We performed non-radioactive in situ hybridization histochemistry in several areas that include both the association areas and the lower sensory areas of monkey cerebral cortex, and investigated the localization of neurons expressing two growth-associated proteins: GAP-43 and SCG10. Both GAP-43 and SCG10 mRNAs were observed in both pyramidal and non-pyramidal neurons. Prominent hybridization signals for GAP-43 mRNA were observed in layers II–VI of the adult association areas: the prefrontal areas (FD), the temporal (TE) and the parietal (PG) association areas. The signals for GAP-43 mRNA were weak in layers I–III of the adult primary somatosensory area (PB) and primary (OC) and secondary (OB) visual areas, and cells with prominent signals were observed in layers IV–VI. The prominent signals for SCG10 mRNA were observed in layers IV–VI of all areas examined. These results suggest that the expression pattern of GAP-43 mRNA, but not that of SCG10 mRNA, may be related to the functional difference between association and lower sensory areas of adult cortex. In the infant cortex (postnatal days 2, 8 and 31), the signals for both mRNAs were intense in layers II–VI of all areas. Therefore, layer-specific expressions of GAP-43 and SCG10 mRNAs are established after the first postnatal month.

Differentiation between association areas and primary areas is a prominent feature of the primate cerebral cortex. The growth-associated protein GAP-43 is a protein that characterizes the association areas. It participates in signal transduction during axonal elongation (Akers and Routtenberg, 1985; Alexander et al., 1987; Stritmatter et al., 1990; for review see Benowitz and Routtenberg, 1997). The present results provide insight into the neuronal components that have high levels of plasticity in the association areas.

We also performed an in situ hybridization study of another growth-associated protein, SCG10. Although SCG10 (Stein et al., 1988a) and GAP-43 (Basi et al., 1987; Karnaš et al., 1987; Rosenthal et al., 1987) have dissimilar amino acid sequences, the subcellular localization of SCG10 is quite similar to that of GAP-43 (Stein et al., 1988a). SCG10 is thought to participate in axonal elongation (Stein et al., 1988b; McNeill et al., 1992; Hannan et al., 1996) by regulating the instability of microtubules (Riedler et al., 1997). Therefore, we compared the expression patterns of SCG10 with those of GAP-43.

Furthermore, we investigated the expression of these growth-associated proteins in the cerebral cortex of infant monkeys. Both GAP-43 mRNA (Neve and Bear, 1989; Mower and Rosen, 1993; Sugiuira and Mori, 1995; Kanazir et al., 1996; Oishi et al., 1998) and SCG10 mRNA (Anderson and Axel, 1985; Stein et al., 1988a; Sugiuira and Mori, 1995; Higo et al., 1996) are highly expressed in the infant nervous system. There are no data available, however, on the localization of GAP-43 and SCG10 mRNAs in the cerebral cortex of infant primate. Therefore, we compared the localization of GAP-43 and SCG10 mRNAs between mature and infant monkey cerebral cortices to determine whether the region-specific expression of these growth-associated proteins is established postnatally. Preliminary results have been reported elsewhere (Higo et al., 1998b,c).

Materials and Methods

Animals and Tissue Preparation

Brain tissue was obtained from seven Japanese monkeys (Macaca fuscata): four adults and three infants (postnatal days 2, 8 and 31). Adult monkeys were purchased from a local provider. Infant monkeys were bred as follows: 2-day-olds in the Tokyo Metropolitan Institute for Neuroscience, 8-day-olds in Juntendo University School of Medicine and 31-day-olds in the Primate Research Institute of 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.

The animals were pretreated with an i.m. injection of ketamine hydrochloride (10 mg/kg) and deeply anesthetized by i.v. administration of pentobarbital sodium (Nembutal; 30 mg/kg). They were then perfused through the ascending aorta with 0.5 l of ice-cold saline containing 2 ml (2000 units) of heparin sodium, followed by 1–2 l of ice-cold fixative (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 0.15 M phosphate buffer). During perfusion, the heads were chilled with
crushed ice. After perfusion, the brains were immediately removed and blocked in the coronal plane (5 mm thick). Then they were immersed in a post-fixative solution containing 4% PFA and 5% sucrose in 0.15 M phosphate buffer for several hours, followed by sucrose in 10, 20 and 50% sucrose in 0.15 M phosphate buffer. The brain blocks were mounted in O.C.T. compound (Miles Inc., Elkhart, IN) and frozen rapidly in a dry ice/acetone bath, then stored at –80°C until dissection.

Synthesis of Probes

For the detection of GAP-43 and SCG10 mRNAs, digoxigenin labeled DNA probes were used. Human cDNA clones for GAP-43 (pGR20, 500 bp; a gift from Dr R.L. Neve of Harvard Medical School, Boston) and SCG10 (pSG601, 550 bp; a gift from Dr N. Mori of National Institute for Longevity Sciences, Japan) were labeled with digoxigenin using a random priming method according to the manufacturer’s instructions (DIG-DNA Labeling Kit, Boehringer Mannheim Biochemica, Germany). Before use, the labeled probes were precipitated with ethanol, then washed to remove unincorporated digoxigenin labeled nucleotides.

