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Radial glia is a progenitor of neocortical neurons in the developing cerebral cortex

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Abstract

Neocortical neurons are produced by cell division of neural stem cells in the ventricular zone of the cerebral cortex. We investigated the production of neurons by infecting neuroepithelial cells with a modified GFP-recombinant adenovirus. The adenovirus DNA is inherited by only one daughter cell at each cell division and travels one way from the progenitor to the progeny. Since the ventricular zone (VZ) of the embryo neocortex expressed an adenovirus receptor, CAR ubiquitously, morphology and cell-lineage of cells in the VZ could be revealed by the adenovirus infection. Radial glias, cells with a bipolar shape, and spherical cells were found as modified-GFP-positive (mGFP+) in the VZ. The bipolar cells (radial cells) had a radial process not in contact with the pia mater and a growth-cone-like structure at the edge of their radial process, while the radial glias had a process spanning all the cortical layers. Ten hours after viral infection, most mGFP+ cells were radial cells. In the following 8 h, the percentage of mGFP+ radial glias in mGFP+ neocortical cells increased from 18 to 50%, while that in radial/spherical cells decreased from 75 to 19%. The radial glias often divided asymmetrically and produced spherical cells and neuronal precursors. The spherical cells seemed to become radial cells by extending a radial process. The spherical cells, radial cells and radial glias seemed to constitute a proliferating cell cycle during which postmitotic neuronal precursors are produced. The neuronal precursors that inherited the radial processes migrated radially and developed into neocortical neurons. Four days after the viral infection, 97% of mGFP+ cells were neocortical neurons. Here, we propose that the radial glia is a progenitor of neocortical neurons, and that a significant number of radially migrating neurons is guided by their own radial processes connected to the pia mater.

Keywords: Adenovirus; Cell migration; GFP; Neural stem cell; Progenitor; Pyramidal cell; Radial glia

1. Introduction

In the developing cerebral cortex, radial glias have been considered to play an important role in guiding radially migrating neurons from the ventricular zone (VZ) to the cortical plate and contribute to the specification of cerebral cortical areas (Rakic, 1972, 1988, 1990). Recently, however, radial glias were suggested to have proliferative activity to be considered as multipotent progenitor cells (Chanas-Sacre et al., 2000; Malatesta et al., 2000; Hartfuss et al., 2001; Noctor et al., 2001). The radial glial system is not static. Proliferation, extension of new fibers, and translocation of radial glial cell bodies are common features found in the radial glial system across the entire developing brain (Misson et al., 1988b, 1991; Voigt, 1989; Takahashi et al., 1990). Thus, it should be a very important issue to understand the in vivo behavior of radial glias in developing brains. In order to address this issue, first we should have a tool for identifying the radial glias in vivo. Although radial glias are immunoreactive for several molecular markers, such as RC2 (Misson et al., 1988a; Gaiano et al., 2000), vimentin (Kamei et al., 1998), and nestin (Lendahl et al., 1990; Gaiano et al., 2000; Super et al., 2000), these molecular markers are

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also used for identification of neural stem cells in vitro (Malatesta et al., 2000; Hartfuss et al., 2001) and in vivo (Yamaguchi et al., 2000). Furthermore, RC2 immunoreactivity was observed not only in radial glias but also in radial cells (Alvarez-Buylla et al., 1990), which are found in the VZ and had radial processes not spanning all the cortical layers (Gadisseux et al., 1992). Therefore, at present, no reliable molecular markers are available for the identification of the radial glia. Hence, we went back to the original definition of the radial glia, i.e. glial cells with a soma in the VZ and with a radial fiber spanning all the cortical layers (Rakic, 1972, 1988, 1990). In this study, we applied a recombinant adenovirus-expressing-membrane-targeted green fluorescent protein (mGFP) (Moriyoshi et al., 1996) to the lateral ventricle of the mouse embryo. This technique was revealed to be useful in investigating the detailed structures of the infected cells in the brain (Tamamaki et al., 2000). We infected neuroepithelial cells in vivo at the stage when most of the cortical neurons were produced and followed the fate of mGFP-positive (mGFP +) cells at various developmental stages. Here, we report that radial glias proliferate not only to produce glial progeny (Kamei et al., 1998; Misson et al., 1988b, 1991; Voigt, 1989), but also to supply neocortical neurons in vivo (Tamamaki, 2000).

2. Materials and methods

2.1. Viral construction and infection

The present experiments were approved by the Committee for Animal Care and Use, and the committee for recombinant-DNA experiments of the Graduate School of Medicine in Kyoto University. AxCAG-GAP43-EGFP was constructed by inserting cDNA of GAP43 palmitoylation site and that of EGFP into the SwaI site in an adenovirus vector, pAxcw (Adenovirus kit, TAKARA, Japan). Then the recombinant adenovirus vector was introduced into 293 cells according to the manufacturer's protocol. After three amplifications, we obtained a viral solution at a titer of 6×10^8 PFU/ml. Thirty pregnant C57BL/6J mice were anesthetized by pentobarbital injection (50 mg/kg i.p.). One-quarter to one-half microliter of the adenovirus solution (2×10^6–6×10^8 PFU/ml) was injected into one side of the lateral ventricle of the brain of the mouse embryos at embryological day (E) 14–15 by pressure through glass capillary pipettes. E0 was the day vaginal plug was found. E19 (P0) was the day of birth. At predetermined periods from 8 h to 25 days after the injection, the pregnant and infant mice were anesthetized with pentobarbital. Embryos and the infant mice were perfused transcardially with a fixative (4% formaldehyde in 0.1 M phosphate buffer, pH 7.4). Brains were removed and postfixed with the same fixative for 2 h and then immersed in 30% sucrose in PBS overnight.

