Activity-dependent Expression of occ1 in Excitatory Neurons Is a Characteristic Feature of the Primate Visual Cortex

occ1 is a gene whose expression is particularly abundant in neurons in the macaque primary visual cortex (V1). In the present study, we report that the expression of occ1 mRNA in the macaque neocortex can be classified into two modes. The first mode is associated with excitatory neurons distributed in the major thalamocortical recipient layers that exhibit strong cytochrome oxidase activity. This is highly prominent in V1. The second mode is associated with parvalbumin-positive GABAergic interneurons and is distributed across the macaque neocortex. In V1, monocular deprivation showed that occ1 mRNA expression in excitatory neurons was markedly dependent on affective activity, whereas in GABAergic interneurons was not. Cross-species comparison showed specific differences in expression. In marmosets, a strong expression was observed in V1 similarly to macaques. The occ1 mRNA expression, however, was generally weak in the mouse neocortex. In rabbit and ferret cortices, the strong expression was observed only in GABAergic interneurons. We conclude that activity-dependent occ1 mRNA expression in the excitatory neurons of V1 was caused by a novel mechanism acquired by primates after their separation from other lineages.

Keywords: brain evolution, in situ hybridization, Macaca fuscata, TSC-36, V1-specific

Introduction
The mammalian neocortex is subdivided into functionally and anatomically distinct areas (Brodmann, 1909). There have been long-standing debates as to what extent the specification of neocortical areas is genetically programmed or experience dependent (Rakic, 1988; O'Leary, 1989). Recent studies have revealed that the early regionalization of the neocortex is primarily determined by gradients of signal molecules and transcriptional factors before thalamocortical projections arrive (Donoghue and Rakic, 1999a,b; Rubenstein, 1999; O'Leary and Nakagawa, 2002; Dufour et al., 2003; Seibt et al., 2003), although interactions between extrinsic factors and thalamocortical projections may be important in the establishment of a functional architecture (Rakic, 1988; Schlaggar and O'Leary, 1991; Katz and Shatz, 1996; White et al., 2001). Knowledge of the genetic basis of brain architectures, however, is still limited, particularly regarding the genes contributing to functional differences between primate and nonprimate brains (Dorus et al., 2004).

To investigate the evolutionary aspects of areal specification of the primate neocortex, we have taken the approach of identifying molecular markers that exhibit a highly area- and region-specific expression in the adult primate neocortex (Watakabe et al., 2001a,b; Komatsu et al., 2005). occ1, the gene encoding the monkey orthologue of follistatin-related protein (Frp) and transforming growth factor (TGF)-β1-stimulated clone 36 (TSC-36), was identified as a gene preferentially expressed in the monkey visual cortex (Tochitani et al., 2001). Nonradioactive in situ hybridization (ISH) revealed that occ1 mRNA is particularly abundant in the major thalamocortical recipient layers of the primary visual cortex (V1) (Tochitani et al., 2001; Komatsu et al., 2005), and monocular deprivation markedly downregulates occ1 mRNA expression in adult macaques (Tochitani et al., 2001). Developmentally, the occ1 mRNA expression level is low at birth and markedly increases postnatally (Tochitani et al., 2003). These observations suggest that occ1 mRNA expression is regulated by the activity of visual afferents from the thalamus and plays a role in activity-dependent plasticity in adult macaque V1.

Comparative neuroanatomy has revealed many differences in the cortical architecture across species. In particular, the sensory cortices of species are specialized in size and organization for adapting to the environment. For example, rodents and some lagomorphs possess ordered multineuronal units called barrels in the primary somatosensory cortex, which represent the topographical organization of connections with the contralateral vibrissae (Woolsey et al., 1975; Wong-Riley and Welt, 1980). Cetaceans communicate by echo-location and have a prominent acoustic system, which is manifested in their enlarged auditory cortices (Revischchin and Garay, 1991; Glezer et al., 1998). The visual cortex is particularly prominent in primates, presumably because diurnal primates depend greatly on vision. They possess a large and highly organized V1 that has many features not found in other areas or in V1 of other species (Lund et al., 1979, 1994; Rockel et al., 1980; Casagrande and Kaas, 1994).

We thus considered that the abundant expression of occ1 mRNA in V1 would hold clues to the specific characteristics of the primate visual cortical. In this report, we further analyzed detailed features of occ1 mRNA expression in the adult macaque neocortex and compared them with those in other species. We found two distinguishable modes of occ1 mRNA expression in the macaque neocortex. Furthermore, there were clear differences in the patterns of occ1/Frp in primates other than mammals as 'occ1/Frp' in this paper) mRNA expression among New World monkeys (common marmosets), rodents (mice), lagomorphs (rabbits) and carnivores (ferrets). Some of the data has been reported elsewhere in abstract form (Takahata et al., 2004).