Pretreatment of Sections

The blocks including cortical areas prefrontal area (FD), the temporal association area (TE), the parietal association area (PG), the primary somatosensory area (PB) and primary (OC) and secondary (OB) visual areas (Fig. 1) of von Bonin and Bailey (1947) were sectioned at a 10 µm thickness on a cryostat (CRYOCUT 3000, Leica, Nussloch, Germany). Because of the condition of the preservation and the need to distribute the preparations among other researchers, we could not investigate all cortical areas of every animal. Three adult and two infant (postnatal days 2, 8 and 30) cortices were used for TE; and four cortical areas of every animal. Three adult and three infant (postnatal days 2, 8 and 30) cortices were used for FD, PG and PB, three adult and three infant (postnatal days 2, 8 and 30) cortices were used for TE, and four adult and three infant (postnatal days 2, 8 and 30) cortices were used for OB and OC.

The sections were mounted on slides coated with Vectabond Reagent (VECTOR Laboratories, Burlingame, CA), dried and pretreated for in situ hybridization by successive incubations in: 4% PFA in 0.15 M phosphate buffer for 15 min at room temperature; 30 µg/ml Proteinase K (Boehringer Mannheim Biochemica, pH 8.0) for 30 min at 37°C; and 4% PFA in 0.15 M phosphate buffer for 10 min at room temperature. After a wash of 0.15 M phosphate buffer, the sections were dehydrated through 70, 80, 90 and 100% ethanol series (1 min at each concentration), then dried.

Hybridization and Detection

Sections were prehybridized in 45% formamide, 4% SSC (standard saline citrate), 500 µg/ml denaturated salmon sperm DNA for 3 h at 45°C. Following prehybridization, sections were transferred to fresh hybridization buffer containing an additional 10% dextran sulfate and 200 ng/ml digoxigenin-labeled DNA probe, then covered with a cover glass, heated on a dry block at 95°C for 10 min and chilled on ice for 10 min. Hybridization was performed for at least 16 h at 45°C.

The hybridized sections were incubated in 2× SSC, 0.2% SDS to remove the cover glasses, rinsed four times in 2× SSC, 0.2% SDS to remove the hybridization mixture (room temperature), then washed four times in pre-heated 0.5× SSC, 0.2% SDS at 55°C, each time for 15 min.

The buffer was changed to 0.1 M maleic acid, 0.15 M NaCl and 0.2% Tween 20 (pH 7.5) for 10 min at room temperature. Sections were then incubated in 2% blocking reagent (DIG Nucleic Acid Detection Kit, Boehringer Mannheim Biochemica), 0.1 M maleic acid, 0.15 M NaCl (pH 7.5) for 1 h at room temperature, then incubated in diluted (1:500) anti-digoxigenin Fab-fragments conjugated with alkaline phosphatase (DIG Nucleic Acid Detection Kit, Boehringer Mannheim Biochemica), 2% blocking reagent, 0.1 M maleic acid and 0.15 M NaCl (pH 7.5) for 5 h at room temperature. Unbound antibody conjugate was removed by washing three times for 10 min each with 0.1 M maleic acid, 0.15 M NaCl and 0.2% Tween 20 (pH 7.5).

Finally, the sections were preincubated in 0.1 M Tris–HCl buffer (pH 9.5) containing 0.1 M NaCl and 0.05 M MgCl₂ for 5 min, then incubated in the blocking buffer containing the substrates nitroblue tetrazolium (NBT, 340 µg/ml; Boehringer Mannheim Biochemica) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 170 µg/ml; Boehringer Mannheim Biochemica) for 20 h in the dark. Color development was stopped by incubation in 0.1 M Tris–HCl buffer (pH 7.5) containing 0.01 M EDTA for 10 min. The sections were incubated in 4% PFA in 0.15 M phosphate buffer for 10 min at room temperature to prevent fading. Following this, sections were dehydrated through 70, 80, 90 and 100% ethanol series (1 min at each concentration); transferred to xylene for three washes of 5 min each, then covered with a coverslip and Permount histological mounting medium (Fisher Scientific, Fair Lawn, NJ).

Quantification

The relative amount of mRNA in each cortical layer was evaluated by measuring optical density rather than by cell counting because the intensity of the hybridization signals for both GAP-43 mRNA and SCG10 mRNA were highly variable among the neurons.

Images were captured with a microscope (BX60, Olympus, Tokyo, Japan) using a 5CCD color video camera (DXC-950, Sony, Tokyo, Japan) and digitized by an image analysis system (MCID; Imaging Research Inc., St. Catharines, Canada). Using this image analysis system, we overlaid in situ hybridized sections onto the adjacent Nissl (creysl violet)-stained sections, and identified each cortical layer. Optical density was measured in each layer of a 300-µm-wide column that sampled all layers of cortex (Fig. 5), and optical density of the background staining was measured in the subjacent white matter. As presented by Mcberg and Rountenberry (1991) in their study of the rat hippocampus, the optical density of a given cortical layer, expressed as a percentage of the optical density of the background staining, was used to define the staining intensity (SI). Twelve columns from each cortical area (four columns from each of three sections or six columns from each of two sections) were measured for each monkey. SIs from in situ hybridized sections of GAP-43 mRNA and SCG10 mRNA (in situ-SI) were compared with SIs from Nissl-stained sections (Nissl-SI), which represent the cell density in each layer of cortical area (Tables 1 and 2).

