Paraneoplastic Antigen-Like 5 Gene (PNMA5) Is Preferentially Expressed in the Association Areas in a Primate Specific Manner

To understand the relationship between the structure and function of primate neocortical areas at a molecular level, we have been screening for genes differentially expressed across macaque neocortical areas by restriction landmark cDNA scanning (RLCS). Here, we report enriched expression of the paraneoplastic antigen-like 5 gene (PNMA5) in association areas but not in primary sensory areas, with the lowest expression level in primary visual cortex. In situ hybridization in the primary sensory areas revealed PNMA5 mRNA expression restricted to layer II. Along the ventral visual pathway, the expression gradually increased in the excitatory neurons from the primary to higher visual areas. This differential expression pattern was very similar to that of retinol-binding protein (RBP) mRNA, another association-area-enriched gene that we reported previously. Additional expression analysis for comparison of other genes in the PNMA gene family, PNMA1, PNMA2, PNMA3, and MOAP1 (PNMA4), showed that they were widely expressed across areas and layers but did not have the differentiated pattern of PNMA5. In mouse brains, PNMA1 was only faintly expressed and PNMA5 was not detected. Sequence analysis showed divergence of PNMA5 sequences among mammals. These findings suggest that PNMA5 acquired a certain specialized role in the association areas of the neocortex during primate evolution.

Keywords: association area evolution, cortical structure, in situ hybridization, neocortex, primate specific

The neocortex has undergone a marked increase in volume and complexity during the course of primate evolution (Stephan et al. 1981; Haug, 1987; Preuss and Goldman-Rakic 1991c; Finlay and Darlington 1995). Brodmann, who conducted comprehensive surveys of the mammalian cytoarchitecture, concluded that the granular frontal cortex is rudimentary or absent in mammals other than primates, but in primates it underwent considerable expansion with the concomitant addition of new areas during evolution (Brodmann 1909, 1912). Consistent with this classic study, Preuss and Goldman-Rakic also suggested that anthropoid primates evolved additional cortical areas during their phylogenetic history (Preuss and Goldman-Rakic 1991a, 1991b, 1991c). This idea was based on the more elaborate cytoarchitectonic and connectional subdivisions in the frontal, parietal and temporal areas in Macaca as compared with Galago, particularly in the granular frontal and superior temporal polysensory cortices. The new areas that emerged during primate evolution correspond to higher-order association areas, the regions that receive inputs from sensory and other association areas (Pandya and Kuypers 1969; Jones and Powell 1970; Mesulam et al. 1977; Pandya and Seltzer 1982), and which mediate integrative aspects of somatosensory and visuospatial (Friedman et al. 1986; Andersen 1989), auditory (Leinonen et al. 1980; Galaburda and Pandya 1983), polysensory (Bruce et al. 1981; Baylis et al. 1987), and memory processes (Van Hoesen 1982). These structures influence perception, cognition, or behavior in part through their strong connections with the frontal lobe (Bignall and Imbert 1969; Milner and Petrides 1984; Goldman-Rakic 1988; Pandya and Yeterian 1990). Because of such divergence of functions, it still remains a challenge to answer "what is the nature of association areas?". To solve this problem, applying molecular genetic approaches is potentially very effective (Crick 1999).

Previous studies have demonstrated the heterogeneous distribution of various neurochemicals and neurotransmitter receptors within the adult primate neocortex (Mash et al. 1988; Bloom et al. 1997; Pimenta et al. 2001; Zilles et al. 2002). In our own studies, we previously found 3 types of genes that show characteristically different expression patterns across the neocortical areas of adult macaques (Watakabe et al. 2006; Yamamori and Rockland 2006). 1) ecc1, testican-1, testican-2, serotonin 1B, and serotonin 2A receptor genes exhibit enriched expression in the primary visual cortex (Tochitani et al. 2001; Takahata et al. 2008; Watakabe et al. 2008). 2) Growth/differentiation factor 7 is preferentially expressed in the primary motor area of the neocortex of African green monkeys (Watakabe, Fujita, et al. 2001). 3) Retinol-binding protein gene (RBP) is highly expressed in higher-order association areas of macaque neocortex (Komatsu et al. 2005). Interestingly, RBP mRNA distribution shows a highly layer-specific pattern. In the primary sensory areas, it is weak and restricted to layer II, but its expression increases toward the deeper layers along the ventral visual pathway. In the higher-order association areas, it is expressed widely in layers II–VI, except layer IV. Although the RBP gene is a good candidate to study the relationship between the structure and function of primate association areas, we think it is unlikely that RBP is the only gene that shows a pattern of association area-specific expression (Watakabe, Sugai, et al. 2001; Evans et al. 2003; Sato et al. 2007). It would help us to understand the features of association area-specific genes in primates if other genes with a comparable expression pattern could be identified and characterized.

We therefore performed additional rounds of screening for genes differentially expressed in adult macaque neocortical areas using a cDNA display method, namely, restriction landmark cDNA scanning (RLCS) (Suzuki et al. 1996; Shintani et al. 2004). By this method, we succeeded in identifying paraneoplastic antigen-like 5 gene (PNMA5) as a gene that is conspicuously and selectively expressed in primate association...
areas. The PNMA5 gene in humans is a member of a putative gene family that consists of 6 genes known as PNMA1, PNMA2, PNMA3, PNMA4 (also called modulator of apoptosis-1, MOAP1), PNMA5, and PNMA6A (Schüller et al. 2005). The functions of this PNMA family of genes in the brain are unknown.

