Cell Type- and Region-specific Expression of Neurogranin mRNA in the Cerebral Cortex of the Macaque Monkey

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Neurogranin is a postsynaptic substrate for protein kinase C (PKC). It has been identified in the central nervous system, and the expression has been related to postsynaptic plasticity. Using non-radioactive *in situ* hybridization histochemistry, we investigated whether mRNA expression of neurogranin varied among the cerebral region and cell types. In most areas of the neocortex excluding area OC (the primary visual area), intense signals were observed in the pyramidal cells in layers III, V and VI. In area OC, intense signals were observed in layers IV as well as layers III and VI. We previously showed that intense signals for GAP-43, a presynaptic PKC substrate, were observed in relay neurons of the lateral geniculate nucleus. From this result and the present result in area OC, we conclude that both pre- and postsynaptic PKC substrates (GAP-43 and neurogranin) are abundant in the geniculocortical synapses. In the hippocampus, intense signals were observed in the pyramidal cells in the subiculum. Taken together with our previous study showing intense signals for GAP-43 in Ammon's horn, the result indicates that both PKC substrates are abundant in the connections between neurons in Ammon's horn and in the subiculum.

Keywords: GAP-43, plasticity, primate, protein kinase C, RC3

Introduction

Protein kinase C (PKC) and PKC-associated signal transduction play an important role in synaptic plasticity (for reviews, see Nishizuka, 1986, 1995). Our previous studies investigated the gene expression of GAP-43 and MARCKS (myristoylated alanine-rich C-kinase substrate), which are major PKC-substrates, in the monkey central nervous system (Higo *et al.*, 1998, 1999, 2002d, 2003; Oishi *et al.*, 1998). GAP-43 (also known as B50, F1, neuromodulin, pp46, P-57 and GAP-48; for reviews, see Benowitz and Routtenberg, 1987, 1997) is known to accumulate in presynaptic axon terminals (Nelson and Routtenberg, 1985; Meiri *et al.*, 1986, 1988; Skene *et al.*, 1986). MARCKS has been localized to presynaptic axon terminals and small dendrites (Aderem *et al.*, 1988; Ouimet *et al.*, 1990; Swierczynski and Blackshear, 1995). There are several lines of evidence that relate the expression of both GAP-43 and MARCKS mRNAs with structural synaptic reorganization in the mature nervous system (Van der Zee *et al.*, 1989; Tetzlaff *et al.*, 1991; Linda *et al.*, 1992; Levin and Dunn-Meynell, 1993; Chong *et al.*, 1994; Aigner *et al.*, 1995; Holtmaat *et al.*, 1995; Bendotti *et al.*, 1997; McNamara *et al.*, 2000; McNamara and Lenox, 2000). We previously reported that mRNAs of GAP-43 and MARCKS were highly expressed in specific regions of the adult monkey brain, such as the association areas of the cerebral neocortex and Ammon's horn of the hippocampus (Higo *et al.*, 1998, 1999, 2002d; Oishi *et al.*, 1998). Furthermore, we previously reported that GAP-43 and MARCKS mRNAs were expressed in the monkey lateral geniculate nucleus (LGN) in an activity-dependent manner (Higo *et al.*, 2000, 2002a). These results have enhanced our understanding of the molecular basis of plasticity in each region of the monkey brain.

In the present study, we focused on the gene expression of neurogranin (also known as RC3, canarigranin, BICKS and p17; for a review, see Gerendasy and Sutcliffe, 1997), another major neuron-specific PKC substrate, which has been localized to postsynaptic dendrites (Represa *et al.*, 1990; Watson *et al.*, 1992). Among the PKC substrates, GAP-43 and neurogranin are the most closely related molecules having identical PKC phosphorylation sites and calmodulin-binding domains (Watson *et al.*, 1990; Baudier *et al.*, 1991; for reviews, see Gerendasy and Sutcliffe, 1997; Chakravarthy *et al.*, 1999). Both GAP-43 and neurogranin bind calmodulin when they are not phosphorylated by PKC and release calmodulin as calcium-calmodulin in response to Ca^{2+} influx. Thus, GAP-43 and neurogranin are presynaptic and postsynaptic counterparts of calmodulin storage proteins. Like GAP-43, neurogranin and its mRNA are expressed at high levels in the developing rat brain. While the expressions decrease in most regions of the mature rat brain, high levels of expression remain in specific regions such as the hippocampus and cerebral neocortex (Represa *et al.*, 1990; Watson *et al.*, 1990). Although the precise role of neurogranin in the mature nervous system is not yet clear, there are several lines of evidence that relate the expression level with plasticity and memory function in the adult nervous system. Phosphorylation of neurogranin is enhanced during long-term potentiation and depression (Klann *et al.*, 1993; Pasinelli *et al.*, 1995; Ramakers *et al.*, 1995, 2000a,b; Chen *et al.*, 1997) and reduced expression has been observed in Alzheimer's disease (Chang *et al.*, 1997; Davidsson and Blennow, 1998). Further, neurogranin knockout mice have been shown to have impaired spatial learning (Pak *et al.*, 2000). Thus, the region-specific neurogranin expression in the adult central nervous system might reflect functional specialization which relates to postsynaptic plasticity in each region. In the present study, we performed a non-radioactive *in situ* hybridization histochemical analysis of the cerebral cortex of the macaque monkey to determine the localization and type of cells expressing neurogranin mRNA. We then quantified the expression levels of neurogranin mRNA in each area of the cerebral neocortex and each subregion of the hippocampal formation. Taken together with our previous studies investigating the expression of GAP-43 mRNA (Higo *et al.*, 1998, 1999; Oishi *et al.*, 1998), we suggest that both pre- and postsynaptic PKC substrates (GAP-43 and neurogranin) are abundant in several specific connections of the monkey brain.

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The preliminary results of these studies have been reported elsewhere (Higo *et al.*, 2002b,c).

Materials and Methods

Animals and Tissue Preparation

Brain tissue was obtained from 11 macaque monkeys (six *Macaca fuscata* and five *M. mulatta*) aged 2 or more years. Monkeys were purchased from a local provider, or were bred in the Primate Research Institute, Kyoto University. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals established by the Institute of Laboratory Animal Resources and the Guide for the Care and Use of Laboratory Primates established by the Primate Research Institute, Kyoto University. To investigate the effect of visual deprivation on the expression of neurogranin mRNA, five monkeys received tetrodotoxin (TTX, 15 μg in 10 μl of normal saline; Sigma, St Louis, MO), injected intravitreously into the right eye every fifth day for a total of 5 (n = 2), 10 (n = 2), and 30 (n = 1) days before they were killed. The monkeys were anesthetized with ketamine hydrochloride (10 mg/kg, i.m.) and pentobarbital sodium (Nembutal, 20 mg/kg, i.v.), prior to TTX administration. The pupillary light reflex of these monocularly deprived monkeys remained suppressed throughout the deprivation period.

