The gene, occ1, is preferentially expressed in the primary visual cortex in an activity-dependent manner: a pattern of gene expression strikingly related to the functional area in macaque neocortex.

by

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General Introduction

The mammalian central nervous system is composed of quite various types of neurons. Nowhere is this neuronal diversity more apparent than in the cerebral neocortex. The neuronal diversity in the neocortex may be, at least in part, reflected in molecular characteristics. The discovery of molecular markers unique to specific neuronal groups can allow us to see and study those groups in isolation, which would be effective in deciphering neuronal organizations and functions in the neocortex.

The adult mammalian neocortex is subdivided into functional areas which have distinct cytoarchitectonic characteristics. With a notion that I might come across such a marker as to visualize a specific subset of neurons comparing molecular properties among the neocortical subdivisions, I started to compare genes expressed as mRNA in the functionally and structurally distinct areas of adult macaque neocortex. Macaque monkeys were selected as the samples because cortical differentiation is more fully expressed in macaques than in the other mammalian model organisms such as mice and rats. The comparison of
mRNAs was performed using differential display, with which you can compare many kinds of mRNAs at the same time with relatively small amount of mRNA. As a result of the search, a cDNA fragment which was preferentially transcribed in the occipital neocortex was obtained, and it has been named occ1. In part I, the identification of occ1 and the characterization of the neurons specified by occ1 expression will be described.

The distribution of occ1-expressing neurons shows a marked correlation with the cytoarchitectonic borders, which raises the following questions. What is the function of occ1 in the neurons? How does the region-selective expression of occ1 contribute to the function of neocortex? Answering these questions will help us to understand how the neocortex is formed to function. As a first step toward addressing these questions, the spatial and temporal expression pattern of occ1 during the postnatal developmental events of visual cortex was examined. The description on this study will be presented in part II.
Part I

Identification and characterization of the gene occ1, which is preferentially expressed in the primary visual cortex in an activity-dependent manner.
Summary

Marker molecules to visualize specific subsets of neurons are useful for studying how the neocortex is organized to function. One approach to identify such molecular markers is to examine the differences in molecular properties among morphologically and physiologically distinct neuronal cell types. I applied differential display to compare mRNA expression in the anatomically and functionally distinct areas of the adult macaque neocortex. I found that a gene designated occ1 was preferentially transcribed in the posterior region of the neocortex, especially, area 17. Complete sequencing analysis revealed that occ1 encodes a macaque homologue of a secretable protein, TSC-36/follistatin-related protein (FRP). In situ hybridization histochemistry confirmed the characteristic neocortical expression pattern of occ1 and showed that occ1 transcription is high in layers II, III, IVA and IVC of area 17. In addition, occ1 transcription was observed selectively in cells of the magnocellular layers in LGN and specific subset of neurons in the hippocampal formation. Dual labeling immunohistochemistry showed that the occ1-positive neurons in area 17 include
both GABA-positive aspiny inhibitory cells and α subunit of type II calcium/calmodulin-dependent protein kinase (CaMKII α)-positive spiny excitatory cells. With brief periods of monocular deprivation, the occ1-mRNA level markedly decreased in deprived ocular dominance columns of area 17. From these, I conclude that the expression of occ1 mRNA is marked to a subset of neurons which are preferentially localized in particular laminae of area 17 and consist of various morphological and physiological neuronal types, and, furthermore, occ1 transcription is subject to visually driven activity-dependent regulation.
Introduction

The distributions of specific molecules show striking correlations with particular physiological organizations in the primate visual system. The Cat-301 antibody, which recognizes a chondroitin sulfate proteoglycan (Zaremba et al., 1989; Hockfield et al., 1990), primarily stains the magnocellular layers (layers 1-2) of the lateral geniculate nucleus (LGN) (Hendry et al., 1984). Immunostaining for calcium/calmodulin-dependent protein kinase II α subunit (CaMK II α in combination with neuronal tracing techniques revealed that the koniocellular layers (intercalated layers) in macaque LGN send direct afferent inputs to blobs in area 17 (Hendry and Yoshioka, 1994). Although functional modules of the neocortex are even more complicated than those in thalamic nuclei, some molecules spatially coincide with the fundamental functional subdivisions of the neocortex, such as layers, columns and areas. Cytochrome oxidase (CO) is enriched in the blobs in layers II and III of the primary visual cortex (area 17) (Wong-Riley, 1979; Horton and Hubel, 1981) and in the stripe-shaped structures in the secondary visual cortex (area 18) (Livingstone and Hubel, 1982, 1983; Horton, 1984). Synaptic zinc is enriched in CO-poor interblob regions (Dyck and Cynader, 1993). The pattern of m2 muscarinic acetylcholine receptor immunoreactivity is also reciprocal to the pattern of CO histochemical staining in layers II and III, the strongest immunoreactivity being observed in layers IVA and IVCβ of area 17 where projections from the parvocellular layers (layers 3-6) of the LGN selectively terminate (Mrzljak et al., 1996). Regional variations among
higher visual areas exist in terms of density and laminar distribution of neurons marked by SMI-32, a monoclonal antibody against the medium- and high-molecular-weight subunits of neurofilament protein (Hof and Morrison, 1995). The antigen recognized by Cat-301 is more abundantly expressed in the areas of the dorsal stream than in those of the ventral stream of the visual processing pathway (Hendry et al., 1988b).

The results of these studies provide impressive examples of molecular parcelations of the nervous system although the number of available molecular markers is still limited. It is expected that as more molecular markers that allow visualization of specific neuronal subsets in the neocortex are obtained, more information about how the neocortex is organized in relation to function will arrive. New histochemical markers may allow us to recognize new populations of neurons and/or new functional subdivisions of the neocortex. In addition, it may be noted that most of the known markers and the neurons marked by them have been discovered by chance. This implies that an extensive and systematic approach to identify such molecular markers for determining specific neuronal subsets would be more fruitful (Hendry & Calkins, 1998).

I applied differential display (DD) (Liang and Pardee, 1992) to compare mRNA expression in structurally and functionally distinct areas of the adult macaque neocortex. I cloned a cDNA named occ1, which showed a high transcription level in the occipital cortex. In this report, I describe the
identification of *occ1* and the characterization of the neurons specified by *occ1* expression.
Materials and methods

Tissue dissection, total RNA extraction and DD PCR.

The brains were removed from three adult macaques (Macaca fascicularis) under deep Nembutal anesthesia at the Japan Poliomyelitis Research Institute. The brains were dissected and frozen on dry ice. Total RNA was obtained by a single-step RNA isolation method by guanidine thiocyanate-urea-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The DD PCR was performed following the protocol of the RNA Image kit (GeneHunter, Nashville, TN, USA) with minor modifications. The reverse transcription (RT) reaction was carried out using an anchor oligo-dT primer, followed by arbitrarily primed PCR with the 5'-end-32P-labeled anchor oligo-dT primer and an arbitrary 13-mer primer by KlenTaq Polymerase (Clontech Laboratories, Palo Alto, CA, USA). The PCR parameters were 1 cycle of 94°C (5 min), 40°C (5 min) and 68°C (5 min), 6 cycles of 94°C (2 min), 40°C (5 min) and 68°C (5 min), and 33 cycles of 94°C (1 min), 40°C (2 min) and 68°C (1 min), followed by the final elongation step at 72°C for 20 min. The PCR products were then separated by electrophoresis on 4% polyacrylamide sequencing gels. The bands that showed differential expressions among the areas were reamplified by PCR using the same primer set that generated them. The PCR parameters for reamplification were 95°C for 5 min, 30 cycles of 95°C (1 min), 40°C (1 min) and 68°C (1 min), and finally at 72°C for 5 min.
RT-PCR analysis.

Total RNAs (2.0 μg) from five regions (areas FDΔ, FA, TE, OA and OC; see Fig. I-1) of Macaca fascicularis neocortex were reverse-transcribed using Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) with Oligo (dT) 12-18 primer (Life Technologies) in a final reaction volume of 40 μl. Simultaneously, the samples subject to the same preparations without reverse transcriptase were prepared and used as negative control samples (RT-) to show the absence of contaminated genomic DNA in the total RNAs. PCR was performed using a primer set corresponding to the end sequences of the cloned occ1 DD band (5'-GGAAGAGATTTAATCTTACAAAAGG-3' and 5'-TATACAGTCAAAGAGGTTGCAACAG-3'). PCR conditions were 95°C for 5 min, 20 cycles of 94°C (30 sec), 60°C (30 sec) and 72°C (30 sec), and finally at 72°C for 5 min. After separation on a 1.0 % agarose gel, the products were blotted and detected by hybridization to the 32P-labeled occ1 probe. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (g3pdh) was performed with primers 5'-AGCGGATCCCTCCAAAATCAAGTG-3' and 5'-GCCATGCGAGTGCTCCCGTTCA -3' as an internal control.

Northern blot analysis.

Poly (A)+ RNA (12 μg) was purified from the total RNA from neocortical area OC, electrophoresed on a 1.2% agarose gel and transferred to Hybond N+ nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The hybridization probe was prepared from an occ1 cDNA clone (1024 bp + poly (A)+
tail), firstly obtained by screening a cDNA library, and radiolabeled with $^{32}$P dCTP.

**Construction of cDNA library and isolation of occ1 full-length clone.**

Using poly (A)$^+$ mRNA from *Macaca fascicularis* neocortical area OC, cDNAs were synthesized with ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) and ligated to EcoRI/XhoI-digested $\lambda$ ZAPII vector (Stratagene), followed by packaging with Gigapack III Gold packaging extract (Stratagene). The nucleotide sequences determined on both strands are in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB039661. Alignments of the sequences were performed using CLUSTAL W (Thompson et al., 1994).

**Generation of antiserum against OCC1.**

Rabbit OCC1 antisera were raised against a fusion protein. OCC1-GST (amino acids (aa) 87-308 of OCC1 fused to GST) was constructed by subcloning this segment of the occ1 cDNA into pGEX 2TK (Amersham Pharmacia Biotech) and expressed in *Escherichia coli* BL21. For antiserum production, three New Zealand white rabbits were immunized, and the antisera were passed through the affinity resin Affi-gel 15 (Bio-Rad Laboratories, Hercules, CA, USA) coupled with the crude lysates of *E. coli* BL21 transfected with mock pGEX2TK vector to exclude the antibodies against GST and bacterial proteins.
Construction of expression vector, transient expression of OCC1 in COS-7 cells and Western blot analysis.

The expression plasmid, pOCC1, was constructed as follows: the complete sequence of the occ1 cDNA was amplified by PCR using a 5′-primer (5′-CCGCTCCAGATGGAAACGCTGGCTCGCTC-3′) which introduced an XhoI cleavage site at the N terminus and a 3′-primer (5′-AAACTGCAGTCATTAGATCCTTTGGTGTCAC-3′) introducing two consecutive stop codons and a PstI cleavage site at the C-terminus of the occ1 cDNA. The XhoI/PstI fragment of the PCR product was cloned into the CMV-promoter driven pEGFP-N1 (Clontech Laboratories). COS-7 cells were transiently transfected with pOCC1 using Lipofectamine Plus (Life Technologies) and cultured in DMEM/10% FCS. Two days after transfection, the cells were washed with DMEM/1% FCS and cultured for another day. Aliquots of cell lysates and conditioned medium were subjected to electrophoresis on 15% SDS-polyacrylamide gels and transferred to Immobilon P transfer membrane (Millipore, Bedford, MA, USA) using standard protocols. After blocking, the blot was incubated with anti-OCC1 antiserum (1:800). The blots were then immunoreacted with goat anti-rabbit antibody coupled to peroxidase (Organon Teknika, Durham, NC, USA; 1:2000) and the immunoreactivity was detected using an ECL detection kit (Amersham Pharmacia Biotech). The blotting performed with the antiserum that had been preabsorbed with 20 μg/ml OCC1-GST fusion protein did not display any band (data not shown).
Tissue preparation for in situ hybridization and immunohistochemistry.

Ten adult macaques (three *Macaca fascicularis* and seven *Macaca fuscata*) weighing 2.9-9.1 kg were used. In five, tetrodotoxin (TTX; 15 μg in 10 μl of normal saline) was injected under Ketamine anesthesia into the vitreous cavity of the left eye twice a week for a total of 7, 10, 14 (two monkeys) or 21 days prior to sacrifice. Retinal activity in the injected eye remains to be suppressed at least for five days following a single injection of this dose of TTX (Hendry et al., 1988a). The other five were untreated. All monkeys were given overdoses of Nembutal and perfused through the hearts with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The brains were removed, post fixed 3-6 h at room temperature in the fixative, cut into blocks and cryoprotected in 30% sucrose in 0.1M phosphate buffer. The blocks were cut as frozen sections on a sliding microtome. The blocks of occipital lobes were sectioned at 30 μm thickness. In three monkeys, either parasagittal (45 μm thickness), horizontal (35 μm) or frontal (40 μm) slices were prepared through one entire hemisphere. In addition, the cortices posterior to the lunate sulcus of the right hemispheres of all monocularly-deprived and one normal monkeys were dissected out, flattened between glass slides during postfixation and cut parallel to the opercular surface at 25 μm thickness.

In situ hybridization.