For double staining with BrdU and mGFP, BrdU (50 μg/g in 0.007 N NaOH) was injected three times 10–16 h after viral injection. After BrdU injection, the pregnant mouse was anesthetized and embryos were perfused 16 h after viral injection.

2.2. Staining of tissue sections

Fixation of the infected animals, preparation of frozen sections, and immunoperoxidase detection of mGFP have been described recently (Tamamaki et al., 2000). For double immunofluorescence staining with mGFP and nestin, or mGFP and RC2, frontal frozen sections of 30–50 μm thickness were simultaneously incubated with the primary antibodies to them. mGFP-immunoreactive sites were labeled with FITC, and nestin- or RC2-immunoreactive sites were labeled with Alexa-594 (Molecular probe, USA). For double staining with BrdU and mGFP, sections were incubated with an anti-GFP antibody and a biotinylated secondary antibody, and fixed briefly with the same fixative (Tamamaki et al., 2000). The sections were treated with 3 N HCl to denature DNA and incubated with an anti-BrdU antibody. mGFP-immunoreactive sites were labelled with FITC, and -BrdU-immunoreactive sites were also labeled with Alexa-594. The primary antibodies used were as follows: purified rabbit anti-GFP (1:200–500), purified rabbit anti-CAR (1:400, a gift from Dr Kuwahara), mouse anti-RC2 (IgM, supernatant, 1:2, DSHB, Iowa City, IA); mouse anti-nestin (IgG1, 1:1000, Chemicon, USA); and mouse anti-BrdU (1:50, Sigma, USA). The secondary fluorescent antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania) and were raised in donkeys. Fluorescent images were obtained using a confocal microscope (LSM, Zeis, Germany).

2.3. Electron microscopy

For electron microscopy of mGFP + structures, embryos were perfused with a fixative (0.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) and postfixed with the same fixative for 2 h. Frontal sections of 50 μm thickness were cut with a microslicer and treated with 0.1% sodium bromohydride for 30 min. The sections were immersed in a cryoprotective solution (10% glycerol and 25% sucrose in phosphate-buffered saline (PBS) pH 7.4) and frozen quickly on dry ice. After several rinses in PBS, the sections were transferred to a serial incubation solution for GFP-immunoperoxidase staining (Tamamaki et al., 2000). Triton X-100 was not added to any of the incubation solutions. After visualizing the GFP-immunoreactive sites by DAB reaction, the sections were

osmificated in buffered 1% OsO_4 solution for 2 h, dehydrated in an ethanol series, and then embedded in epon. Ultrathin sections were stained with uranil acetate and lead citrate, and observed under an electron microscope (Hitachi 7100, Japan).

2.4. Culture of infected brain slices

Thirty-six hours after the viral injection, embryo brains were removed, embedded in 7% low-melting-point agarose, and cut into slices of 200 μm thickness. The slices were transferred to a culture dish for brain slices (Transwell, Corning Costar, USA) and submerged in a culture medium (DMEM + 10% FCS). The brain slices were observed under a fluorescent microscope at 30-min intervals for 3 h. Then the slices were fixed by adding buffered 10% formalin in the culture medium, rinsed in PBS and 30% sucrose solution, then resectioned into 20 μm thickness using a freezing microtome and mounted serially on gelatin-coated glass slides. Then, mGFP-immunoreactive sites in the serial sections were detected and traced.

Alternate brain slices were collected from the serial slice obtained from the embryo brain 10 h after viral infection. They were cultured for 8–10 h in collagen gel and fixed for 2 h. The gel with the sliced tissue was processed for immunohistochemistry as a whole preparation as described above except for the extended incubation period for each antibody.

3. Results

To investigate the proliferation of neural stem cells and the production of postmitotic neurons, we injected recombinant adenovirus AxCAG-GAP43-EGFP into the lateral ventricle of embryos at E14–15, the stage when the layers V and IV neocortical neurons are produced (Fig. 1A). mGFP has a palmitoylation site at the N terminus, and is distributed on the plasma membrane to produce Golgi-stain-like labeling (Tamamaki et al., 2000). After the viral injection, the embryos were allowed to develop from 8 h to 25 days. The mGFP expression in the cortical cells was detected based on its fluorescence or by immunohistochemistry. It should be noted that the adenovirus DNA does not duplicate in the infected cell, since it lacks a eukaryotic DNA origin in its construct. Thus the viral DNA is inherited only by one daughter cell when the infected cell proliferates and is transferred one way from progenitors to the progeny in vivo. This was confirmed by the in vitro control experiments using proliferating COS-1 cells; after viral infection, the number of mGFP + COS cells remained relatively constant during culture for up to 1 week (Tamamaki et al., unpublished data). This result further indicates that mGFP was quickly lost from the daughter cell which did not inherit the viral DNA.