The animals were pretreated with i.m. injections of ketamine hydrochloride (10 mg/kg) and deeply anesthetized by intravenous administrations of pentobarbital sodium (Nembutal, 35 mg/kg). The animals were then perfused through the ascending aorta with 0.5 l of ice-cold saline containing 2 ml (2000 U) of heparin sodium, followed by 2–5 l of ice-cold fixative containing 2% paraformaldehyde (PFA) and 0.5% glutaraldehyde in 0.15 M phosphate buffer (pH 7.4) or 2% PFA, 0.5% glutaraldehyde and 0.2% picric acid in phosphate buffer. During perfusion, the heads were chilled on crushed ice. After perfusion, the brains were immediately removed and blocked in the coronal plane (5 mm thick). The blocks were postfixed in 4% PFA and 5% sucrose and immersed in 30% sucrose for cryoprotection. The brain blocks were mounted in O.C.T. compound (Miles Inc., Elkhart, IN), rapidly frozen in a dry ice-acetone bath and stored at –80°C until dissection.

In Situ *Hybridization*

Brain segments from seven areas of the neocortex and the hippocampal formation were sectioned coronally to a thickness of 16 μm on a cryostat. We identified individual neocortical areas, including the prefrontal area (FD), the temporal association area (TE), the parietal association area (PG), the primary motor area (FA), the primary auditory area (TC), the primay somatosensory area (PB), and the primary visual area (OC), using the cytoarchitectonic criteria of von Bonin and Bailey (1947).

The procedure for *in situ* hybridization was performed as described in our previous report (Higo *et al.*, 2003). Briefly, the sections on the slides were fixed in 4% PFA, treated with 30 μg/ml proteinase K (Roche Diagnostics, Germany) and dehydrated through a graded series of ethanols. Sections were prehybridized in 50% formamide, 600 mM NaCl, 1× Denhardt's solution, 0.25% SDS, 10 mM Tris–HCl (pH 7.6), 1 mM EDTA and 200 μg/ml tRNA for 3 h at 50°C. Following prehybridization, sections were transferred to hybridization buffer containing an additional 10% dextran sulfate and 1 μg/ml digoxigenin-labeled RNA probe, which was made from human RC3/neurogranin cDNA (pHRC3, 1.3 kb; a gift from Dr J.B. Watson, University of Calfornia, Los Angeles, CA). Hybridization was performed for at least 16 h at 50°C. The hybridized sections were washed with SSC and then treated with RNase A (30 μg/ml; Roche Diagnostics). Sections were then incubated for 1 h in diluted (1:500) anti-digoxigenin-alkaline phosphatase conjugate (DIG Nucleic Acid Detection Kit; Roche Diagnostics). Finally, the sections were incubated in color-developing buffer containing NBT and BCIP for 5 h.

The specificity of the probes was confirmed by Northern blot analysis, in which a specific 1.3 kb RNA transcript was observed (Fig. 1*A*). Intense signals for neurogranin mRNA were observed in all neocortical areas examined and weak signals were seen in the hippocampus. No signal was detected in the cerebellum. The lack of expression in the cerebellum is consistent with our previous report using a histochemical technique (Higo *et al.*, 2003). In addition, control sections were hybridized by the method described above, using the sense probe for each mRNA. These control sections showed no specific signals (Fig. 1*C*).

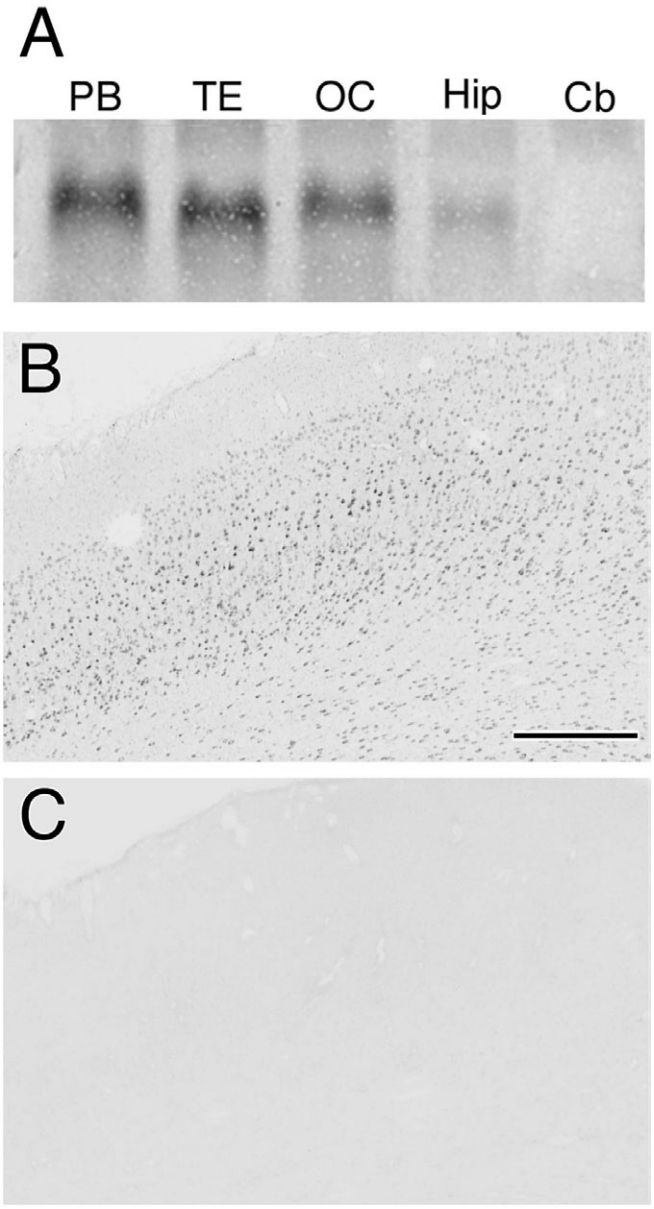

Figure 1. (*A*) Northern hybridization study to confirm probe specificity. The probe was hybridized to specific RNA transcripts (1.3 kb). Intense signals were observed in all neocortical areas examined (areas PB, TE and OC) and weak signals were observed in the hippocampus. No signal was detected in the cerebellum. (*B, C*) Control experiments to confirm the specificity of the signals for neurogranin mRNA. The adjacent coronal sections including cortical area TE are shown. Normal reaction for neurogranin mRNA (*B*) produced positive hybridization signals. No signal was observed in the section hybridized with a sense probe (*C*). Scale bar = 500 μm.