Digoxigenin-labeled antisense and sense riboprobes were prepared from the nucleotide positions 333-999 (aa 87-308) of the *oc1* cDNA with digoxigenin-
dUTP labeling kit (Roche Diagnostics, Indianapolis, IN, USA). All the sections were stained with these probes, except the horizontal sections of a whole cerebral hemisphere, which were stained with the antisense and sense probes prepared from the nucleotide positions 4632-5675. *In situ* hybridization was carried out as described (Liang et al., 2000). Briefly, free-floating sections were treated with 1 μg/ml proteinase K for 30 min at 37°C. After acetylation, sections were incubated in a hybridization buffer containing 1.0 μg/ml digoxigenin-labeled riboprobes at 50°C. Hybridized sections were washed by successively immersing once (unless otherwise indicated) in 2×SSC/50% formamide/0.1% N-lauroylsarcosine (50°C, 20 min, twice), RNase A buffer (room temperature, 10 min), RNase A buffer containing 20 μg/ml RNase A (37°C, 30 min), 2×SSC/0.1% N-lauroylsarcosine (room temperature and 50°C, 15 min each), 1×SSC/0.1% N-lauroylsarcosine (room temperature, 5 min), 0.5×SSC/0.1% N-lauroylsarcosine (room temperature, 5 min) and 0.2×SSC/0.1% N-lauroylsarcosine (room temperature and 50°C, 20 min each). Hybridization signals were visualized by alkaline-phosphatase immunohistochemistry with the digoxigenin detection kit (Roche Diagnostics). In the series of parasagittal or frontal sections of whole hemisphere, every 12th section was processed with the antisense probe, every 48th (parasagittal) or 24th (frontal) section was processed with the sense probe. The series of horizontal sections at about 2 mm and 8 mm intervals were stained with the antisense probe and the sense probe, respectively. In control sections hybridized with the sense probes, the neocortex
gave no staining above background. The results obtained from both species of macaques were indistinguishable.

The signal intensity of *in situ* hybridization in the processed tissues was quantified with NIH image software by taking optical density meanings of the digitized image. Optical density readings were taken from at least 20 areas (each approximately 1100-6500 μm² in size) around the centers of perturbed and non-perturbed columns, which were identified by matching them to columns showing reduced and normal staining in adjacent CO-stained sections, in layer III or IV/Cβ of area 17 in a section. Background readings, taken from the almost-unstained regions just suprajacent to the white matter because the white matter itself exhibit the pale staining by endogenous alkaline phosphatase activity. These were subtracted and the optical densities were averaged. Data from three sections of each monocularly deprived monkey were then averaged. The significance of difference was examined using the two-tailed Student's *t* test.

*Double immunohistochemical staining.*

For dual labeling, two antiserum/antibody combinations were used: rabbit anti-OCC1 (1:100) and mouse anti-CaMKII α (Roche Diagnostics; 1:400), rabbit anti-OCC1 and mouse anti-GABA (GB-69, Sigma, St. Louis, MO, USA; 1:400). Selected 30 μm-thick sections of normal monkey visual cortex were preincubated in 0.25% Triton X-100 in phosphate buffered saline (PBS) at r. t. for 2 h before being placed in the blocking buffer (1% blocking reagent from Roche Diagnostics, 5% normal goat serum and 0.1% Triton X-100 in PBS) at room
temperature for at least 2 h. They were then transferred to the blocking buffer containing each primary antiserum/antibody combination. After 36-48 h at 4°C, the sections were washed and incubated in a mixture of Alexa 488 conjugated goat anti-rabbit IgG (Molecular probe, Eugene, OR, USA; 1:100) and Alexa 594 conjugated goat anti-mouse IgG (Molecular probe; 1:100) in PBS containing 5% normal goat serum and 0.1% Triton X-100. For controls, the same procedures were performed without primary antiserum or antibody. The control sections showed no fluorescent staining.

All the experiments described here were performed in compliance with the guidelines for animal experiments at Okazaki National Research Institute.
Results

Identification of occ1, a transcript expressed preferentially in macaque occipital neocortex.

Total RNAs from five anatomically distinct regions of the adult cynomolgus monkey neocortex (areas FDΔ, FA, TE, OA and OC according to the classification of von Bonin and Bailey (von Bonin and Bailey, 1947) shown in Fig. I-1) were converted to cDNA with anchor oligo-dT primers, and the mRNA expression in these regions was compared by DD. Among the bands of the PCR-amplified fragments, I identified a 190 base pair (bp) cDNA fragment that showed the highest transcription level in area OC (Fig. I-2a). RT-PCR analysis using a primer set corresponding to the both ends of the clone confirmed this characteristic regional expression pattern (Fig. I-2b).

The cDNA sequence of the clone obtained from DD shared no homology with any DNA sequences available on the GenBank database. Because the clone was likely to represent a 3'-noncoding region adjacent to the poly (A) tail, I isolated a full-length cDNA from a cDNA library from area OC of cynomolgus monkey neocortex. Northern blotting showed that the full-length occ1 mRNA is about 5.7 kb long (Fig. I-3). The first screen of a cDNA library with the DD cDNA fragment resulted in the isolation of a cDNA clone including a 1024 bp sequence upstream from the poly (A) tail. I obtained a full-length clone after another screening of a cDNA library using the elongated clone as a probe. The entire sequence of the clone consisted of 5688 nucleotides, and a putative open
reading frame of 308 amino acids (calculated molecular mass of 34,999 Da) was found upstream from the nucleotide sequence for the DD clone (Fig. I-4 a and b).

I named the gene occ1 (for occipital). Homology search revealed that this gene encodes the macaque homologue of human, rat and mouse follistatin-related protein (FRP)/TSC-36 (Fig. I-5 and I-6). The sequence comparisons showed that the identity of the coding DNA sequence of occ1 to human, mouse and rat frp/tsc-36 was 98.6 %, 88.8% and 88.5 %, respectively, while the deduced amino acid sequence identity of OCC1 to human, mouse and rat FRP/TSC-36 was 99.7 %, 91.6 % and 92.5 %, respectively (Table I-1). FRP/TSC-36 was originally isolated from the cells of a mouse osteoblastic cell line treated with TGF β1 (Shibanuma et al., 1993), and contains a cystein-rich follistatin motif, three putative N-glycosylation sites and various phosphorylation sites (Fig. I-6; Zwijsen, 1994; Patel, 1996; Ohashi, 1997; Tanaka, 1998; Okabayashi, 1999). The follistatin motif is shared by various proteins, such as the activin-inhibitor FOLLISTATIN, the protein which induces the aggregation of nicotinic acetylcholine receptors AGRIN, the multifunctional extracellular glycoprotein SPARC/OSTEONECTIN/BM-40, the rat brain protein SC1 and the quail retina specific protein QR1 (Johnston et al., 1990; Guermah et al., 1991; Patthy and Nikolics, 1993; Maurer et al., 1995; Phillips and de Kretser, 1998; Motamed, 1999).

The sequence analysis of the 3'-untranslated region (UTR) of the full-length clone revealed that the clone has the cytoplasmic polyadenylation element (CPE; TTTTTAT) residing 22 nucleotides upstream of the sequences similar to
the typical polyadenylation signal hexanucleotides AATAAA sequences (HEX) in its 3' end (Fig. I-7). The CPE, which usually resides about 20 nucleotides 5' of HEX, stimulates cytoplasmic polyadenylation and translation during oocyte maturation (Fox et al., 1989; McGrew et al., 1989). In neurons, the CPE is also thought to mediate polyadenylation-induced translation, which is necessary for controlling local and rapid translation of specific mRNAs in neuronal processes (Wu et al., 1998; Wells et al., 2000). As shown in Fig. I-9, the occ1 mRNA were occasionally observed to be localized in the neuronal processes. Taking this observation and the presence of the CPE in occ1 mRNA into consideration, it is speculated that occ1 mRNA undergoes cytoplasmic local translation in the neuronal processes.

OCC1 has putative N-terminal signal peptides (Fig. I-6; Zwijsen, 1994 #6). I performed Western blot analysis with the cell lysates and the supernatant of conditioned medium of COS-7 cells transfected with OCC1 expression vector, and found that the molecular weight of the major product detected in the medium (arrow in Fig. I-8; about 43 kDa) is larger than that in the cell lysates (arrowhead in Fig. I-8; about 36 kDa). This result showed that OCC1 was released into the medium with posttranslational modifications.

**Neocortical distribution of the cells specified by occ1.**

In the monkey neocortex, most occ1-positive signals in *in situ* hybridization preparation were observed in neurons. This is judged from the following observations: i) Little signal was observed in the white matter. ii) The cells
stained by the probe had large somata in general (7-12 μm in diameter in layers III and IVC of area 17). iii) occ1 mRNA was occasionally observed to be localized in the processes of some pyramidal cells, resulting in contoured cell shapes (Fig. I-9).

*In situ* hybridization histochemistry of the serial sections of the entire cerebral hemisphere demonstrated that the distribution of occ1-positive neurons was as expected on the basis of the results of DD and RT-PCR experiments (Fig. I-10). The strongest and densest hybridization signals were observed in area 17. The other occipital areas showed moderate expression of occ1 (Fig. I-10a and e). occ1-positive neurons were distributed with relatively weak labeling basically throughout the temporal and the posterior half of the parietal cortex (Fig. I-10a, c and d). In these cortices, certain cortical areas could be identified by densities and patterns of staining. The primary somatosensory cortex (area 3b) show some dense expression in layer IV and the deeper stratum of layer III (Fig. I-10c, d and I-11a). The primary auditory cortex (AI) also exhibited relatively dense expression in layer IV and the deeper half of layer III (Fig. I-10d and I-11c). The intensity of labeling and the frequency of the signals was even lower in the areas anterior to the central sulcus than in the postcentral regions. In the precentral regions, no obvious cortical areas could be identified by the occ1 expression pattern (Fig. I-10a and b).

Laminar distribution of occ1-positive neurons in visual cortex and visual thalamus.
Examination of occ1 mRNA positive neurons in areas 17 and 18 revealed distinctive laminar distributions of occ1-expressing neurons in these regions. In area 17 (Fig. I-12a), signal was most densely distributed and cells were most intensely labeled in layers IVCα and IVCβ. Layers II, III and IVA exhibited many intensely labeled neurons. In the intervening layer, IVB, a relatively smaller number of moderately labeled neurons existed. The distribution of occ1-positive neurons divided layer V into a superficial, lightly stained sub-layer and a deeper, even more lightly stained sub-layer. In layer VI and the deeper half of layer V, a few moderately stained neurons were rather sparsely found. The positive neurons in layer I were lightly stained and sparsely present.

The laminar distribution of occ1-positive neurons in area 18 (Fig. I-12c) was completely different from that in area 17. Many intensely labeled neurons with large somata were found in the lower one third of layer III. In the upper two thirds of layer III and layer IV, many lightly labeled cells and some moderately labeled cells were observed. Layer II exhibited many lightly labeled neurons. In layers V and VI, a few relatively lightly labeled cells were present. Weak signal was sparsely distributed in layer I.

The subset of neurons specified by occ1 includes functionally and morphologically distinct types.

To determine whether the neurons marked by the expression of occ1 can be classified into a single functional and morphological class of cells, I performed dual labeling immunohistochemistry on the neurons in area 17 with the antisera
to OCC1 and a monoclonal antibody against either CaMKII α or GABA. CaMKII α immunoreactivity is found only in a population of glutamatergic, excitatory cells in the cerebral cortex (Benson et al., 1991; Benson et al., 1992; Liu and Jones, 1996), which includes the spiny stellate cells in layer IV and pyramidal cells in the other layers of area 17 (Hendry and Kennedy, 1986; Tighilet et al., 1998). On the other hand, GABAergic neurons of the primate neocortex belong to a class of inhibitory and aspiny nonpyramidal neurons (Houser et al., 1983; Jones et al., 1994). No GABA-immunoreactive neuron shows CaMKII α immunoreactivity in area 17 (Tighilet et al., 1998).

The OCC1 immunoreactivity is diffusely present within the cell bodies, especially around the cell nuclei, and seen in a punctate pattern along the neuronal processes (Fig. I-13 left panels). These observations are consistent with the possibility that the product of occ1 can be released from neurons as well as from the transfected COS-7 cells. The great majority of CaMKII α-expressing neurons show OCC1 immunoreactivity (Fig. I-13a and b). On the other hand, OCC1 immunoreactivity was found in not all, but in a large population of GABAergic neurons (Fig. I-13c). Each type of neurons, double immunoreactive for OCC1 and CaMKII α and double immunoreactive for OCC1 and GABA, was observed in all layers of area 17 except for layer I in which virtually all neurons are GABA-positive (Hendry et al., 1987). These observations show that the neuronal subset revealed by occ1 expression includes physiologically and morphologically different classes of neurons in area 17.
occ1 is expressed by the neurons in area 17 in an activity-dependent manner.

The neurons marked by occ1 expression were preferentially distributed in area 17. A question arose: does visual experience play a role in the regulation of occ1 expression in the adult brain? Area 17 of the Old World primates is divided into alternating ocular dominance columns (Hubel and Wiesel, 1972; Wiesel et al., 1974). I can examine the alterations in neuronal phenotypes based on changes in neuronal activity in the brains of monocularly deprived animals using immunohistochemical techniques (Hendry and Jones, 1986; Hendry and Kennedy, 1986; Hendry and Jones, 1988).

Following monocular deprivation by TTX injection into the vitreous cavity (7, 10, 14 or 21 days), dramatic changes were detected in the in situ hybridization pattern of occ1 in area 17. Alternating lightly and darkly stained stripes appeared (Fig. I-14a, c, e and g), irrespective of the length of deprivation. Comparison with the adjacent sections stained for CO (Wong-Riley, 1979; Horton and Hubel, 1981), which exhibited the same pattern of alternating dark and light stripes, showed that occ1 transcription was reduced in the deprived columns. The change was observed in layers III, IVA, IVB, IVCα, IVCβ and V, being most apparent in layers III and IVCβ (Fig. I-14a). The signal intensity measurements confirmed this observation that the average level of mRNA in the perturbed columns were 57% lower in layer III (range, 45-68%; p<0.0005), and 74% lower in layer IVCβ (66-82%; p<0.0001) than those in the non-perturbed columns.
In the tangential sections cut through layer III of area 17 of TTX-injected animals, the wide row of occ1 signal was seen along the line of blobs in the undeprived columns, and faint patchy stainings coinciding with the CO periodicities were observed in the perturbed columns (Fig. I-14c, d, e and f; arrows in e and f indicate the patchy CO and corresponding occ1 stainings in the perturbed columns). The occ1 expression in the undeprived columns was beyond the confines of the undeprived blobs to form an almost continuous signal, and the extent of the expression in the deprived columns was even less than that of the shrunken blobs observed in the CO sections. In the sections cut through layer IVCβ, the ocular dominance columns observed in the sections stained for occ1 were even more obvious than those seen in the CO sections (Fig. I-14g and h). These observations also suggest that the occ1 expression in layers III and IVC was significantly decreased by the blockade of visual input. From these results, I conclude that occ1 mRNA is transcribed in an activity-dependent manner in area 17. An obvious change was not observed in area 18.