To clarify the difference in the expression patterns of each cortical area, we compared the SI of the outer pyramidal layer (layer III) and the inner pyramidal layer (layer V) of each cortical area. We first normalized each in situ-SI with reference to its cellular density (Nissl-SI), then determined the ratio:

\[ \text{II} / \text{V} = \frac{\text{in situ-SI in layer II}}{\text{Nissl-SI in layer II}} / \frac{\text{in situ-SI in layer V}}{\text{Nissl-SI in layer V}} \]

When the expression of GAP-43 mRNA or SCG10 mRNA is higher in layer V than in layer III, this ratio is <1. Because we could not fix the boundary between layers II and III of area OC, we used the SI from layers II–III instead of the SI from layer III in the study of area OC. This ratio was useful for confirming any difference in expression patterns for GAP-43 mRNA or SCG10 mRNA among cortical areas and between adult and infant cortices (Figs 7 and 10).

Results

Control Experiments

The specificity of the probes was confirmed by Northern blot
analysis, in which specific bands for both GAP-43 and SCG10 mRNAs were observed (Higo et al., 1998a). In addition, two kinds of control experiments were carried out in the same way as the previous in situ hybridization study in the monkey hippocampus (Higo et al., 1998a). A series of adjacent sections that included area TE were treated with ribonuclease A before in situ hybridization (Fig. 2C,D). The sections were incubated with ribonuclease A (20 µg/ml) for 30 min at 37°C, then processed through the hybridization as described above. Another series of adjacent sections were used as a competition control (Fig. 2E,F), in which >800-fold unlabeled probe was added to the hybridization buffer along with the digoxigenin-labeled probe (200 ng/ml). The sections that were treated normally with the digoxigenin-labeled probe (Fig. 2A,B) had positive hybridization signals in the cytoplasm and the proximal dendrites of the neuron. Only background levels of signals were observed in the sections pretreated with ribonuclease A (C,D), and signals were dramatically reduced in the sections of the competition control (E,F). Scale bar = 500 µm.

Figure 2. Control experiments to confirm the specificity of the signals from GAP-43 (A,C,E) and SCG10 (B,D,F) mRNAs. Six adjacent coronal sections of cortical area TE were studied. Normal reactions for GAP-43 mRNA (A) and SCG10 mRNA (B) mRNAs produced positive hybridization signals. Only background levels of signals were observed in the sections pretreated with ribonuclease A (C,D), and signals were dramatically reduced in the sections of the competition control (E,F). Scale bar = 500 µm.

Expression Patterns of GAP-43 mRNA in the Adult Cerebral Cortex

In areas FD, TE and PG, many GAP-43 mRNA-positive cells with moderate to intense hybridization signals were observed in layers II–VI (Figs 3B,E,H and 4B). Intense hybridization signals were frequently observed in the larger pyramidal cells in layers III, V and VI (Fig. 5A, 15–30 µm in diameter), but we also observed intense hybridization signals in the smaller pyramidal cells (Fig. 5C, 5–15 µm in diameter) and the non-pyramidal cells in these layers. Some of them resembled the bipolar cells that had vertically oriented dendrites (Fig. 5E; 15–30 µm in length; Jones, 1975; Fairén et al., 1984; Peters, 1984; Lund and Lewis, 1993; Gabbott and Bacon, 1996; Peters and Sethares, 1997). The intensities of the hybridization signal were almost the same in outer pyramidal layer (layer III) and inner pyramidal layer (layer V), and the III_{OD}/V_{OD} ratios were close to 1 (1.03 ± 0.02 in area FD, 0.99 ± 0.04 in area TE and 1.06 ± 0.11 in area PG; Fig. 7A).