Materials and Methods

Tissue preparation
Eight macaques (Macaca fuscata, adult, either sex, 2.9–4.7 kg), three common marmosets (Callithrix jacchus, adult, either sex, 300–500 g), Toru Takahata, Yusuke Komatsu, Akiya Watakabe, Tsutomu Hashikawa, Shiro Tochitani and Tetsuo Yamamori.
six mice (Mus musculus, ICR, SLC, Japan, 10–13 weeks old, either sex, 20–40 g), two rabbits (Oryctolagus cuniculus, adult, either sex, 2.8–3.9 kg) and five ferrets (Mustela putorius, adult, male, 1.1–1.5 kg) were used in this study. In four of the eight macaques, tetrodotoxin (TTX; 15 µg in 10 µl of normal saline) was injected repeatedly (twice a week) under ketamine anesthesia into the vitreous cavity of the left eye and the macaques were kept alive for 7, 10 or 14 days. These monocularly deprived macaque cortices were used also in our previous study (Tochitani et al., 2001). All animals were administered an overdose of Nembutal (at least 100 mg/kg body wt) and perfused intracardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4). The brain was then removed from the skull, postfixed for 3–6 h at 4°C (mice, rabbits and ferrets) or room temperature (marmosets and macaques) in the same fixative, cut into several blocks and cryoprotected in 30% sucrose in 0.1 M PB at 4°C. The blocks were cut as frozen sections on a sliding microtome at 15–40 µm thick. The sections were maintained in a cryoprotectant solution [30% glycerol, 30% ethylene glycol and 40% 0.1 M phosphate-buffered saline (PBS), pH 7.4] at −30°C when they were not used for more than 24 h after sectioning until experiment. Frozen block samples were stored at −30°C.

The protocols of the present study were approved by the Animal Research Committee of the National Institute for Basic Biology and the National Institute for Physiological Science, Japan, and followed the animal care guidelines of NIH, USA.

**Histological Analysis**

For colorimetric ISH, digoxigenin (DIG)-labeled antisense and sense riboprobes were prepared using a DIG-dUTP labeling kit (Roche Diagnostics, Indianapolis, IN). Probes and primers used for this study are listed in the supplementary table. For marmosets, mice, rabbits and ferrets, more than two types of probe for occ1/Frp were prepared. Since each probe exhibited essentially the same patterns of signal distribution when used separately (data not shown), we mixed the probes to enhance signals. Sense probes detected no signals higher than the background signal. ISH was carried out as described previously (Tochitani et al., 2001). Briefly, free-floating sections (35–40 µm thick) were soaked in 4% PFA at 0.1 M PB (pH 7.4) overnight at 4°C and treated with 1–10 µg/ml proteinase K for 30 min at 37°C. After acetylation, the sections were incubated in hybridization buffer [5 × SSC, 2% blocking reagent, 50% formamide, 0.1% N-lauroylsarcosine (NLS), 0.1% SDS] containing 1.0 µg/ml DIG-labeled riboprobe at 60–65°C overnight. Hybridized sections were washed by successively immersing in washing buffer (2 × SSC, 50% formamide, 0.1% NLS; 60–65°C, 20 min, twice), RNase A buffer (10 µg/ml Tris-HCl, 10 mM EDTA, 0.5 M NaCl, pH 8.0) containing 20 µg/ml RNase A (37°C, 15 min), 2 × SSC/0.1% NLS (37°C, 20 min, twice) and 0.2 × SSC/0.1% NLS (37°C, 15 min, twice). Hybridization signals were visualized by alkaline phosphatase (AP) immunohistochemical staining using a DIG detection kit (Roche Diagnostics). After mounting onto glass slides, the sections were dehydrated through a graded series of increasing ethanol concentration followed by xylene, and then coverslipped with Entellan new. The ISH signals in marmoset samples were generally weak; and the signal/noise ratio was not as high as that for the other mammalian samples and double ISH data were unavailable.

Fluorescence double ISH was performed as described previously (Komatsu et al., 2005). Fluorescein (FITC)-labeled riboprobes for γ-aminobutyric acid (GABA)-ergic marker genes [glutamic acid decarboxylase 67 (GAD67), parvalbumin (PV), calbindin D-28k (CB) and calretinin (CR)] and a glutamatergic marker, vesicular glutamate transporter 1 (VGLUT1) were prepared (see supplementary table for probes used). Brain sections (15 µm thick) were hybridized with the DIG-labeled occ1/Frp probe and either the FITC-labeled GABAergic probe or the glutamatergic marker probe. The hybridization protocol was the same as that of colorimetric single ISH. DIG was detected by staining with AP-conjugated anti-DIG antibodies and using a HNPP Fluorescence Detection kit (Roche Diagnostics). FITC was detected by staining with horseradish peroxidase-conjugated anti-FITC antibodies (Roche Diagnostics) followed by enhancement using a TSA-Plus DNP system (PerkinElmer Life Sciences, Boston, MA) and staining with Alexa 488-conjugated anti-DNP antibodies (Molecular Probes, Eugene, OR). After mounting onto glass slides, sections were air-dried and then covered with the PermaFluor Aqueous mounting medium (Thermo, Pittsburgh, PA).