The characteristic distribution of occ1-positive neurons in the other brain regions.

I examined the occ1 expression in LGN to ask whether occ1 has a specific pattern of laminar distribution in the thalamic nucleus in which visual system channels are segregated into distinct layers, and found that relatively weak signal was present selectively in cells of the magnocellular layers (layers 1 and 2; Fig. I-15a, b and d).
I next examined the distribution of occ1-expressing neurons in the hippocampal formation which is also divided into functionally and structurally distinct subregions. I could find rather many intensely-labelled cells scattered in the stratum oriens and, occasionally, the most outer region of pyramidal cell layer in CA1 and CA2 (Fig. I-16a and c). The positive cells usually contained large somata and labelled processes (arrows in Fig. I-16e). In addition to these positive cells, quite many rather intensely-stained pyramidal cells were selectively localized in the inner fourth of the CA2 pyramidal cell layer (Fig. I-16a, c and e). In CA3, some intensely-labeled cells were scattered throughout the width of the pyramidal cell layer. The pyramidal cell layer of subiculum contained some rather intensely-labeled cells (Fig. I-16a). In the presubiculum, many rather intensely-labeled neurons were found in the lamina principalis interna, and some rather-intensely-labeled cells were sparsely present in the lamina principalis externa (Fig. I-16a). In the dentate gyrus, almost no signal was observed except for the light signals which were detected in most cells of the granule cell layer (Fig. I-16a and c).
Discussion

I have screened for genes that are transcribed differentially among structurally and functionally distinct areas of macaque neocortex, and identified occ1. occ1-positive neurons were selectively distributed in the posterior region of the neocortex, especially, in area 17.

occ1 expression and neocortical neuronal organization

It has been considered that neocortex is basically homogeneous based on the number and density (Rockel et al., 1980), and the morphology (Fairen et al., 1984) of its neuronal components. The ratios between pyramidal and nonpyramidal neurons are very similar in the motor, somatosensory and visual cortices (Sloper, 1973; Tombol, 1974; Sloper et al., 1978). In studies of the chemical properties of neurons, an extensive quantitative survey of the distribution of a major neurotransmitter, GABA, demonstrated that its distribution varies little among neocortical areas (Hendry et al., 1987). However, in these neuron counting studies, there was an exception, area 17, in which the number of neurons through the full depth of the cortex is more than twice than in the other areas (Rockel et al., 1980) and the proportion of GABAergic neurons to total neurons is lower than that in the other areas (Hendry et al., 1987). Therefore, the distribution of occ1 mRNA, preferentially observed in the posterior region of neocortex with regional variations, is a dramatic example of neocortical neurons' heterogeneity, which coincides with the functional subdivisions, and the
high occ1 transcription in area 17 reveals the unique neuronal organization of area 17 in the primate neocortex. Furthermore, as the results shown in Fig. 1-14 demonstrate, occ1 mRNA levels can be remarkably downregulated by blockade of afferent activity in area 17. High occ1 expression is not only spatially coincident with area 17, but also subject to regulation dependent on neuronal activity in area 17. These results show the important features of the expression of occ1 that can be used as a marker for neurons in area 17 as well as a good indicator of neural activities in the particular laminae of area 17.

The further characterization of the neurons specified by occ1 in area 17 showed that they include morphologically and functionally distinct classes of neurons, spiny excitatory cells and aspiny inhibitory cells. This result suggests the other unique aspect of occ1 expression in neocortical neurons that occ1 is expressed not in a single type of neurons but in various types of neurons in a region-selective manner.

**occ1 expression in neocortex and thalamocortical connectivity**

The present results show that the transcription pattern of occ1 relate to the cytoarchitectonic area of neocortex. At present, I cannot explain how such a difference in gene expression among neocortical neurons is generated during the brain development. In regard to the neocortical region where occ1 shows its high transcription, however, I can find some correlation between the laminar distribution of occ1 expressing neurons and the pattern of thalamic afferent innervation.
I found the strong \textit{occ1} transcription in layers II, III, IVA and IVC of area 17. The major inputs to area 17 in primates and some other mammals are from the (dorsal) LGN and the minor ones are from the nuclei of the pulvinar complex. Thalamic inputs from LGN in primates terminate mainly in layer IVC and, to a lesser degree, in layer IVA of area 17 (Hubel and Wiesel, 1972; Hendrickson et al., 1978; Blasdel and Lund, 1983). The pulvinar axons and those from intercalated layers of LGN provide thalamocortical innervation to the cells in layer I and the blobs of layers II and III of area 17 (Fitzpatrick et al., 1983; Hendry and Yoshioka, 1994). In area 18, I observed strong signals in the deep stratum of layer III. The pulvinar afferent terminations in area 18 are dense in the deep part of layer III and layer IV (Curcio and Harting, 1978; Livingstone and Hubel, 1982). In addition to these thalamic recipient regions, \textit{occ1} also shows its high transcription in the regions on which thalamic inputs have strong influences in indirect ways. \textit{occ1} is transcribed in the interblob regions of layer II and III of area 17 where no apparent termination of direct thalamic input has been observed (Livingstone and Hubel, 1982; Itaya et al., 1984). I also found moderate and light signals in layer IVB and the upper stratum of layer V, respectively. Neither layer IVB or V receive direct geniculate inputs. On the other hand, the interblob regions of layers II and III receive indirect inputs from the LGN through layers IVA and IVC. Neurons in layer IVB receive strong inputs from layer IVC\textalpha. The upper subdivision of layer V makes prominent connections to the thalamic recipient layers IVA, IVC\textalpha, IVC\textbeta and VI (Lund, 1988; Callaway, 1998).
occ1 expression and a functional subdivision of visual thalamus and hippocampus.

I observed that the occ1 expression in LGN is observed more frequently in the magnocellular layers in which the cells with broad band spectral qualities are selectively localized (Wiesel and Hubel, 1966) than in the other layers. occ1 is also preferentially expressed in a functional subdivision of a thalamic nucleus. Some markers, for example Cat-301 and SMI-32, are known to primarily labels the magnocellular layers (Hendry et al., 1988b; Chaudhuri et al., 1996). occ1 can also be used as a marker to visualize a subset of neurons in those layers of LGN and contribute to the classification of neurons in LGN.

As shown in the Fig. I-16, occ1 is expressed in a specific type of neurons in the hippocampal formation in a region-specific manner. The neuronal subtypes in the hippocampal formation can be classified by using calcium-binding proteins, Calbindin-D28K, Calretinin and Parvalbumin, as markers (Berger and Alvarez, 1996; Freund and Buzsaki, 1996). Judging from their laminar distribution and somal size, it could be inferred that the population of cells in the Ammon's horn and subiculum marked by occ1 overlaps with that of the cells specified by a calcium-binding protein parvalbumin (Ribak et al., 1990; Seress et al., 1991; Ribak et al., 1993; Berger et al., 1999). The experiments to determine the cell type which expresses occ1 are needed. However, in any case, the occ1, labeling the cells in limited number with a characteristic laminar distribution, can be used as a new marker.
The function of occ1 in cortical plasticity

The data presented here clearly indicate that occ1 mRNA is preferentially expressed in posterior regions of the neocortex, especially in area 17, in an activity-dependent manner. Activity-dependent gene expression, resulting in changes in neuronal phenotypes, plays an important role in neuronal plasticity of brain function and development (Marty et al., 1997; Lee and Sheng, 2000). Many molecules have been reported to be expressed in an activity-dependent manner, but none of them shows such a characteristic regional expression pattern in the neocortex as that of occ1. Although the function of OCC1 (FRP, TSC-36) remains unknown to date, it has been suggested that follistatin motifs of follistatin, agrin and SC1 might play similar functions in the differentiation of the nervous system by accumulating, protecting and modulating the activity of growth factors (Patthy and Nikolics, 1993). This suggestion also presents the possibility that OCC1 may function by binding other molecule(s) and affecting its (their) activity(ies). The results of the expression study with COS-7 cells suggest that OCC1 may function in a secreted form. These together imply that OCC1 may work to mediate activity-dependent interactions between specific subsets of neurons through modulating the functions of other proteins in response to changes in neuronal activity in particular neuronal circuits. Furthermore, the presence of CPE sequence in 3'-UTR of occ1 mRNA clone suggests the possibility that the occ1 mRNA undergo the rapid local translation in the neuronal processes in response to change in the neuronal activities. Further studies on
the nature of OCC1 and to elucidate the role of the molecule in neuronal function will shed new light on the functional structure of the cerebral neocortex.

A few molecular markers for specific neuronal subtypes in the rodent brain have been identified and characterized, such as Limbic System-Associated Membrane protein (LAMP) and Latexin (Levitt, 1984; Zacco et al., 1990; Arimatsu et al., 1992; Arimatsu, 1994). LAMP is a glycoprotein that is expressed in the subsets of neurons in the adult rat brain that are associated with classic limbic structures (Reinoso et al., 1996). Latexin-immunoreactive neurons are confined essentially to the infragranular layers of lateral cortical areas in the rat neocortex (Arimatsu et al., 1999). Among these markers, the distribution of LAMP in the primate brain has been examined, and it has been shown that LAMP can also be effective in visualizing neuronal subsets in the primate limbic system (Cote et al., 1996). This result suggests that the characterization of occ1-positive neurons in rodents and other mammals' brains may show the similarity and the difference of the neuronal organization between the primate and other mammalian brains. This kind of knowledge will contribute to understanding the nature of occ1-expressing neurons in future studies.

A systematic survey for marker molecules in the primate neocortex described here presents a successful example of such an approach. Histochemical analyses using new molecular markers combined with various other techniques would become powerful tools in deciphering the functional organization of the
neocortex.
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Figure legends

Fig. I-1. The five neocortical regions (areas FDΔ, FA, TE, OA and OC; colored) from which tissues were dissected. ari, inferior ramus of arcuate sulcus (s); ars, superior ramus of arcuate s; ce, central s; itp, intraparietal s; lat, lateral s; lu, lunate s; oci, inferior occipital s; oct, occipito-temporal s; prn, principal s; ti, inferior temporal s; ts, superior temporal s.

Fig. I-2. Differential display and RT-PCR analysis of occ1 expression in the five neocortical regions. (a) Total RNAs from five areas of cynomolgous monkey neocortex were compared by differential display. A cDNA fragment, named occ1, was differentially detected (arrow; 190 bp). Lane 1, area FDΔ; lane 2, area FA; lane 3, area TE; lane 4, area OA; lane 5, area OC. (b) RT-PCR confirmation of differential transcription of occ1 in the five neocortical areas. cDNAs or RT-s from area FDΔ (lane 1), area FA (lane 2), area TE (lane 3), area OA (lane 4) and area OC (lane 5) were used. occ1 is transcribed at a high level selectively in area OC. PCR using serial dilutions (1/2-1/16) of cDNA from area OC shows the cycles employed (20 cycles for both primers) were within the range in which amplification occurred linearly. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (g3pdh), which is known to be ubiquitously expressed in various tissues and cells, was used as a control reaction to show the quantity and quality of the cDNAs applied.
Fig. I-3. Northern blot analysis of occ1 expression in neocortical area OC. The lane contained 12 μg of poly (A)$^+$ RNA(a) purified from Macaca fascicularis neocortical area OC. The blot was hybridized with a probe prepared from an occ1 cDNA clone, firstly obtained by screening a cDNA library (1024bp + poly (A)$^+$ tail). RNA length is indicated by the numbers at the left (kb). A single band, about 5.7 kb in size, was detected.

Fig. I-4. Nucleotide and deduced amino acid sequences of the full-length clone of occ1 cDNA. (a) The entire sequence of the clone, composed of 5688 nucleotides, contained a putative open reading frame of 927 nucleotides (308 amino acids; bolded). The sequence of the band firstly detected in the differential display (208 bp.) was found at the 3' end of the clone (underlined). (b) Schematic structure of the full length clone. The open reading frame (927 bp.) is shown as a box. Sequences for the clone detected in the differential display is found at the 3' end of the full-length clone as indicated by a black bar. The nucleotide sequences are in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB039661.

Fig. I-5. occ1 is a homologue of human, mouse and rat frp (follistatin-related protein)/tsc-36. The coding region of occ1 cDNA is aligned with the corresponding region of human, mouse and rat frp/tsc-36. Asterisks indicate nucleotides invariant between the sequences.
Fig. I-6. *occ1* encodes a homologue of a secretable protein, FRP/TSC-36. Alignment of OCC1 deduced amino acid sequences with human, mouse and rat FRP/TSC-36. Identical residues are indicated by asterisks. The putative signal sequences at the N-terminus are bolded. The follistatin motif is boxed and shadowed, while the common cystein residues are in bold and marked by stars. Putative recognition sites for N-glycosylation are marked by open triangle, for CaMKII phosphorylation by filled and inverted triangles, for protein-kinase C by open squares and for tyrosine kinase phosphorylation by closed squares.

