution of *Er81* in P7 rat brain was visualized by ISH. Coronal sections at three antero-posterior levels (A–C) and a sagittal section (D) are shown. Amy, basal amygdaloid nuclei; Cg, cingulate cortex; Hb, medial habenular nucleus; IO, inferior olivary nuclei; Me5, mesencephalic trigeminal nucleus; PRh, perirhinal cortex; RS, retrosplenial cortex; Sb, subiculum; SC, intermediate gray layer of SC. For ventromedial thalamic nucleus (VM), see Fig. 1A. Only the edge of VM is shown in B. The layer 5 of the middle and the posterior part of the cerebral cortex is labeled more heavily than that of anterior part (D) because the section contains isocortex in the anterior part while it contains Cg or RS in the middle or the posterior part. Scale bar=1 mm.

B and D in Voelker et al., 2004). The cells in the primary somatosensory cortex retrogradely labeled from SC were also restricted to layer 5 (Fig. 2E, F and H in Voelker et al., 2004). The cells projecting to the contralateral cortex hemisphere through the corpus callosum (Fig. 2I in Voelker et al., 2004) and were located in all cortical layers except layer 1. Layers 2, 3 and 5 showed the greatest number of cells projecting to the contralateral cortex (Fig. 2J and L in Voelker et al., 2004). To investigate the expression of ER81 in these retrogradely labeled cells, brain sections through the retrogradely labeled cortices were subjected to IHC for ER81 (Fig. 6). The most cells retrogradely labeled from SpC or SC were positive for ER81 (Fig. 6A and 6B, respectively). On the other hand, the cells retrogradely labeled from CPSC consisted of both ER81-positive and -negative cells (Fig. 6C and 6D, respectively). Layer 5 neurons that contained microbeads were quantitatively analyzed for ER81 expression. The percentages of double-labeled cells (cells that contained beads and expressed ER81 in the same section) per total bead-labeled cells for each injection paradigm were 96.6±4.5% for SpC, 98.1±1.6% for SC and 33.9±6.4% for CPSC projecting neurons (mean±SD, n=3 for SpC, n=4 for SC and CPSC).

To detect expression of ER81 with higher sensitivity, we performed single-cell RT-PCR experiments designed to selectively amplify *Er81* mRNAs from retrogradely labeled neurons identified and recorded in acute slices of adult mice. We observed that seven of 10 SC-projecting

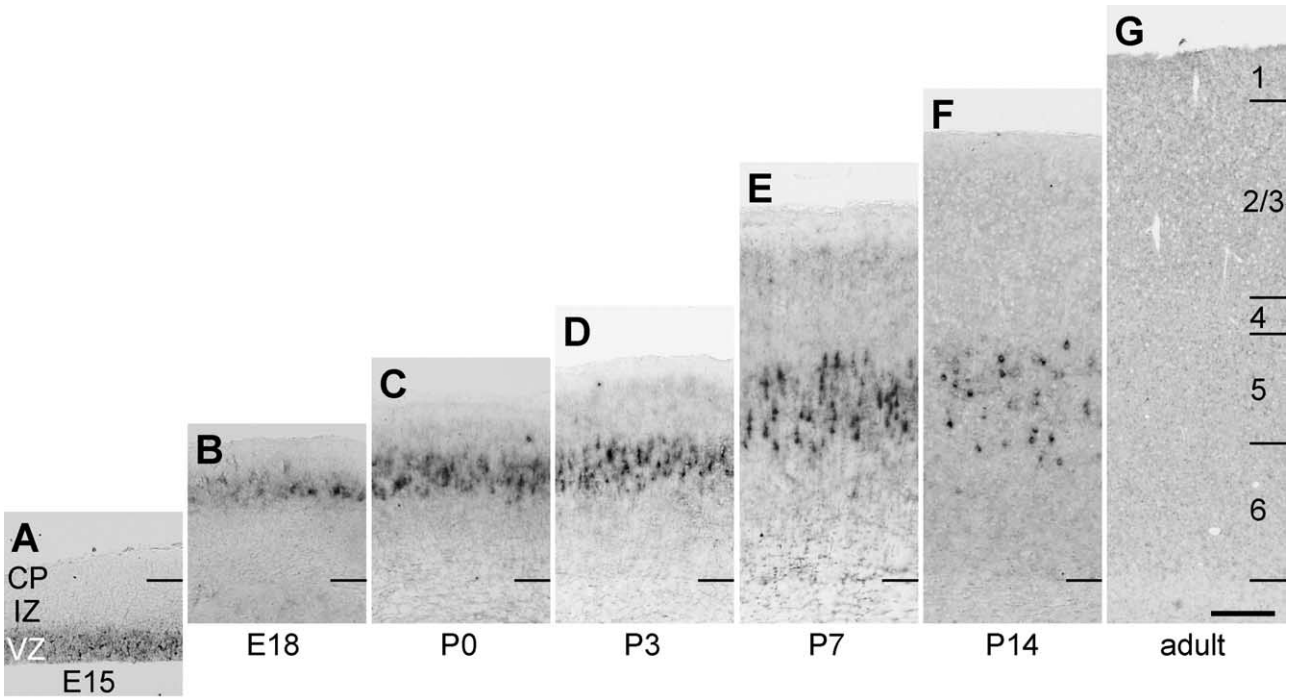

Fig. 5. Developmental *Er81* expression pattern in rat neocortex. The expression of *Er81* in E15 (A), E18 (B), P0 (C), P3 (D), P7 (E), P14 (F) and adult (G) rat brain was visualized by ISH. Panels are aligned at the bottom of the cortical plates or cerebral cortices (thin bars). At E15, *Er81* was expressed in the ventricular zone (A). From E18 to P14, the expression corresponding to the putative position of layer 5 neurons (B–F). No significant expression was detected in the adult (G). CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone. Scale bar=200 μm.

neurons expressed *Er81* (Fig. 7A), while an amplification product was obtained in only two of seven CPSC-projecting neurons (Fig. 7B).