To understand the role of PNMA5 and its gene family, we performed detailed expression analyses of these genes by in situ hybridization (ISH) in macaques, marmosets, and mice. We also performed northern blot hybridization and reverse transcription polymerase chain reaction (RT-PCR) in humans, African green monkeys, mice, and rats for gross expression analyses in these species. We found that PNMA5 mRNA exhibited a pattern of area and laminar expression strikingly similar to that of RBP mRNA. Other family members were expressed in the macaque brains, but did not show such conspicuous area and laminar differences. Interestingly, among the PNMA family of gene, PNMA5 and PNMA1 were not expressed in the mouse brains. Comparisons between human and mouse sequences revealed moderate to high conservation in the amino acid sequences of PNMA1, PNMA2, PNMA3, and MOAP1, but considerable divergence of the sequences of PNMA5 was observed. These results suggest an important role of PNMA5 in the specialization of association areas during primate evolution.

Materials and Methods

Experimental Animals and Tissue Preparation
Three adult Japanese monkeys (Macaca fuscata) and 3 adult common marmosets (Callithrix jacchus) were used for histological analyses as previously reported (Takahata et al. 2000; Watakabe et al. 2007). Five adult mice (Mus musculus, C57BL/6, SLC, Japan) were also used. For tissue fixation, the mice were deeply anesthetized with Nembutal (100 mg/kg body weight, intraperitoneally) and perfused intracardially with 4% paraformaldehyde in 0.1 M phosphate buffer at 4 °C. The brains were postfixed for 5 h at room temperature and then cryoprotected with 30% sucrose in 0.1 M phosphate buffer at 4 °C.

Adult African green monkeys (Cercopithecus aesthiopis; 1 female and 1 male for testis), adult mice (M. musculus, C57BL/6), and adult rats (Rattus norvegicus, Wistar) were used for northern blot hybridization and RT-PCR. The adult African green monkeys were used as previously reported (Watakabe, Fujita, et al. 2001). Mice and rats were deeply anesthetized with an overdose of Nembutal (100 mg/kg body weight, intraperitoneally) and sacrificed. All the experiments followed the animal care guidelines of the National Institute for Basic Biology and the National Institute for Physiological Sciences, Japan, and the National Institute of Health, United States.

RNA Isolation
Total RNAs were isolated from frozen tissues by the acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987). Total RNA of a human brain was purchased from Clontech (Mountain View, CA). Poly(A) RNAs were purified from the total RNA using Oligotex-dT30 (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer’s recommended procedure.

RLCS Analysis
RLCS analyses were carried out essentially as described previously (Suzuki et al. 1996; Shintani et al. 2004), using poly(A) RNAs purified from 4 areas (area 46, primary motor area, temporal area, and primary visual area) of the African green monkeys. The double-stranded cDNAs were synthesized using the poly(A) RNA with a biotin-anchored primer and digested with a first restriction enzyme, Bcl. The biotin-tagged fragments were radioisotope labeled. These fragments were collected and subjected to 1D agarose gel electrophoresis. After electrophoresis, the cDNA fragments in the gel were again digested with a second restriction enzyme, HindIII. The digested fragments were then subjected to 2D acrylamide gel electrophoresis.

The cDNA fragments separated in 2D electrophoresis were displayed as spots by autoradiography. A comparison of the intensity of each spot on the corresponding set of autoradiograms was performed, and cDNA spots showing different intensities among areas were eluted from the gel, subcloned, and their sequences determined.

In Situ Hybridization
For single-colored ISH, coronal sections were cut to 35-μm thickness. The digoxigenin (DIG)-labeled riboprobes were produced using template plasmids, which included the polymerase chain reaction (PCR) fragments generated using the primers listed in Supplementary Table S1. ISH was carried out as previously described (Liang et al. 2000; Komatsu et al. 2005). Briefly, free-floating sections were treated with proteinase K (5 μg/ml for macaque monkey and marmoset sections, and 1 μg/ml for mouse sections) for 30 min at 37 °C, acetylated, then incubated in a hybridization buffer (5× SSC, 2% blocking regent [Roche Diagnostics, Basel, Switzerland], 50% formamide, 0.1% N-lauroylsarcosine, 0.1% SDS) containing 0.5 μg/ml DIG-labeled riboprobes at 60 °C. The sections were sequentially treated in 2× SSC/0.5% formamide:0.1% N-lauroylsarcosine for 15 min at 60 °C twice, 30 min at 37 °C in RNase buffer (10 mM Tris-HCl [pH 8.0], 1 mM ethylenediaminetetraacetic acid [EDTA], 500 mM NaCl) containing 20 μg/ml RNase A (Sigma Aldrich, Saint Louis, MI), 15 min at 37 °C in 2× SSC/0.1% N-lauroylsarcosine twice, and 15 min at 37 °C in 0.2× SSC/0.1% N-lauroylsarcosine twice. The hybridization probe was detected with an alkaline phosphatase-conjugated anti-DIG antibody using DIG nucleic acid detection kit (Roche Diagnostics). For the ISH of PNMA family genes, 2 probes were prepared for each gene of 1 species. We confirmed that the 2 probes for each of the genes exhibited essentially the same hybridization signal patterns and there were no signals above the background with the sense probes. After confirming these points, the 2 probes were mixed together to intensify the signals.

Fluorescence double-colored ISH was carried out using DIG- and fluorescein-labeled riboprobes as described previously (Watakabe et al. 2007). The sections were cut to 15-μm thickness. The hybridization and washing were carried out as described above, except that both DIG- and fluorescein-labeled probes were used for the hybridization. After blocking in 1% blocking buffer (Roche Diagnostics) for 1 h, the probes were detected in 2 different ways. For the detection of fluorescein probes, the sections were incubated with an anti-fluorescein antibody conjugated with horseradish peroxidase (Roche Diagnostics, 1:2000 in the blocking buffer) for 3 h at room temperature. After washing in TNT buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% Tween20) 3 times for 15 min, the sections were treated with 1:100-diluted TSA-Plus reagents (Perkin Elmer, Boston, MA) for 30 min according to the manufacturer’s instruction, and the fluorescein signals were converted to dinitrophenol (DNP) signals. After washing with TNT buffer 3 times for 10 min, the sections were incubated overnight at 4 °C with an anti-DNP antibody conjugated with Alexa488 (1:500, Molecular Probes, Eugene, OR) in 1% blocking buffer for the fluorescence detection of the DNP signals. At this point, an anti-DIG antibody conjugated with alkaline phosphatase (1:1000, Roche Diagnostics) was also incubated for the detection of the DIG probes. The sections were washed 3 times in TNT buffer, once in TS 8.0 (0.1 M Tris-HCl [pH 8.0], 0.1 M NaCl, 50 mM MgCl2), and the alkaline phosphatase activity was detected using HNPP fluorescence detection kit (Roche Diagnostics) according to the manufacturer’s instruction. The incubation for this substrate was carried out for 30 min and stopped in PBS containing 10 mM EDTA. The sections were then counterstained with Hoechst 33342 (Molecular Probes) to intensify the signals.