Fig. I-7. Schematic representation of 3' untranslated region (3' UTR) of the *occ1* full-length cDNA. The CPE sequence (TTTTTAT) is localized 22 nucleotides (nt) upstream from the HEX-like sequence (ATTAAA), which resides 19 nucleotides 5' from poly (A)* tail.

Fig. I-8. Western blot analysis with anti-OCC1 antiserum of lysates and conditioned medium of COS 7 cells transfected with OCC1 expression vector. Untransfected COS 7 cells were used as a control. l, cell lysates; m, conditioned medium. The molecular weight of the major product detected in the medium of OCC1-expressing COS 7 cells (arrow; about 43 kDa) is larger than that in the cell lysates (arrowhead; about 36 kDa) indicating that the product is secreted after being modified.

Fig. I-9. A photomicrograph for neurons stained by *in situ* hybridization
histochemistry with the occ1 antisense probe in layer IVA of area 17. occ1 mRNA was occasionally found in the processes of some neurons, which resulted in contoured pyramidal-shaped cell bodies (arrow). Pial surface is to the top. Bar, 20 μm.

Fig. I-10. Neocortical distribution of the neurons marked by occ1-mRNA expression. (a-e) Parasagittal (a) and frontal (b-e) whole-brain sections processed by in situ hybridization. In (a), rostral is to the left, and the arrows indicate the borders between areas 17 and 18. In (b-e), medial is to the right. Arrows in (c) indicate the borders of area 3b. Arrowheads and arrows in (d) indicate the borders of area 3b and Al, respectively. (f) Lateral view of macaque neocortex, rostral is to the left. The lines indicate the coronal planes sliced for the sections (b-e). The signals were localized preferentially in the posterior region of the neocortex, in particular, area 17. Bar, 5 mm. Abbreviations are as used in Fig. 1. cc, sulcus of corpus callosum.

Fig. I-11. Area 3b and Al can be identified by densities and patterns of occ1 expression. (a-d) Sections through area 3b (a; the region indicated by arrows in Fig. 10c at higher magnification) and Al (c; the region indicated by arrows in Fig. 10d at higher magnification), stained for occ1 (a and c) and with thionin (b and d). Area 3b and Al show relatively dense expression of occ1 in layer III and IV. Bar, 500 μm.
Fig. I-12. Laminar distribution of occ1-positive neurons in areas 17 and 18. (a-d) Sections through area 17 (a and b) and area 18 (c and d), stained for occ1 (a and c) and with thionin (b and d). In areas 17 and 18, the strongest signals are found in layer IVC and in the deeper stratum of layer III, respectively. Bar, 200 μm.

Fig. I-13. Neuronal subset revealed by occ1 includes two different classes of neurons. (a-c) Sections were double labeled with antiserum against OCC1 (left panels) and antibody against either CaMKII α (a and b; middle panels) or GABA (c; middle panel), and imaged to determine colocalization (right panels, OCC1 in green, CaMKII α or GABA in red). (a) from layer II, (b) and (c) from layer IVCβ of area 17. Pial surface is to the top. Arrowheads indicate the representative cells double-positive for each antiserum/antibody combination. Note that, owing to the difference of the subcellular localization pattern of OCC1 from that of GABA, the confocal images to show colocalization of them (c) had to be acquired in a slice plane in which optimal staining of OCC1 might not be yielded, which resulted in the difference of OCC1 staining patterns in layer IVCβ in the two images (b and c; left panels). Bar, 20 μm.

Fig. I-14. Change in the occ1 transcription in adult area 17 following monocular deprivation. Sections are stained for occ1 (left panels) and CO (right panels). (a,
b) A coronal section through area 17 of a monkey monocularly deprived for 14 days. The change was detected in layers III, IVA, IVB, IVCα, IVCβ and V. (c-f) Tangential sections through layer III. The boxed areas in (c) and (d) are magnified in (e) and (f), respectively. (g, h) Tangential sections through layer IVCβ. By comparing the positions of the same blood vessel profiles in these sections (arrowheads), the remarkable reduction of occ1 mRNA was found in the perturbed columns. Arrows in (e, f) indicate the representative patchy stainings in the perturbed columns in each section. Bars, 500 μm.

Fig. I-15. Distribution of occ1-expressing neurons in the LGN. (a, c) Frontal sections through LGN, stained for occ1 (a) and with thionin (c). (b) Drawing of the positive signals (dots) in (a). (d) The boxed area in (a) is magnified. (e) The boxed area in (c) is magnified. In LGN, occ1 mRNA is expressed selectively in the cells of the magnocellular layers (layers 1 and 2). Bar in (c) for (a-c), 500 μm; bar in (e) for (d, e), 200 μm.

Fig. I-16. Distribution of occ1-expressing neurons in the hippocampal formation of the macaque monkey. Frontal sections through the hippocampus, stained for occ1 (a, c and e) and with thionin (b, d and f). CA1-3, subfields of Ammon’s horn;
DG, dentate gyrus; Sub, subiculum; Prs, presubiculum. The arrows in (a) and (b) indicate the borders between CA1 and CA2 and between CA2 and CA3. The CA2 and CA3 subregions in (a) and (b) were magnified in (c) and (d), respectively. The CA2 subregions in (a) and (b) were at further higher magnification in (e) and (f), respectively. occ1 mRNA is observed to be localized in the processes of the positively-stained neurons; arrows in (e) mark the cells whose processes were visualized by in situ hybridization. gc, granule cell layer; ml, molecular layer; pc, pyramidal cell layer; pm, polymorphic layer; s l-m, stratum lacunosum-moleculare; lpe, the lamina principalis externa; lpi, the lamina principalis interna; so, stratum oriens; sr, stratum radiatum. Bars in (b) and (d), 500 μm; bar in (f), 100 μm.

Table I-1. The sequence identity between occ1 and human, mouse and rat FRP/TSC-36. Proportion of identical nucleotide sequences (top right) and identical amino acid sequences (bottom left), calculated with GENETYX-Mac ver 8.0 (SOFTWARE DEVELOPMENT).
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Table I-1.
Part II

The expression pattern of occ1 in neonatal and adult monkeys: occ1 mRNA expression increases in macaque primary visual cortex during postnatal development.
Summary

As shown in part I, occ1 is preferentially expressed in the primary visual cortex of adult monkeys in an activity-dependent manner. How does the region-selective expression of occ1 contribute to the function of neocortex? Before answering the question, it is necessary to examine the function of this molecule in neurons. It is known that there happen two major developmental events in the primary visual cortex of macaque during the first several months after birth, the duration of the sensitivity of ocular dominance columns to monocular deprivation and the increase and peak of synaptogenesis. If occ1 is involved in these postnatal developmental events, these events become good model systems to clarify the function of occ1 in neurons. I analyzed the expression pattern of occ1 mRNA in the primary visual cortex in newborn (1-2 day old), 3-month old (92-97 day old) and adult monkeys in order to examine whether there is a periodical correlation between these events and the change in occ1 mRNA expression. In situ hybridization experiment showed that the laminar expression pattern of occ1 changes during development. Optical density measurements showed that the
relative amount of occ1 mRNA ever increases gradually during postnatal
development and get the highest at adult. These data suggest the possibility that
occ1 expression is increased in an activity-dependent manner during postnatal
development, and further imply that occ1 plays a role in the postnatal
developmental events. Further examination on the occ1 expression pattern in
area 18 of newborn and 3-month old monkeys suggested that the boundaries
between areas 17 and 18 can be revealed by occ1 expression at early stages as
they are in adult monkeys, and the relative mRNA level and the laminar pattern
of occ1 expression change also in area 18 during postnatal development.
Introduction

It is well known that sensory experience in early postnatal life influence the maturation of the mammalian brain (Berardi et al., 2000). In the visual system, after a brief period of monocular deprivation during an early postnatal period, input from the deprived eye to the primary visual cortex is physiologically weakened and morphologically decreased in size (Shatz and Stryker, 1978; LeVay et al., 1980; Antonini and Stryker, 1993). It has been reported that some secretable molecules, such as neurotrophic factors, are involved in establishing this experience-dependent plasticity (Lo, 1995; Thoenen, 1995; Bonhoeffer, 1996). The facts that the expressions and secretions of neurotrophins are influenced by neuronal activity raise the hypothesis that a reciprocal relation between neurotrophin, functioning as a retrograde messenger, and neural activity may give an instructive factor by which frequently used neuronal connections are selectively strengthened (Isackson et al., 1991; Castren et al., 1992; Goodman et al., 1996; Heymach et al., 1996; Marty et al., 1997)).

In primates, the ocular dominance columns with an adult-like pattern emerge
within the last few weeks in gestation and are already formed at birth (Rakic, 1977; Horton and Hocking, 1996). The already-segregated columns are most vulnerable to shrinkage due to monocular deprivation for the first week of life, and the vulnerability is gradually decreased and disappears at latest by age 12 weeks (Horton and Hocking, 1997).

During the development of nervous system, the synapses are transiently overproduced and, the density of synapses then declines as the development proceeds, which is caused by both neuronal cell death and pruning the useless connections (Lund et al., 1977; Oppenheim, 1985; Acebes and Ferrus, 2000). In the development of macaque neocortex, the density of synapse increases at a rapid rate, reaches the highest level at 2-4 months after birth and, thereafter, declines gradually (Rakic et al., 1986; Hayashi, 1992).

As shown in Part I, the gene, occ1, which encodes a secretable protein, is selectively expressed in adult area 17 at high level, and its expression is subject to the activity-dependent regulation. It might be possible that occ1 plays a role in the visual cortex during the early postnatal development. As a first step toward
addressing this possibility, I examined the expression pattern of occ1 in area 17 of newborn (1-2 postnatal days), 3 month-old (92-97 postnatal days) and adult monkeys. In situ hybridization experiment showed that the laminar patterns of occ1 expression were different at the stages examined. I compared the change of occ1 mRNA level in each layer at these developmental stages. The results showed that the expression of occ1 ever increases gradually as the development proceeds.
Materials and methods

Animals and tissue preparation.

Brain tissues were obtained from nine macaques (*Macaca fascicularis* or *Macaca fuscata*) : three adult monkeys weighing 3.0 kg-9.2 kg, three newborn monkeys (postnatal day(s) 1, 1 and 2) and three 3-month old monkeys (postnatal days 92, 96 and 97). The animals were given an overdose of Nembutal and transcardially perfused with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were blocked and cryoproected in 30% sucrose in 0.1 M phosphate buffer. Sections from blocks that included the primary visual cortex were cut at 40 μm thickness. Every third section was processed with the occ1 antisense probe, for Nissl staining or with the occ1 sense probe.

*In situ hybridization histochemistry.*

The occ1 digoxigenin-labeled antisense and sense riboprobes were transcribed from a cDNA that corresponds to nucleotide positions 333-999 (aa 87-308) of the
occ1 full-length mRNA (in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB039661) with digoxigenin-dUTP labeling kit (Roche Diagnostics, Indianapolis, IN, USA). In situ hybridization was carried out as described (Liang et al., 2000). Free-floating sections were rinsed three times in phosphate buffered saline (PBS, pH 7.4) and then treated once at room temperature (unless otherwise indicated) successively in 0.75% glycine in PBS (15 min, three times), 0.3% Triton X 100 in PBS (5 min), 1 µg/ml proteinase K in protease K buffer (0.1 M Tris-HCl, pH 8.0 and 50 mM EDTA; 30 min, 37°C) and 0.25% acetic anhydrate in 0.1 M triethanolamine, pH 8.0 (10 min). After two washes in 2× saline sodium citrate (SSC), the sections were incubated in the hybridization buffer containing 50% deionized formamide, 5× SSC, 2% blocking reagent (Roche Diagnostics) and 0.1% N-lauroylsarcosine at least for 1 hr at 50°C, transferred to the hybridization buffer containing 1.2 µg/ml digoxigenin-labeled riboprobes and incubated for at least 40 hr at 50°C. Following hybridization, sections were washed sequentially in 2× SSC/50% formamide/0.1% N-lauroylsarcosine (20 min, 50°C, twice), RNase A buffer (10
mM Tris-HCl, pH 8.0, 0.5 M NaCl and 1 mM EDTA; 10 min, room temperature),
RNase A buffer containing 20 μg/ml RNase A (30 min, 37°C), 2× SSC/0.1% N-
lauroylsarcosine (15 min, room temperature), 2× SSC/0.1% N-lauroylsarcosine
(15 min, 50°C, 1× SSC/0.1% N-lauroylsarcosine (5 min, room temperature), 0.5×
SSC/0.1% N-lauroylsarcosine (5 min, room temperature), 0.2× SSC/0.1% N-
lauroylsarcosine (20 min, room temperature) and 0.2× SSC/0.1% N-
lauroylsarcosine (20 min, 50°C).

Hybridization signals were visualized by alkaline-phosphatase
immunohistochemistry. The sections were rinsed twice in 0.1% Tween 20 in
Tris-HCl buffered saline (TBS; 0.1 M Tris-HCl, pH 7.4 and 0.1 M NaCl),
incubated in the blocking buffer (1% blocking reagent, 3% normal sheep serum
and 2 mM levamisole in TBS) for at least 60 min, and then incubated in anti-
digoxigenin Fab-fragments conjugated with alkaline phosphatase (Roche
Diagnostics) diluted (1:500) in the blocking buffer for 2 hr. After extensive wash
in TBS, the sections were preincubated in TBS (pH 9.5) containing 0.1% Tween
20, 5 mM MgCl₂ and 2 mM levamisole followed by incubation in the color
development solution (0.033% nitroblue tetrazolium, 0.017% 5-bromo-4-chloro-3-indolyl phosphate, 5 mM MgCl₂, 4 mM levamisole in TBS, pH 9.5) for 24 hours in the dark.

For quantification, tissues from nine monkeys at three different ages were processed simultaneously.

Quantification.