We also examined the ratio of ER81-expressing cells to the entire population of layer 5 cells in the primary sensory cortices with IHC for ER81 and DAPI counterstaining.

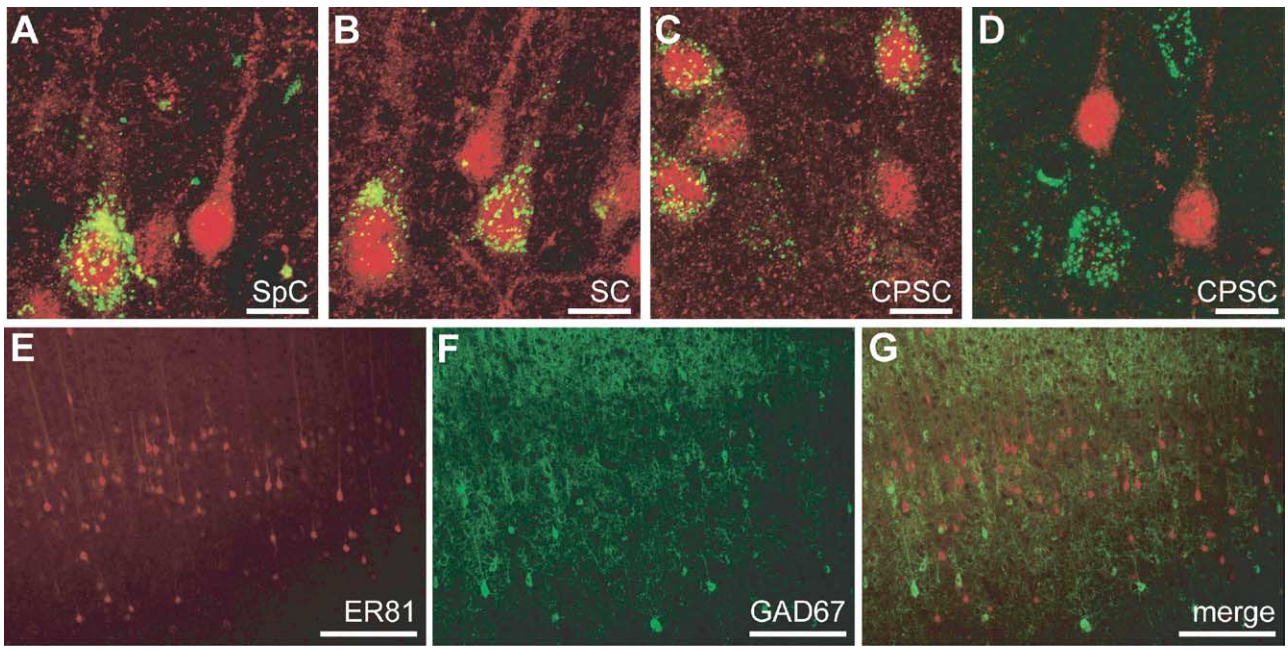

Fig. 6. Projection types and identity of ER81-expressing cells. Cortical neurons that were retrogradely labeled (green) by injecting fluorescent microbeads into either the SpC (A), SC (B) or CPSC (C, D) were stained for ER81 (red) by IHC in primary motor cortex (A) or primary somatosensory cortex for SC and CPSC (B–D). The percentage of double-labeled cells (cells that contained microbeads and expressed ER81) per total beads-labeled cells was 96.6±4.5% for SpC (A), 98.1±1.6% for SC (B) and 33.9±6.4% for CPSC (C, D) projecting cells (mean±SD, $n=3$ for SpC, $n=4$ for SC and CPSC). By ER81 (red, E) and GAD67 (green, F) IHC, no double-labeled cell was found (G). Scale bars=20 μm (A–D); 200 μm (E–G).

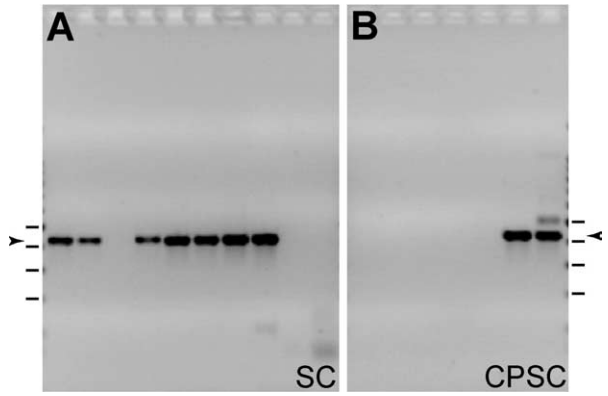

Fig. 7. Expression of *Er81* in layer 5 neurons projecting to SC and to CPSC. *Er81* mRNA was detected by single-cell RT-PCR from neurons retrogradely labeled from SC (A) or CPSC (B). *Er81* corresponding amplification products (426 bp long, arrowheads) were observed in seven of 10 SC-projecting neurons and in two of seven CPSC-projecting neurons. Bars indicate the positions of DNA size markers (500, 400, 300 or 200 bp, from top to bottom).