A significant area of the neocortical ventricular surface is occupied by radial glias (Rakic, 1972, 1988, 1990) and radial cells (Alvarez-Buylla et al., 1990; Gadisseux et al., 1992), which are radial glia-like and bipolar in shape with an ascending radial process not contacting the pia mater. By an adenovirus injection into the lateral ventricle, these radial glias and radial cells would be exposed to the viral solution from the ventricular surface. However, adenovirus does not necessarily infect all the cells in contact with virus particles. In order to infect the cells in contact, a proper adenovirus receptor on the surface of cells is required. Thus, we investigated the expression of an adenovirus receptor, CAR, in the embryo neocortex (Tomko et al., 2000). The immunoreactivity of CAR was highest in the VZ, while it covered the other layers with various strengths. In the VZ, CAR was expressed ubiquitously in all cells (Fig. 1B and C). Thus, the adenovirus was expected to have a high probability of infecting all cell types in the neocortical VZ. In all the experiments, we used viral solutions with various titers. The highest titer we used was 6×10^8 PFU/ml. By reducing the concentration and volume of the vial solution, we could observe from several hundred to a few mGFP + cells in a 50 μm thick section from 8 h after viral injection. Ten hours after viral injection, about 3/4 of mGFP + cells in the neocortex were cells with a bipolar shape (Fig. 1D) and about 1/5 were radial glias. On the other hand, most mGFP + cells in the ganglionic eminence (GE) (Fig. 1E) and thalamus were radial-glia-like in shape and had a long radial process. However, we could not trace the long process up to the pia mater. The rest of the mGFP + cells in the neocortex were spherical cells (Fig. 1E, G) and multipolar cells (Fig. 1K and L) (Stensaas and Stensaas, 1968). The radial glias were identified based on morphological criteria, such as the localization of soma in the VZ and a long radial process spanning all the cortical layers in serial sections or in sections cut parallel to the radial process (Fig. 1G and H) (Rakic, 1972, 1988, 1990). The radial processes often had lateral enlargements on their surface and came into contact with the pia mater by end-feet (Fig. 1I). In contrast, the radial cells had a growth-cone-like structure at the tip of the apical process (arrow in Fig. 1D). Many mGFP + radial glias were found with radial cells (Fig. 1J) or spherical cells (Fig. 1E and arrow in Fig. 1G).

Eighteen hours after viral injection, the percentage of mGFP + radial glias in mGFP + neocortical cells increased from 18 to 50% (Fig. 1G and P), while that of radial/spherical cells decreased from 75 to 19%. This observation and that in a cell cycle study (Takahashi et al., 1995) suggest that the radial cells observed at 10 h became radial glias, which were observed 18 h after viral injection without proliferation. We also investigated the change by obtaining serial brain slices from