In areas PB, OB and OC, the hybridization signals for GAP-43 mRNA were weak in layer II (Fig. 6B,E,H). Though signals were generally weak in layer III, gradients of signal intensity were
Figure 3. The areas FD (A–C), TE (D–F) and PG (G–I) of the adult cortex. (A, D, G) Nissl-stained sections. (B, E, H) Localization of GAP-43 mRNA. (C, F, I) Localization of SCG10 mRNA. To quantify the hybridization signal, the optical density was measured in a 300-µm-wide column sampling all layers of the cortex, and the optical density of the background staining was measured in the subjacent white matter (A–C; see Materials and Methods for detail). Scale bar = 200 µm.
observed (Fig. 6B,E,H): the lower part of layer III contained more intense hybridization signals than the upper part of layer III. The larger pyramidal cells at the lowermost part of layer III often had more intense signals than any cells in layer IV. In layers V and VI, we observed a large number of cells with moderate-to-intense hybridization signals. Intense signals were frequently observed in the larger pyramidal cells. As in the association areas, intense hybridization signals were also observed in the smaller pyramidal cells and the non-pyramidal cells in layers V and VI. The hybridization signals were weaker in layer III than in layer V (Fig. 6B,E,H). In areas PB and OB, there were only slight differences between the in situ-SI of layer III and the in situ-SI of layer V (Table 1). Because the neuronal densities were higher in layer III than in layer V (see Nissl-SI in Table 1), the IIIOD/VOD ratios were <1 (0.92 ± 0.06 in area PB and 0.87 ± 0.06 in area OB; Fig. 7A). The IIIOD/VOD ratio for area OC (0.77 ± 0.07) may have been underestimated because the neurons in layer II tended to contain less intense signals than the neurons in layer III, and we used the SI from layers II–III instead of the SI from layer III in the study of area OC (see Materials and Methods). The IIIOD/VOD ratios were significantly lower in the lower sensory areas (areas PB and OB; we excluded area OC because the IIIOD/VOD ratio for
area OC may have been underestimated) than in the association areas (areas FD, TE and PG: P < 0.01, Mann–Whitney U-test; Fig. 7A). In area OC, intense hybridization signals were often observed in the large cells in layer IVB (Fig. 5G, outer Meynert cell; Lund, 1973; Valverde, 1985; Peters and Sethares, 1991; Peters, 1994) and layers V and VI (Fig. 5I, inner Meynert cell; Lund, 1973; Chan-Palay et al., 1974; Winfield et al., 1981; Valverde, 1985; Payne and Peters, 1989; Peters and Sethares, 1991; Peters, 1994).

In layer I of all areas examined, weak-to-moderate GAP-43 mRNA-positive signals were observed in the small (5–10 µm in diameter), round neurons (Fig. 5K), but we could not detect GAP-43 mRNA-positive Cajal–Retzius neurons in this layer (Marin-Padilla, 1984). We also observed GAP-43 mRNA-positive neurons with weak hybridization signals in the white matter.

Expression Patterns of SCG10 mRNA in the Adult Cerebral Cortex

Intense hybridization signals for SCG10 mRNA were preferentially observed in layers V and VI of all areas examined (Figs 5C, F, I and 6C, F, I). As shown in Figure 4, SCG10 mRNA as well as GAP-43 mRNA was localized in most neurons in layers V and VI, suggesting that a number of neurons in these layers contain both GAP-43 and SCG10 mRNAs. In layer II, the signals for SCG10 mRNA were weak. In layer III, the signals were generally weak and gradients of signal intensity were observed: the lower part of layer III contained more intense hybridization signals than the upper part of layer III. The larger pyramidal cells at the lowermost part of layer III often had more intense signals than any cells in layer IV. Intense signals tended to be localized in larger pyramidal cells in layers V and VI (Fig. 5B), but intense

Figure 5. High-magnification photomicrographs of cortical neurons that have hybridization signals for GAP-43 mRNA (A, C, E, G, J, K) and SCG10 mRNA (B, D, F, H, J, L). (A, B) The large pyramidal cells in layer V of area TE. The arrows show the apical dendrites of the pyramidal cells. (C, D) The small pyramidal cells in layer V of area TE. The arrows show the apical dendrites of the pyramidal cells. (E, F) The bipolar cells in layer V of area TE. The arrows show the vertically oriented dendrites of the bipolar cells. (G, H) The outer Meynert cells in layer IVB of area OC. (I, J) The inner Meynert cells in layer VI of area OC. (K, L) The small neurons that had weak to moderate signals for GAP-43 mRNA (the arrows in K) and weak signals for SCG10 mRNA (the arrows in L) in layer I of area TE. Scale bar = 20 µm.
Figure 6. The areas PB (A–C), OB (D–F) and OC (G–I) of the adult cortex. (A,D,G) Nissl-stained sections. (B,E,H) Localization of GAP-43 mRNA. (C,F,I) Localization of SCG10 mRNA. Scale bar = 200 µm.
expression patterns between the outer pyramidal layer (layer III) and the inner pyramidal layer (layer V) of each cortical area (see Materials and Methods for detail). (A) The IIIOD/VOD ratio of GAP-43 mRNA in the adult cortices. #The IIIOD/VOD ratio of SCG10 mRNA in the adult cortices. *The IIIOD/VOD ratio for area OC may have been underestimated.

Figure 7. The IIIOD/VOD ratio was calculated to clarify the differences in the expression patterns between the outer pyramidal layer (layer III) and the inner pyramidal layer (layer V) of each cortical area (see Materials and Methods for detail). (A) The IIIOD/VOD ratio (± SD) of GAP-43 mRNA in the adult cortices. #The IIIOD/VOD ratio of SCG10 mRNA in the adult cortices. *The IIIOD/VOD ratio for area OC may have been underestimated.

hybridization signals were also observed in the smaller pyramidal cells (Fig. 5D) and the non-pyramidal cells in layers V and VI (Fig. 5F). In all areas examined, the hybridization signals were weaker in layer III than in layer V (the IIIOD/VOD ratios were: 0.97 ± 0.05 in area FD, 0.93 ± 0.04 in area TE, 0.94 ± 0.02 in area PG, 0.90 ± 0.09 in area PB, 0.91 ± 0.05 in area OB, and 0.79 ± 0.03 in area OC; Fig. 7B). In areas PB and OC, as we found for GAP-43 mRNA, there were only slight differences between the in situ SI of SCG10 mRNA of layer III and the in situ SI of layer V (Table 1).