The result showed that 13.4+0.8% and 13.0+0.0% out of DAPI-stained cells were ER81-positive in layer 5 of the primary somatosensory cortex of a P21 and an adult rat, respectively (not shown, mean±SD, $n=3$ each). To further investigate the identity of ER81-expressing cells, IHC for ER81 and GAD67 was performed. No double-labeled cell was found in the primary sensory cortex of the P21 (Fig. 6E–G) and the adult (not shown) rat.

Er81 expression in monkey cerebral cortices

In order to determine whether the layer-specific distribution pattern of *Er81* was preserved across different mammalian species, we analyzed the expression of *Er81* in the cerebral cortices of monkeys using ISH. First, we examined the occipital, parietal and temporal cortices of a P2 Japanese monkey (Fig. 8). The section of occipital and parietal cortices included the primary visual cortex (V1), prestriate cortices (V2, V4, and MT) parietal visual cortex (7b) and somatosensory cortex (Fig. 8A). The temporal cortex section included the insular cortex, superior temporal cortex (auditory association cortex), inferior temporal cortex (TEp, TEa, TEO), and entorhinal cortex (area 28) (Fig. 8B). TEa and TEO are not shown. *Er81*-expressing cells were sparsely distributed within the entire width of layer 5 in all cortical areas examined and a few cells in layer 6 also expressed *Er81*. Many of the *Er81* expressing neurons showed radially-oriented pyramidal shape with a thick apical process, which was similar to what was observed in rats (Figs. 3, 5, 6). Next we examined visual (in the occipital, parietal, temporal cortex), somatosensory and motor areas as well as the frontal cortex in two adult Japanese monkeys and a cynomolgus monkey. In contrast to the P2

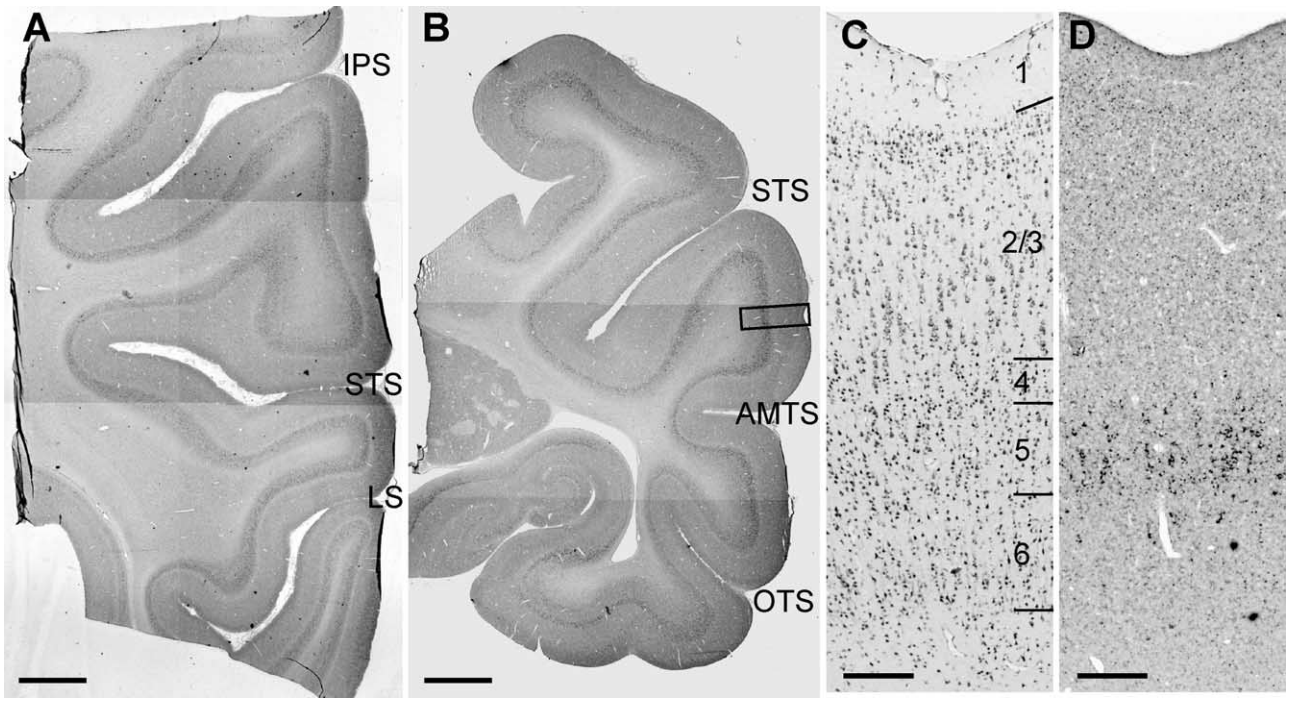

Fig. 8. Expression of *Er81* in monkey cerebral cortex. The distribution of *Er81* in P2 Japanese monkey brain was visualized by ISH (A, B, D). D shows the higher magnification in the box in B. Nissl staining of an adjacent section to D is shown for layer assignment (C). *Er81* expressing cells were sparsely distributed within the entire width of layer 5 in all cortical areas examined. Many of the *Er81* expressing neurons showed radially-oriented pyramidal shape with a thick apical process, which is similar to what was observed in rats. AMTS, anterior middle temporal sulcus; IPS, intraparietal sulcus; LS, lateral sulcus; OTS, occipitotemporal sulcus; STS, superior temporal sulcus. Scale bars=2 mm (A, B); 200 μm (C, D).

monkey, no significant *Er81* expression was detected throughout the cortices in the adult monkeys (not shown).