Quantification and Image Analysis

We quantified the expression patterns in the neocortex and the hippocampus using the same method that we used for the quantification of GAP-43 and MARCKS mRNAs (Higo *et al.*, 1999, 2002d). Briefly, optical density (OD) was measured in each layer of a 300-μm-wide column that sampled all layers of the neocortex or each hippocampal subregion. The OD of the background staining in each section

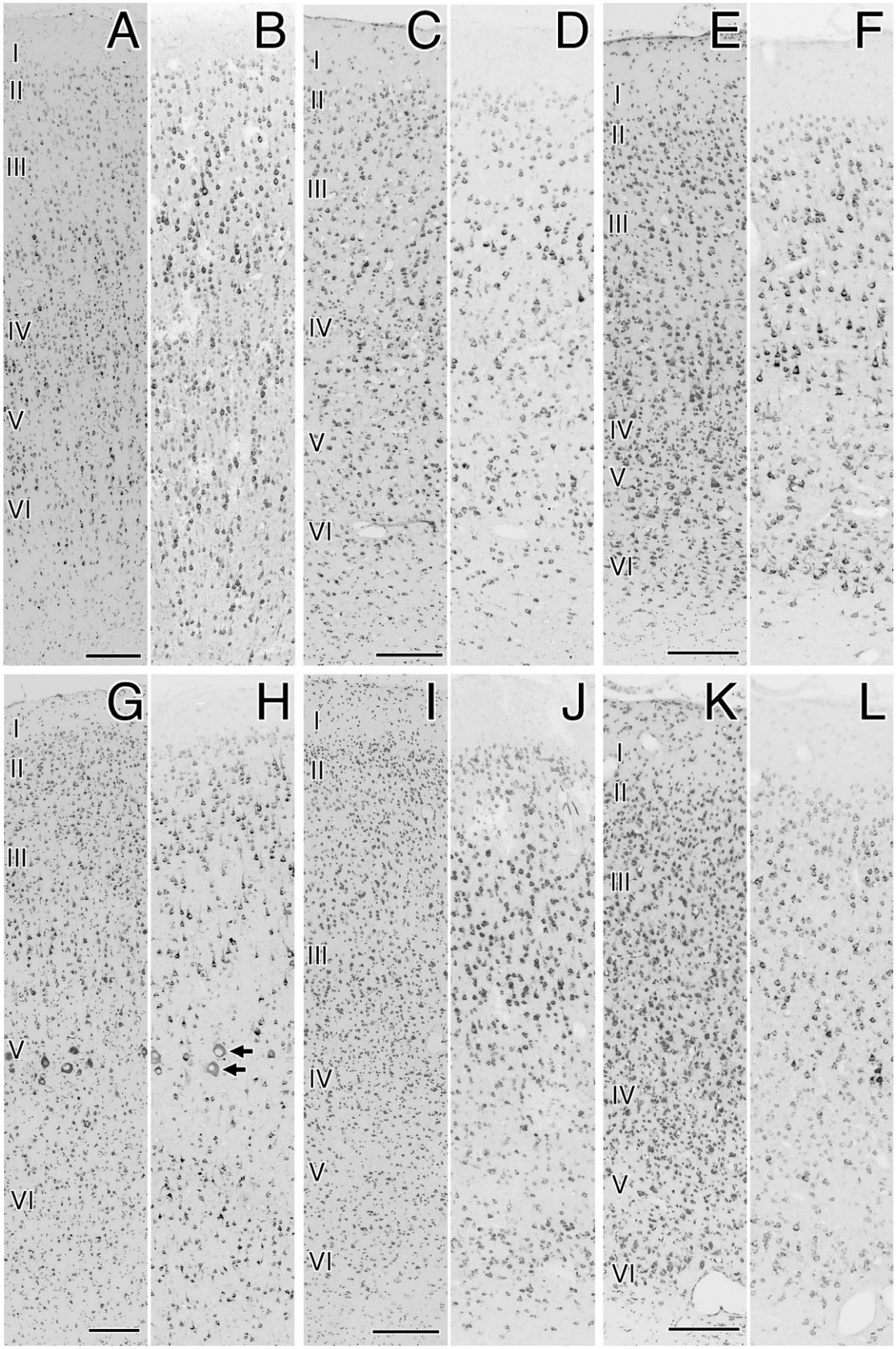

Figure 2. Areas FD (*A*, *B*), TE (*C*, *D*), PG (*E*, *F*), FA (*G*, *H*), TC (*I*, *J*) and PB (*K*, *L*) of the cortex. (*A*, *C*, *E*, *G*, *I*, *K*) Nissl-stained sections. (*B*, *D*, *F*, *H*, *J*, *L*) Localization of neurogranin mRNA. In areas FD, TE, PG, FA, TC and PB, intense hybridization signals for neurogranin mRNA were observed in layers III, V and VI. No signal was observed in layer I. Arrows in *H* indicate the large pyramidal cells (Betz cells) in layer V of area FA, in which the signals were weaker than in neighboring smaller pyramidal cells in layer V and in pyramidal cells in layers III and VI. Scale bar = 200 μm.

was measured in the subjacent white matter. Six columns from each cortical area and each hippocampal subregion (three columns from each of two sections) were measured for each monkey. We calculated the normalized OD of each layer or each subregion from *in situ* hybridized sections (*in situ* OD_{signal} – *in situ* $OD_{background}$). We also calculated the normalized OD from Nissl-stained sections (Nissl OD_{signal} – Nissl $OD_{background}$), which represent the cell density in each region. To compensate for differences of cell density among layers or subregions, we determined the ratio as follows (Figs 4, 5*E* and 8):

$$\frac{\text{Neurogranin OD}}{\text{Nissl OD}} = \frac{(\textit{in situ}\ \text{OD}_{signal} - \textit{in situ}\ \text{OD}_{background})}{(\text{Nissl OD}_{signal} - \text{Nissl OD}_{background})}$$

We also represented the relative expression levels of mRNAs among subregions of the hippocampal formation by dividing the OD in each pixel of the digitized images of *in situ* hybridized sections by that of the adjacent Nissl-stained sections (Fig. 7), as described previously (Higo *et al.*, 1998, 2002a). The OD was measured in each pixel of the digitized *in situ* hybridized (*in situ* pOD_{signal}) and Nissl-stained sections (Nissl pOD_{signal}). The $OD_{background}$ was estimated as the average of the ODs from five randomly sampled local regions of the white matter.