As with GAP-43 mRNA, because of the differences in neuronal densities between layer III and layer V, the IIIOD/VOD ratios were <1 also in these areas. The IIIOD/VOD ratio for area OC may have been underestimated because the neurons in layer II tended to contain less intense signals than the neurons in layer III. The difference between the association and lower sensory areas was not significant (P > 0.05, Mann–Whitney U-test). In area OC, the outer Meynert cells (Fig. 5H) and inner Meynert cells (Fig. 5J) contained intense signals. The hybridization signals for SCG10 mRNA in these Meynert cells were more intense than those in neighboring cells (Fig. 5H, J).

Weak SCG10 mRNA-positive signals were sometimes observed in the smaller neurons in layer I (Fig. 5L) and in the white matter of all areas examined.

Expression Patterns of GAP-43 mRNA in the Infant Cerebral Cortex

Intense hybridization signals for GAP-43 mRNA were observed in layers II–VI of all areas of the infant cortex examined (Figs 8B, E, H and 9B, E, H). Intense signals for GAP-43 mRNA tended to be observed in the pyramidal cells, but were sometimes observed in non-pyramidal cells. The expression pattern was identical among the infant cortices of three different ages (postnatal days 2, 8 and 31), so we combined quantifying data from these three monkeys (Table 2, Fig. 10A). The hybridization signals in layer III were higher than those in layer V in most cases (Figs 8B, E, H, 9B, E, H and 10A, Table 2). The IIIOD/VOD ratio for area OC (0.95 ± 0.05; Fig. 10A) may have been underestimated because the neurons in layer II tended to contain less intense signals than the neurons in layer III. In layer I and the white matter of all areas examined, moderate signals for GAP-43 mRNA were observed.

In areas FD, TE and PG, in situ-SIs for GAP-43 mRNA in all layers of the infant cortex were higher than those of the adult cortex (Tables 1 and 2). In layers II–IV of areas PB and OB, and layers II–IVA of area OC, in situ-SIs for GAP-43 mRNA were higher in the infant cortex than in the adult cortex (Tables 1 and 2), whereas in layers V and VI of areas PB and OB, and layers IVB to VI of area OC, in situ-SIs for GAP-43 mRNA were almost the same between infant and adult cortices (Tables 1 and 2).

Expression Patterns of SCG10 mRNA in the Infant Cerebral Cortex

Intense hybridization signals for SCG10 mRNA were observed from layers II–VI of all areas of the infant cortex examined (Figs 8C, F, I and 9C, F, I). As we saw with GAP-43 mRNA, intense signals for SCG10 mRNA were frequently observed in the pyramidal cells. In the study of SCG10 mRNA, the pyramidal cells tended to be more densely stained than those in the study of GAP-43 mRNA (Figs 8C, F, I and 9C, F, I). The expression pattern was identical among the infant cortices of three different ages (postnatal days 2, 8 and 31), so we combined quantifying data from these three monkeys (Table 2, Fig. 10B). The hybridization signals in layer III were higher than those in layer V in most
Figure 8. The areas FD (A–C), TE (D–F) and PG (G–I) of the infant cortex (postnatal day 8). (A,D,G) Nissl-stained sections. (B,E,H) Localization of GAP-43 mRNA. (C,F,I) Localization of SCG10 mRNA. Scale bar = 200 µm.
Figure 9. The areas PB (A–C), OB (D–F) and OC (G–I) of the infant cortex (postnatal days 8). (A,D,G) Nissl-stained sections. (B,E,H) Localization of GAP-43 mRNA. (C,F,I) Localization of SCG10 mRNA. Scale bar = 200 µm.
GAP-43 mRNA, the IIIOD/VOD ratio for area OC (1.00 ± 0.13; Fig. 10).

Previously, we had investigated the developmental changes of GAP-43 mRNA and SCG10 mRNA in the monkey cerebral cortex. Our results revealed that the distribution patterns of GAP-43 mRNA were different between infant and adult cortices. We also showed that the distribution patterns of GAP-43 mRNA were different between the association areas and the lower sensory areas of the adult cortex.

Comparison with Northern Blot Study in Monkeys

Discussion

This report describes the exact location and types neurons labeled positively for GAP-43 mRNA and SCG10 mRNA in the monkey cerebral cortex. Our results revealed that the distribution patterns of both GAP-43 and SCG10 mRNAs were different between infant and adult cortices. We also showed that the distribution patterns of GAP-43 mRNA were different between the association areas and the lower sensory areas of the adult cortex.