The relative amount of mRNA in each cortical layer was quantified by taking optical density meanings of the digitized image. Images were obtained using a digital camera (DP11; Olympus, Tokyo, Japan) with a microscope (BX50; Olympus). Optical density readings were taken in each layer of 200 μm width in the sections hybridized with the occ1 antisense probe using NIH image software. Background readings were taken from the neighboring sections stained with the occ1 sense probe. The signal intensity (SI) of a given cortical layer was determined as the optical density of the layer expressed as a percentage of the optical density of the background staining as Meberg and Routtenberg's
presented (Meberg and Routtenberg, 1991). Twelve areas (four areas from each of three sections) in each layer were measured for each animal. The significance of difference was examined using the two-tailed Student's t test.

All the experiments described here were performed in compliance with the guidelines for animal experiments at Okazaki National Research Institute.
Results

I examined the occ1 mRNA expression in the primary visual cortex (area 17) of newborn monkeys (postnatal day(s) 1, 1 and 2), 3-month old monkeys (postnatal days 92, 96 and 97) and adult monkeys weighing 3.0 kg-9.2 kg by in situ hybridization. To confirm the changes in the occ1 mRNA level and the distribution in the cortical layers, I quantified the relative mRNA level in the cortical layers by taking optical density meanings of the digitized images of the sections processed by in situ hybridization.

occ1 mRNA expression patterns in area 17 of newborn monkey.

In situ hybridization showed that occ1 is expressed in area 17 of newborn monkey at a certain level with a characteristic laminar pattern (Fig. II-1 a). Many neurons hybridized with antisense occ1 cRNA probe with moderate intensity were found in layers IVCα and IVCβ. Layers II and III contained many rather moderately labeled neurons. In layer IVB, some relatively moderately-labeled neurons were present. In layers V and VI, a few moderately stained neurons
were sparsely present, and very light signals were observed throughout the layers. The positive signals in layer I were lightly stained and sparsely present.

*occ1* mRNA expression patterns in area 17 of 3-month old monkey.

The pattern of *occ1* expression in 3-month old monkey appeared basically similar to that observed in newborn monkeys, while some changes in laminar pattern were observed (Fig. II-1c). Many moderate signals were observed in layers IVCα and IVCβ. Some rather intensely-labeled neurons were also scattered in layers IVCα and IVCβ. In layers II, III and IVA, I observed moderately labeled neurons. In layer IVB, rather moderate hybridization signals were distributed with relatively low density. Layers V and VI contained a few relatively moderately-stained cells. Very light signals which were equivalent to those I observed throughout layers V and VI of newborn monkeys were even weaker. In layer I, only a few very lightly-labeled neurons were observed.
occ1 mRNA expression patterns in area 17 of adult monkey.

occ1 is expressed in a pattern different from that in newborn or 3-month old monkey (Fig. II-1e). The major difference was found in the striking dense signals of layer IVC. occ1 hybridization signals were most preferentially localized in layers IVCα and IVCβ, in which signals were very dense and intense. Many intensely labeled neurons were found in layers II, III and IVA. In layer IVB, relatively intense signals were observed. Moderate occ1 hybridization signals were present throughout the upper half of layer V. In layer VI and the deeper half of layer V, a few moderately stained neurons were rather sparsely found. Very light signals which I observed in layers V and VI of newborn monkeys were not observed. The positive neurons in layer I were light and sparse.

Change of level of occ1 mRNA in area 17 during development.

To clarify the change in the level of occ1 mRNA, I measured the optical densities of the sections hybridized with the occ1 riboprobes. I then introduced the signal intensity (SI) defined by Meberg and Routtenberg (Meberg and Routtenberg,
1991) to analyze the differences in the intensities of hybridization signals in layers II-IVCβ of area 17 at newborn, 3-month old and adult stages (Fig. II-2). For quantification, tissues from nine monkeys at three different stages were processed simultaneously with the same condition of hybridization. The calculated standard deviation (SD) of the average of SI in each layer at each stage was generally less than 10% of the value of each average, ranging from 5.8% to 12.1% (Fig. II-2). I then judged that the SIs were enough reliable to analyze the differences of the signal intensities at the stages examined, and the difference in the value of SI reflect that of the amount of the expressed occ1 mRNA. The SIs in layers IVCα and IVCβ in 3-month old monkeys were significantly higher than those at newborn stage (Fig. II-2 double asterisks, P<0.01). The SIs in the other layers in 3-month old monkeys appeared to change slightly comparing to those in the same layers in newborn monkeys, although the differences in the levels did not reach statistical significance.

I observed the marked increase in the intensity of the occ1 hybridization signals in adult monkeys comparing to the signals detected in newborn or 3-
month old monkey. The SIs in layers III-IVCβ were significantly higher than those in the same layers at early developmental stages (Fig. II-2 asterisks, $P<0.01$).

The intensity in layer II was slightly higher than that in newborn or 3-month old monkey, while the level was significantly higher only than that in 3-month old monkey ($P=0.025$; between newborn and adult monkeys, $P=0.222$).

**Change of laminar pattern of occ1 expression during development.**

To clarify the difference in the laminar expression pattern of occ1 at the stages examined, I employed the SI ratio. The SI ratio of a given cortical layer was determined as the SI of each layer divided by the SI of layer IVCβ. Thus, I can gain the relative intensity of each layer to that of layer IVCβ at each stage (Fig. II-3). Comparing the ratios between the stages examined, I can know the difference in the laminar patterns. In newborn monkeys, the SI ratios were relatively similar, ranging from 0.881 (IVB/IVCβ) to 0.960 (II/IVCβ). In 3-month old monkey, the SI ratios of layers II, III, IVA and IVB were significantly lower than those in newborn monkeys (Fig. II-3 double asterisks, $P<0.01$). In adult, the
ratios in all the layers examined were even lower than those in 3-month old or newborn monkeys (Fig. II-3 asterisks, P<0.01). These data suggest that the levels of the expressed occ1 mRNA at layers II-IVCβ are rather similar at the newborn stage, and the levels in these layers increase gradually as development proceeds while the increase in layer IVCβ is the largest.

Differential pattern of occ1 expression in areas 17 and 18 in newborn, 3-month old and adult monkeys.

As shown in Part I or Fig. II-4e, area 17 in adult monkeys displayed a characteristic pattern of occ1 mRNA expression that allowed for its well-defined discrimination from the adjacent area 18. The intense signals were observed in layers II, III, IVA and IVC of area 17, while intensely stained neurons with large somata were observed in the lower one third of layer III in area 18. I examined the patterns of occ1 mRNA expression in area 18 in newborn and 3-month old monkeys (Fig. II-4). The signal intensity throughout the layers in area 18 of newborn or 3-month old monkey was much lower than that in area 17, which
allowed us to define the border between the areas easily at these stages (Fig. II-4a and c).

In area 18 of newborn monkeys, a few moderately labeled neurons were found in layer III, V and VI (Fig. II-4a). Some rather moderately-labeled neurons were present in layer II. Many light signals were observed in layer IV. Light signals were sparsely distributed in layer I.

In area 18 of 3-month old monkeys, the signals throughout the layers appeared to be slightly weaker than those observed in newborn monkeys (Fig. II-4c). Some moderate signals were observed in layers II and the lower one third of layer III. The upper two thirds of layer III exhibited many light signals. A few rather moderate signals were present in layer IV, V and VI. The signals in layer I were sparse and light.

These data showed that the difference in the relative amount and laminar pattern of occ1 expression between areas 17 and 18 allow for clear definition of the cortical field both in newborn and 3-month old monkeys. Furthermore, these data also suggest that the changes in laminar expression pattern of occ1 during
postnatal development occur in area 18, and the expression level changes in
area 18 as the neocortical development proceeds.
Discussion

I examined the change in occ1 mRNA expression pattern and the relative amount of occ1 expression during postnatal development of the macaque. The measurements of signal intensity suggested that occ1 mRNA increases in its level during the first three postnatal months. In the later development, occ1 expression further increases and gets the highest level at adult.

Change in the laminar pattern of occ1 expression in area 17 during postnatal development.

The sections hybridized with occ1 cRNA probe showed differential laminar pattern of occ1 mRNA expression at the stages examined. As shown in Fig. II-2, the signal intensities in all the layers examined except for layer II in adult monkeys are significantly higher than those at the earlier developmental stages, which suggests that the occ1 mRNA level increases during postnatal development. The change of signal intensity in layer IVcβ during postnatal development was the most marked among the changes. The ratio of signal
intensity in each layer to that in layer IVCβ examined in Fig. II-3 confirmed that the increase of occ1 expression during development is the largest in layer IVCβ, which is reflected by the SI ratio much smaller than 1.0 in each layer at adult while the ratios are relatively close to 1.0 in all layers examined at newborn. The difference in the level of increase in occ1 mRNA expression in each layers appeared to result in the differences in the laminar pattern of in situ hybridization at the stages examined. Then, how does the differential increase happen during development in occ1 mRNA level in each layer? As presented in Part I, I applied in situ hybridization to the monocularly-deprived animals. I observed that apparent decrease in occ1 mRNA expression occurred only in layers III, IVA, IVB, IVCα, IVCβ and V. Furthermore, the extent of decrease is different in each layer such as 57% and 74% in layer III and layer IVCβ, respectively. These observations suggest that the extent of dependence of occ1 expression on neuronal activity is different in each layer at adult. The change in layer IVCβ by monocular deprivation was the largest, which indicates that the extent of the dependency of occ1 mRNA expression on neuronal activity is the largest in the
layer. To answer the question presented above, being hypothesized that the increase in occ1 expression during postnatal development would be mainly due to increase in neuronal activity in area 17, the differential increase in each layer is conceivable. In other words, occ1 expression during postnatal development increases mostly in an activity-dependent manner, and the increase during development is the largest in layer IVCβ, therefore the extent of activity-dependent expression of occ1 becomes the largest in layer IVCβ at adult. There is another fact supporting this hypothesis. The SI in layer II are almost constant throughout development (Fig. II-2). On the other hand, in monocularly-deprived adult animals, almost no apparent change was observed in layer II, which implied that the extent of dependence of occ1 expression on neuronal activity is little in layer II. Provided that the increase of occ1 expression during development is dependent on activity, it can make sense that there was little increase of occ1 expression in layer II. From these, I should suggest that the increase in occ1 expression is principally due to increase in neuronal activity in area 17 during postnatal development, which resulted in the differential increase
of occ1 expression in each layer during development.

**Temporal correlation between change in occ1 mRNA level and postnatal development.**

In primates, among the developmental events of the visual cortex, the ocular dominance plasticity and synaptogenesis are well examined. Visual deprivation during early postnatal life causes shrinkage in the ocular dominance column serving the deprived eye and expansion in the column serving the other normal eye in area 17. The shrinkage and expansion of the ocular dominance columns by retraction and sprouting of their geniculocortical terminal arbors occur only for the early period in postnatal development, which is called ‘critical period’. In monkeys, this plasticity of ocular dominance columns peaks at the first postnatal week and halts at latest by postnatal 3 months (Horton and Hocking, 1997). On the other hand, synaptic density in area 17 increase during the last 2 months of gestation and the first postnatal months, get the highest level at postnatal 4 months and gradually decrease (Lund et al., 1977; Rakic et al., 1986).
The results of signal intensity measurements shown in Fig. II-2 suggested that the expression of occ1 mRNA in area 17 increases gradually during the postnatal development, and its level becomes the highest at adult. The increase of occ1 expression does not show such a clear periodical correlation with the postnatal developments described above as the peak of expression is timed with the events and the expression declines later. But it is certain that the level increases during postnatal development, especially during the postnatal developmental events. And, as discussed in the previous section, the increase of occ1 expression during postnatal development is thought to be basically dependent on the increase of neuronal activity in area 17. Regarding the involvement of neurotrophic factors in activity-dependent plasticity or synaptogenesis, the expression and the retrograde release of neurotrophic factors stimulated by neuronal activity lead to selective strengthening of specific active synapses and circuits (Lo, 1995; Wang et al., 1995). The results suggest that occ1 expression is stimulated by neuronal activity in area 17 during the developmental events. Therefore, I should present the possibility that occ1 is
involved in the postnatal development of visual cortex.

The data presented in Fig. II-4 showed that \textit{occ1} is preferentially expressed in area 17 of newborn and 3-month old monkeys. These data show that \textit{occ1} can be used as a molecular marker to reveal a specific neuronal subset in neocortex in newborn and 3-month old monkeys as it can be in adult neocortex. Furthermore, the data support the possibility that \textit{occ1} plays a role in the development of the visual cortex because it shows \textit{occ1} is preferentially expressed in area 17 while the developmental events occur in area 17.

However, many important questions remain to be answered. How and where is the product released? Is the product is released in an activity-dependent manner? If so, from what subcellular region is the product released? Furthermore, most importantly, does the product have the effect on the efficacy of neuronal synapse or neuronal morphologies? It is necessary to answer each questions utilizing appropriate biological systems.
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Figure legends

Fig. II-1. Laminar distribution of occ1 mRNA-expressing neurons in area 17 in newborn (a and b), 3 month-old (c and d) and adult (e and f) monkeys. Sections through area 17, stained for occ1 (a, c and e) and with thionin (b, d and f). The laminar expression pattern was observed to change slightly as the development proceeds. Bar, 200 μm.

Fig. II-2. Histograms of signal intensity (SI) of occ1 in situ hybridization in layer II-IVCβ at three different developmental stages. Means ± Standard Deviations are shown. NB, new born; 3M, 3 month-old; AD, adult. The SIs in layers III-IVCβ in adult monkeys are higher than in new born monkeys (*, P<0.01; Student's t test), and the intensities in layers IVCα and IVCβ in 3 month-monkey are higher than those in newborn monkey (**, P<0.01).