Quantification of ISH Signals
We analyzed the optical densities of the ISH signals of PNMA5 and RBP mRNAs to investigate the layer distribution. First, the layer borders
were determined based on the Nissl staining patterns of the adjacent sections. The images of the ISH-stained sections of PNMA5 and RBP were then transformed using Adobe Photoshop, so that the heights of the images became equal. The optical density of the adjusted images was measured using ImageJ image analysis software (Abramoff et al. 2004) after binarization of the signals with an equable criterion, mean minus standard deviation (SD) in the gray scale image.

Furthermore, we manually counted the double positive cells for PNMA5 mRNA and either one of the VGLUT1, GAD67 or RBP mRNAs. For the counting, 15-μm-thick sections containing several areas were used for the double ISH. These areas were identified in reference to a standard atlas (Paxinos et al. 1999). The following procedures were carried out using Adobe Photoshop. The images taken in 3 channels (ISH signals in red/green channels and Hoechst nuclear staining in the blue channels) were layered into a single file. Eight 200 × 200 μm² windows were selected within layer II and upper region of layer III of each area for counting. On the basis of Hoechst staining, the cells positive for red or green ISH fluorescent signals were plotted manually onto a blank layer as dots for later counting. The numbers and proportions of double positive cells for PNMA5 mRNA and 1 of VGLUT1, GAD67 or RBP mRNAs were counted, and then calculated for each window of each area and averaged.

Northern Blot Hybridization and RT-PCR
Northern blot hybridization was carried out essentially as described previously (Sambrook et al. 1989). Briefly, glyoxylated poly(A) RNA was electrophoresed in 1.2% agarose gel and transferred onto a Hybond N+ nylon membrane (GE Healthcare, Little Chalfont, Buckinghamshire, England). For each probe, regions between the primers listed in Supplementary Table S1 were labeled with α-P by random priming. After 2 h of prehybridization in a hybridization buffer (50 mM Tris–HCl [pH 7.5], 10 mM EDTA [pH 8.0], 1% sodium N-dodecylsulfate, 1 M NaCl, 0.2% bovine serum albumin, 0.2% ficoll 400, 0.2% polyvinylpyrrolidone, 100 μg/mL salmon sperm DNA), 32P-labeled probes were added to a final concentration of 5 × 10⁵ cpm/mL. Hybridization was carried out at 65 °C overnight. After hybridization, the membrane was washed twice in 0.2× SSC (16.65 mM NaCl: 16.65 mM trisodium citrate dihydrate) with 1% sodium dodecyl sulfate (SDS) and autoradiographed. The membrane blotted with the mRNAs of the African green monkey was used in the previous report (Watakanbe, Fujita, et al. 2001). For RT-PCR, 1.0 μg of DNase-treated total RNA was reverse-transcribed using Superscript II RNase H Reverse Transcriptase (Invitrogen, Carlsbad, CA). After the reaction was terminated, the reaction mixtures were diluted 2-fold with distilled water. PCR was performed in 10 μL of reaction mixtures, containing 1 μL of the reaction mixture, 2 pmol of gene-specific primers (Supplementary Table S1), 1× KOD-Plus buffer, 2 nmol each of dNTP, 10 nmol MgSO4, 5% DMSO and 0.2 units of KOD-Plus polymerase (TOYOBO, Osaka, Japan). An initial denaturation step of 5 min at 95 °C was followed by predetermined optimal cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 40 s. PCR products obtained with the same primer set for RNAs derived from different tissues were loaded and separated on the same 1% agarose gel and visualized with ethidium bromide.

Sequence Determination of PNMA5 and the PNMA Family Genes
We cloned cDNAs of all known PNMA family genes in the human, African green monkey, and mouse and determined their nucleotide sequences. For the African green monkey, library screening and isolation of positive clones were carried out according to the standard screening procedure. The cDNA inserts of the positive clones were PCR-amplified from cDNAs of all known family genes in the human, African green monkey, and mouse and determined their nucleotide sequences. For the African green monkey, library screening and isolation of positive clones were carried out according to the standard screening procedure. The cDNA inserts of the positive clones were PCR-amplified from cDNAs of all known family genes in the human, African green monkey, and mouse.

Results
Identification of PNMA5 as an Association Area-Specific Gene
For a systematic large-scale screening of area-specific genes in the adult macaque neocortex, we carried out RLCS (Suzuki et al. 1996; Shintani et al. 2004) using mRNAs purified from 4 distinct cortical areas (area 46, primary motor, temporal, and primary visual areas; Fig. 1A) as described in the Materials and Methods section. Using a pair of the restriction enzymes of BclI and Hinfl, we found several spots showing different expression profiles among neocortical areas. One of these spots was most abundant in the association areas of the frontal (area 46) and temporal areas but was almost absent in the visual area (Fig. 1C). This spot was excised from the gel, and the corresponding gene was identified as PNMA5. RT-PCR analysis confirmed the area-specific expression of PNMA5 mRNA (data not shown).