Comparison with Northern Blot Study in Monkeys

Previously, we had investigated the developmental changes of GAP-43 mRNA (Oishi et al., 1998) and SCG10 mRNA (Higo et al., 1996) by Northern blot analyses. We have shown that both GAP-43 and SCG10 mRNAs are more abundant in all examined areas of infant monkeys (postnatal days 1, 8 and 30) than in those of the adult monkey (Higo et al., 1996; Oishi et al., 1998). These results are consistent with the present results: more intense hybridization signals were observed in all cortical areas of all three infant monkeys than in adult cortical areas. We have also shown in Northern blot analyses that the amount of SCG10 mRNA decreases more steeply from the infant cortex to the adult cortex than does the amount of GAP-43 mRNA (N. Higo et al., unpublished observation). In the present results, the differences in signal intensity between infant and adult cortices were remarkable, especially for SCG10 mRNA (Tables 1 and 2). Therefore, the results from the previous Northern blot analyses and the results from the present in situ hybridization studies are also consistent on this point.

In the present study, we showed that the amount of GAP-43 mRNA decreased in every layer of areas FD, TE and PG during postnatal development (Figs 3B,E,H and 8B,E,H, Tables 1 and 2). In contrast, the amount of GAP-43 mRNA decreased selectively in layers II–IV of areas PB, OB and OC (Figs 6B,E,H and 9B,E,H). The amount of GAP-43 mRNA did not decrease much in layers V and VI of areas PB, OB and OC, and the SIs in these layers of the adult cortex were almost the same as those in the same layers of the infant cortex (Tables 1 and 2). The amount of SCG10 mRNA decreased in every layer of all areas examined during postnatal development (Figs 3C,F,I, 6C,F,I, 8C,F,I and 9C,F,I). The amount of SCG10 mRNA decreased more steeply in layers II–IV than in layers V and VI (Tables 1 and 2).

In a Northern blot analysis of the adult cortex, we have shown that the amount of GAP-43 mRNA is higher in the association areas than does the amount of SCG10 mRNA (N. Higo et al., 1996). The present results show that the amount of GAP-43 mRNA is higher in the association areas than does the amount of SCG10 mRNA. These results are consistent with the present results: more intense hybridization signals were observed in all cortical areas of all three infant monkeys than in adult cortical areas. We have also shown in Northern blot analyses that the amount of SCG10 mRNA decreases more steeply from the infant cortex to the adult cortex than does the amount of GAP-43 mRNA (N. Higo et al., unpublished observation). In the present results, the differences in signal intensity between infant and adult cortices were remarkable, especially for SCG10 mRNA (Tables 1 and 2). Therefore, the results from the previous Northern blot analyses and the results from the present in situ hybridization studies are also consistent on this point.

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In a Northern blot analysis of the adult cortex, we have shown that the amount of GAP-43 mRNA is higher in the association areas (areas FD, TE and PG) than in the lower sensory areas (areas PC, OB and OC; Oishi et al., 1998). The present results showed that the distribution patterns of GAP-43 mRNA were also different between the association and lower sensory areas: prominent hybridization signals for GAP-43 mRNA were restricted to layers IV–VI of the lower sensory areas (Fig. 6B,E,H), but were observed from layers II–VI of the association areas (Fig. 3B,E,H) of the adult cortex. Therefore, the difference in the amount of GAP-43 mRNA between the association areas and the lower sensory areas may be due to the differential distribution patterns of GAP-43 mRNA between them.

There was no difference in the amount of SCG10 mRNA.
among adult cortical areas in the Northern blot analysis (Higo et al., 1996). The present results showed no difference in the laminar distribution of SCG10 mRNA among cortical areas (Figs 3C, F, I and 6C, F, I). Thus, results from these two studies are consistent.

From Northern blot analyses and in situ hybridization studies, we confirmed that both the quantity and the distribution pattern of GAP-43 mRNA, but not those of SCG10 mRNA, are different between the association and lower sensory areas of the adult cortex. Nelson et al. (1987) reported that the phosphorylation of GAP-43 is higher in the visual association area (TE) than in the primary (OC) and secondary (OB) visual areas. The present result suggests that this gradient of the phosphorylation is regulated, at least in part, by the level of mRNA.

Comparison with Studies of Other Animals
Several in situ hybridization studies of the rat cerebral cortex have examined GAP-43 mRNA (Kruger et al., 1993; Yao et al., 1993; Sugiuira and Mori, 1995) and SCG10 mRNA (Himi et al., 1994b; Sugiuira and Mori, 1995). Both GAP-43 mRNA (Kruger et al., 1993; Yao et al., 1993) and SCG10 mRNA (Himi et al., 1994b) are preferentially expressed in layer IV, V or VI of most of the mature rat cerebral cortex. The observation that GAP-43 mRNA is expressed in layer II as well as layer VI of the anterior limbic (cingulate) field of the rat cortex (Kruger et al., 1993) corresponds with our findings in monkeys that GAP-43 mRNA is expressed in the supragranular layers as well as the infragranular layers only in the associative regions (Fig. 7A). In the postnatal development of the rat cerebral cortex, the hybridization signals for both GAP-43 and SCG10 mRNAs are more intense in the supragranular layers than the infragranular layers (Sugiura and Mori, 1995), which are similar to the present findings in the monkey cerebral cortex (Fig. 10A, B). Previous studies revealed that GAP-43 mRNA exists in the hippocampal dentate granule cells of the monkey (Higo et al., 1998a), but not in that of the rat (Meberg and Routtenberg, 1991; Jacobs et al., 1993; Kruger et al., 1993; Yao et al., 1993). Conversely, SCG10 mRNA exists in the granule cells of the rat (Himi et al., 1994a), but not in that of the monkey (Higo et al., 1998a). We did not detect these kinds of striking differences between the monkey and the rat cerebral cortex.