To visualize cytochrome oxidase (CO) activity, sections were immersed in 0.33 mg/ml cytochrome C type III (Sigma, Tokyo, Japan), 0.54 mg/ml 3’3-diaminobenzidine (DAB), and 4.5% sucrose in 0.1M PB (pH 7.4) and incubated in the dark at 37°C for 4–5 h (Wong-Riley, 1979). For Nissl staining, sections were mounted onto glass slides, air-dried and stained with thionine. In both procedures, the sections were dehydrated through a graded series of increasing ethanol concentration followed by xylene and coverslipped with Entellan new.

The cortical areas in macaques and mice were identified using brain atlases (Paxinos et al., 2000; Paxinos and Franklin, 2001) and by comparing adjacent Nissl-stained sections. The cortical areas of marmosets, rabbits and ferrets were determined by Nissl staining, parvalbumin immunostaining and CO staining of adjacent sections. The scale bars in figures are corrected for shrinkage caused by ISH.

**Statistical Analysis**

To quantify double ISH data, we counted the number of cells exhibiting signals. The data for each animal were represented by one or two bins (690 µm width) from layers I to VI in vertical sections. The areas in which cells were counted were calculated, and cell numbers were normalized to per mm². Signals were magnified 20 times under a dark-field microscope and counted manually. Signals not arising from neurons were excluded from the calculation. To estimate objectively, two of the authors (T. T. and A. W.) counted the numbers of signals separately. Since the count error was sufficiently small (the errors between the two counters were 12.9 ± 2.7, 5.5 ± 1.4 and 5.2 ± 0.8 for the number of GABAergic markers, number of double-positive cells and ratios, respectively), results obtained by the first author were used in this paper. The significance of differences between results from the nondeprived column and those from the deprived column in the experiment of monocular deprivation of macaque V1 was analyzed by paired Student’s T-test.

**Results**

### Two Distinct Modes of occ1 mRNA Expression in Macaque Neocortex

Although occ1 mRNA is highly abundant in V1 (Tochitani et al., 2001), occ1 mRNA is also expressed at low levels across other neocortical areas. Because occ1 mRNA distribution outside of the visual pathways has not yet been well studied (Tochitani et al., 2001; Komatsu et al., 2005), we further analyzed the cortical occ1 mRNA expression in macaques.

As described previously (Tochitani et al., 2001; Komatsu et al., 2005), occ1 mRNA signals were most intense in V1 in the macaque neocortex (Fig. 1A). Signals were also detected in the middle layers of the extrastriate cortices but progressively more weakly along the ventral visual pathway (Fig. 1B–D). The middle temporal area (MT) showed patterns of signals similar to those in V2 (data not shown). The primary somatosensory and auditory cortices also exhibited moderately intense occ1 mRNA signals in the middle layers, with markedly intense signals that are sparsely distributed in layers IV–VI (Figs 1E,F). There were sparse but intense signals in the motor cortex (Fig. 1G). In association cortices (e.g. area 46), sparse signals were observed, but the density of occ1 mRNA-expressing cells and the intensity of signals were still lower than those in early sensory and motor cortices (Fig. 1H).

Previously, we reported that both glutamatergic excitatory neurons and GABAergic interneurons express the OCC1 protein in V1 (Tochitani et al., 2001). We further examined whether the cell-type specificity of occ1 expression differs
among areas by a double ISH technique using probes for GAD67 and VGLUT1. GAD67 is a GABA-synthesizing enzyme and a useful marker of GABAergic interneurons (Fitzpatrick et al., 1987; Hendrickson et al., 1994). VGLUT1 is a marker of glutamatergic neurons in the cerebral cortex (Takamori et al., 2000; Fremeau et al., 2001).

Darkfield microscopy images in Figure 2A,B are from macaque V1 sections hybridized with the occ1 probe and either the VGLUT1 (A) or GAD67 (B) probe. Consistent with our previous report (Tochitani et al., 2001), occ1 mRNA signals colocalized well with both VGLUT1 and GAD67 mRNA signals. Thus, both glutamatergic excitatory neurons and GABAergic inhibitory interneurons express occ1 mRNA in V1. occ1 mRNA signals in layers II–IVβ overlapped with both VGLUT1 and GAD67 mRNA signals, but the sparse occ1 mRNA signals in infragranular layers predominantly overlapped with GAD67 mRNA signals, rather than with VGLUT1 mRNA signals. The signals in excitatory neurons were observed in pyramidal-like cells and layer IV spiny stellate-like cells among excitatory neurons in V1. In V2, there were also neurons doubly positive both for occ1/VGLUT1 mRNA in layers II–V although rare in layer VI, and for occ1/GAD67 mRNA in layers II–VI (Fig. 2C,D).