Distribution of PNMA5 mRNA in the Macaque Brain
To examine the distribution of PNMA5 mRNA in detail, we carried out ISH using adult macaque brains. Figure 2 shows the distribution patterns of PNMA5 mRNA in various coronal sections. Overall, the cortical expression of PNMA5 mRNA was high and the expressions in the subcortical nuclei were low. Consistent with the results of the RLCS analysis, we observed a heterogeneous expression pattern within the neocortex: that is, we observed strong expression in the prefrontal (Fig. 2A, area 46) and temporal association areas (Fig. 2D, TE), whereas we observed weak expression in the occipital cortex. It was particularly weak in the primary visual area (Fig. 2G, V1). In addition to the neocortical areas, strong expression was observed in the limbic regions such as the insular (Fig. 2C), cingulate (Fig. 2C), perirhinal (Fig. 2D), and entorhinal cortices (Fig. 2D), as well as the amygdala (Fig. 2C) and hippocampus (Fig. 2F).

The expression patterns of PNMA5 mRNA showed area-specific differences within layers (Fig. 3). In the frontonal association areas such as area 46 (Fig. 3A), strong PNMA5 mRNA expression was observed throughout layers II–VI, with dense staining in layer II, but low expression in layer IV. In the temporal association areas such as TE (Fig. 3H), perirhinal (Fig. 3I) and entorhinal cortices (Fig. 3J), strong PNMA5 mRNA expression was observed (see below). In contrast, PNMA5 mRNA expression was weak and mainly restricted to layer II in the primary sensory areas such as the primary auditory (Fig. 3C, A1), somatosensory (Fig. 3D, area 3b), and visual (Fig. 3E, V1) areas. In the primary motor area (Fig. 3B, area 4), the PNMA5 mRNA signals were mainly located in layer II as in the primary sensory areas. However, sparse signals were also present in layers III and V. Along the ventral visual pathway toward higher-order areas, we observed gradual increases of PNMA5 mRNA expression in both of the intensity and laminar distribution. In V1 (Fig. 3E), PNMA5 mRNA expression was restricted mainly to a narrow layer at the top of layer II as described earlier. However, in V2 (Fig. 3F), its expression extended over the entire layer II, but still restricted only within the supragranular layer. In V4 (Fig. 3G), PNMA5 mRNA expression was also observed predominantly in layer II, but weakly detected in layers III, V, and VI as well. In TE (Fig. 3H), the PNMA5 mRNA expression in layers III, V, and VI was stronger than that in V4, but absent in layer IV. In the
perirhinal cortex (Fig. 3I), PNMA5 mRNA was strongly expressed in layers II and V with no expression in layer IV. In the entorhinal cortex (Fig. 3J), PNMA5 mRNA expression was strongly observed in all the layers. In the hippocampus (Fig. 3K), PNMA5 mRNA was expressed through CA1–CA4 and the dentate gyrus except that the expression was low in CA2 and absent in the Subiculum.

In summary, PNMA5 mRNA showed strong expression in layers II, III, V, and VI of the association areas and in the limbic regions, but was weak and restricted only to layer II in the primary sensory areas and subcortical nuclei in macaque brains.

PNMA5-mRNA-Positive Cells Are Mostly Excitatory Neurons

To determine the type of cells that express PNMA5 mRNA, we examined what percentages of excitatory and inhibitory neurons express PNMA5 mRNA. For this purpose, we performed double ISH of PNMA5 mRNA with either vesicular glutamate transporter 1 (VGLUT1) mRNA as a glutamatergic excitatory neuronal marker (Takamori et al. 2000) or glutamate decarboxylase 1 (GAD67) mRNA as a GABAergic inhibitory neuronal marker. Figure 4 shows a typical example of such double ISH in area TE. At a first glance, it was clear that most cells that expressed PNMA5 mRNA also coexpressed VGLUT1 mRNA (Fig. 4A, lower panels for coexpression). In our counting, over 95% of the PNMA5-mRNA-positive neurons coexpressed VGLUT1 mRNA in layer II (Table 1). Conversely, most of the excitatory neurons in layer II (ca. 70%) expressed PNMA5 mRNA in area 9, area 4 and TE (Table 1). On the other hand, we observed little coexpression of PNMA5 mRNA with GAD67 mRNAs (Fig. 4B, lower panels for coexpression). Less than 4% of the PNMA5-mRNA-positive neurons expressed GAD67 mRNA (Table 1). It is also worth noting that the expression of PNMA5 mRNA per cell varied consecutively from undetectable to abundant levels and not in all-or-none fashion. In the deeper layers and in the primary sensory areas, PNMA5 mRNAs showed generally low levels of expressions even in the positive cells.

Distribution of PNMA5 mRNA Is Similar to That of RBP mRNA

The distribution of PNMA5 mRNA in the adult macaque brains as mentioned above was strikingly similar to that of RBP mRNA (Komatsu et al. 2005). Thus, we directly compared expressions of PNMA5 and RBP mRNAs in the adjacent sections. As shown in Figure 5A, the overall expression patterns of the 2 mRNAs in the adult brain were very similar (Fig. 5A). Both the mRNAs showed strong expressions in the
showed that the mRNAs of subpopulations for either mRNA. and concentration, and not necessarily confined to particular areas, both mRNAs showed generally low levels of expressions both mRNAs. In the deeper layers and in the primary sensory expressions PNMA5 expression of in the liver than brain (Pfeffer et al. 2004). The mRNA different expression patterns. RBP (Table 1). Thus, some excitatory neurons in layer II seemed to express RNAs. For example, in the association areas, the expression of several differences between the expression patterns of the 2 genes range from 38.5% (between PNMA5 and PNMA6A) to 56.6% (between PNMA1 and MOAP1) (Supplementary Table S2). The percent identity of the amino acid sequences of PNMA5 between the human and mouse was low (57.0%), although that of PNMA1 was highly conserved (92.1%), and those of PNMA2, PNMA3 and MOAP1 were moderately conserved (78.7%, 74.5%, and 77.1%, respectively) (Table 2). The low conservation of PNMA5 sequence led us to determine the sequences of PNMA family genes in the African green monkey. The amino acid sequence identity between human and African green monkey PNMA5 was 93.6% (Table 2). This was substantially lower than those of the other PNMA family genes (99.7%, 97.8%, 98.3%, and 97.4%, respectively).