The OD of each pixel in the *in situ* hybridized sections was normalized by the following formula:

$$pOD_{normalized} = \frac{(\textit{in situ}\ pOD_{signal} - \textit{in situ}\ OD_{background})}{(\text{Nissl}\ pOD_{signal} - \text{Nissl}\ OD_{background})}$$

Results

Neocortex

The laminar expression patterns of neurogranin mRNA were similar among areas FD, TE, PG, FA, TC and PB (Fig. 2*B*,*D*,*F*,*H*,*J*,*L*). Prominent hybridization signals were observed in the cell body and proximal dendrites of pyramidal cells in layers III, V, and VI (Figs 2*B*,*D*,*F*,*H*,*J*,*L* and 3*A*). We also observed prominent signals in the horizontal cells (Fig. 3*B*) and fusiform cells in these layers (Fig. 3*C*). Cells with the most intense signals were frequently observed in layer III (Fig. 2*B*,*D*,*F*,*H*,*J*,*L*). While the neurons in layers V and VI contained weaker hybridization signals than those in layers III, the proportion of positive neurons in layers V and VI tended to be

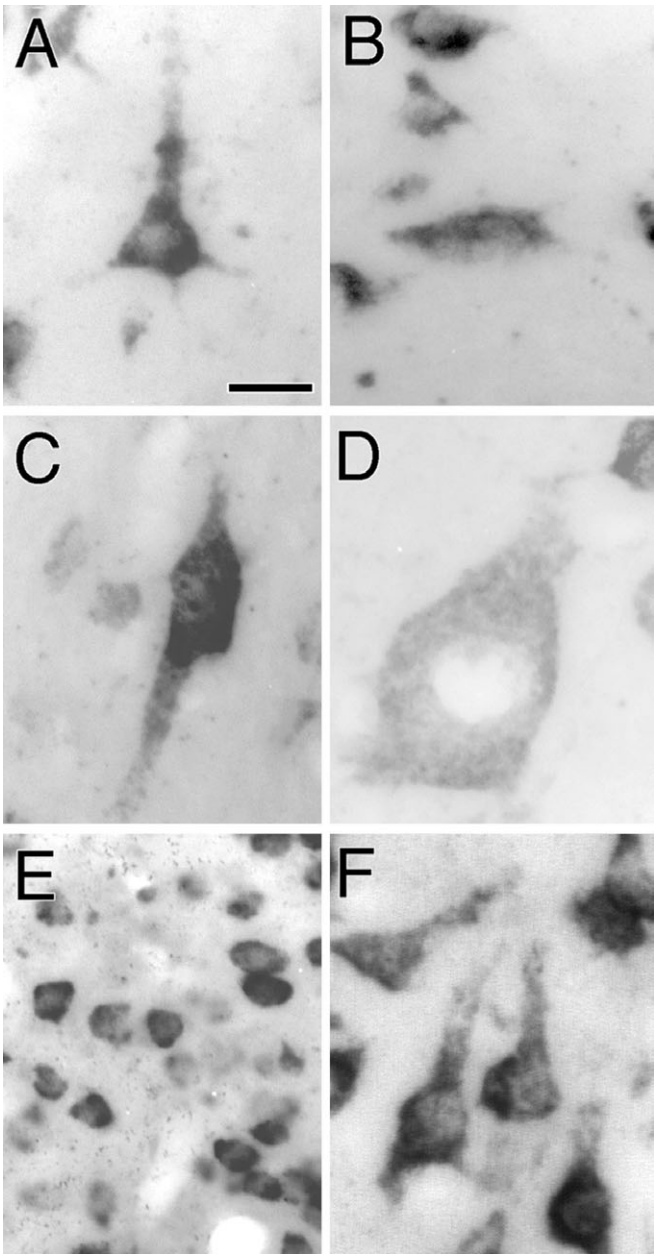

Figure 3. High-magnification photomicrographs of cortical neurons that have hybridization signals for neurogranin mRNA. (*A*) The pyramidal cell in layer III of area TE. (*B*) The horizontal cell in layer VI of area TE. (*C*) The fusiform cell in layer VI of area TE. (*D*) The large pyramidal cell in layer V of area FA. (*E*) The small round cells in layer IV of area OC. (*F*) The pyramidal cell in the subiculum. Scale bar = 20 μm.

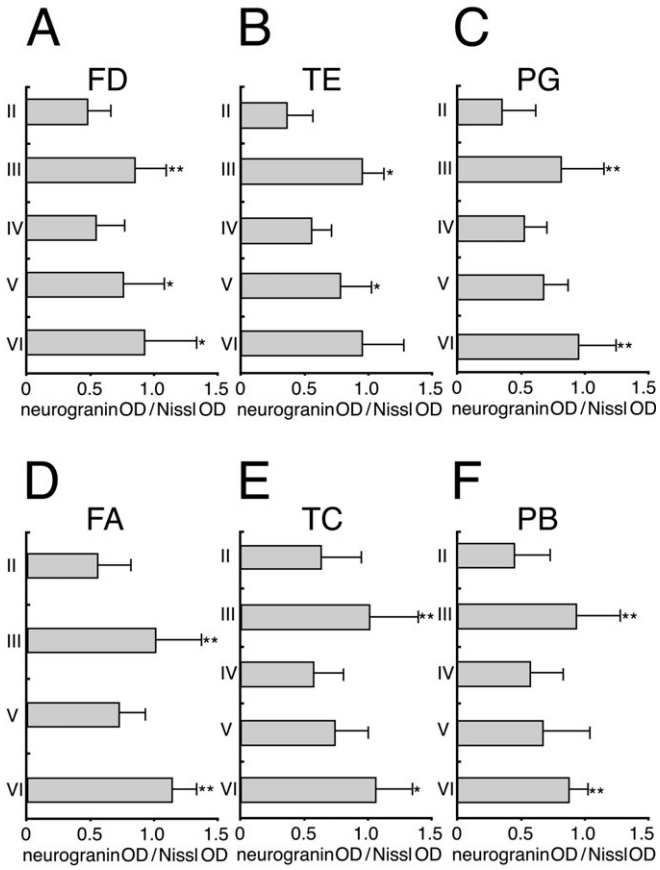

Figure 4. To quantify the hybridization signal, the neurogranin OD/Nissl OD ratios in each of six cortical areas were calculated (see Materials and Methods for details). The ratios were higher in layers III and VI than in the remaining layers. Asterisks indicate significant difference from the lowest value in each area (*$P < 0.02$; **$P < 0.01$, one sample *t*-test, $n = 6$).

larger than that in layer III. Signals were weak in the small round cells in layers II and IV. The large pyramidal cells in layer V of area FA also contained the hybridization signals (arrows in Figs 2*H* and 3*D*), but the signals were weaker than in neighboring smaller pyramidal cells in layer V and pyramidal cells in layers III and VI. No signal was observed in layer I (Fig. 2*B*,*D*,*F*,*H*,*J*,*L*). Quantitative analysis indicated that the hybridization levels (the neurogranin OD/Nissl OD ratios) in these areas were higher in layers III, V and VI than in the remaining layers (Fig. 4*A*–*F*). Although detailed quantitative analysis has not been performed, most of the other neocortical areas excluding area OC showed similar laminar expression patterns to those in areas FD, TE, PG, FA, TC and PB.