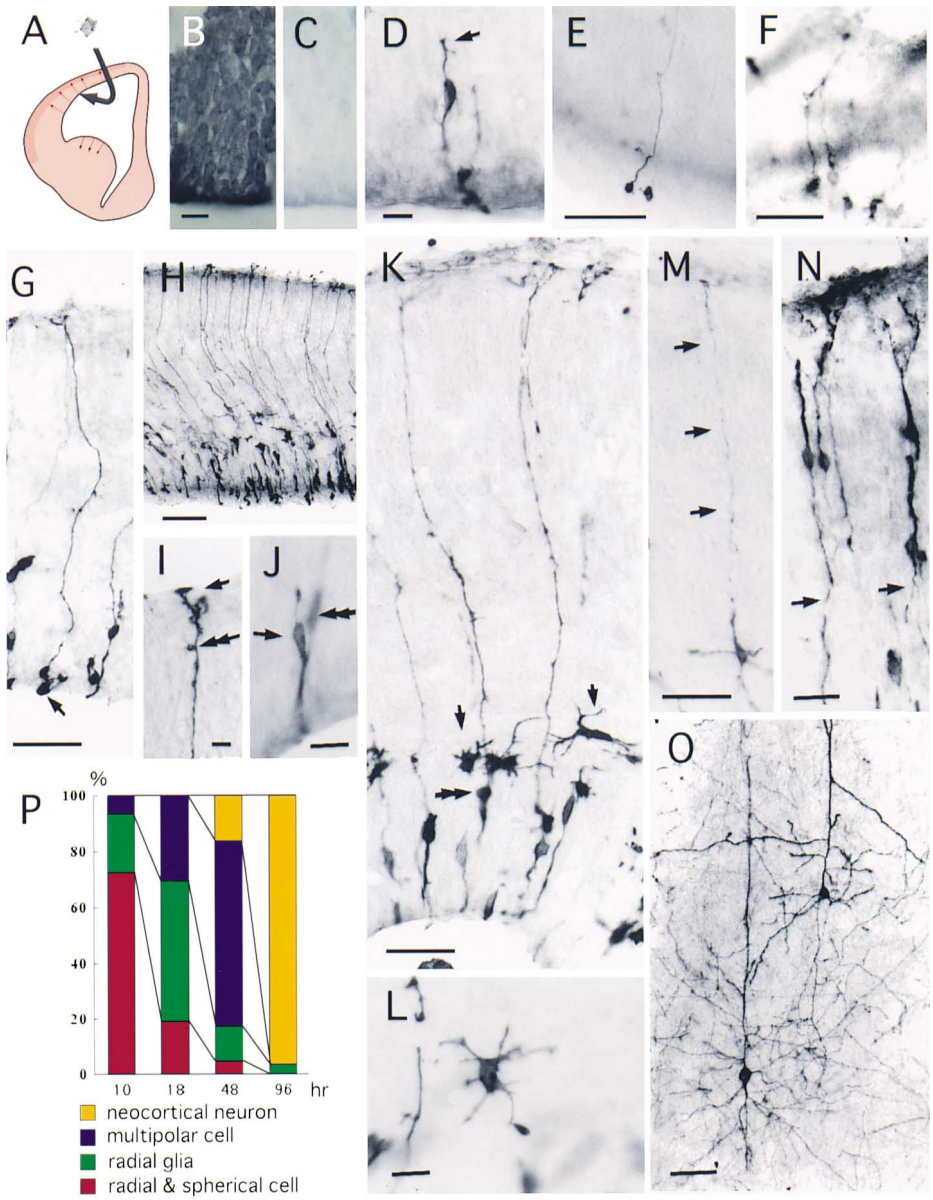

Fig. 1. Neocortical cells changed with time after viral injection as revealed by mGFP expression. (A) Schematic diagram of the strategy of adenovirus infection. (B) CAR immunoreactivity in the E14 mouse neocortical VZ. (C) VZ processed for immunohistochemistry with normal rabbit serum. (D) A radial cell observed in the VZ of the neocortex 10 h after viral injection. The arrow indicates a growth-cone-like structure at the tip of the radial process. (E) A mGFP+ radial glia and a spherical cell in the lateral ganglionic eminence of the same embryo. (F) mGFP+ radial glias observed in the brain slice which was obtained from embryo brains 10 h after viral infection and cultured for 8–10 h in collagen gel. (G) mGFP+ radial glias 18 h after viral injection. The arrow indicates a spherical cell, which accompanies a radial glia. (H) A micrograph of the same embryo neocortex at low magnification. (I) End-foot (arrow) and lateral expansion (double arrow) observed on a radial process. (J) A pair of a radial cell (arrow) and a radial glia (double arrow) 24 h after viral injection. (K) mGFP+ cells observed in the neocortex 24 h after viral injection. Arrows indicate multipolar cells in the SVZ. A double arrow indicates a radial glia in the SVZ, which lost its contact to the ventricular surface. (L) A multipolar cell in the SVZ of the same embryo. (M) mGFP+ cells in the lower IZ 48 h after viral injection. Arrows indicate its radial process connected to the pia mater. A drawing of the same cell is shown in Fig. 3e-2. (N) mGFP+ neocortical neurons 4 days after viral injection. Arrows indicate descending axons. (O) Mature layer-III and -V pyramidal cells observed in the neocortex 25 days (P20) after viral injection. (P) Composition of the mGFP+ cells found in each embryo neocortex at different observation times after viral injection. The percentages of radial and spherical cells, radial glias, multipolar cell, and neocortical neurons were determined in the serial frontal sections or in several frontal sections cut parallel to the radial process in each embryo brain. Ten, 18, 48, and 96 h after viral injection, they were (74.8 ± 9.7, 19.2 ± 9.4, 6.0 ± 2.6, 0%; $n = 5$), (19.1 ± 6.2, 50.5 ± 6.6, 30.4 ± 4.0, 0%; $n = 3$), (4.5 ± 2.2, 12.6 ± 1.9, 66.2 ± 16.3, 16.5 ± 5.9%; $n = 3$), and (3.5 ± 4.3, 0, 0, 96.5 ± 0.4%; $n = 3$), respectively. Calibration bars in B, E, F, G, H, K, M, N, and O are 50 μm, and those in D, I, J, and L are 10 μm.

embryos 10 h after viral infection, fixing the alternate slices immediately and transferring the alternate slices to a collagen-gel culture system (Fig. 1F). We used this experimental paradigm to investigate the change in composition of mGFP + cells under the same extent of viral infection. The composition of mGFP + cells in the embryos 10 h after viral injection was confirmed by immunohistochemistry of brain slices fixed immediately and the increase of mGFP + radial glias was investigated by immunohistochemisty of brain slices cultured for 8–10 h. As a result of this experiment, we found that more than half of the mGFP + cells in the cultures were radial glias (Fig. 1F), while the radial cells were dominant in the slices that were fixed immediately. From these results, we concluded that radial cells become radial glias by extending their radial processes, as earlier suggested (Ramon y Cajal, 1911; Stensaas and Stensaas, 1968; Morest, 1970; Takahashi et al., 1990).