In the motor cortex, however, the majority of the intense scattered occ1 mRNA signals colocalized with GAD67 mRNA signals (Fig. 2F), and VGLUT1 mRNA signals rarely overlapped with occ1 mRNA signals (Fig. 2E). The intense sparse signals in infragranular layers of the primary somatosensory and primary auditory cortices overlapped with GAD67 mRNA signals, rather than with VGLUT1 mRNA signals, while moderately intense signals in layer III corresponded to both marker signals. Both modes of occ1/Prp mRNA signals were observed in layer IV (Supplementary Fig. 1A–D). Weak and sparse occ1 mRNA signals in association areas overlapped mainly with GAD67 mRNA signals (Supplementary Fig. 1E).

**Preferred Expression of occ1 mRNA in PV-positive Subpopulation**

To determine whether occ1 mRNA is preferentially expressed in a certain subtype of GABAergic interneuron, we performed double ISH to examine the expression of occ1 mRNA and the three intracellular Ca\(^{2+}\)-binding proteins (PV, CB and CR) in the macaque neocortex. Our ISH results conserving the areal and laminar distributions of these markers were consistent with those of previously reported immunohistochemical studies (Van Brederode et al., 1990; Kondo et al., 1994; DeFelipe et al., 1999). Photomicrographs in Figure 3 show macaque sections hybridized with the occ1 probe and either the PV probe or the CB probe. In V1, occ1 mRNA signals coincided well with PV mRNA signals (Fig. 3A), but neither with CB mRNA signals (Fig. 3B), nor with CR mRNA signals (data not shown). A similar tendency was observed in V2 (Fig. 3C,D), the motor cortex (Fig. 3E,F), and the somatosensory and auditory cortices (data not shown) in spite of the areal differences in occ1 mRNA expression. The number of double-positive neurons was counted for each marker in V1, V2 and the motor cortex. Since some pyramidal cells in the primate neocortex are suggested to express certain levels of these Ca\(^{2+}\)-binding proteins (Conde et al., 1994; Preuss and Kaas, 1996; Kondo et al., 1999; Ichinohe et al., 2004), we excluded weak signals in pyramidal-like cells from our analysis. We found that 26.9 ± 2.8, 20.8 ± 2.4 and 24.7 ± 0.5% of GAD67 mRNA-positive cells were occ1 mRNA-positive in V1, V2 and the motor cortex, respectively (Table 1). Moreover, in these same areas, 46.7 ± 2.9, 47.5 ± 7.6 and 60.6 ± 9.3% of PV mRNA-positive cells were also occ1 mRNA-positive, whereas this was true for only 8.1 ± 1.9, 13.0 ± 6.3 and 8.4 ± 2.6% of CB mRNA-positive cells, and for only 0.6 ± 0.3, 1.6 ± 0.5 and 1.2 ± 0.6% of CR mRNA-positive cells (Table 1). These results suggest that occ1 mRNA is preferentially expressed in the subpopulation of PV-positive GABAergic neurons.
Figure 2. Coronal sections of macaque V1 (A, B), V2 (C, D) or area 4 (E, F) for double ISH of occ1 (red) and either VGLUT1 (A, C, E) or GAD67 (B, D, F) (green). Scale bar = 150 μm.

Figure 3. Coronal sections of macaque V1 (A, B), V2 (C, D) or area 4 (E, F) for double ISH of occ1 (red) and either PV (A, C, E) or CB (B, D, F) (green). Scale bar = 150 μm.
Table 1
Statistics of double ISH of several areas in macaques and ferrets. The numbers of cells that exhibit signals of each GABAergic marker (mm²), the numbers of cells that exhibit double-positive signals for marker and occ1/Fr, and the number ratios of double-positive to marker-positive cells are shown. The values are means ± SEM for three individuals.