Next, we examined the mRNA expression patterns of PNMA family genes in several tissues from the African green monkeys and mice by northern blot hybridization (Fig. 6A,C). In the African green monkeys, PNMA5 mRNA was expressed specifically in the neocortex and testis, whereas RBP mRNA was expressed in several other tissues (Supplementary Fig. S1 and see below).

Comparative Analyses of PNMA5 and the Putative Family Genes
PNMA1, PNMA2, PNMA3, MOAP1 (PNMA4), PNMA5, and PNMA6A genes are considered to form a gene family (Schüller et al. 2005). The amino acid identities between these family genes range from 38.5% (between PNMA5 and PNMA6A) to 56.6% (between PNMA1 and MOAP1) (Supplementary Table S2). The percent identity of the amino acid sequences of PNMA5 between the human and mouse was low (57.0%), although that of PNMA1 was highly conserved (92.1%), and those of PNMA2, PNMA3 and MOAP1 were moderately conserved (78.7%, 74.5%, and 77.1%, respectively) (Table 2). The low conservation of PNMA5 sequence led us to determine the sequences of PNMA family genes in the African green monkey. The amino acid sequence identity between human and African green monkey PNMA5 was 93.6% (Table 2). This was substantially lower than those of the other PNMA family genes (99.7%, 97.8%, 98.3%, and 97.4%, respectively).

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expression levels of each gene in the neocortical areas were similar (Fig. 6B). In addition to the neocortex, these 4 genes were expressed in the cerebellum and spinal cord as well. The mRNAs of PNMA1 and PNMA3 were also expressed in other tissues (Fig. 6A). PNMA1 mRNA showed ubiquitous expressions, although the levels of expressions among the tissues were different. PNMA3 mRNA was expressed in the testis.

We next examined the mRNA expressions of the PNMA family genes in the mouse tissues by northern blot hybridization and RT-PCR. Interestingly, we found that the expression patterns in the tissues of mice were quite different from those of the African green monkeys. First, PNMA5 mRNA was detected at a low level only in the testis of mice, and in no other tissues including the brain (Fig. 6C). This expression pattern was confirmed by RT-PCR (Fig. 6D). The RT-PCR experiments also showed that PNMA5 mRNA was not expressed in the brain at any developmental stage tested. Also in rats, PNMA5 mRNA expression was confirmed in the testis, but not in the brain (Fig. 6E). Second, in contrast to the widespread expression of PNMA1 mRNA in the tissues of the African green monkeys, it was detected only in the testis of the mice. Third, PNMA3 mRNA was not detected in the mouse testis, whereas MOAP1 mRNA was strongly expressed. In addition, the expression of MOAP1 mRNA in the mouse was relatively ubiquitous, the pattern of which was similar to that of PNMA1 mRNA in the African green monkey.

In addition to the 5 PNMA family genes described above, the PNMA6A gene is annotated in the human genome. We confirmed its mRNA expression in the human brain by RT-PCR (Fig. 6F). In the human genome, the PNMA6A gene is

Figure 3. Expression pattern of PNMA5 mRNA in the macaque cortex. The cortical areas and hippocampus depicted in Figure 2 are magnified. The layers indicated on the left side of each panel were identified according to the Nissl staining of the nearby section. Left of each panel shows Nissl staining and right shows the expression patterns of PNMA5 mRNA by ISH. Abbreviations: DG, dentate gyrus; S, subiculum.
located next to PNMA3 gene, and they are separated by approximately 12 kb. There is also a very similar PNMA6B gene (99.2% amino acid identity), which is separated by 0.6 kb from the PNMA6A gene and positions in the reverse orientation. However, PNMA6A gene orthologue is not present in the corresponding location in the mouse genome compilation of the NCBI database. Furthermore, we could not clone the PNMA6A gene fragments of either macaque or mouse by either RT-PCR or genomic PCR. By southern blot hybridization, we detected clear hybridizing bands for PNMA6A in the human and slowloris (prosimian) genomes, but not in the African green monkey (Old World monkey) and common marmoset (New World monkey) genomes (data not shown). Thus, although PNMA6A gene could be functional in humans, it appears to be absent in macaques, and we therefore refrained from further investigation.

Expression Patterns of PNMA Family Genes in Monkeys and Mice

To examine the distribution of the mRNAs of PNMA family genes in more detail, we carried out ISH. Figure 7 shows the mRNA expression patterns of 4 PNMA family genes in the adult macaque neocortex. In the macaque neocortex, the mRNAs of the 4 genes were observed in all the areas and layers examined, although the layers where these genes were preferentially expressed slightly differed from each other. The differential expressions of these genes were most notable in V1. In V1, PNMA1 mRNA showed a relatively weak expression below layer IVC and was particularly weak in layer V. On the other hand, PNMA2 mRNA showed relatively weak signals in layer IVC. PNMA3 and MOAP1 mRNAs showed patterns similar to that of PNMA1 mRNA, although the signal intensities of these 2 genes were weaker than that of PNMA1 mRNA. In the other primary sensory areas, such as area 3b, PNMA3 mRNA seemed to be expressed strongly in the upper layers than lower layers. This was also observed in the primary auditory area (data not shown). In the subcortical regions, we observed relatively ubiquitous expressions of the 4 mRNAs. However, PNMA3 mRNA was expressed only in sparsely distributed cells of the caudate nucleus (Fig. 7A).

To determine the cell types that express these mRNAs in the neocortex, we performed double ISH of each of these genes with VGLUT1 or GAD67 mRNAs. Unlike PNMA5 mRNA, the mRNAs of these 4 family genes were all colocalized with both VGLUT1 and GAD67 mRNAs (Supplementary Fig. S2). Thus, these genes appear to be expressed in almost all the neurons in the neocortical areas, although the expression levels per cell varied.