We called radial-glia-like cells with an ascending radial process that does not reach the pia as radial cells. In addition, we also found radial-glia-like cells not in contact with the ventricular surface. They seemed to migrate to the subventricular zone (SVZ), leaving a spherical cell or a radial cell in the VZ (double arrow in Fig. 1K) (Voigt, 1989). Multipolar cells (Stensaas and Stensaas, 1968) in the SVZ became the major cells (72–66%) 24–48 h after viral injection (Fig. 1K–M). The short processes of the multipolar cells appeared to interact with the surrounding structures (Fig. 1L), while a long and thin radial process was retained by many multipolar cells (Fig. 1M). Many multipolar cells remained in the SVZ more than 3 days after viral injection. Four days after the viral injection, more than 95% of the mGFP + cells were found to be neocortical neurons, which was identified by the presence of an axon extending from the bottom of the mGFP + cells (Fig. 1N and P) toward the thalamus or the corpus callosum. Finally, many mature neocortical neurons and a few astrocytes were observed as mGFP + 25 days after the viral injection (20 days after birth; Fig. 1O).

The composition analysis of mGFP + cells suggested that radial glias are precursors of multipolar cells which grow into neocortical neurons (Fig. 1P). Production of multipolar cells by proliferation of a radial glia was also shown by a clonal analysis with a recombinant retrovirus in the neocortex (Noctor et al., 2001). Our data further suggested that the radial-glia-like cells not in contact with the ventricular surface developed into multipolar cells similar to the case of astrocyte production (Voigt, 1989). The recombinant adenovirus would infect the VZ of the GE simultaneously. However, considering the periods of incorporation of viral particles into the progenitor cells, proliferation to produce postmitotic neurons, and migration of neurons from the GE to the neocortex, there would be no contamination of neurons originating in the GE that will affect our observation for 24 h. As a summary of the flow of viral DNA, it labeled radial cells, radial glias, multipolar cells, and neocortical neurons.

To examine the proliferative activity of radial glias and radial cells, we conducted a confocal microscopic observation. We examined the colocalization of mGFP with nestin and RC2, which are radial glial markers as well as neural stem markers. As a result, we found a significant number of mGFP and netin (Fig. 2A), and mGFP and RC2 (Fig. 2B) double-labeled cells in the VZ. Interestingly, mGFP + radial cells incorporated BrdU injected 10 h after the viral injection (Fig. 2C), indicating that the radial cell synthesized DNA for proliferation. We further found mGFP + radial glias under cell division (Fig. 2D–G). The radial glia in Fig. 2D–F was in the anaphase of mitosis and had two clusters of chromosomes in the soma and one radial process. Some radial glias seemed to divide symmetrically (Fig. 2G) or asymmetrically (Fig. 2H) (Chenn and McConnell, 1995), and only one daughter cell appeared to inherit the radial process (Fig. 2H and I). However, if we take the retained radial process even during the cell division into account, the cell division of all radial glias was asymmetric, regardless of cleavage angle. As the result of cell division, a radial glia and a spherical cell seemed to be produced (Fig. 2H and I). From these observations, both the characteristics of the radial glias and neural stem cells coexist in the mGFP + cells in the VZ. Thus far, however, we did not find any mGFP + radial cells and spherical cells in cell division. If spherical cells become radial cells by extending a radial process (Fig. 1G and J), as recently reported (Miyata et al., 2000), they seemed to constitute, together with radial cells and radial glias, a proliferating cell cycle during which postmitotic neuronal precursors are produced.

In addition to the composition analysis in mGFP + cells, we carried out a statistical analysis on viral DNA distribution. The number of mGFP + neocortical cells revealed by a single viral injection was roughly proportional to the volume and concentration of the viral solution (data not shown), indicating that the viral DNA was not lost during cell division and that mGFP expression in each cell depended on a single-copy viral DNA. The latter was in agreement with the Poisson distribution with a small mean value in probability of viral infection. We counted the total percentage of progenitor cells (radial glias in different phases of cell mitosis; that is, radial cells + spherical cells + radial glias) at several points after the viral injection, and approximated them with a single exponential decay curve (red line in Fig. 2J). The blue line in Fig. 2J is the predicted reduction curve estimated from the parameters of the progenitor cell cycle under an assumption that all mGFP + progenitor cells are actively prolifera-

tive (Takahashi et al., 1996b). The exponential reduction was expected from the adenovirus DNA construct that does not contain a eukaryotic origin, while retrovial DNA was inherited in a different manner (Kirkwood et al., 1992; Walsh et al., 1992). If there were any quiescent mGFP+ radial glias, the red line must have been above the blue line. The fact that the red line is always under the blue line indicates that the proliferation of progenitor cells is faster than expected, or that the reduction is accelerated by other factors such as apoptosis and preferred inheritance of viral DNA into multipolar cells.