<table>
<thead>
<tr>
<th>Area</th>
<th>Marker (mm²)</th>
<th>Double-positive (mm²)</th>
<th>Ratio (%)</th>
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<tbody>
<tr>
<td>Macaque V1</td>
<td></td>
<td></td>
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<tr>
<td>GAD67</td>
<td>400.7 ± 38.4</td>
<td>106.7 ± 11.7</td>
<td>26.9 ± 2.8</td>
</tr>
<tr>
<td>PV</td>
<td>204.6 ± 15.8</td>
<td>96.1 ± 11.4</td>
<td>46.7 ± 2.9</td>
</tr>
<tr>
<td>CB</td>
<td>63.8 ± 7.8</td>
<td>5.3 ± 1.4</td>
<td>8.1 ± 1.9</td>
</tr>
<tr>
<td>CR</td>
<td>40.7 ± 9.7</td>
<td>0.6 ± 0.3</td>
<td>1.6 ± 0.7</td>
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<tr>
<td>Macaque V2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GAD67</td>
<td>328.7 ± 17.1</td>
<td>67.1 ± 4.8</td>
<td>20.8 ± 2.4</td>
</tr>
<tr>
<td>PV</td>
<td>57.5 ± 16.4</td>
<td>24.9 ± 4.2</td>
<td>47.5 ± 7.6</td>
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<tr>
<td>CB</td>
<td>66.1 ± 18.1</td>
<td>7.1 ± 2.9</td>
<td>13.0 ± 6.3</td>
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<tr>
<td>CR</td>
<td>65.7 ± 10.3</td>
<td>1.6 ± 0.5</td>
<td>3.1 ± 1.5</td>
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<tr>
<td>Macaque motor</td>
<td></td>
<td></td>
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<tr>
<td>GAD67</td>
<td>200.3 ± 15.1</td>
<td>40.4 ± 3.5</td>
<td>24.7 ± 0.5</td>
</tr>
<tr>
<td>PV</td>
<td>40.1 ± 10.1</td>
<td>23.5 ± 5.0</td>
<td>60.6 ± 9.3</td>
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<tr>
<td>CB</td>
<td>69.6 ± 16.3</td>
<td>4.7 ± 0.7</td>
<td>8.4 ± 2.6</td>
</tr>
<tr>
<td>CR</td>
<td>43.7 ± 4.6</td>
<td>1.2 ± 0.6</td>
<td>2.9 ± 1.4</td>
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<tr>
<td>Ferret V1</td>
<td></td>
<td></td>
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<tr>
<td>GAD67</td>
<td>211.2 ± 38.6</td>
<td>72.4 ± 15.4</td>
<td>36.7 ± 8.7</td>
</tr>
<tr>
<td>PV</td>
<td>95.3 ± 13.6</td>
<td>57.5 ± 3.3</td>
<td>64.7 ± 11.0</td>
</tr>
<tr>
<td>CB</td>
<td>10.9 ± 4.2</td>
<td>1.8 ± 0.6</td>
<td>18.4 ± 1.2</td>
</tr>
<tr>
<td>CR</td>
<td>22.3 ± 6.0</td>
<td>1.1 ± 0.4</td>
<td>5.6 ± 3.0</td>
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mRNA-positive GABAergic interneurons throughout primate neocortical areas.

Differential Effect of Monocular Deprivation on occ1 mRNA Expression Between Two Types of Neuron in Macaque V1

We previously reported that occ1 mRNA signal intensity is significantly decreased in layers II–V by monocular deprivation in macaque V1 (Tochitani et al., 2001). To determine whether occ1 mRNA expression in the two populations of excitatory and inhibitory neurons reported above is equally affected by visual deprivation, we carried out double ISH of V1 of macaques monocularly injected with TTX.

Monocular deprivation decreased occ1 mRNA signal intensity in the deprived columns as reported previously (Fig. 4A, Tochitani et al., 2001). Whereas occ1 mRNA signals in VGLUT1 mRNA-positive cells were strong in the nondeprived column, those in the deprived column were abolished both in pyramidal-like cells and spiny stellate-like cells (Fig. 4B, C). Consistent with previous reports that GAD67 expression level of mRNA and cellular PV expression in macaque V1 are not affected by monocular deprivation (Benson et al., 1991; Blümcke et al., 1994), GAD67 and PV mRNA signal intensities were not affected by monocular deprivation in our present study (Fig. 4A). In contrast to occ1 mRNA signals in VGLUT1 mRNA-positive cells, those in GAD67 mRNA- and PV mRNA-positive cells were not significantly different between the nondeprived column and neighboring deprived column (Fig. 4D, E). To quantify this observation, we counted the numbers of occ1/GAD67 mRNA-positive and occ1/PV mRNA-positive neurons in each column of all layers. This confirmed that the numbers of GAD67 mRNA-positive neurons, PV mRNA-positive neurons, and those double labeled with occ1 mRNA in deprived columns were not significantly different from those in nondeprived columns (Fig. 4F, G). Moreover, the ratios of the number of occ1/GAD67 mRNA- or occ1/PV mRNA-positive cells to the total number of GAD67 mRNA- or PV mRNA-positive cells in deprived columns did not significantly differ from those in nondeprived columns (nondeprived versus deprived = 28.7 ± 2.3% versus 28.8 ± 4.3%, P > 0.9, for occ1/GAD67, and 40.1 ± 2.5% versus 43.6 ± 2.8%, P > 0.2, for occ1/PV mRNA-positive cells, respectively), and were similar to those in untreated V1 (Table 1).