We next examined the mRNA expressions of PNMA family genes in the mouse brain by ISH (Fig. 8). In our analysis, PNMA5 and PNMA1 mRNAs were poorly expressed in any part of the mouse brains, the results of which were consistent with those of northern blot hybridization. No PNMA5 mRNA was expressed anywhere in the brain, whereas the PNMA1 mRNA was observed at a very low level in the piriform cortex, hippocampus and some subcortical nuclei. PNMA2, PNMA3, and MOAP1 mRNAs were abundantly expressed in the mouse brain. In the neocortex, PNMA2, PNMA3, and MOAP1 mRNAs were expressed throughout all the layers, although the expression levels of the genes differed slightly among the layers (Fig. 8B). For example, the expression level of PNMA2

Figure 4. Double ISH of PNMA5 and VGLUT1 or GAD67 mRNAs in area TE. (A) PNMA5 mRNA (left, red) was expressed in most of the VGLUT1-mRNA-positive cells (middle, green) in layer II (bottom: higher magnification). (B) PNMA6 mRNA was barely expressed in GAD67-mRNA-positive cells (middle, green).
mRNA was low in layer IV. Although the expression of PNMA3 mRNA was strong in the upper layers but relatively weak in the lower layers, that of MOAP1 mRNA was strong in layer V. In the subcortical regions, PNMA2 mRNA was expressed in the striatum and hypothalamus, but was relatively weak in the thalamus. PNMA3 mRNA was expressed weakly in the striatum but strongly in the hypothalamus. MOAP1 mRNA was expressed weakly in the striatum but strongly in the thalamus and hypothalamus. The mRNA expression patterns of PNMA2, PNMA3, and MOAP1 in the brain of macaques and mice were basically conserved.

The different expression patterns of PNMA5 and PNMA1 mRNAs between macaques and mice raised an interesting possibility that the expression of the 2 genes may be primate specific. To test this, we examined the expressions of all PNMA family genes including PNMA5 in the neocortex of marmosets, which belong to the New World monkey that is of different lineage from the Old World monkey including macaques. In the marmosets, the expression patterns of these 5 PNMA family genes showed similarity to those in the macaques (Supplementary Fig. S3). PNMA5 mRNA showed area-specific expression patterns similar to those in the macaques. It was strong in the frontal and temporal association cortices and weak in the parietal and occipital cortices. The mRNAs of the other PNMA family genes were expressed in all the areas and layers, although the expression levels of the genes differed among the layers, particularly in the occipital cortex (V1). These expression patterns of the PNMA family genes in marmosets were all similar to those in the macaques.

**Discussion**

In this study, we have identified a novel gene, PNMA5, whose transcript is preferentially expressed in the primate association areas. The expression pattern of PNMA5 mRNA in the macaque neocortex was similar to that of RBP mRNA, which we previously reported as being enriched in the excitatory neurons of association areas (Komatsu et al. 2005). The very similar expression patterns of the PNMA5 and RBP genes suggest that these 2 genes have roles in closely related cortical circuits, which may well be involved in fundamentally associative functions of the neocortex. The comparative expression analyses showed that PNMA5 mRNA is expressed in the primate brains (macaques and marmosets), but not in mouse brains. The sequence comparison indicated that the PNMA5 sequences among mammals are significantly diverged even within primates. Divergences in the amino acid sequences and mRNA expression of PNMA5 suggest that it acquired a specialized role in the association areas of neocortex during primate evolution.

**Common Laminar Pattern for PNMA5 and RBP mRNA Expressions**

The expression patterns of PNMA5 and RBP mRNAs were very similar in terms of laminar specificity across areas of the macaque neocortex. One common feature is that PNMA5 and RBP mRNAs were expressed in layer II across neocortical areas including V1. As we discussed previously (Komatsu et al. 2005), several lines of evidence suggest a specialized role, possibly related to integration, for layer II in the macaques. For example, the neurons in layer II generally receive the convergence of several feedback cortical inputs (Kennedy and Bullier 1985; Rockland and Virga 1989; Rockland et al. 1994), as well as amygdalo-cortical projections (Freese and Amaral 2005). Physiologically, there are cells near the border between blob and interblob regions in layers II and III that have mixed receptive field properties (Nealey and Maunsell 1994). Shipp and Zeki (2002) indicate that the responses for visual stimuli in layer II of V2 are generalized for directional, orientational and
spectral sensitivities, and speculate that multimodal association neurons in layer II induce sets of functionally unimodal projection neurons to adopt correlated rates of firing. There is, thus, a possibility that both PNMA5 and RBP contribute to specifically associative properties of layer II neurons. Several studies have shown that layer II neurons are contributing to feedback projections, whereas neurons in the deeper part of the supragranular layers (layer III) are associated with feedforward projections in visual areas (Tigges et al. 1973; Kennedy and Bullier 1985). Physiological differences are reported between the neurons in layer II and layer III of the primary visual cortex of macaque monkeys (Gur and Snodderly 2008). This observation may be related to the theory that layer III neurons are involved in generating and transmitting information about image features, whereas layer II neurons may participate in top-down influences from higher cortical areas (Ullman 1995). Connectionally and physiologically different properties of layer II and III neurons in visual areas may be reflected in the expression of PNMA5 and RBP in the occipital areas where they are expressed only in the upper part of the supragranular layers.