DISCUSSION

By screening the subtraction cDNA library, we identified *Er81* as a layer 5-enriched gene. The expression of *Er81* was restricted to a subset of layer 5 neurons and a small number of upper layer 6 neurons throughout the cerebral cortex and found in putative prospective layer 5 neurons for the entire period of cortical development starting at E15 in the rat ventricular zone. ER81 was expressed in nearly all SpC and SC-projecting neurons while only one-third of CPSC-projecting neurons in layer 5 expressed the protein. We also found that *Er81* was expressed in various cortical areas in an infant Japanese monkey with essentially the same laminar distribution as found in rodents.

These findings suggest that ER81 is involved in (a) process(es) of cell differentiation of a subset of layer 5 projection neurons and that this mechanism is conserved among rodents and primates and maybe among mammalian species. The subpopulation defined by the expression of ER81 did not segregate with the previously reported neuron types (Kasper et al., 1994) according to the projection target. Neurons may be classified into greater diversity of repertoire by combination of distinct criteria.

Expression of Er81 in neocortex and other brain structures

ER81 is a member of the ETS transcription factors (de Launoit et al., 1997). ETS proteins have been shown to contribute to the specification of various vertebrate and invertebrate cell types (Wasylyk et al., 1998). In vertebrate neuronal system, ER81 and another member of the ETS transcription factors, PEA3, are expressed by developing proprioceptive sensory neurons and motor neurons in the SpC. 'Motor neuron pools' and subsets of muscle sensory afferents can be defined by the expression of ER81 and/or PEA3. There is a matching in ER81 and/or PEA3 expression by motor and sensory neurons that are functionally interconnected and are connected to the same muscle (Lin et al., 1998; Arber et al., 2000). *Er81* mutant mice exhibited severe defects in motor coordination, which resulted from a failure of group Ia proprioceptive afferents to form proper projections (Arber et al., 2000). ER81 also marked a subpopulation of neurons in the subventricular zone of the lateral ganglionic eminence, rostral migratory stream and olfactory bulb interneuron progenitors (Stenman et al., 2003). In the present study, we showed that *Er81* marks a subpopulation of neighboring cortical layer 5 neurons (Figs. 1, 4, 5, 6, 7, 8). The morphology of ER81-expressing cells further suggests that the subpopulation consists of pyramidal cells. The finding that ER81-positive cells did not contain GABAergic interneurons (Fig. 6) also supports this view. Several other brain structures (Fig. 4) also expressed *Er81* including the SC, which is a major target of layer 5 projecting neurons. ER81 may be involved in the formation of specific neural circuits composed of these layer 5 pyramidal neurons and the SC cells as observed in spinal motor neurons and proprioseptive sensory neurons (Lin et al., 1998; Arber et al., 2000; Livet et al., 2002). The expression of ER81 in layer 5 (Sugitani et al., 2002; Beggs et al., 2003; Hevner et al., 2003; Watakabe et al., 2004) and the inferior olive (Zhu and Guthrie, 2002) were also previously reported.

Developmental change of *Er81* expression

Er81 was expressed in putative prospective layer 5 neurons for the entire period of cortical development (Fig. 5) starting at E15 in the rat ventricular zone (Fig. 5A). This result may suggest that the fate of *Er81*-expressing cortical neurons is already determined in the ventricular zone.

What determines the expression of *Er81*? As we have shown in this study, *Er81* is first expressed at E15 in the ventricular zone (Fig. 5A), which is a relatively early stage in the course of various events of cortical development. Layer 5 neurons are born around E15 and complete their migration to the cortical plate by E20 (Miller, 1988). The first corticofugal axons enter the internal capsule at E17 and reach the targets between E19 and the early postnatal period (De Carlos and O'Leary, 1992). Thalamo-cortical afferents invade the prospective somatosensory cortical plate between E18.5 and P0 (Senft and Woolsey, 1991; López-Bendito et al., 2002; Rebsam et al., 2002). Layers 2–4 neurons settle in the cortical plate at E20 and later (Miller, 1988). These neurons then form synapses with the layer 5 neurons that are already located within the cortical plate. None of these events take place as early as E15. After invading the prospective somatosensory cortical plate between E18.5 and P0, thalamo-cortical afferents establish functional connections with cortical neurons. Coincident with this period, orphan nuclear receptor genes, RORα and RORβ and COUP-TF1, change the expression pattern in cerebral cortex drastically during the first postnatal week (Nakagawa and O'Leary, 2003; Liu et al., 2000). In contrast, the *Er81* distribution remained relatively stable until P14 (Fig. 5), when the expression dramatically decreased across the cortex. Taken together, it is likely that the expression of *Er81,* both in the ventricular zone and the cortex, is regulated independent of synaptic connections with subcortical afferent fibers, other cortical neurons or their projection targets. The expression may be determined by their intrinsic program or mutual interactions through cell surface proteins, such as Notch and Delta (Artavanis-Tsakonas et al., 1995).