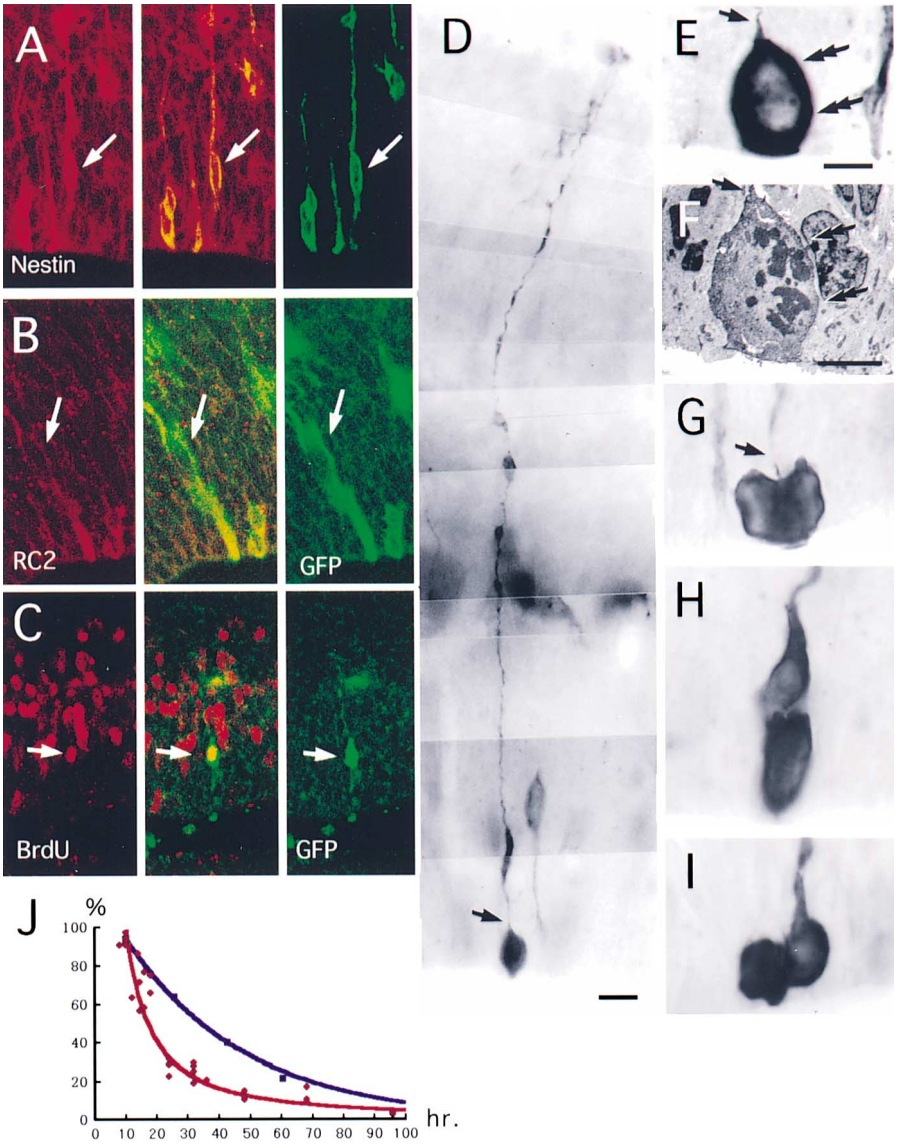

Fig. 2. Proliferation of mGFP+ radial glias in vivo. (A) Double-labeling study for mGFP and nestin. Arrows in A, B, and C, indicate double-labeled cells with bipolar shape. Calibration bar is 10 μm, which applies to B and C. (B) Double-labeling study for mGFP and RC2. (C) Double-labeling study for mGFP and BrdU. (D) Composite photomicrograph of a radial glia in mitosis 24 h after viral injection. The radial glia with two nuclei and one radial process was observed in a section embedded in epon, which was processed further for EM observation. Arrows in D, E, F, and G indicate the retained radial process during mitosis. Calibration bar is 10 μm. (E) The soma of the radial glia in D had two nuclei. The soma was observed in a 5-μm-thick semithin section made by sectioning the epon block in D perpendicularly to the surface. Double arrows in E and F indicate the two nuclei in the soma. Calibration bar is 5 μm, which applies to G–I. (F) Electron microscopy of the same cell. Two clusters of chromosomes without a nuclear membrane were observed in the soma. Calibration bar is 5 μm. (G) Symmetrically dividing soma of a radial glial. The arrow indicates a retained radial process during mitosis. (H) A radial glia and a spherical cell seemed to be produced by an asymmetric division of a radial glia. (I) A radial glia and a spherical cell seemed to be produced by a symmetric division of a radial glia. (J) Reduction curve of percentage of mGFP+ radial glias in mGFP+ cells predicted from the parameters of neural stem cell cycle (a blue line and dots) and obtained by direct measurement using tissue sections (a red line and dots). The predicted reduction after cell division of n times was obtained using the equation: $Rn = R_0(p_1 + q_1/2)(----)(p_n + q_n/2)$. The following values of parameters are obtained from the study by Takahashi et al. (1995, 1996b) (Tc1 = 15.7, Tc2 = 17.1, Tc3 = 17.7, $p_1 = 0.68$, $q_1 = 0.32$, $p_2 = 0.75$, $q_2 = 0.25$, $p_3 = 0.92$, $q_3 = 0.08$).