Fig. II-3. Histograms of the ratio of SI in each layer to SI in layer IVCβ at each developmental stage. Means ± Standard Deviations. NB, new born; 3M, 3
month-old; AD, adult. Asterisks (*) and double asteriskes (**) denote the significant differences from 3 month-old and adult and from newborn and adult monkeys, respectively (P<0.01; Student's t test).

Fig. II-4. Staining patterns at the boundary between areas 17 and 18 in newborn (a and b), 3 month-old (c and d) and adult (e and f) monkeys. Sections were stained for occ1 (a, c and e) and with thionin (b, d and f). Contrasting patterns in areas 17 and 18 were observed at all the stages examined. Arrows indicate the boundaries between areas 17 and 18. Bar, 200 µm.
Fig. II-2
Fig. II-3
General discussion and conclusion

In part I, the identification of the gene, occ1, was described. I applied differential display to compare mRNAs expressed in the distinct neocortical areas in macaque neocortex and found that occ1 was preferentially expressed in the occipital cortex. Further histological analysis showed that occ1 is selectively expressed in area 17. Although I have already known some molecules which show regional localization in the mammalian neocortex, none of them shows such a spatial pattern strikingly related to the known cytoarchitectonic borders as that of occ1. It is surprising that there is a regulation of gene expression which spatially correlates with a specific functional area within neocortex. The further histochemical analysis revealed the other aspect of occ1 expression in area 17 that occ1 is expressed in various types of neurons in an activity-dependent manner. This feature, at least, makes occ1 as an good indicator of neuronal activity in visual cortex because the change of occ1 expression due to molecular deprivation was prominent among the conventional histochemical markers such as cytochrome oxidase and zif268. Moreover, many molecules have been
reported to be expressed in an activity-dependent manner, but none of them shows such a characteristic regional expression pattern in the neocortex as that of occ1. Activity-dependent gene expression, resulting in changes in neuronal phenotypes, plays an important role in neuronal plasticity of brain function and development. So, occ1 is all the more outstanding in this feature of expression. Furthermore, it was shown that occ1 labels specific types of neurons in the areas other than neocortex such as LGN and hippocampus. These results show that occ1 will be useful as the markers not only for neurons in area 17, but for specific subsets of neurons in the other regions of the brain.

In part II, it was shown that the level of occ1 mRNA is increased during postnatal development. The laminar pattern of occ1 mRNA expression changes as the development proceeds. As discussed in Part II, these data suggest that occ1 might be involved in the events of the development of visual cortex. It is necessary to further examine the effects of occ1 in the developmental events by overproduction or disruption of the product. To examine the function of occ1 in development will provide an information which would help us to answer the
essential question about the occ1 localization in adult neocortex, 'how does the region-selective expression of occ1 in neocortex contribute to the function of neocortex?' If the function of occ1 had been clarified, an insight into the functional organization of neocortex would be obtained.

To conclude the present thesis, I would like to mention that the first systematic survey for marker molecules in the neocortex could give a successful example of such an approach. I should like to also emphasize that the further investigation on the function of occ1 will provide a clue to understanding how the neocortex is organized in relation to function.
Publication

Abstracts


The \textit{occ1} gene is preferentially expressed in the primary visual cortex in an activity-dependent manner: a pattern of gene expression related to the cytoarchitectonic area in adult macaque neocortex

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The occ1 gene is preferentially expressed in the primary visual cortex in an activity-dependent manner: a pattern of gene expression related to the cytoarchitectonic area in adult macaque neocortex

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Abstract

Marker molecules to visualize specific subsets of neurons are useful for studying the functional organization of the neocortex. One approach to identify such molecular markers is to examine the differences in molecular properties among morphologically and physiologically distinct neuronal cell types. We used differential display to compare mRNA expression in the anatomically and functionally distinct areas of the adult macaque neocortex. We found that a gene, designated occ1, was preferentially transcribed in the posterior region of the neocortex, especially in area 17. Complete sequence analysis revealed that occ1 encodes a macaque homolog of a secretable protein, TSC-36/follistatin-related protein (FRP). In situ hybridization histochemistry confirmed the characteristic neocortical expression pattern of occ1 and showed that occ1 transcription is high in layers II, III, IVa and IVc of area 17. In addition, occ1 transcription was observed selectively in cells of the magnocellular layers in the lateral geniculate nucleus (LGN). Dual labeling immunohistochemistry showed that the occ1-positive neurons in area 17 include both γ-aminobutyric acid (GABA)-positive aspiny inhibitory cells and the α-subunit of type II calmodulin-dependent protein kinase (CaMKII α)-positive spiny excitatory cells. With brief periods of monocular deprivation, the occ1 mRNA level decreased markedly in deprived ocular dominance columns of area 17. From this we conclude that the expression of occ1 mRNA is present in a subset of neurons that are preferentially localized in particular laminae of area 17 and consist of various morphological and physiological neuronal types, and, furthermore, occ1 transcription is subject to visually driven activity-dependent regulation.

Introduction

The distribution of specific molecules shows a striking correlation with particular physiological organizations in the primate visual system. The Cat-301 antibody, which recognizes a chondroitin sulfate proteoglycan (Hockfield et al., 1990; Zaremba et al., 1989) primarily stains the magnocellular layers (layers I–II) of the lateral geniculate nucleus (LGN; Hendry et al., 1984). Immunostaining for calcium/calmodulin-dependent protein kinase II α-subunit (CaMKII α) in combination with neuronal tracing techniques revealed that the koniocellular layers (intercalated layers) in macaque LGN send direct afferent inputs to area 17 (Hendry & Yoshioka, 1994). Although the functional modules of the neocortex are even more complicated than those of the thalamic nuclei, some molecules coincide spatially with the fundamental functional subdivisions of the neocortex, such as layers, columns and areas. Cytochrome oxidase (CO) is enriched in the blobs in layers II and III of the primary visual cortex (area 17) (Wong-Riley, 1979; Horton & Hubel, 1981) and in the stripe-shaped structures in the secondary visual cortex (area 18) (Livingstone & Hubel, 1982, 1983; Horton, 1984). Synaptic zinc is enriched in CO-deficient interblob regions (Dyck & Cynader, 1993). The pattern of m2 muscarinic acetylcholine receptor immunoreactivity is also reciprocal to the pattern of CO histochemical staining in layers II and III, the strongest immunoreactivity being observed in layers IVa and IVcβ of area 17 where projections from the parvocellular layers (layers 3–6) of the LGN terminate selectively (Mrzljak et al., 1996). Regional variations in higher visual areas exist in terms of density and laminar distribution of neurons marked by SMI-32, a monoclonal antibody against the medium and high molecular weight subunits of neurofilament protein (Hof & Morrison, 1995). The antigen recognized by Cat-301 is expressed more abundantly in the areas of the dorsal stream than in those of the ventral stream of the visual processing pathway (Hendry et al., 1988b).

These studies provide impressive examples of molecular bundles of the nervous system, although the number of available molecular markers is still limited. It is expected that as more molecular markers that allow visualization of specific neuronal subsets in the neocortex are obtained, more information on how the neocortex is organized in relation to function will be gained. New histochemical markers could allow us to recognize new populations of neurons and/or new functional subdivisions of the neocortex. In addition, it should be
noted that most of the known markers and the neurons marked by them have been discovered by chance. This implies that an extensive and systematic approach to identify such molecular markers for determining specific neuronal subsets would be more fruitful (Hendry & Calkins, 1998).

We applied differential display (DD; Liang & Pardee, 1992) to compare mRNA expression in structurally and functionally distinct areas of the adult macaque neocortex. We cloned a cDNA named occ1, which showed a high transcription level in the occipital cortex. In this report, we describe the identification of occ1 and the characterization of the neurons specified by occ1 expression.

Materials and methods

Tissue dissection, total RNA extraction and DD polymerase chain reaction

The brains of three adult macaques (Macaca fascicularis) were removed under deep Nembutal anesthesia at the Japan Policymyelitis Research Institute. The brains were dissected and frozen on dry ice. Total RNA was obtained by a single-step RNA isolation method by guanidine thiocyanate–urea–phenol–chloroform extraction (Chomczynski & Sacchi, 1987). The DD polymerase chain reaction (PCR) was performed following the protocol of the RNA Image kit (GeneHunter, Nashville, TN, USA) with minor modifications. The reverse transcription (RT) reaction was carried out using an anchor oligo-dT primer, followed by arbitrarily primed PCR with the 5'-end-32P-labelled anchor oligo-dT primer and an arbitrary 13-mer primer by KlenTaq polymerase (Clontech Laboratories, Palo Alto, CA, USA). The PCR parameters were 1 cycle at 94 °C (5 min), 40 °C (5 min) and 68 °C (5 min), 6 cycles at 94 °C (2 min), 40 °C (5 min) and 68 °C (5 min), and 33 cycles at 94°C (1 min), 40 °C (2 min) and 68 °C (1 min), followed by the final elongation step at 72 °C for 20 min. The PCR products were then separated by electrophoresis on 4% polyacrylamide sequencing gels. The bands that showed differential expressions among the areas were reamplified by PCR using the same primer set used to generate them. The PCR parameters for reamplification were 95 °C for 5 min, 30 cycles at 95 °C (1 min), 40 °C (1 min) and 68 °C (1 min), and finally 5 min at 72 °C.

RT–PCR analysis

Total RNAs (2.0 µg) from five regions (areas FDα, FA, TE, OA and OC; see Fig. 4) of Macaca fascicularis neocortex were reverse-transcribed using Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) with Oligo (dT)12–18 primer (Life Technologies) in a final reaction volume of 40 µL. Simultaneously, the samples subject to the same preparations without reverse transcriptase were prepared and used as negative control samples (RT–) to show the absence of contaminated genomic DNA in the total RNAs. PCR was performed using a primer set corresponding to the end sequences of the cloned occ1 DD band (5'-GGAGAG-GATTAACTTACAAAGG-3' and 5'-TATAAGTCGAGAG-GTTGCCAAGC-3'). PCR conditions were 95 °C for 5 min, 20 cycles of 94 °C (30 s), 60 °C (30 s) and 72 °C (30 s), and finally at 72 °C for 5 min. After separation on a 1.0% agarose gel, the products were blotted and detected by hybridization to the 32P-labeled occ1 probe. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (g3pdh) was performed with primers 5'-AGCGGATGCTCCCTCAAATCAGTG-3' and 5'-GCCATGCCATGAGCCTCCGGTTCA-3' as an internal control.

Fig. 1. Differential display and RT-PCR analysis of occ1 expression in the five neocortical regions. (a) Total RNAs from five areas of cynomolgus monkey neocortex were compared by differential display. A cDNA fragment, named occ1, was differentially detected (arrow; 190 bp). Lane 1, area FDα; lane 2, area FA; lane 3, area TE; lane 4, area OA; lane 5, area OC. (b) RT-PCR confirmation of differential transcription of occ1 in the five neocortical areas. cDNAs or RT-α from area FDα (lane 1), area FA (lane 2), area TE (lane 3), area OA (lane 4) and area OC (lane 5) were used. occ1 is transcribed at a high level selectively in area OC. PCR using serial dilutions (1/2–1/16) of cDNA from area OC shows the cycles employed (20 cycles for both primers) were within the range in which amplification occurred linearly. RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (g3pdh), which is expressed ubiquitously in various tissues and cells, was used as a control reaction to show the quantity and quality of the cDNAs applied.

Northern blot analysis

Poly (A)+ RNA (12 µg) was purified from the total RNA from neocortical area OC, electrophoresed on a 1.2% agarose gel and transferred to Hybond N nylon membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The hybridization probe was prepared from an occ1 cDNA clone (1024 bp + poly (A)+ tail), firstly obtained by screening a cDNA library, and radiolabeled with [32P]dCTP.

Construction of cDNA library and isolation of occ1 full-length clone

Using poly (A)+ mRNA from Macaca fascicularis neocortical area OC, cDNAs were synthesized with ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) and ligated to EcoRI/XhoI-digested λ ZAPII vector (Stratagene), followed by packaging with Gigapack III Gold packaging extract (Stratagene). The nucleotide sequences determined on both strands are in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB039661.

Generation of antiserum against OCC1

Rabbit OCC1 antisera were raised against a fusion protein. OCC1–glutathione-S-transferase (OCC1–GST) (amino acids 87–308 of OCC1 fused to GST) was constructed by subcloning this segment of the occ1 cDNA into pGEX 2TK (Amersham Pharmacia Biotech) and expressed in Escherichia coli (E. coli) BL21. For the production of antiserum, three New Zealand white rabbits were immunized and the antisera passed through Affigel 15 affinity resin (Bio-Rad Laboratories, Hercules, CA, USA) coupled with the crude lysates of E. coli BL21 transfected with mock pGEX2TK vector to exclude the antibodies against GST and bacterial proteins.

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Construction of expression vector, transient expression of OCCI in COS-7 cells and Western blot analysis

The expression plasmid, pOCCI, was constructed as follows: the complete sequence of the OCCI cDNA was amplified by PCR using a 5'-primer (5'-CTGCTCAAGTGGGAAAACCGTGGCTCGGTC-3') which introduced an Xhol cleavage site at the N-terminus and a 3'-primer (5'-AACTGCACTTAGATCTTGTCTGC-3') introducing a PstI cleavage site and two consecutive stop codons at the C-terminus of the ocell cDNA. The Xhol/PstI fragment of the PCR product was cloned into the CMV promoter driven pEGFP-N1 (Clontech Laboratories). COS-7 cells were transiently transfected with pOCCI using Lipofectamine Plus (Life Technologies) and cultured in DMEM/10% fetal calf serum (FCS). Two days after transfection, the cells were washed with DMEM/1% FCS and cultured for another day. Aliquots of cell lysates and conditioned medium were subjected to electrophoresis on 15% SDS-polyacrylamide gels and transferred to Immobilon P transfer membrane (Millipore, Bedford, MA, USA) using standard protocols. After blocking, the blot was incubated with anti-OCCI1 antiseraum (1:800). The blots were then immunoreacted with goat anti-rabbit antibody coupled to peroxidase (Organon Teknika, Durham, NC, USA; 1:2000) and the immunoreactivity was detected using an ECL detection kit (Amersham Pharmacia Biotech). The blotting performed with the antiseraum that had been preabsorbed with 20 μg/mL OCCI-GST fusion protein did not display any band (data not shown).