Future work needs to examine if the ER81 protein is functional in the ventricular zone cells at E15. Otx1, another layer 5-enriched transcriptional factor, is also expressed in the ventricular zone of E15 rats (Weimann et al., 1999). Otx1 is mostly located in the cytoplasm of the ventricular zone neurons and is translocated to the nucleus when layer 5 neurons enter a period of axonal refinement (Weimann et al., 1999). The activity of a transcription factor can be mediated by translocation into nucleus (Liou and Baltimore, 1993). *Er81* may undergo the same translocation process and mediate neuron differentiation in much the same fashion.

Correlation between ER81 expression and projection targets

Virtually all neurons projecting to SpC or SC expressed ER81 while only one-third of the neurons projecting to the CPSC expressed the protein (Figs. 6, 7). In accordance with the present finding, Hevner et al. (2003) also reported that all of the layer 5 neurons projecting to the SpC were ER81-positive. However, our results with the neurons projecting to the SC or CPSC were inconsistent with previous studies (Weimann et al., 1999; Hevner et al., 2003). According to the data by Hevner et al. (2003), that Otx1 and ER81 are expressed in different layer 5 neurons in early postnatal cortex, the sum of the ratio of ER81-positive neurons per SC-projecting neurons and that of Otx1-positive neurons per SC projecting neurons should be no more than 100%. However, in our study, 98.1±1.6% (mean±SD, $n=4$) of layer 5 neurons projecting to the SC expressed ER81 (Figs. 6, 7) and Weimann et al. (1999) reported that 37.6% or 51.0% (one animal each) of layer 5 neurons projecting to the SC expressed Otx1. The sum of 98.1% (adult, our data) and 37.6% or 51.0% (P16 and P1 respectively, Weimann et al., 1999) makes well over 100%. Furthermore, our study showed that one-third of layer 5 neurons projecting to the CPSC were ER81-posive (Figs. 6, 7), while Hevner et al. (2003) report that all of the cortico-cortical layer 5 neurons express the protein. These discrepancies may be related to the ages of the animals. According to our preliminary data, very few SpC- or SC-projecting cells ('type I' layer 5 projecting neurons, Kasper et al., 1994, see below) were ER81-positive before P7 (not shown). The ratio of ER81-positive cells among these projecting cells may drastically increase during the juvenile stage. The discrepancies may also be due to the locations of samplings. We injected the tracer into the defined locations and counted the defined areas of the sections, and as for CPSP projecting neurons for example, we counted neurons only in primary somatosensory area (see Experimental Procedures) and noticed that most of cortico-cortical neurons were located not in the somatosensory area but in the cingulate cortices (not shown). To understand the relation between neuron classes and molecular expression, sampling should be conducted in an appropriately defined area because distribution of a molecule is not necessarily homogeneous throughout cortical areas and injected tracer labels only a limited area. We counted about 130 neurons in each animal and about 520 neurons per a paradigm (see Experimental Procedures section). Our result was rather consistent among individual animals.

Kasper et al. (1994) classified projection neurons in layer 5 into two classes, type I and type II. Type I are subcortical projecting neurons (projecting to SpC, SC and pontine nuclei) that have tufted apical dendrites terminating in layer 1 and generate bursts of action potentials in response to a depolarizing current pulse. Type II are CPSC projecting neurons that have non-tufted apical dendrites, which arborize in layers 2–5, and do not fire bursts of action potentials (Kasper et al., 1994). However, in the present study, the ER81-expressing neurons were found both in SpC- or SC-projecting neurons and in CPSC-projecting neurons, which means the expression of ER81 did not segregate with 'type I' or 'type II.' Otx1 is also reported not to segregate with these 'types' (Weimann et al., 1999). Therefore, among layer 5 neurons, ER81 and Otx1 chalked out new boundaries that have been missed by morphological, hodological or electrophysiological criteria.

It is still unknown whether a layer 5 neuron consistently expresses ER81 or Otx1 from E15 to juvenile stage (in rat, for example) and whether these molecules define consistent neuron classes during this period. Alternatively, the presence of ER81 may not correlate with certain developmental processes. For example, Otx1-knockout mice are defective in the pruning of the aberrant axonal projections of layer 5 neurons (Weimann et al., 1999) that persist only transiently in normal animals. Because pruning should occur in all the aberrant projections, the expression of Otx1 may not occur in a fixed subpopulation of layer 5 neurons but may be dynamically regulated to occur transiently only in neurons undergoing some specific refinement process. Since ER81 is suggested to be expressed in a separate layer 5 neuron population than Otx1 (Hevner et al., 2003), ER81 may function antagonistically with Otx1 and may be dynamically regulated as well. A diachronic study to correlate the molecular expression patterns and developmental events at the single-cell resolution may help to reveal the regulations of expression and the functions of these molecules.

In the adult rat cortex, *Er81* mRNA was not detected by ISH (Fig. 5), but the protein was detected by IHC (Fig. 6). This discrepancy is most likely due to the relatively low sensitivity of the ISH method. The result of the single-cell PCR experiment (Fig. 7) supports this assumption.