In area OC, the laminar expression pattern was different from those in the remaining areas. In contrast to the other neocortical areas, the intense hybridization signals were observed in the small round cells in layer IV as well as in the pyramidal cells in layers III and VI (Figs 3*E* and 5*B*). Signals were weak in layers II and V. Figure 5*C*,*D* shows the results in the border region between area OC and area OB (the secondary visual area). Intense signals were observed in layer IV of area OC, but not in layer IV of area OB. Quantitative analysis indicated that the hybridization level (the neurogranin OD/Nissl OD ratio) was higher in layers IV and VI than in the remaining layers (Fig. 5*E*). In layer IV, the ratio was higher in layers IVB and IVCα, which receive inputs from magnocellular neurons in the LGN (Lund *et al.*, 1994), than in layers IVA and IVCβ, which receive inputs from parvocellular neurons. As in the other cortical areas, no signal was observed in layer I of area OC (Fig. 5*B*).

To investigate the effect of visual input on the expression of neurogranin mRNA in area OC, we performed *in situ* hybridization histochemistry in monkeys that had been deprived of monocular visual input via intraocular injections of TTX. After monocular deprivation for 5, 10, or 30 days, we observed a periodic pattern of staining for cytochrome oxidase in layer IV of area OC (Fig. 6*B*), indicating reduced neuronal activity of the ocular dominance columns that received visual input from the TTX-injected eye. However, we did not detect such a periodic ocular dominance pattern of staining for neurogranin mRNA after monocular deprivation for each period (Fig. 6*C*). Unequal distribution of neurogranin mRNA in layer IV correlated with the cell density revealed by Nissl-stained section (Fig. 6*A*). The hybridization pattern for neurogranin mRNA was

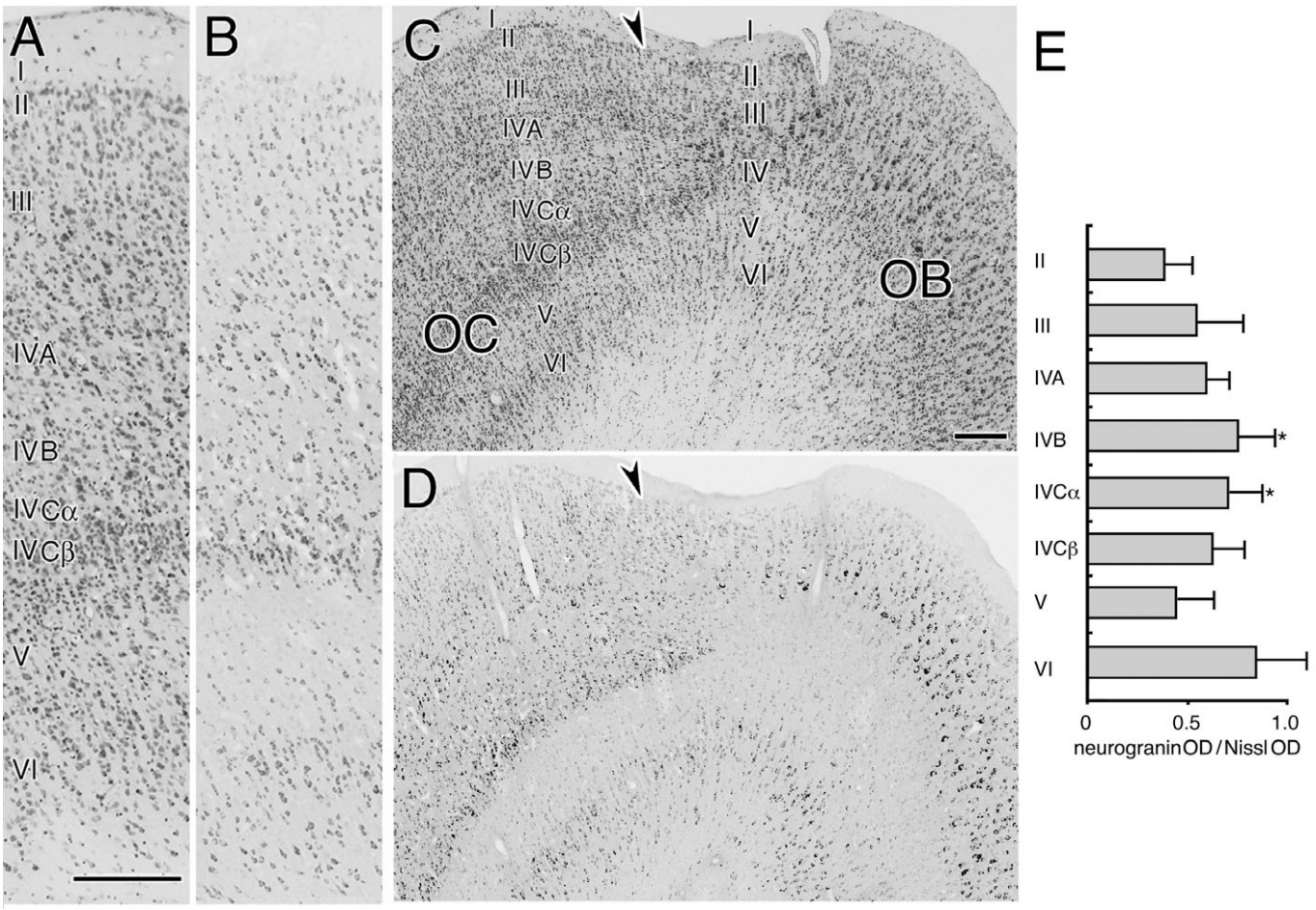

Figure 5. (*A*, *B*) Nissl-stained (*A*) and *in situ* hybridized sections (*B*) of area OC. In the primary visual area, intense signals for neurogranin mRNA were observed in the small round cells in layer IV as well as in the pyramidal cells in layers III and VI. No signal was observed in layer I. Scale bar = 200 μm. (*C*, *D*) Nissl-stained (*C*) and *in situ* hybridized sections (*D*) of the border region between area OC and area OB (the secondary visual area). Arrowheads indicate the border. Intense signals were observed in layer IV of area OC, but not in layer IV of area OB. Scale bar = 500 μm. (*E*) The neurogranin OD/Nissl OD ratio in area OC. The ratio was higher in layers IV and VI than in the remaining layers. *Significantly different from the lowest value (layer II; $P < 0.02$, one sample *t*-test, $n = 6$).