In humans, the distribution of neurons expressing GAP-43 mRNA has been studied in only a few cortical areas (Neve et al., 1996). The present results showed no difference in the laminar distribution of SCG10 mRNA among cortical areas (Figs 3C, F, I and 6C, F, I). Thus, results from these two studies are consistent.

From Northern blot analyses and in situ hybridization studies, we confirmed that both the quantity and the distribution pattern of GAP-43 mRNA, but not those of SCG10 mRNA, are different between the association and lower sensory areas of the adult cortex. Nelson et al. (1987) reported that the phosphorylation of GAP-43 is higher in the visual association area (TE) than in the primary (OC) and secondary (OB) visual areas. The present result suggests that this gradient of the phosphorylation is regulated, at least in part, by the level of mRNA.

Comparison with Studies of Other Animals
Several in situ hybridization studies of the rat cerebral cortex have examined GAP-43 mRNA (Kruger et al., 1993; Yao et al., 1993; Sugiuira and Mori, 1995) and SCG10 mRNA (Himi et al., 1994b; Sugiuira and Mori, 1995). Both GAP-43 mRNA (Kruger et al., 1993; Yao et al., 1993) and SCG10 mRNA (Himi et al., 1994b) are preferentially expressed in layer IV, V or VI of most of the mature rat cerebral cortex. The observation that GAP-43 mRNA is expressed in layer II as well as layer VI of the anterior limbic (cingulate) field of the rat cortex (Kruger et al., 1993) corresponds with our findings in monkeys that GAP-43 mRNA is expressed in the supragranular layers as well as the infragranular layers only in the associative regions (Fig. 7A). In the postnatal development of the rat cerebral cortex, the hybridization signals for both GAP-43 and SCG10 mRNAs are more intense in the supragranular layers than the infragranular layers (Sugiura and Mori, 1995), which are similar to the present findings in the monkey cerebral cortex (Fig. 10A, B). Previous studies revealed that GAP-43 mRNA exists in the hippocampal dentate granule cells of the monkey (Higo et al., 1998a), but not in that of the rat (Meberg and Routtenberg, 1991; Jacobs et al., 1993; Kruger et al., 1993; Yao et al., 1993). Conversely, SCG10 mRNA exists in the granule cells of the rat (Himi et al., 1994a), but not in that of the monkey (Higo et al., 1998a). We did not detect these kinds of striking differences between the monkey and the rat cerebral cortex.

In humans, the distribution of neurons expressing GAP-43 mRNA has been studied in only a few cortical areas (Neve et al., 1996; Perrone-Bizzozero et al., 1996). In one human study, Neve et al. (1998) showed that the most intense hybridization signals were concentrated in layer II of both the association area (Brodmann’s area 20, corresponding to area TE) and the primary area (area 17, corresponding to area OC); this is not consistent with our results in monkeys. Perrone-Bizzozero et al. (1996) showed, however, that the expression of GAP-43 mRNA was robust in layers II–VI of the association area (area 10), while a few GAP-43 mRNA-positive cells were also detected in layer I of the area. These results are consistent with our results in the association areas of monkeys (Fig. 3B, E, H). The laminar distribution patterns of GAP-43 mRNA in some areas might be different between monkeys and humans. Because human data are currently scarce and only qualitative, further experiments in the various areas of human cerebral cortex are necessary to confirm the laminar distribution of GAP-43 mRNA.

Benowitz et al. (1989) performed an immunohistochemical study of GAP-43 protein in several areas that included both the association areas and the primary sensory areas of the adult human cerebral cortex. Because GAP-43 immunoreactivity is localized primarily in the nerve terminals of the neuropil, the result tells nothing about the density and the distribution of cell bodies expressing GAP-43. However, GAP-43 immunoreactivity also showed specific laminar distribution and more localization in restricted layers of the lower sensory areas than in the association areas (Benowitz et al., 1989).

Comparison with Other Molecules Regarding Changes in Laminar Distribution during Postnatal Development
In this study, we showed the laminar distribution patterns for both GAP-43 and SCG10 mRNAs in both infant and adult cerebral cortices. There are several kinds of molecules whose laminar distribution patterns change during development.