Pattern of occ1 mRNA Expression in Excitatory Neurons Corresponds to CO Staining Pattern

As shown in Figures 1 and 2, occ1 mRNA expression in excitatory neurons was mostly restricted to particular layers in sensory cortices in the macaque neocortex. In V1, layer IVC exhibited the strongest occ1 mRNA expression, although the signals in excitatory neurons were also observed in layers II, III, IVA and less prominently in layer IVB. Deeper layer III and layer IV of extrastriate cortices, and the middle layers of the primary somatosensory and auditory areas also showed occ1 mRNA signals in excitatory neurons. The distribution pattern of excitatory neurons expressing occ1 mRNA corresponded well to the laminar-specific CO staining pattern (Fig. 5). We further noted a blob-like distribution of occ1 mRNA signals in layers II/III in V1 (see Fig. 1A). This signal distribution corresponds to blobs in a tangential section in layer III of V1, as verified by comparing with an adjacent CO-stained section (Fig. 6).

occ1/Fr mRNA Distribution in Other Mammalian Neocortices

The primary visual area is highly specialized in primates with massive thalamocortical inputs (Hubel and Wiesel, 1972). In contrast, most nonprimate mammalian species depend more on nonvisual or multimodal senses, and the organization of neurons and patterns of neuronal activity in their cortices are highly diversified (Kaas, 1989). Therefore, the preferential occ1/Fr mRNA expression in V1 could be restricted to primates. To clarify this point, we examined occ1/Fr mRNA expression patterns in the neocortices of marmosets, mice, rabbits and ferrets.

Although the marmoset is a phylogenetically distant primate from the macaque, the V1 structure is relatively well conserved between New and Old World monkeys (Spatz et al., 1994; Krubitzer, 1995). We found a similar specificity of occ1 mRNA expression in marmoset V1, although not as prominent as in macaque V1. Strong laminar-specific occ1 mRNA signals were observed, which were not observed in the case of using the control sense probe in layer IVC in marmoset V1 (Fig. 7B, E, F). The distribution pattern of these signals corresponded well with dense CO staining in layer IVC (Fig. 7C). Layers II and III also showed occ1 mRNA signals. However, the occ1 mRNA expression in the somatosensory, auditory and extrastriate visual areas in marmosets was not as specific to the thalamocortical recipient layers as in macaques: that is, moderately intense signals were observed throughout the layers with slightly intense signals in the superficial layers. The pattern was similar to that in the motor area. Although data from a double ISH was not available, the diffuse distribution of occ1 mRNA signals is consistent with the existence of GABAergic interneurons in these areas (Fitzpatrick et al., 1987) (Fig. 7B, H). Higher association areas showed weaker signals than any other areas (data not shown).

The selective expression of occ1 mRNA in V1, which is conserved between macaques and marmosets, was not observed in nonprimate species. In mouse V1, only weak signals were observed except for some cells in deeper layer V1 with...
Figure 4. (A) Coronal section of monocularly deprived macaque V1 for double ISH of occ1 (red) and PV (green) (the survival time after deprivation was 10 days). Scale bar = 300 μm. N, nondeprived column; D, deprived column. (B–E) Highly magnified sections of monocularly deprived macaque V1 in layer IVC with survival time of 14 days for double ISH. Sections were hybridized with the occ1 probe (red) and either the VGLUT1 (B, C) or PV (D, E) probe (green). B and D depict cells in the nondeprived columns and C and E in the neighboring deprived columns. Arrows indicate cells that showed VGLUT1 mRNA signals. Circles indicate cells that showed PV mRNA signals. Scale bar in B = 50 μm. F and G represent the normalized numbers (1/mm²) of interneuron marker-positive cells and double-positive cells with occ1 mRNA in both deprived and nondeprived columns of monocularly deprived macaque V1 (n = 4) with GAD67, G with PV. Each mean ± SEM in the nondeprived columns and deprived columns are compared. P values are presented over each bar.
moderately intense signals (Fig. 8B). In the rabbit visual cortex, weak signals were observed in the upper part of layer V, and neurons in layers II and III were also weakly stained. Some cells, which were presumably interneurons and showed intense signals, were sparsely observed in all layers (Fig. 8F). Unlike in primates, area-specific differences in occ1/Frp mRNA expression were not pronounced either in mice or rabbits, even in the barrel field that receives strong thalamocortical inputs (Fig. 8D; Wong-Riley and Welt, 1980). In the ferret visual cortex, laminar-specific signals were not observed. occ1/Frp mRNA signals were sparsely distributed in all layers (Fig. 8J), which appeared to be in interneurons. These signals were most abundant in the caudal, visual cortex, both in terms of cell number and signal intensity, and signal intensity gradually decreased toward the rostral, frontal cortex (Fig. 8H).

Double ISH revealed that most of the sparsely distributed signals in the ferret visual cortex overlapped with GAD67 mRNA signals (Supplementary Fig. 2A), which is consistent with our interpretation that these signals were in GABAergic interneurons. Further analysis demonstrated that these signals were most frequently detected in PV mRNA-positive GABAergic interneurons, rather than in CB or CR mRNA-positive neurons, as similarly observed in macaques (Fig. 9 and Table 1). Scattered signals in rabbits also overlapped with GAD67 mRNA signals (Supplementary Fig. 2B), but in this species, these scattered signals were evenly observed in all three GABAergic subtypes (data not shown).