Conserved expression of *Er81* among rodents and primates

Although the mammalian cerebral cortex shows some structural diversity among species (DeFelipe et al., 2002; Krubitzer, 2000; Preuss, 2001; Kaas and Collins, 2001), the overall areal and laminar organization remains similar. The cerebral cortices of different mammals contain recognizably similar cell types (Cajal, 1911), the overall design of the neocortical map is conserved (Krubitzer, 1995) and most mammalian neocortices show the isocortical organization containing six layers of cells (Jacobson, 1991). Furthermore, the basic process of neocortical development is shared among species with slight variations (Rakic, 2003).

What is the molecular mechanism responsible for conferring these similarities and diversities onto neocortices? A disruption of a single molecule, which is involved in the conserved developmental process of the cortices, is known to have a profound influence on the conserved organization (Bishop et al., 2000, 2002, 2003; Fukuchi-Shimogori and Grove, 2001; Chenn and Walsh, 2002; Piao et al., 2004) and can cause cortical malformations (Ross and Walsh, 2001). Mutations of these molecules are also suggested to explain the emergence of new cortical fields during evolution (Grove and Fukuchi-Shimogori, 2003). In this study we showed that *Er81* expression in layer 5 neurons is con-

served among mice, rats, and monkeys. The *Er81* signal was weaker in neonatal monkey brain than rats and mice (Figs. 3, 5, 6). This difference may be because macaques are born with more mature cerebral cortices than rodents. Neurons in the macaque visual cortex are born two months before the delivery of the animal (Rakic, 1974, 1977) while, in rat, prospective cortical neurons are still undergoing their final division on the last day of gestation (Jacobson, 1991). Another possibility is that the difference was caused by the difference of the sequence of the gene among primates and rodents. The sequence of clone 262 was commonly used for all species in the present study. ER81 protein may be detected with a specific antibody. The conservation of layer 5-enriched expression of *Er81* among these species suggests that, in layer 5, ER81 contributes to some cellular process that is conserved among mammalian species.

Acknowledgments—We thank Dr. Thomas M. Jessell (Columbia University) for the generous gift of the ER81 antibody, Dr. Atsushi Hayashi (Osaka University) for the brain of the cynomolgus monkey, and Aurélie Dewaele and Kimu Yoneshima for technical assistance. This work was supported by the Human Frontier Science Program (RG 107/2001 to ZM, EA, NY), the Avenir program of the INSERM (EA), 'Dynamics of Biological Systems' Project Fund under the 21st Century COE Program of MEXT (I.F., H.Y., N.Y.). Funding for CCJV was provided by a Rhodes Scholarship.

REFERENCES

Angevine JB, Sidman RL (1961) Autoradiographic study of cell migration during histogenesis of the cerebral cortex in the mouse. Nature 192:766–768.

Arber S, Ladle DR, Lin JH, Frank E, Jessell TM (2000) ETS gene *Er81* controls the formation of functional connections between group Ia sensory afferents and motor neurons. Cell 101:485–498.

Arimatsu Y, Kojima M, Ishida M (1999) Area- and lamina-specific organization of a neuronal subpopulation defined by expression of latexin in the rat cerebral cortex. Neuroscience 88:93–105.

Artavanis-Tsakonas S, Matsuno K, Fortini ME (1995) Notch signaling. Science 268:225–232.

Beggs HE, Schahin-Reed D, Zang K, Goebbels S, Nave KA, Gorski J, Jones KR, Sretavan D, Reichardt LF (2003) FAK deficiency in cells contributing to the basal lamina results in cortical abnormalities resembling congenital muscular dystrophies. Neuron 40:501–514.

Bishop KM, Garel S, Nakagawa Y, Rubenstein JLR, O'Leary DDM (2003) Emx1 and *Emx2* cooperate to regulate cortical size, lamination, neuronal differentiation, development of cortical efferents, and thalamocortical pathfinding. J Comp Neurol 457:345–360.

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

Bishop KM, Rubenstein JL, O'Leary DDM (2002) Distinct actions of *Emx1*, Emx2, and *Pax6* in regulating the specification of areas in the developing neocortex. J Neurosci 22:7627–7638.

Brown TA, McKnight SL (1992) Specificities of protein-protein and protein-DNA interaction of GABP alpha and two newly defined ets-related proteins. Genes Dev 6:2502–2512.

Cajal R (1911) Histologie du système nerveux de l'homme et des vertébrés. Paris: Maloine.

Cauli B, Audinat E, Lambolez B, Angulo MC, Ropert N, Tsuzuki K, Hestrin S, Rossier J (1997) Molecular and physiological diversity of cortical nonpyramidal cells. J Neurosci 17:3894–3906.

Chenn A, Walsh CA (2002) Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science 297:365–369.

De Carlos JA, O'Leary DDM (1992) Growth and targeting of subplate axons and establishment of major cortical pathways. J Neurosci 12:1194–1211.

DeFelipe J (1993) Neocortical neuronal diversity: chemical heterogeneity revealed by colocalization studies of classic neurotransmitters, neuropeptides, calcium-binding proteins, and cell surface molecules. Cereb Cortex 3:273–289.