Tissue preparation for in situ hybridization and immunohistochemistry

Ten adult macaques (three Macaca fascicularis and seven Macaca fascata) weighing 2.9–9.1 kg were used. In five, tetrodotoxin (TTX; 15 μg in 10 μL of normal saline) was injected under ketamine anesthesia into the virfous cavity of the left eye twice a week for a total of 7, 10, 14 (two monkeys) or 21 days prior to the animals being killed. Retinal analysis in the injected eye was suppressed for at least 5 days following a single injection of this dose of TTX (Hendry et al., 1988a). The other five were untreated. All monkeys were given overdoses of Nembutal and perfused through the hearts with 4% parafomaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, post fixed 3-6 h at room temperature in the fixative, cut into blocks and cryoprotected in 30% sucrose in 0.1 M phosphate buffer. The blocks were cut as frozen sections on a sliding microtome. The blocks of occipital lobes were sectioned at 30 μm thickness. In three monkeys, either parasagittal (45 μm thickness), horizontal (35 μm) or frontal (40 μm) slices were prepared through one entire hemisphere. In addition, the cortexes posterior to the lunate sulci of the right hemispheres of all monocularly deprived and one normal monkey were dissected out. flattened between glass slides during post fixation and cut parallel to the opercular surface at 25 μm thickness.

In situ hybridization

Digoxigenin-labelled antisense and sense riboprobes were prepared from the nucleotide positions 333-999 (amino acids 87-308) of the OCCI cDNA with a digoxigenin–dUTP labeling kit (Roche Diagnostics, Indianapolis, IN, USA). All the sections were stained with these probes, except the horizontal sections of a whole cerebral hemisphere, which were stained with the antisense and sense probes prepared from the nucleotide positions 4632-5675. In situ hybridization was carried out as described (Liang et al., 2000). Briefly, free-floating sections were treated with 1 μg/mL proteinase K for 30 min at 37 °C. After acetylation, sections were incubated in a hybridization buffer containing 1.0 μg/mL digoxigenin-labeled riboprobes at 50 °C. Hybridized sections were washed by successively immersing once (unless otherwise indicated) in 2 × SSC/50% formamide/0.1% N-lauroylsarcosine (50 °C, 20 min, twice), RNase A buffer (room temperature, 10 min). RNase A buffer containing 20 μg/mL RNase A (37 °C, 30 min), 2 × SSC/0.1% N-lauroylsarcosine (room temperature and 50 °C, 15 min each), 1 × SSC/0.1% N-lauroylsarcosine (room temperature, 5 min), 0.5 × SSC/0.1% N-lauroylsarcosine (room temperature, 5 min) and 0.2 × SSC/0.1% N-lauroylsarcosine (room temperature and 50 °C, 20 min each). Hybridization signals were visualized by alkaline phosphatase immunohistochemistry with the digoxigenin detection kit (Roche Diagnostics). In the series of parasagittal or frontal sections of the whole hemisphere, every 12th section was processed with the antisense probe, and every 48th (parasagittal) or 24th (frontal) section was processed with the sense probe. The series of horizontal sections at about 2 mm and 8 mm intervals were stained with the antisense probe and the sense probe, respectively. In control sections hybridized with the sense probes, the neocortex gave no staining above background. The results obtained from both species of macaques were indistinguishable. The signal intensity of in situ hybridization was quantified with NIH image software by taking optical density measurements of the digitized image. Optical density readings were taken from at least 20 areas (each approximately 1100–6500 μm² in size) around the centers of perturbed and nonperturbed columns, which were identified by matching them to columns showing reduced and normal staining in adjacent CO-stained sections, in layer III of IVCB of area 17 in a section. Background readings were taken from the almost unstained regions just suprageniculate to the white matter because the white matter itself exhibited pale staining by endogenous alkaline phosphatase activity. These were subtracted and the optical densities were averaged. Data from three sections of each monocularly deprived monkey were then averaged. The significance of difference was calculated using the two-tailed Student's t-test.

Double immunohistochemical staining

For dual labeling, two antiserum–antibody combinations were used: rabbit anti-OCCI (1:100) and mouse anti-CaMKIIα (Roche Diagnostics; 1:400), rabbit anti-OCCI1 and mouse anti-α-amino- butyric acid (GABA; GB-69, Sigma, St. Louis, MO, USA; 1:400). Selected 30 μm-thick sections of normal monkey visual cortex were preincubated in 0.25% Triton X-100 in phosphate buffered saline (PBS) at room temperature for 2 h before being placed in the blocking buffer (1% blocking reagent from Roche Diagnostics, 5% normal goat serum and 0.1% Triton X-100 in PBS) at room temperature for at least 2 h. The sections were then transferred to the blocking buffer containing each primary antiserum–antibody combination. After 36–48 h at 4 °C, the sections were washed and incubated in a mixture of Alexa 488 conjugated goat anti-rabbit IgG (Molecular Probe, Eugene, OR, USA; 1:100) and Alexa 594 conjugated goat anti-mouse IgG (Molecular probe; 1:100) in PBS containing 5% normal goat serum and 0.1% Triton X-100. For controls, the same procedures were performed without primary antiserum or antibody. The control sections showed no fluorescent staining.

All the experiments described here were performed in compliance with the guidelines for animal experiments at Okazaki National Research Institute.

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**Fig. 2.** Identification of occl as a macaque homolog of a secreted protein. (a) Northern blotting with an occl probe displayed a band at approximately 5.7 kb. (b) Western blot analysis with anti-OCC1 antisera of lysates and conditioned medium of COS-7 cells transfected with OCC1 expression vector. Untransfected COS-7 cells were used as a control. L, cell lysates; m, conditioned medium. The molecular weight of the major product detected in the medium of OCC1-expressing COS-7 cells (arrow; approximately 43 kDa) is larger than that in the cell lysates (arrowhead; approximately 36 kDa) indicating that the product is secreted after being modified.

**Results**

**Identification of occ1**

Total RNAs from five anatomically distinct regions of the adult cynomolgus monkey neocortex (areas F4A, FA, TE, OA and OC according to the classification of von Bonin & Bailey (1947) (see Fig. 4) were converted to cDNA with anchor oligo-dT primers, and the mRNA expression in these regions was compared by DD. Among the bands of the PCR-amplified fragments, we identified a 190 base pair (bp) cDNA fragment that showed the highest transcription level in area OC (Fig. 1a). RT-PCR analysis using a primer set corresponding to the both ends of the clone confirmed this characteristic regional expression pattern (Fig. 1b). The cDNA sequence of the clone obtained from DD shared no homology with any DNA sequences available on the GenBank database. Because the clone was likely to represent a 3'-noncoding region adjacent to the poly (A) + tail, we isolated a full-length cDNA from a cDNA library from area OC of cynomolgus monkey neocortex. Northern blotting showed that the full-length occl mRNA is about 5.7 kb long (Fig. 2a). The first screen of a cDNA library with the DD cDNA fragment resulted in the isolation of a cDNA clone including a 1024-bp sequence upstream from the poly (A) + tail. We obtained a full-length clone after another screening of a cDNA library using the elongated clone as a probe. The entire sequence of the clone consisted of 5688 nucleotides, and a putative open reading frame of 308 amino acids (calculated molecular mass of 34,999 kDa) was found upstream from the nucleotide sequence for the DD clone. We named the gene occl. Homology search revealed that this gene encodes the macaque homolog of human, rat and mouse follistatin-related protein (FRP)/TSC-36, which was originally isolated from the cells of a mouse osteoblastic cell line treated with transforming growth factor β1 (Shibanuma et al., 1993), and contains a cysteine-rich follistatin motif, three putative N-glycosylation sites and several phosphorylation sites (Zwijnsen et al., 1994; Patel et al., 1996; Ohashi et al., 1997; Tanaka et al., 1998; Okabayashi et al., 1999). The follistatin motif is shared by various proteins, such as the activin inhibitor follistatin, agrin, the protein that induces the aggregation of nicotinic acetylcholine receptors, the multifunctional extracellular glycoprotein SPARC/osteonectin/BNM-40, the rat brain protein SCl and the quail retinospecific protein QR1 (Johnston et al., 1986; Guernah et al., 1991; Pathy & Nikolides, 1993; Maudet et al., 1995; Phillips & de Kreiter, 1998; Motamed, 1999). Compatible with the prediction that OCC1 has N-terminal signal peptides (Zwijnsen et al., 1994), Western blot analysis of cell lysates and the supernatant of conditioned medium of COS-7 cells transfected with OCC1 expression vector showed that OCC1 was released into the medium with post-translational modifications (Fig. 2b).

**Neocortical distribution of the cells specified by occ1**

In the monkey neocortex, most occl-positive signals in *in situ* hybridization preparations were observed in neurons. This was judged from the following observations: (i) little signal was observed in the white matter; (ii) the cells stained by the probe had large somata in general (7–12 μm in diameter in layers III and IV of area 17); and (iii) occl mRNA was occasionally observed to be localized in the processes of some pyramidal cells, resulting in contoured cell shapes (Fig. 3). In *in situ* hybridization histochemistry of the serial sections of the entire cerebral hemisphere demonstrated that the distribution of occl-positive neurons was as expected on the basis of the results of

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DD and RT-PCR experiments in which samples were taken from the cytoarchitectonic areas shown in Fig. 4. The strongest and densest hybridization signals were observed in area 17. The other occipital areas showed moderate expression of occl (Fig. 5a and e). Neurons positive for occl were distributed with relatively weak labeling throughout the temporal and the posterior half of the parietal cortex (Fig. 5a, c and d). In these cortices, certain areas could be identified by densities and patterns of staining. The primary somatosensory cortex (area 3b) showed some dense expression in layer IV and the deeper stratum of layer III (Figs 5c and d, and 6a). The primary auditory cortex (AI) also exhibited relatively dense expression in layer IV and the deeper half of layer III (Figs 5d and 6c). The intensity of labeling and the frequency of the signals were even lower in the areas anterior to the central sulcus than in the postcentral regions. In the precentral regions, no obvious cortical areas could be identified by the occl expression pattern (Fig. 5a and b).

Laminar distribution of occl-positive neurons in the visual cortex and visual thalamus

Examination of neurons positive for occl mRNA in areas 17 and 18 revealed distinctive laminar distributions of neurons expressing occl in these regions. In area 17 (Fig. 7a), signal was most densely distributed and cells were most intensely labeled in layers IVa/VIc and IVCb. Layers II, III and IVA exhibited many intensely labeled neurons. In the intervening layer IVB, a lower number of moderately labeled neurons were present. The distribution of occl-positive neurons divided layer V into a superficially stained superlayer and a deeper, even more lightly stained sublayer. In layer VI and the deeper half of layer V, sparse moderately stained neurons were found.

Fig. 4. The five neocortical regions (areas FDA, FA, TE, OA and OC, colored) from which tissues were dissected and used as samples for differential display and RT-PCR experiments. ari, inferior ramus of arcuate sulcus; ars, superior ramus of arcuate sulcus; cc, central sulcus; itp, intraparietal sulcus; lat, lateral sulcus; lu, lunate sulcus; ocl, inferior occipital sulcus; oct, occipito-temporal sulcus; pm, principal sulcus; ti, inferior temporal sulcus; ts, superior temporal sulcus.

The positive neurons in layer I were lightly stained and sparsely present. The laminar distribution of occl-positive neurons in area 18 (Fig. 7c) was different from that in area 17. Many intensely labeled neurons with large somata were found in the lower one-third of layer III. In the upper two-thirds of layer III and layer IV, many lightly

Fig. 5. Neocortical distribution of the neurons marked by occl-mRNA expression. Parasagittal (a) and frontal (b-e) whole-brain sections processed by in situ hybridization. In (a), rostral is to the left, and the arrows indicate the borders between areas 17 and 18. In (b) to (e), medial is to the right. Arrows in (c) indicate the borders of area 3b. Arrowheads and arrows in (d) indicate the borders of area 3b and A1, respectively. (f) Lateral view of macaque neocortex, rostral is to the left. The lines indicate the coronal planes sliced for the sections (b-e). The signals were localized preferentially in the posterior region of the neocortex, in particular, area 17. Abbreviations are as used in Fig. 4. cc, sulcus of corpus callosum. Scale bar, 5 mm.
labeled cells and some moderately labeled cells were observed. Layer II exhibited many lightly labeled neurons. In layers V and VI, a few relatively lightly labeled cells were present. Weak signal was sparsely distributed in layer I.

We further examined the occ1 expression in LGN to ask whether occ1 has a specific pattern of laminar distribution in the thalamic nucleus in which visual system channels are segregated into distinct layers, and found that relatively weak signal was present selectively in cells of the magnocellular layers (layers I and 2; Fig. 8a, b and d).

The subset of neurons specified by occ1 includes functionally and morphologically distinct types

To determine whether the neurons marked by the expression of occ1 can be classified into a single functional and morphological class of cells, we performed dual labeling immunohistochemistry on the neurons in area 17 with the antisera to OCC1 and a monoclonal antibody against either CaMKII α or GABA. CaMKII α immunoreactivity is found only in a population of glutamatergic, excitatory cells in the cerebral cortex (Benson et al., 1991, 1992; Liu & Jones, 1996), which includes the spiny stellate cells in layer IV and pyramidal cells in the other layers of area 17 (Hendry & Kennedy, 1986; Tighilet et al., 1998). By contrast, GABAergic neurons of the primate neocortex belong to a class of inhibitory and aspiny nonpyramidal neurons (House et al., 1983; Jones et al., 1994). No GABA-immunoreactive neurons showed CaMKII α immunoreactivity in area 17 (Tighilet et al., 1998).