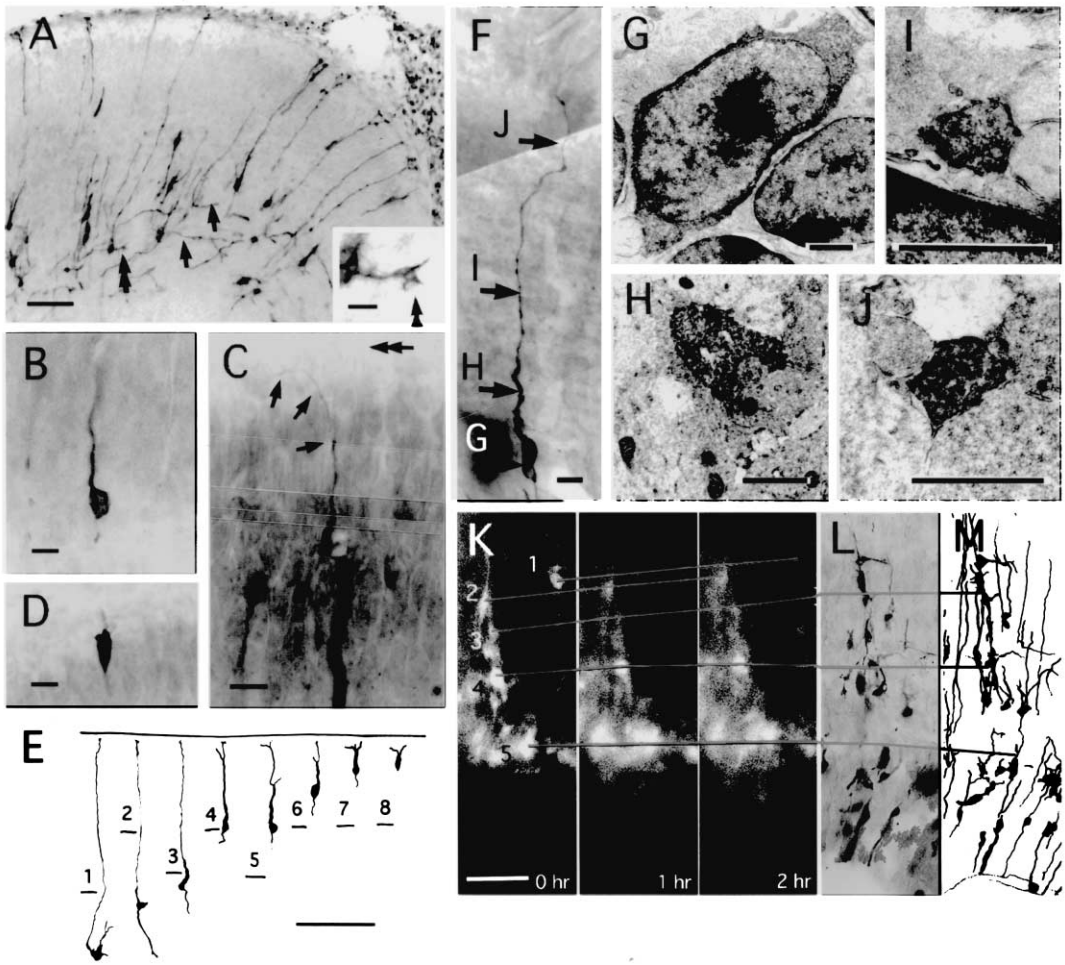

Fig. 3. Radial migration retaining a radial process. (A) mGFP+ neurons in the IZ and in the cortical plate with an axon (arrows) extending in the IZ. The double arrow indicates an axon growth cone shown in the inset at high magnification. (B) A radially migrating neuron in the cortical plate. A drawing of the same cell is shown in E-5. (C) The radial process of the same cell. The proximal thick part is distinct in B, but the distal thin part (arrows) is visible only at higher magnification. The double arrow indicates the level of the pia mater. (D) A radially migrating neuron reached the marginal zone. (E) Drawings of the mGFP+ neurons in the IZ and the cortical plate. Underlines of the numbers indicate the level of subplate. (F) An mGFP+ neuron observed in the IZ and extending long radial fiber which could be traced into the upper half of the cortical plate. (G-J) Transverse sections of the same neurons at four different levels indicated by arrows in (F). The mGFP+ radial process in F is a single-cell process and connected to the cell soma. (K) Radial migration of the mGFP+ cells observed for 2 h in a living slice. Lines in the figure connect the same cells in K, L, and M. (L) mGFP+ structures in a section obtained from the brain slice and visualized by immunoperoxidase technique. (M) Traced drawing of the GFP+ cells in the same section. Calibration bars in A and K are 50 μm, those in B, C, D, and F are 10 μm, those in G, H, I and J are 1 μm, that in the inset of A is 5 μm, and that in E is 100 μm.