Discussion

In this study, we analyzed the expression patterns of occ1/Frp mRNA in the neocortices of macaques, marmosets, mice, rabbits and ferrets. In the macaque neocortex, we found (i) a conspicuous, activity-dependent occ1 mRNA expression in excitatory,
but not GABAergic neurons in infragranular and granular layers of V1; (ii) a moderately strong occ1 mRNA expression in excitatory neurons in the middle layers of primary somatosensory, auditory, and extrastriate visual cortices; and (iii) occ1 mRNA expression in GABAergic (particularly PV-positive) interneurons throughout the neocortex. The distribution of excitatory neurons expressing occ1 mRNA corresponded well with the distribution of CO activity in sensory areas. Whereas occ1/Frp mRNA expression in GABAergic interneurons was also observed in rabbits and ferrets, the strong expression in excitatory neurons was restricted to primates. The difference in the regulation of gene transcription suggests different physiological roles of OCC1/FRP in the brains of these different species. The expression pattern of occ1/Frp mRNA clearly illustrates a unique primate-specific feature in the adult visual cortex at the molecular level.

Possible Function of OCC1/FRP in Brain

occ1/Frp was first identified as a gene whose expression is stimulated by TGF-β in mouse osteoblastoma cell lines (Shibanuma et al., 1993). OCC1/FRP is a secretable glycoprotein and considered to belong to the SPARC (secreted protein, acidic and rich in cystein)/BM-40 family, which shares one follistatin-like (FS) domain followed by one extracellular Ca\(^{2+}\)-binding (EC) domain (Hohenester et al., 1997; Yan and Sage, 1999). Although the physiological function of OCC1/FRP in the brain remains to be elucidated, we can suggest some possible roles of OCC1/FRP.

OCC1/FRP may interact with some extracellular growth factors (neurotrophic factor) or may act as a trophic factor itself and thereby regulate the neuronal phenotype. Follistatin and FSTL3, which have three or two FS domains, bind to some members of the TGF-β family and suppress their activities (Nakamura et al., 1990; Tsuchida et al., 2000). Thus, secreted OCC1/FRP could control the function of molecules similarly to TGF-β.

Alternatively, OCC1/FRP may interact with the extracellular matrix (ECM). Recent studies have suggested that ECM has important roles in activity-dependent synaptogenesis and plasticity both in developing and adult brains (Pizzorusso et al., 2002; Dzvonek et al., 2004; Pavlov et al., 2004). SPARC/BM-40 and SC1/hevin, which are both SPARC/BM-40 family genes, are ECM glycoproteins and participate in the regulation of morphogenesis and cellular differentiation through the modulation of cell–matrix interactions (Tremble et al., 1993; Mendis and Brown, 1994; Mendis et al., 1999; Yan and Sage, 1999; Brekken et al., 2003). Moreover, OCC1/FRP has been suggested to have inhibitory effects on ECM degeneration, such as an inhibition of invasion of tumor cells and downregulation of matrix metalloproteinases expression (Johnston et al., 2000; Tanaka et al., 2003). Therefore, OCC1 could stabilize neuronal connections by stabilizing ECM. If this is the case, the downregulation of occ1 mRNA expression in the absence of neuronal activity may stimulate the reorganization of neuronal connections in primate V1.

Identity of Cells Expressing occ1/Frp mRNA

In the present study, we demonstrated that occ1 mRNA is expressed both in excitatory neurons and GABAergic inhibitory interneurons in the macaque cerebral cortex. Excitatory
neurons mostly consist of spiny stellate cells distributed in layer IV of the primary sensory areas, and pyramidal cells distributed in the supra- and infragranular layers throughout the neocortex (McCormick et al., 1985; DeFelipe and Farinas, 1992; Nieuwenhuys, 1994). As determined from their location and morphology, occ1 mRNA appears to be expressed in both types of excitatory neuron.

We also demonstrated that in macaques and ferrets occ1 mRNA is preferentially expressed in PV-positive subtypes among interneurons. According to the data obtained by previous research, PV-, CB- and CR-positive cortical neuronal subpopulations are mostly GABAergic inhibitory interneurons, and are associated with distinct morphological and electrical properties (Van Brederode et al., 1990; DeFelipe, 1997; Kawaguchi and Kubota, 1997; Markram et al., 2004; Zaitsev et al., 2005). These subtypes are also associated with expressions of other genes, such as neuropeptides (Kawaguchi and Kubota, 1997), ion channels (Sekirnjak et al., 1997; Chow et al., 1999), AMPA receptors (Kondo et al., 1997) and trkB receptors (Cellerino et al., 1996). Subsets of these molecules are suggested to contribute to the morphological and electrical properties of GABAergic neuronal subtypes (Toledo-Rodriguez et al., 2004). It remains, however, to be studied how the function of OCC1 is related to the functions of these genes that have the unique properties in PV-positive interneurons.