DeFelipe J, Alonso-Nanclares L, Arellano JI (2002) Microstructure of the neocortex: comparative aspects. J Neurocytol 31:299–316.

de Launoit Y, Baert JL, Chotteau A, Monte D, Defossez PA, Coutte L, Pelczar H, Leenders F (1997) Structure-function relationships of the PEA3 group of Ets-related transcription factors. Biochem Mol Med 61:127–135.

Fukuchi-Shimogori T, Grove EA (2001) Neocortex patterning by the secreted signaling molecule FGF8. Science 294:1071–1074.

Gilbert CD, Wiesel TN (1985) Intrinsic connectivity and receptive field properties in visual cortex. Vision Res 25:365–374.

Gray PA, Fu H, Luo P, Zhao Q, Yu J, Ferrari A, Tenzen T, Yuk DI, Tsung EF, Cai Z, Alberta JA, Cheng LP, Liu Y, Stenman JM, Valerius MT, Billings N, Kim HA, Greenberg ME, McMahon AP, Rowitch DH, Stiles CD, Ma Q (2004) Mouse brain organization revealed through direct genome-scale TF expression analysis. Science 306:2255–2257.

Grove EA, Fukuchi-Shimogori T (2003) Generating the cerebral cortical area map. Annu Rev Neurosci 26:355–380.

Hasegawa H, Ashigaki S, Takamatsu M, Suzuki-Migishima R, Ohbayashi N, Itoh N, Takada S, Tanabe Y (2004) Laminar patterning in the developing neocortex by temporally coordinated fibroblast growth factor signaling. J Neurosci 24:8711–8719.

Hevner RF, Daza RAM, Rubenstein JLR, Stunnenberg H, Olavarria JF, Englund C (2003) Beyond laminar fate: toward a molecular classification of cortical projection/pyramidal neurons. Dev Neurosci 25:139–151.

Hevner RF, Shi L, Justice N, Hsueh YP, Sheng M, Smiga S, Bulfone A, Goffinet AM, Campagnoni AT, Rubenstein JLR (2001) Tbr1 regulates differentiation of the preplate and layer 6. Neuron 29:353–366.

Jacobson M (1991) Developmental neurobiology. New York: Plenum Press.

Jones EG, Powell TPS (1973) Anatomical organization of the somatosensory cortex. In: Handbook of sensory physiology, vol. 2: Somatosensory system (Iggo A, ed.), pp 579–620. New York: Springer-Verlag.

Kaas JH, Collins CE (2001) Evolving ideas of brain evolution. Nature 411:141–142.

Kasper EM, Lübke J, Larkman AU, Blakemore C (1994) Pyramidal neurons in layer 5 of the rat visual cortex. III. Differential maturation of axon targeting, dendritic morphology, and electrophysiological properties. J Comp Neurol 339:495–518.

Katz LC, Burkhalter A, Dreyer WJ (1984) Fluorescent latex microspheres as a retrograde neuronal marker for in vivo and in vitro studies of visual cortex. Nature 310:498–500.

Koester SE, O'Leary DDM (1992) Functional classes of cortical projection neurons develop dendritic distinctions by class-specific sculpting of an early common pattern. J Neurosci 12:1382–1393.

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

Krubitzer LA (2000) How does evolution build a complex brain? In: Evolutionary developmental biology of the cerebral cortex (Bock GR, Carden G, eds), pp 206–226. Chichester: Weiley.

Lambolez B, Audinat E, Bochet P, Crepel F, Rossier J (1992) AMPA receptor subunits expressed by single Purkinje cells. Neuron 9: 247–258.

Larkman A, Mason A (1990) Correlations between morphology and electrophysiology of pyramidal neurons in slices of rat visual cortex. I. Establishment of cell classes. J Neurosci 10:1407–1414.

Lewis JW, Olavarria JF (1995) Two rules for callosal connectivity in striate cortex of the rat. J Comp Neurol 361:119–137.

Lin JH, Saito T, Anderson DJ, Lance-Jones C, Jessell TM, Arber S (1998) Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate *ETS* gene expression. Cell 95:393–407.

Liou HC, Baltimore D (1993) Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system. Curr Opin Cell Biol 5:477–487.

Liu Q, Dwyer ND, O'Leary DDM (2000) Differential expression of COUP-TFI, CHL1, and two novel genes in developing neocortex identified by differential display PCR. J Neurosci 20:7682–7690.

Livet J, Sigrist M, Stroebel S, De Paola V, Price SR, Henderson CE, Jessell TM, Arber S (2002) ETS gene *Pea3* controls the central position and terminal arborization of specific motor neuron pools. Neuron 35:877–892.

López-Bendito G, Chan CH, Mallamaci A, Parnavelas J, Molnár Z (2002) Role of *Emx2* in the development of the reciprocal connectivity between cortex and thalamus. J Comp Neurol 451:153–169.

Lund JS (1988) Anatomical organization of macaque monkey striate visual cortex. Annu Rev Neurosci 11:253–288.

McConnell SK (1989) The determination of neuronal fate in the cerebral cortex. Trends Neurosci 12:342–349.

McConnell SK, Kaznowski CE (1991) Cell cycle dependence of laminar determination in developing neocortex. Science 254:282–285.

Miller MW (1988) Development of projection and local circuit neurons in neocortex. In: Development and maturation of cerebral cortex (Peters A, Jones E, eds), pp 133–175. New York: Plenum Press.