A second shared feature is the laminar expansion of PNMA5 and RBP mRNAs from superficial to deeper layers, which occurs gradually from the primary sensory areas to higher-order association areas. Elston and coworkers reported that there exists a gradient across areas for dendrite architectures of pyramidal neurons in layer III (Elston et al. 1999; Elston 2000); that is, the size of the basal dendritic field and the spine

Figure 5. Comparison of expression patterns of PNMA5 and RBP mRNAs in macaque brains. (A) Coronal sections at positions A, C, D, E, and F in Figure 2 are shown. (B) Higher magnification views of several areas. The expression patterns of PNMA5 and RBP mRNAs are shown, together with the laminar profiles of ISH signals quantified by measuring the optical density (red, PNMA5 mRNA; green, RBP mRNA). These profiles show the averages of the normalized values of 2 or 3 different macaques. The cortical layers were identified in reference to the Nissl staining of the nearby section. (C) Double ISH of PNMA5 and RBP mRNAs were performed to examine the coexpression of these mRNAs. Note that PNMA5 mRNA (left, red) is expressed in a large population of RBP-mRNA-positive cells (middle, green) in layer II.
density increase in the association areas, which they interpret as signifying differential associative capacities. Interestingly, there are other reports of gradientwise changes, for example, changes in cross-sectional areas of patchy intrinsic terminations along the ventral visual pathway, which are interpreted as reflecting the increase of global computational roles (Amir et al. 1993; Lund et al. 1993; Tanigawa et al. 2005). These observations support the possibility that associative processes gradually occupy more neural space with progression along the sensory pathways. We propose that PNMA5 and RBP are generally involved in associative processes in layer II throughout all layers, but that their role is only gradually extended to other layers in the higher-order association areas.

Third, recent studies in macaques show that the laminar distribution and of synaptic zinc in the occipito-temporal pathway is very similar to those of the PNMA5 and RBP mRNAs. That is, synaptic zinc is very poor in layer IV, but in anterior

<table>
<thead>
<tr>
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<th>AGM</th>
<th>Mouse</th>
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<tbody>
<tr>
<td>PNMA5</td>
<td>93.6</td>
<td>57.0</td>
</tr>
<tr>
<td>PNMA1</td>
<td>96.0</td>
<td>70.4</td>
</tr>
<tr>
<td>PNMA2</td>
<td>98.8</td>
<td>67.4</td>
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<tr>
<td>PNMA3</td>
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<tr>
<td>MOAP1</td>
<td>98.0</td>
<td>78.3</td>
</tr>
<tr>
<td>PNMA6A</td>
<td>98.3</td>
<td>80.3</td>
</tr>
<tr>
<td>PNMA4</td>
<td>97.4</td>
<td>77.1</td>
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<td>% nuc.</td>
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AGM: African green monkey.
% a.a.: percent identity of amino acid sequences.
% nuc.: percent identity of nucleotide sequences.
ND: not determined.

Figure 6. Expression analyses of PNMA5 gene and the PNMA family genes in various tissues by northern hybridization and RT-PCR. (A,C) Northern hybridization of PNMA family genes of African green monkeys (A) and mice (C). A single membrane blotted with various poly(A) mRNAs was serially probed with 5 PNMA family genes and GAPDH gene with repeated stripping and hybridization. No hybridization signals were detected after each stripping. The positions of the size marker are indicated on the left. (B) Quantification of northern hybridization in the neocortical areas of the African green monkey. The X-ray films shown in panel A were scanned and digitized for quantification. This panel shows the results of quantification of the lanes for 5 neocortical areas of the African green monkey for each gene. The expression levels were normalized to the ratio between the PNMA and GAPDH mRNA expression levels in area 46. The expression levels of PNMA3 mRNA are indicated by the averages of the upper and lower bands. Abbreviations: S1, primary somatosensory area; TE, temporal area; 46, area 46; V1, primary visual area; M1, primary motor area. (D-F) RT-PCR analyses of the PNMA family genes expressed in developmental brains and various adult tissues of mice (D), brain and testis of the rat (E), and human brain (F). PCR using reverse-transcribed cDNAs from the same amount of total RNAs was performed with the predetermined optimal cycle for each primer set. RT-PCR products obtained with the same primer sets for RNAs derived from the same species were loaded and separated on the same agarose gel. The GAPDH gene was used as control.
inferotemporal and perirhinal cortices, is widely distributed in layers Ib, II, III, V, and VI (Ichinohe and Rockland 2005; Miyashita et al. 2007). Zn is considered to be a neuromodulator associated with activity-dependent synaptic plasticity (Dyck et al. 2003). The similarity in distribution of \textit{PNMA5} and \textit{RBP} mRNA expressions and of synaptic Zn suggests that the expression patterns for the mRNAs of the 2 genes may represent regions of highly neuronal plasticity (Murayama et al. 1997). The expression patterns of \textit{PNMA5} and \textit{RBP} mRNAs are not identical (i.e., they have varying intensities at the level of single cells) but are overall very similar. This may be interpreted to mean that the expression of the 2 genes reflects extracellular environmental factors, rather than the cell autonomous properties of cortical neurons. That is, the similar expression patterns may be a signal of common responses to microcircuits and/or microenvironments, which are specifically dedicated to associative functions. Therefore, \textit{PNMA5} mRNA expression in monkeys may reflect increasing associative functions of the neocortex during primate evolution.

\textbf{Potential Functions of \textit{PNMA} Family Genes in Mammalian Brains}

What would be the molecular functions of \textit{RBP} and \textit{PNMA} genes in the brain? RBP protein is suggested to have a role in maintaining higher brain function, and disruption of RBP-mediated retinoid metabolism is associated with some mental disorders and dementias, for example, Alzheimer's disease (Goodman and Pardee 2003; Puchades et al. 2003), schizophrenia (Goodman, 1998), and frontotemporal dementia (Davidsson et al. 2002). Despite similarity in the expression pattern, the amino acid sequences of \textit{PNMA5} and its family of genes show no similarity to that of \textit{RBP} or the other genes that are involved in the retinol metabolism.