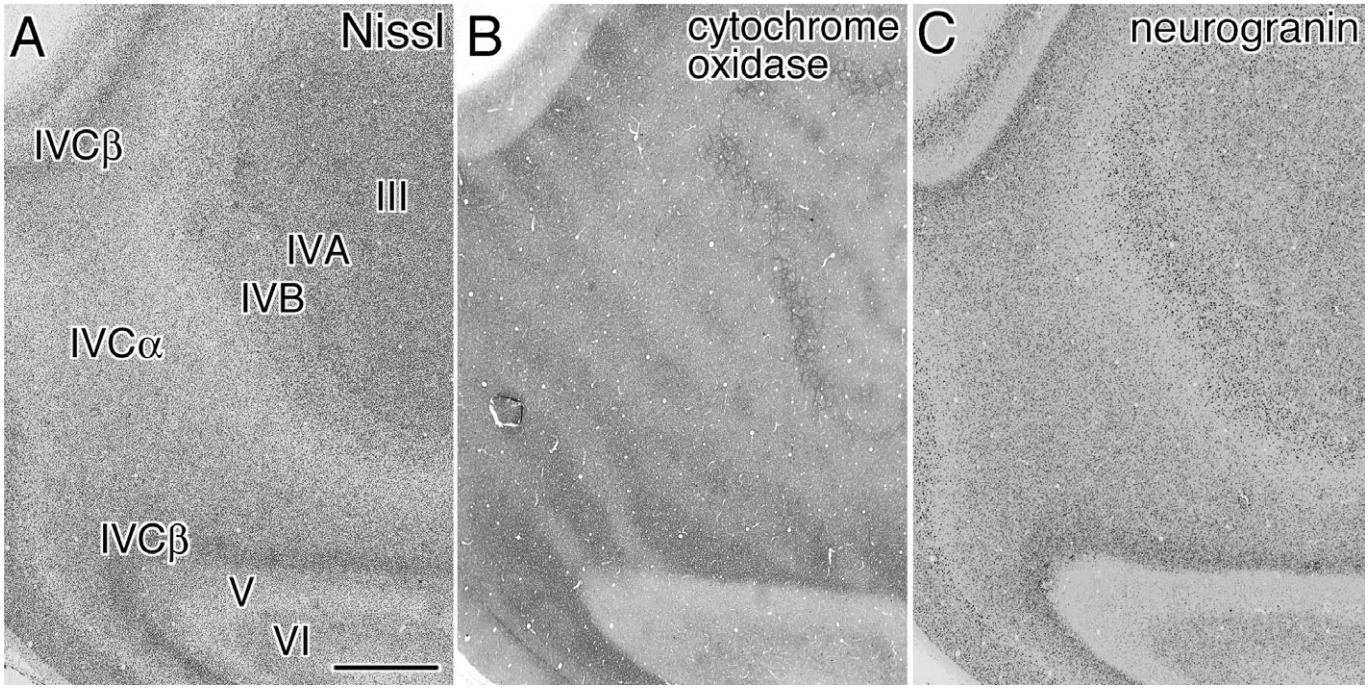

Figure 6. Surface parallel sections through area OC of a monkey that had been monocularly deprived for 30 days. (*A*) Nissl-stained section. (*B*) Cytochrome oxidase-stained section. (*C*) Localization of neurogranin mRNA. No deprivation effect was observed for neurogranin mRNA. Scale bar = 1 mm.

identical to those for normal monkeys. These results indicate that the expression of neurogranin mRNA in area OC is not affected after monocular deprivation for these periods.

Hippocampal Formation and Surrounding Regions

In the entorhinal cortex and perirhinal cortex (areas 35 and 36), the hybridization signals were weaker than in the neighboring neocortical areas (areas TE and TA; Fig. 7*B*,*C*). In the amygdala, intense hybridization signals for neurogranin mRNA were observed in specific nuclei, such as the basal and lateral nuclei (arrowheads and double arrowheads in Fig. 7*B*,*C*). Intense signals were also observed in the claustrum (Fig. 7*B*,*C*).

In the hippocampal formation, the most intense hybridization signals were observed in the subiculum (Fig. 7*E*,*F*; see Fig. 3*F*). In the parahippocampal cortex, the signals were weaker than in the neighboring neocortical area (TEO) and the prominent signals were restricted to the deeper layers. Intense signals were observed in the striatum such as the caudate nucleus, but not in the thalamus such as the LGN (Fig. 7*E*,*F*). Quantitative analysis in the principal layers of the hippocampus indicated that the neurogranin OD/Nissl OD ratio was highest in the subiculum, higher in the CA3 and CA1 subfields of Ammon's horn and weak in the dentate gyrus and CA4 subfield of Ammon's horn (Fig. 8).

Discussion

Comparison with other Animals

At least two previous studies reported the expression of neurogranin and its mRNA in the mature rat brain (Represa *et al.*, 1990; Watson *et al.*, 1990). In general, our results in the monkey brain are consistent with the results in the rat brain in that the prominent mRNA expression for neurogranin was observed in the cerebral neocortex, hippocampus and striatum, but not in the thalamus and the cerebellum (Represa *et al.*, 1990; Watson *et al.*, 1990).

In the rat neocortex, the most intense hybridization signals for neurogranin mRNA were observed in large neurons in layers III, V and VI (Watson *et al.*, 1990). The previous results in the rat neocortex are similar to the present result in most cortical areas of the monkey brain (areas FD, TE, PG, FA, TC and PB). However, the expression of neurogranin mRNA in layer IV of the visual area (area OC), which was observed in the monkey neocortex, has not been reported in the rat neocortex (Watson *et al.*, 1990). The specific laminar distribution of neurogranin mRNA in the visual area may be a characteristic of animals that have highly developed vision, such as primates. In the human, the distribution of neurons expressing neurogranin mRNA has been studied in only a few neocortical areas (temporal and frontal cortex; Chang *et al.*, 1997). The previous results in these areas of the human neocortex showing prominent hybridization signals in the pyramidal cells throughout layers II–VI are similar to our result in the monkey neocortex. A further experiment in the visual cortex is necessary to determine whether the specific laminar distribution of neurogranin mRNA is also present in the human primary visual area (area OC).

In the rat hippocampal formation, prominent signals were observed in the pyramidal cell layer of all regions. Among the hippocampal subregions, the CA3 subfield of Ammon's horn has shown the most intense hybridization signals (Watson *et al.*, 1990). Previous findings in the rat hippocampus differ from those of the present study in the monkey hippocampus, which showed the highest expression in the subiculum. Moreover, Northern and Western blot analyses in the rat have shown that the expression levels of both neurogranin mRNA and neurogranin protein were almost similar between the cerebral neocortex and hippocampus (Represa *et al.*, 1990; Watson *et*