At the newborn stage, two inferior temporo-occipital neocortical cells are transiently distributed at a high density in the supragranular layers of many cortical areas, especially in layer II of areas FD and PE (Yamashita et al., 1989). The expression of somatostatin and its mRNA decrease during postnatal development (Hayashi and Oshima, 1986; Yamashita et al., 1989; Hayashi et al., 1990). These results are similar to our results for GAP-43 and SCG10 mRNAs. Somatostatin has been reported to enhance neurite outgrowth in cultured cells (Ferriero et al., 1994), molluscan neurons (Bulloch, 1987; Grimm-Jørgensen, 1987) and cerebellar granule cells of the rat (Taniwaki and Schwartz, 1995). Thus, somatostatin, as well as GAP-43 and SCG10, may be involved in the axonal elongation in the supragranular layers of infant monkey cortex.

The molecule DARP-P-32, a dopamine- and cAMP-regulated phosphoprotein, also showed differential expression patterns between infant and adult cortical cortices (Berger et al., 1990). Between postnatal days 3 and 42, pyramidal cells containing DARP-P-32 are distributed in layers II–VI of various cortical areas of the macaque monkey. The immunoreactivity for DARP-P-32 remains prominent in the prominent prominent layer V of areas FD and the paralimbic structure in most of the layers. The immunoreactivity for DARP-P-32 is faint in most of the other areas, and localized in more restricted layers of these areas. These results are consistent with our results of GAP-43 mRNA: highly plastic cortical areas keep an extensive laminar distribution, a characteristic of infant cerebral cortex, even in the adult cortex. Further research into the layer-specific development of various molecules will increase our understanding of the maturation of neuronal circuits in the cerebral cortex of primates.

Intensity of mRNA Signals among Cell Types
For both GAP-43 and SCG10 mRNAs, intense hybridization signals were frequently observed in the larger pyramidal cells in layers III, V and VI (Fig. 5A, B). The results suggest that these molecules are highly expressed in the long projection neurons.

We also observed intense signals for both GAP-43 and SCG10 mRNAs in the non-pyramidal cells in layers III, V and VI. Although it was difficult to identify the type of non-pyramidal cells because both GAP-43 and SCG10 mRNA were localized in the cytoplasm and the proximal dendrites of neurons, we observed GAP-43 or SCG10 mRNA-positive cells which have the shape of a bipolar cell (Fig. 5E, F; Jones, 1975; Fairén et al., 1984; Peters, 1984; Lund and Lewis, 1993; Gabbott and Bacon, 1996; Peters and Sethares, 1997). These cells were found especially in layers III, V and VI. These bipolar cells have intrinsic axon terminals within the cortical area. It is likely that the hybridization signals for both mRNAs in these non-pyramidal neurons indicate the plastic nature of their axon terminals.

In area OC, both mRNAs were highly expressed in the outer
Meynert cells in layer IVB (Fig. 5G,H) and the inner Meynert cells in layer V or V1 (Fig. 5I, J). Various studies previously showed that some of these Meynert cells project to area MT of the cortex or the superior colliculus (Lund and Boothe, 1975; Tiggges et al., 1981; Fries and Distel, 1983; Sipp and Zeki, 1989; Peters, 1994). The results might reflect functional specialization regarding the plasticity of these projections.

**Intensity of mRNA Signals among Anatomical Circuits in the Adult Cortex**

Intense hybridization signals for both GAP-43 and SCG10 mRNAs were frequently observed in the pyramidal neurons in layers V and VI of all areas of adult cortex examined. The neurons in layers V and VI extend to various subcortical projections that include corticothalamic or corticostriatal projections (Jones and Wise, 1977; Jones et al., 1977; Catsman and Kuypers, 1978; Arikuni et al., 1983; Arikuni and Kubota, 1986; Goldman-Rakic and Selemon, 1986; Giguere and Goldman-Rakic, 1988; Lund, 1988). Northern blot analysis revealed that the thalamus of the macaque monkey contained a higher level of GAP-43 mRNA than the cerebral cortex and the same level of SCG10 mRNA as the cerebral cortex (T. Oishi et al., unpublished observation). The caudate nucleus and the putamen also contained the same levels of GAP-43 and SCG10 mRNAs as the cerebral cortex. These results indicate the plastic nature of the projections among the cerebral cortex and these subcortical structures.

We detected high levels of GAP-43 mRNA in the supragranular layers (layers II and III) as well as in the infragranular layers of the association areas: areas FD, TE and PG (Tigs 5B,E,H and 7A). The neurons in the supragranular layers of those cortical areas supply both cortico-cortical connections (Barbas and Mesulam, 1981; Schwartz and Goldman, 1984; Friedman et al., 1986; Shiwa, 1987; Vogt and Pandya, 1987; Barbas, 1988; Johnson et al., 1989; Seltzer and Pandya, 1989; Andersen et al., 1990) and intrinsic connections (Amir et al., 1993; Levitt et al., 1993; Kritzer and Goldman-Rakic, 1995; Fujita and Fujita, 1996). Intralaminar local connections in the supragranular layers of the association areas are more laterally widespread than in the primary and secondary visual areas (Kisvarday et al., 1989; Amir et al., 1993; Levitt et al., 1993; Kritzer and Goldman-Rakic, 1995). The abundance of GAP-43 mRNA in the supragranular layers of the association areas may suggest that these intrinsic connections spread laterally within the association areas are functionally specialized regarding plasticity.

**Notes**

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