\textit{PNMA1}, \textit{PNMA2}, and \textit{PNMA3} proteins were first identified as antigens in the sera of patients with paraneoplastic
neurological disorders (Dalmau et al. 1999; Voltz et al. 1999; Rosenfeld et al. 2001). Immunohistochemistry using these sera showed that these 3 proteins mainly localize in the nucleoli, although some of them are in the cytoplasm. On the basis of this subcellular localization and the presence of a zinc-finger motif, PNMA3 protein was suggested to play a role in mRNA biogenesis (Dalmau et al. 1999; Voltz et al. 1999; Rosenfeld et al. 2001). MOAP1 protein (PNMA4) was identified as a binding partner of a proapoptotic Bcl-2 member, Bax, and is capable of triggering cytochrome c-mediated apoptosis on contact with death receptors (TNF-R1 or TRAIL-R1) and tumor suppressor RASSF1A in mammalian cells (Tan et al. 2001; Baksh et al. 2005; Tan et al. 2005; Vos et al. 2006; Foley et al. 2008). However, considering the abundant expression of MOAP1 mRNA in the mature cortical neurons in monkeys and mice, we think that it is unlikely to induce apoptosis in normal adult brains. Consistent with this, a recent study has shown that upregulated MOAP1 protein cannot induce apoptosis in SY5Y human neuroblastoma cells (Fu et al. 2007). In our material, overexpression of the MOAP1 protein also did not trigger apoptosis in Neuro2a mouse neuroblastoma cells (M.T. and T.Y. unpublished observation). We observed that the PNMA5 protein overexpressed in Neuro2a localize in the nucleus (M.T. and T.Y. unpublished observation). To summarize, there is good evidence for the subcellular localizations of these PNMA family genes (i.e., the PNMA family proteins are likely to be present in the cytoplasm or nucleus unlike RBP protein, which is mainly in the plasma), but the molecular functions in the brain are still largely unknown.

What is intriguing about the PNMA family genes is that their mRNA expression patterns differ markedly across different mammalian species. For example, although PNMA1 mRNA is expressed widely in various monkey tissues including the brain, its expression in mice is restricted to the testis, despite the

Figure 8.ISH of PNMA5 and the PNMA family genes in the mouse brains. (A) Coronal sections at several different planes for each gene are shown. The boxed region (V1) of each gene is shown in B at higher magnification. (B) The layers indicated on the left side are identified according to the Nissl staining of the nearby section.
high level of PNMA1 gene sequence conservation (92.1% identity between human and mouse amino acid sequences). Similarly, the mouse orthologue of the PNMA5 gene, which shows a characteristic expression pattern in monkey brains, is not detected in the mouse brain. Whereas PNMA3 mRNA is not expressed in mouse testis, MOAP1 mRNA expression is higher in the mouse testis than in the monkey testis. One possibility for this expression difference would be that PNMA family genes share common features that can compensate for each other. On the other hand, the species-specific expression also suggests that each gene in the PNMA family evolved specialized functions. The common functions and species-specific roles of the PNMA family gene in neural tissues remain to be elucidated.

**Evolution of PNMA5 Sequence and Expression along with Evolution of the Brain**

There have been long-standing debates as to whether important evolutionary changes have occurred predominantly in the gene regulatory sequences or coding sequences (King and Wilson 1975; Carroll 2003; Olson and Varki 2003). Several lines of evidence suggest that both aspects are important. For example, on the basis of human-chimpanzee comparison, King and Wilson (1975) propose that evolutionary changes in the anatomy and lifestyle are more often based on changes in the mechanisms of gene expression than on changes in the amino acid sequences. Recent microarray studies provide further evidence for large changes in the pattern of gene expression across species and suggest that such changes have played roles in evolution (Enard, Khaitovich, et al. 2002; Cáceres et al. 2003; Preuss et al. 2004; Uddin et al. 2004). On the other hand, there are also studies that support the importance of amino acid sequence changes for primate brain evolution. For example, nervous system–related genes display significantly higher rates of protein evolution in primates than in rodents (Dorus et al. 2004). Furthermore, ASPM and MCPH1 genes, which are involved in regulating brain size during development (Bond et al. 2002; Jackson et al. 2002), show significantly accelerated rates of amino acid changes along the lineages leading to humans (Zhang et al. 2003; Evans, Anderson, Vallender, Choi, et al. 2004; Evans, Anderson, Vallender, Gilbert et al. 2004; Kouprina et al. 2004; Wang and Su 2004). The FOXP2 gene, whose mutation is associated with a speech and language disorder in humans (Lai et al. 2001), also shows significantly accelerated amino acid changes along the lineages leading to humans (Enard, Przeworski, et al. 2002; Zhang et al. 2002). These results suggest a possible link between alterations in protein sequences and the phenotypic evolution of the human brain.

In this study, we observed differences in the PNMA family genes in terms of both amino acid sequences and mRNA expressions between primates and rodents. Of particular interest is the divergence of the PNMA5 sequence and expression pattern. The human and mouse amino acid sequences encoded by the PNMA5 gene are only 57.0% identical, which is far below the average human-mouse homology (86.4%, Makalowski and Boguski 1998). This suggests that PNMA5 has acquired a unique function during primate evolution. The characteristic area and lamina expression patterns of PNMA5 mRNA and the sequence divergence in mammals suggest its involvement in functions particular to association areas in the neocortex of primates.

**Supplementary Material**

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/

**Notes**

We thank Drs Hitoshi Horie, Shinobu Abe, and Sou Hashizume of the Japan Poliomyelitis Research Institute for supplying monkey tissues. We thank Drs Junichi Yuasa-Kawada and Masaharu Noda of the National Institute for Basic Biology, and Shingo Akiyoshi of the JFCR Cancer Institute for help with the RLCs method. We thank Dr Yuriko Komine and Tetsuya Sasaki, and Dr Junya Hirokawa in our laboratory for providing mRNAs of developmental mouse brain and rats, and for helping in image analyses and cell counting, respectively. We thank Dr Kathaleen S. Rockland of RIKEN for critical reading and valuable suggestions. This research was supported by a Grant-in Aid for Scientific Research on Priority Areas (A, 17024055 to T.Y.) and partly by the Strategic Research Program for Brain Sciences from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Conflict of Interest: None declared.

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