The OCC1 immunoreactivity is diffusely present within the cell bodies, especially around the cell nuclei, and seen in a punctate pattern along the neuronal processes (Fig. 9 left panels). These observations are consistent with the possibility that the product of occ1 can be released from neurons as well as from the transfected COS-7 cells. The great majority of neurons expressing CaMKII α show OCC1 immunoreactivity (Fig. 9a and b). By contrast, OCC1 immunoreactivity was found in not all, but in a large population of GABAergic neurons (Fig. 9c). Each type of neuron, double immunoreactive for OCC1 and CaMKII α and double immunoreactive for OCC1 and GABA, were observed in all layers of area 17 except for layer I in which virtually all neurons are GABA-positive.

![Fig. 6. Area 3b and AI can be identified by densities and patterns of occ1 expression. (a-d) Sections through area 3b (a; the region indicated by arrows in Fig. 5e at higher magnification) and AI (c; the region indicated by arrows in Fig. 5d at higher magnification), stained for occ1 (a and c) and with thionin (b and d). Area 3b and AI show relatively dense expression of occ1 in layer III and IV. Scale bar, 500 μm.]

![Fig. 7. Laminar distribution of occ1-positive neurons in areas 17 and 18. (a-d) Sections through area 17 (a and b) and area 18 (c and d), stained for occ1 (a and c) and with thionin (b and d). In areas 17 and 18, the strongest signals are found in layer IVC and in the deeper stratum of layer III, respectively. Scale bar, 200 μm.]

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Fig. 8. Distribution of occ1-expressing neurons in the LGN. (a and c) Frontal sections through LGN, stained for occ1 (a) and with thionin (c). (b) Drawing of the positive signals (dots) in (a). (d) The boxed area in (a) is magnified. (e) The boxed area in (c) is magnified. In LGN, occ1 mRNA is expressed selectively in the cells of the magnocellular layers (layers 1 and 2). Scale bar, 500 μm (a-c) and 200 μm (d and e).

Fig. 9. Neuronal subset revealed by occ1 includes two different classes of neurons. (a-c) Sections were double labelled with antiserum against OCC1 (left panels) and antibody against either CaMKII α (a and b; middle panels) or GABA (c; middle panel), and imaged to determine colocalization (right panels. OCC1 in green, CaMKII α or GABA in red). (a) From layer II; (b and c) from layer IVCb of area 17 double stained with OCC1/CaMKII and OCC1/GABA, respectively. Pial surface is to the top. Arrowheads indicate the representative cells double-positive for each antiserum–antibody combination. Note that owing to the difference of the subcellular localization pattern of OCC1 from that of GABA in (c), the confocal images to show colocalization of these markers had to be acquired in a slice plane in which optimal staining of OCC1 might not be yielded; this resulted in the difference of OCC1 staining patterns in layer IVCb in the two images (b and c; left panels). Scale bar, 20 μm.
Hendry et al., 1987). These observations show that the neuronal subset revealed by occ1 expression includes physiologically and morphologically different classes of neurons in area 17.

**occ1 is expressed by the neurons in area 17 in an activity-dependent manner**

The neurons marked by occ1 expression were preferentially distributed in area 17. This posed the question of whether visual experience plays a role in the regulation of occ1 expression in the adult brain. Area 17 of the Old World primates is divided into alternating occular dominance columns (Hubel & Wiesel, 1972; Wiesel et al., 1974). We examined the alterations in neuronal phenotypes based on changes in neuronal activity in the brains of monocularly deprived animals using immunohistochemical techniques (Hendry & Jones, 1986; Hendry & Kennedy, 1986; Hendry and Jones, 1988).

Following monocular deprivation by TTX injection into the vitreous cavity (7, 10, 14 or 21 days), dramatic changes were detected in the in situ hybridization pattern of occ1 in area 17. Alternating lightly and darkly stained stripes appeared (Fig. 10a, c, e, and g), irrespective of the length of deprivation. Comparison with the adjacent sections stained for CO (Wong-Riley, 1979; Horton & Hubel, 1981), which exhibited the same pattern of alternating dark and light stripes, showed that occ1 transcription was reduced in the deprived columns. The change was observed in layers III, IVa, IVb, IVCα, IVCβ and V, being most apparent in layers III and IVCβ (Fig. 10a). The signal intensity measurements confirmed this observation that the average level of mRNA in the perturbed columns were 57% lower in layer III (range, 45-68%; P < 0.0005), and 74% lower in layer IVCβ (66-82%; P < 0.0001) than those in the nonperturbed columns.

In the tangential sections cut through layer III of area 17 of TTX-injected animals, the occ1 signal was seen along the line of blobs in the deprived columns, and faint patchy stainings coinciding with the CO periodicities were observed in the perturbed columns (Fig. 10e-f, arrows in e and f indicate the patchy CO and corresponding occ1 stainings in the perturbed columns). The occ1 expression in the deprived columns was beyond the confines of the under deprived columns to form an almost continuous signal, and the extent of the expression in the deprived columns was even less than that of the shrunken blobs observed in the CO sections. In the sections cut through layer IVCβ, the occular dominance columns observed in the sections stained for occ1 were even more obvious than those seen in the CO sections (Fig. 10g and h). These observations also suggest that the occ1 expression in layers III and IVC was significantly decreased by the blockade of visual input. From these results, we conclude that occ1 mRNA is transcribed in an activity-dependent manner in area 17. An obvious change was not observed in area 18.

**Discussion**

We have screened for genes that are transcribed differentially among structurally and functionally distinct areas of macaque neocortex and identified occ1. Neurons positive for occ1 were selectively distributed in the posterior region of the neocortex, especially in area 17.

**Occ1 expression and neocortical neuronal organization**

It has been considered that the neocortex is basically homogeneous on the basis of the number and density (Rockel et al., 1980), and morphology (Fairen et al., 1984) of its neuronal components. The ratios between pyramidal and nonpyramidal neurons are very similar in the motor, somatosensory and visual cortices (Sloper, 1973; Tombol, 1974; Sloper et al., 1978). In studies of the chemical properties of neurons, an extensive quantitative survey of the distribution of a major neurotransmitter, GABA, demonstrated that its distribution varies little among neocortical areas (Hendry et al., 1987). However, in these neuron counting studies, there was an exception; in area 17 the number of neurons through the full depth of the cortex is more than twice than in the other areas (Rockel et al., 1980) and the proportion of GABAergic neurons to total neurons is lower than in the other areas (Hendry et al., 1987). Therefore, the distribution of occ1 mRNA, preferentially observed in the posterior region of neocortex with regional variations, is a dramatic example of the heterogeneity of neocortical neurons, which coincides with the functional subdivisions, and the high occ1 transcription in area 17 reveals the unique neuronal organization of area 17 in the primate neocortex. Furthermore, as the results shown in Fig. 10 demonstrate, occ1 mRNA levels can be downregulated remarkably by blockade of afferent activity in area 17. High occ1 expression is not only spatially coincident with area 17, but also subject to regulation dependent on neuronal activity in area 17. These results show the important features of the expression of occ1 that can be used as a marker for neurons in area 17 as well as a good indicator of neural activities in the particular laminae of area 17.

The further characterization of the neurons specified by occ1 in area 17 showed that they include morphologically and functionally distinct classes of neurons, spiny excitatory cells and aspiny inhibitory cells. This result suggests the other unique aspect of occ1 expression in neocortical neurons that occ1 is expressed not in a single type of neurons but in various types of neurons in a region-selective manner.

**occ1 expression in neocortex and thalamocortical connectivity**

The present results show that the transcription pattern of occ1 relates to the cytoarchitectonic area of the neocortex. At present, we cannot explain how such a difference in gene expression among neocortical neurons is generated during brain development. In regard to the neocortical region where occ1 shows its high transcription, however, we can find some correlation between the laminar distribution of occ1 expressing neurons and the pattern of thalamic afferent innervation.

We found strong occ1 transcription in layers II, III, IVA and IVC of area 17. The major inputs to area 17 in primates and some other mammals are from the (dorsal) LGN and the minor inputs are from the nuclei of the pulvinar complex. Thalamic inputs from LGN in primates terminate mainly in layer IVC and, to a lesser degree, in layer IVA of area 17 (Hubel & Wiesel, 1972; Hendrickson et al., 1978; Blasdel & Lund, 1983). The pulvinar axons and those from intercalated layers of the LGN provide thalamocortical innervation to the cells in layer I and the blobs of layers II and III of area 17 (Fitzpatrick et al., 1983; Hendry & Yoshioka, 1994). In area 18, we observed strong signals in the deep stratum of layer III. The pulvinar afferent terminations in area 18 are dense in the deep levels of layer III and layer IV (Cuccolo & Harting, 1978; Livingstone & Hubel, 1982). In addition to these thalamic recipient regions, occ1 also shows high transcription in the regions on which thalamic inputs have strong influences in indirect ways. occ1 is transcribed in the interblob regions of layer II and III of area 17 where no apparent termination of direct thalamic input has been observed (Livingstone & Hubel, 1982; Li et al., 1984). We also found moderate and light signals in layer IVC and the upper stratum of layer V, respectively. Neither layer IV B nor V receives direct geniculate inputs. On the other hand, the interblob regions of layers II and III receive indirect inputs from the LGN through layers IVA and IVC. Neurons in layer IVC receive strong inputs from layer IVCα. The upper subdivision of layer V

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Fig. 10. Change in the transcription of occl in adult area 17 following monocular deprivation. Sections are stained for occl (left panels) and CO (right panels). (a and b) A coronal section through area 17 of a monkey monocularly deprived for 14 days. The change was detected in layers III, IVA, IVB, IVCo, IVCB, and V. (c-f) Tangential sections through layer III. The boxed areas in (c and d) are magnified in (e and f), respectively. (g and h) Tangential sections through layer IVCB. By comparing the positions of the same blood vessel profiles in these sections (arrowheads), the remarkable reduction of occl mRNA was found in the perturbed columns. Arrows in (e and f) indicate the representative patchy stainings in the perturbed columns in each section. Scale bars, 500 μm.

makes prominent connections to the thalamic recipient layers IVA, IVcα, IVcβ, and VI (Lund, 1988; Callaway, 1998).

**occ1 expression and a functional subdivision of the visual thalamus**

We observed that the occ1 expression in LGN is more frequent in the magnocellular layers in which the cells with broad band spectral qualities are selectively localized (Wiesel & Hubel, 1966) than in the other layers. occ1 is also preferentially expressed in a functional subdivision of a thalamic nucleus. Some markers, for example Cat-301 and SMI-32, are known to primarily label the magnocellular layers ( Hendry et al., 1988b; Chaudhuri et al., 1996). occ1 can also be used as a marker to visualize a subset of neurons in those layers of the LGN and contribute to the classification of neurons in the LGN.

**The function of occ1 in cortical plasticity**

The data presented here clearly indicate that occ1 mRNA is preferentially expressed in posterior regions of the neocortex, especially in area 17, in an activity-dependent manner. Activity-dependent gene expression, resulting in changes in neuronal phenotypes, plays an important role in neuronal plasticity of brain function and development (Marty et al., 1997; Lee & Sheng, 2000). Many molecules are expressed in an activity-dependent manner, but none of them shows such a characteristic regulatory expression pattern in the neocortex as that of occ1. Although the function of OCC1 (FRP, TSC-56) remains unknown to date, it has been suggested that the follistatin motifs of follistatin, agrin, and SC1 might play similar functions in the differentiation of the nervous system by accumulating, protecting and modulating the activity of growth factors (Pathy & Nikolaus, 1993). This suggestion also presents the possibility that OCC1 could function by binding other molecule(s) and affecting its (their) activity(ies). The results of the expression study with COS-7 cells suggest that OCC1 might function in a secreted form. These together imply that OCC1 can work to mediate activity-dependent interactions between specific subsets of neurons through modulating the functions of other proteins in response to changes in neuronal activity in particular neuronal circuits. Further studies on the nature of OCC1 and to elucidate the role of the molecule in neuronal function will shed new light on the functional structure of the cerebral neocortex.

A few molecular markers for specific neuronal subtypes in the rodent brain have been identified and characterized, such as the limbic system-associated membrane protein (LAMP) and latentin (Levitt, 1984; Zacco et al., 1990; Arimatsu et al., 1992; Arimatsu, 1994). LAMP is a glycoprotein that is expressed in subsets of neurons in the adult rat brain that are associated with classic limbic structures (Reinso et al., 1996). Latexin-immunoreactive neurons are confined essentially to the infragranular layers of lateral cortical areas in the rat neocortex (Arimatsu et al., 1996). Among these markers, the distribution of LAMP in the primate brain has been examined, and it has been shown that LAMP can also be effective in visualizing neuronal subsets in the primate limbic system (Cote et al., 1996). This result suggests that the characterization of occ1-positive neurons in the brains of rodents and other mammals could show the similarity and the difference of the neuronal organization between the primate and other mammalian brains. This kind of knowledge will contribute to understanding the nature of neurons expressing occ1 in future studies. A systematic survey for marker molecules in the primate neocortex described here presents a successful example of such an approach. Histochemical analyses using new molecular markers combined with various other techniques could become powerful tools in deciphering the functional organization of the neocortex.

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**Abbreviations**

CaMKII α, the α-subunit of type II calcium/calmodulin-dependent protein kinase; bp, base pairs; CO, cytochrome oxidase; DD, differential display; FRP, follistatin-related protein; LGN, lateral geniculate nucleus; PBS, phosphate-buffered saline.

**References**


Neurons expressing occl in macaque neocortex