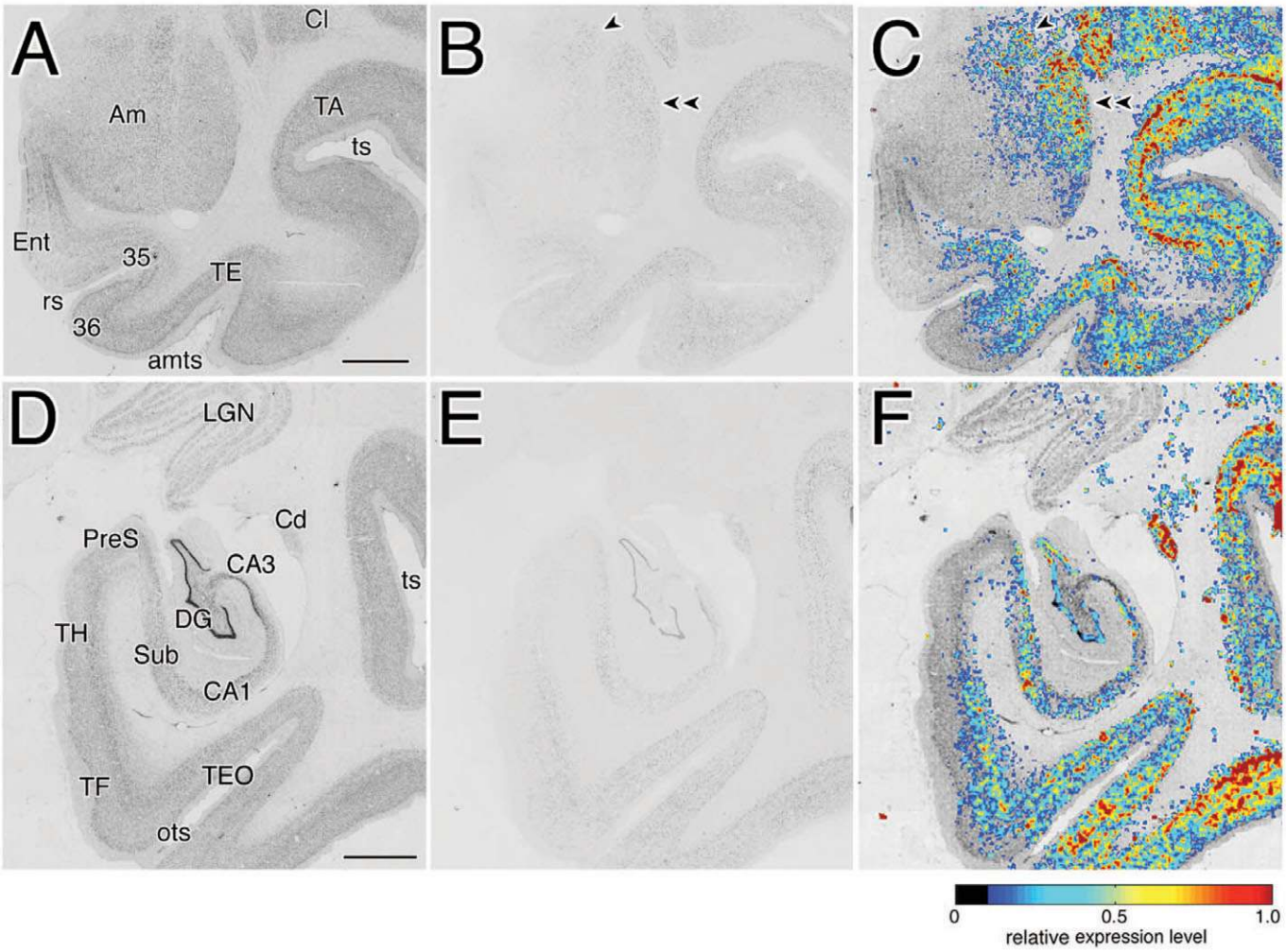

Figure 7. Photographs of Nissl-stained (*A*, *D*) and *in situ* hybridized (*B*, *E*) sections for the detection of neurogranin mRNA. The relative expression levels of neurogranin mRNA in each section of the cerebral cortex are also shown (*C*, *F*). The relative expression levels (0.1–1) of neurogranin mRNA were superimposed in pseudocolor on the digitized images of Nissl-stained sections (see Materials and Methods for details). (*A–C*) Sections including the amygdala, the entorhinal cortex, areas 35 and 36 (the perirhinal cortex), TE, TA and claustrum. The arrowheads and double arrowheads in *B* and *C* indicate hybridization signals in the basal and lateral nuclei of the amygdala, respectively. (*D–F*) Sections including the dentate gyrus, Ammon's horn, subiculum, presubiculum, TF and TH (the parahippocampal cortex), TEO, TA, the caudate nucleus and the lateral geniculate nucleus. Am, amygdala; Cd, caudate nucleus; Cl, claustrum; DG, dentate gyrus; Ent, entorhinal cortex; LGN, lateral geniculate nucleus; PreS, presubiculum; Sub subiculum; amts, anterior middle temporal sulcus; ots, occipitotemporal sulcus; rs, rhinal sulcus; ts, temporal sulcus. Scale bar = 2 mm.

al., 1990). The results in the rat are inconsistent with the present result from both the Northern blot analysis and *in situ* hybridization histochemistry in the monkey brain, which showed more intense hybridization signals in the cerebral neocortex than in the hippocampus. We previously reported that the expression patterns of GAP-43 and MARCKS, which are other PKC substrates, in the monkey hippocampus (Higo *et al.*, 1998, 2002d) were also different from those in the rat hippocampus (Kruger *et al.*, 1992; Yao *et al.*, 1993; McNamara and Lenox, 1997). The phylogenetically different expression of these PKC-substrates may be related to the differential hippocampal function between these species.

Comparison with PKC and Type II Calcium/Calmodulin-dependent Protein Kinase

PKC consists of at least 11 isoforms. Previous histochemical studies of the monkey cerebral neocortex reported the region-specific distributions of several isoforms of PKC (Tominaga *et al.*, 1993; Fukuda *et al.*, 1994). Among them, the expression of PKCγ is similar to the expression of neurogranin. PKCγ immunoreactivity is observed in the dendrites of pyramidal cells in layers II, III and VI of the primary motor area (area FA; Tominaga *et al.*, 1993). In the primary visual area (area OC), PKCγ immunoreactivity is observed in layers II, IV and VI (Fukuda *et al.*, 1994). A previous knockout mouse study showed that stimulation of PKC with a phorbol ester increased neurogranin phosphorylation in wild type mice but failed to affect the phosphorylation in mice lacking PKCγ (Ramakers *et al.*, 1999). Thus, phosphorylation of neurogranin in cortical neurons may be accomplished by PKCγ.

Neurogranin is involved in the activity regulation of type II calcium/calmodulin-dependent protein kinase (CAMKII; Pak *et al.*, 2000). CAMKII consists of four isoforms (α, β, γ, δ) encoded by separate genes. Among them, the expression of CAMKIIα in the monkey cerebral neocortex is similar to the expression of neurogranin mRNA. CAMKIIα immunoreactivity is observed in the dendrites of pyramidal cells in layers II–VI of the monkey sensory-motor cortex (areas FA and PB; Jones *et al.*, 1994). In