Nakagawa Y, O'Leary DDM (2003) Dynamic patterned expression of orphan nuclear receptor genes *ROR*α and *ROR*β in developing mouse forebrain. Dev Neurosci 25:234–244.

O'Leary DDM, Bicknese AR, De Carlos JA, Heffner CD, Koester SE, Kutka LJ, Terashima T (1990) Target selection by cortical axons: alternative mechanisms to establish axonal connections in the developing brain. Cold Spring Harb Symp Quant Biol 55:453–468.

Paxinos G, Watson C, Pennisi M, Topple A (1985) Bregma, lambda and the interaural midpoint in stereotaxic surgery with rats of different sex, strain and weight. J Neurosci Methods 13:139–143.

Peters A, Jones EG (eds) (1984) Cerebral cortex vol. 1: Cellular components of the cerebral cortex. New York: Plenum Press.

Piao X, Hill RS, Bodell A, Chang BS, Basel-Vanagaite L, Straussberg R, Dobyns WB, Qasrawi B, Winter RM, Innes AM, Voit T, Ross ME, Michaud JC, Descarie JC, Barkovich AJ, Walsh CA (2004) G protein-coupled receptor-dependent development of human frontal cortex. Science 303:2033–2036.

Preuss TM (2001) The discovery of cerebral diversity: An unwelcome scientific revolution. In: Evolutionary anatomy of the primate cerebral cortex (Falk D, Gibson K, eds), pp 138–164. Cambridge: Cambridge University Press.

Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. J Comp Neurol 145:61–83.

Rakic P (1974) Neurons in rhesus monkey visual cortex: systematic relation between time of origin and eventual disposition. Science 183:425–427.

Rakic P (1977) Prenatal development of the visual system in rhesus monkey. Philos Trans R Soc Lond B Biol Sci 278:245–260.

Rakic P (2003) Developmental and evolutionary adaptations of cortical radial glia. Cereb Cortex 13:541–549.

Rebsam A, Seif I, Gaspar P (2002) Refinement of thalamocortical arbors and emergence of barrel domains in the primary somatosensory cortex: a study of normal and monoamine oxidase a knock-out mice. J Neurosci 22:8541–8552.

Rice DS, Curran T (2001) Role of the reelin signaling pathway in central nervous system development. Annu Rev Neurosci 24: 1005–1039.

Ross ME, Walsh CA (2001) Human brain malformations and their lessons for neuronal migration. Annu Rev Neurosci 24:1041–1070.

Senft SL, Woolsey TA (1991) Growth of thalamic afferents into mouse barrel cortex. Cereb Cortex 1:308–335.

Sharrocks AD, Brown AL, Ling Y, Yates PR (1997) The ETS-domain transcription factor family. Int J Biochem Cell Biol 29:1371–1387.

Stenman J, Toresson H, Campbell K (2003) Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. J Neurosci 23:167–174.

Sugitani Y, Nakai S, Minowa O, Nishi M, Jishage K, Kawano H, Mori K, Ogawa M, Noda T (2002) Brn-1 and Brn-2 share crucial roles in the production and positioning of mouse neocortical neurons. Genes Dev 16:1760–1765.

Takahashi T, Goto T, Miyama S, Nowakowski RS, Caviness VS Jr (1999) Sequence of neuron origin and neocortical laminar fate: relation to cell cycle of origin in the developing murine cerebral wall. J Neurosci 19:10357–10371.

Voelker CCJ, Garin N, Taylor JSH, Gähwiler BH, Hornung JP, Molnár Z (2004) Selective neurofilament (SMI-32, FNP-7 and N200) expression in subpopulations of layer V pyramidal neurons in vivo and in vitro. Cereb Cortex 14:1276–1286.

Wasylyk B, Hagman J, Gutierrez-Hartmann A (1998) Ets transcription factors: nuclear effectors of the Ras-MAP-kinase signaling pathway. Trends Biochem Sci 23:213–216.

Watakabe A, Ohsawa S, Komatsu Y, Hashikawa T, Yamamori T (2004) A comparative analysis of layer-specific genes in mammalian neocortex. Neurosci Res 50:S139.

Weimann JM, Zhang YA, Levin ME, Devine WP, Brûlet P, McConnell SK (1999) Cortical neurons require Otx1 for the refinement of exuberant axonal projections to subcortical targets. Neuron 24: 819–831.

Welker E, Armstrong-James M, Bronchti G, Ourednik W, Gheorghita-Baechler F, Dubois R, Guernsey DL, Van der Loos H, Neumann PE (1996) Altered sensory processing in the somatosensory cortex of the mouse mutant barrelless. Science 271:1864–1867.

Xu B, Zang K, Ruff NL, Zhang YA, McConnell SK, Stryker MP, Reichardt LF (2000) Cortical degeneration in the absence of neurotrophin signaling: dendritic retraction and neuronal loss after removal of the receptor TrkB. Neuron 26:233–245.

Zhong Y, Takemoto M, Fukuda T, Hattori Y, Murakami F, Nakajima D, Nakayama M, Yamamoto N (2004) Identification of the genes that are expressed in the upper layers of the neocortex. Cereb Cortex 14:1144–1152.

Zhu Y, Guthrie S (2002) Expression of the ETS transcription factor *ER81* in the developing chick and mouse hindbrain. Dev Dyn 225: 365–368.

(Accepted 23 August 2005)
(Available online 14 November 2005)