PV-positive GABAergic interneurons are mostly basket cells that innervate the somas of pyramidal cells, or chandelier cells that innervate the axon initial segment of pyramidal cells with a strong inhibitory effect on these cells (Williams et al., 1992; Conde et al., 1994; Markram et al., 2004). Both types of cell occasionally express CB in the rat somatosensory cortex (Wang et al., 2002), and a subpopulation of chandelier cells coexpress PV and CB in the human temporal cortex (del Rio and DeFelipe, 1997). These reports may be consistent with our data that
8-13% of CB-positive cells express occ1 mRNA in the macaque neocortex (see Table 1), if we assume that parts of basket and chandelier cells also express CB in macaques.

Expression of occ1/Frp mRNA shows area and species differences within the PV-positive population as follows: (i) occ1 mRNA is expressed only in 46-60% of PV-positive cells in areas examined; (ii) occ1 mRNA expression in PV-positive neurons seems strongest in the sensorimotor cortex and weakest in association areas in macaques; (iii) occ1/Frp mRNA is only weakly expressed throughout the neocortex in mice; and occ1/Frp mRNA is not preferentially expressed in PV-positive cells in rabbits. This may reflect a further subpopulation of PV-positive neurons, and inter-areal, inter-species diversity of interneurons (Hof et al., 2000; Preuss, 2000). Moreover, as previously shown, most of the primate interneurons comes from the enlarged subventricular zone while almost all rodent interneurons come from the ganglionic eminence (Anderson et al., 1999; Letinic et al., 2002).

Brain Evolution and occ1/Frp

occ1/Frp orthologues have been identified in the mouse, rat, chick, macaque and human (Shibanuma et al., 1993; Zwisjsen et al., 1994; Patel et al., 1996; Tochitani et al., 2001). Taking the macaque as a standard, the homologies of the nucleotide sequences of the coding regions in these species are high (99, 89, 89 and 78% for the human, mouse, rat and chick, respectively), suggesting conserved function among species. However, the transcriptional regulation of occ1/Frp in the cerebral cortices obviously differs. While unique inter-areal and inter-species differences have been reported for the distribution patterns of Ca2+-binding proteins (PV, CB and CR) and nonphosphorylated neurofilaments (DeFelipe, 1993; Glezer et al., 1993; Hof et al., 2000), the difference in occ1/Frp mRNA expression was more notable even within Euarchontoglires (Murphy et al., 2001). Furthermore, it should be noted that the most characteristic occ1/Frp mRNA expression pattern was observed in primate V1, one of the most evolutionarily distinct cortical areas (e.g. Nieuwenhuys, 1994).

Recent studies have suggested that nonsynonymous nucleotide substitutions with significant amino acid substitutions are associated with the evolution of the human brain (Clark et al., 2003; Dorus et al., 2004). On the other hand, microarray studies also suggested that changes in the temporal and spatial patterns of gene expression are important in brain evolution (Cáceres et al., 2003; Preuss et al., 2004). We consider that occ1/Frp is a typical example of modifications of transcriptional regulation in a cell-type specific and activity-dependent manner without significant changes in the coding sequence.

We illustrated that the distribution of excitatory neurons expressing occ1 mRNA corresponded to CO activity. A high CO activity is generally related to prominent neuronal activity derived by thalamocortical inputs into sensory cortices (Wong-Riley, 1979; Wong-Riley and Welt, 1980). CO blobs in layers II/III of macaque V1 receive projections from koniocellular layers in the dLGN (Livingstone and Hubel, 1982; Ding and Casagrande, 1998). Taken together with the observation that occ1 mRNA expression in excitatory neurons of V1 is strongly regulated by retinal projection, the significant correspondence between occ1 mRNA expression and CO activity implies that excitatory neurons express occ1 mRNA in response to strong neuronal activity throughout the sensory cortices in macaques. However, it seems that the excitatory neurons of V1 uniquely possess a distinct genetic program for expressing particularly abundant occ1 mRNA in an activity-dependent manner, since the occ1 mRNA expression was much more prominent in V1 than in other sensory areas: occ1 mRNA expression in the primary somatosensory and auditory cortices was much weaker than that in V1, while CO activity in these areas was strong (see Fig. 5) (Jones and Friedman, 1982; Jones et al., 1995). Furthermore, it appears that the genetic program of the activity-dependent expression of occ1/Frp mRNA is acquired only in the primate lineage, since in other species, regions that exhibit a high CO activity, such as the rodent barrel field (Wong-Riley and Welt, 1980), did not show a strong occ1/Frp mRNA expression. It is therefore suggested that both the genetic program and sensory input are important to determine the characteristic pattern of occ1 mRNA expression in primate V1. Further studies on the mechanisms underlying this activity-dependent regulation of occ1 mRNA expression will elucidate molecular characteristics underlying the unique features of the primate neocortex.

Supplementary Material

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

Notes

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