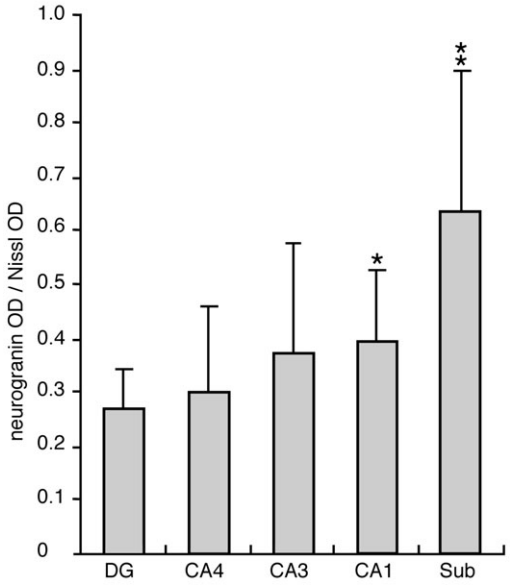

Figure 8. The neurogranin OD/Nissl OD ratio in the hippocampal principal layers. Asterisks indicate significant difference from the lowest value (DG; $*P < 0.02$; $**P < 0.01$, one sample *t*-test, $n = 6$). Abbreviations as in Figure 7.

the primary visual area (area OC), CAMKIIα immunoreactivity is observed in layer IV nonpyramidal cells as well as in pyramidal cells (Tighilet *et al.*, 1998). Thus, neurogranin may regulate CAMKIIα activation in monkey cortical neurons. When monocular visual input is deprived for several (7–16) days by TTX injection, CAMKIIα mRNA expression is increased in deprived ocular dominance columns (Tighilet *et al.*, 1998). This previous result is in contrast to our present result showing no deprivation effect on the expression of neurogranin mRNA. The expression level of CAMKIIα mRNA and neurogranin mRNA may be regulated by different mechanisms.

The Expression of PKC Substrates in the Specific Connection of the Monkey Brain

In previous studies, we investigated the gene expression of GAP-43, another PKC substrate that accumulates only in presynaptic axon terminals (Nelson and Routtenberg, 1985; Meiri *et al.*, 1986, 1988; Skene *et al.*, 1986). From the subcellular distribution of neurogranin and GAP-43 proteins and our findings summarized in Table 1, we suggest that both postsynaptic PKC substrate (neurogranin) and presynaptic PKC substrate (GAP-43) are abundant in some specific connections (Fig. 9). (i) Intense hybridization signals for GAP-43 mRNA were observed in pyramidal cells in superficial layers as well as deep layers of the association areas (Higo *et al.*, 1999). The present result showed high levels of expression of neurogranin mRNA in layers III, V and VI of the neocortical areas. Thus, GAP-43 may be abundant in the presynaptic membrane and neurogranin may be abundant in the postsynaptic membrane of the corticocortical connections that originate from the association areas of the neocortex and terminate outside layer IV, which are the presumed feedback connections (Felleman and Van Essen, 1991). (ii) We previously showed that intense hybridization signals for GAP-43 were observed in excitatory relay neurons in the LGN (Higo *et al.*, 2000), indicating that GAP-43 protein is localized in the geniculocortical axon terminals.

Table 1
Summary of neurogranin and GAP-43 mRNA expression in the monkey cerebral neocortex and hippocampus

	Neurogranin	GAP-43
Cerebral neocortex		
Areas FD, TE and PG		
Layer I	–	+
Layer II	+	++
Layer III	+++	+++
Layer IV	+	++
Layer V	++	+++
Layer VI	+++	+++
Areas FA, TC and PB		
Layer I	–	+
Layer II	+	+
Layer III	+++	+
Layer IV	+	++
Layer V	++	+++
Layer VI	+++	+++
Area OC		
Layer I	–	+
Layer II	+	+
Layer III	++	+
Layer IV	+++	++
Layer V	+	+++
Layer VI	+++	+++
Hippocampus		
Dentate gyrus	+	++
CA4	+	++
CA3	++	+++
CA1	++	++
Subiculum	+++	+
Presubiculum	+	+

The relative levels of neurogranin and GAP-43 mRNA expression were classified as follows: +++, intense; ++, moderate; +, weak; –, no signal. The expression pattern of neurogranin mRNA is different between area OC and the remaining areas of the cerebral neocortex. The expression pattern of GAP-43 mRNA is different between the association areas (FD, TE and PG) and the primary motor and sensory areas (FA, TC, PB and OC). The relative expression of GAP-43 mRNA was taken from our previous reports (Higo *et al.*, 1998, 1999; Oishi *et al.*, 1998).

Taken together with the present result in area OC showing intense hybridization signals for neurogranin mRNA in thalamic recipient neurons in layer IV, we concluded that both pre- and postsynaptic PKC substrates are abundant in the geniculocortical connections. (iii) In the hippocampus, intense signals for GAP-43 mRNA were observed in the pyramidal cell layer of Ammon's horn (Higo *et al.*, 1998). In the present study, we observed intense hybridization signals for neurogranin mRNA in the subicular pyramidal cells. These results indicate that both PKC substrates are abundant in the connections between Ammon's horn and the subiculum.

There is accumulating evidence indicating that the formation of new synapses is the structural basis of long-term potentiation and memory function (Chang and Greenough, 1984; Buchs and Muller, 1996; Kleim *et al.*, 1996; Toni *et al.*, 1999).

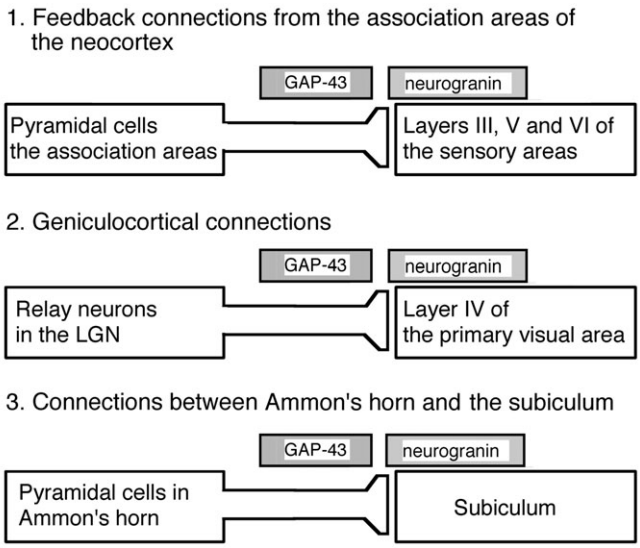

Figure 9. Schematic diagram showing the expression of neurogranin and GAP-43 mRNAs in the neurons subserving the specific connections. The expression of GAP-43 mRNA was taken from our previous report (Higo *et al.*, 1998, 1999, 2000). Taken together with the present results about the expression of neurogranin mRNA, we suggest that both postsynaptic PKC substrate (neurogranin) and presynaptic PKC substrate (GAP-43) are abundant in the connections shown above.

GAP-43 and neurogranin are thought to be involved in structural changes accompanying the induction of long-term potentiation and depression (Ramakers *et al.*, 1995, 2000a,b). Although further understanding of the intracellular roles of neurogranin and GAP-43 is a prerequisite for an appropriate interpretation, we suggest that connections containing both presynaptic GAP-43 and postsynaptic neurogranin may be capable of forming new synapses in the mature cerebral cortex, because new synapse formation should involve both pre- and postsynaptic morphological changes.

Notes

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