After production in the VZ, the postmitotic neurons developed with a change in their morphology as they migrate radially. We observed that a significant number of multipolar cells had a growth cone at the tip of one of their lateral processes (inset of Fig. 3A) and developed an axon. From this point, we could regard the multipolar cells as neurons based on the possession of an axon. The axons projected subcortically or medially as those of layer V pyramidal cells in a similar stage did (Schwartz et al., 1991). At the restart of radial migration from the SVZ, a significant number of multipolar cells lost short lateral processes except for an axon and a long radial process. As the neurons migrated radially toward the cortical surface, the proximal part of the radial process grew thick and the distal part of the process became thinner (Fig. 3A–E). The radial processes of three mGFP+ neurons with the somata in the IZ were examined by electron microscopy. The cell shown in Fig. 3F was serially resectioned in the transverse direction and examined if the labeled radial process belonged to the cell itself or to another radial glia. At all points (Fig. 3G–J), we confirmed that the radial process is derived from a single process that is connected to the cell soma in the IZ. Thus, we concluded that a significant number of neurons in the IZ maintain their own radial process, which is attached to the pial surface (Fig. 3E), as shown in earlier studies (Ramon y Cajal, 1911; Berry and Rogers, 1965; Morest, 1970; Book and Morest, 1990).

The radial migration of the neurons in the IZ was examined using cultured slices. mGFP+ neurons with long radial processes in the IZ were observed based on its fluorescence. Several mGFP+ cells in the IZ were investigated at 30-min intervals for 2 h (Fig. 3K). The mGFP+ cells in the IZ migrated radially at a speed of 10 μm/h (cells 1–3), while the mGFP+ cells in the SVZ remained at the same place throughout the observation period (cells 4 and 5). After the observation in vitro, the brain slice was fixed and resectioned in order to detect mGFP by the immunoperoxidase technique (Fig. 3L and M). The radially migrating cells (1–3) had a radial process almost penetrating the cortical plate similar to the cell shown in Fig. 1M.

4. Discussion

Development of mGFP+ radial cells into radial glias and production of spherical cells by cell division of radial glias suggested that spherical cells, radial cells, and radial glias together constituted a proliferating cell cycle in the VZ (Fig. 4). Furthermore, the present data strongly suggested that radial glias are progenitors of neocortical neurons. Radial glias have long been considered to be nonproliferating supporting cells that serve as a guide for migrating neurons (Rakic, 1972, 1988, 1990). However, a growing body of evidence has recently indicated that radial glias are neural stem cells or cells in a phase of the neural stem cell cycle (Chanas-Sacre et al., 2000; Malatesta et al., 2000; Hartfuss et al., 2001; Noctor et al., 2001). In the adult avian brain, the region filled with radial cells corresponded to the site of adult neurogenesis (Alvarez-Buylla et al., 1990). Lineage analysis of the chick tectum and neocortex with retrovirus infection revealed that clusters of labeled cells contained a single radial glia and progeny of other cell types (Gray and Sanes, 1992; Noctor et al., 2001). Activation of Notch1 signaling, which expands the progenitor pool, in the neuroepithelium induced the formation of radial glias (Gaiano et al., 2000). Furthermore, radial glias which were revealed by immunohistochemistry of phosphorylated vimentin (Kamei et al., 1998) and DiI labeling (Miyata et al., 2000) showed cell division under in vivo and in vitro conditions, respectively. It is well known that radial glias produce astrocytes at late embryonic stages (Voigt, 1989). Collectively, these data indicate that the radial glia is the neural stem cell in the telencephalon. The proliferation of cells with a long process does not seem unusual, since contact with the basement membrane (the pia mater in the brain) is a common feature of epithelial cells including neuroepithelial cells.

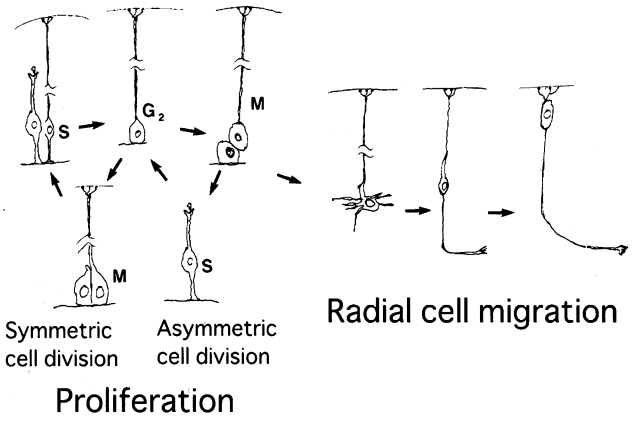

Fig. 4. A schematic diagram showing the proliferative cell cycle of the neural stem cell and the radial migration of postmitotic neurons.

It was reported that radial migration of postmitotic cells paused for about 2 months in the SVZ of the developing monkey brain after pulse labeling of the cells with ^{3}H-thymidine, and that the cells were considered to be radial glias (Schmechel and Rakic, 1979). Recently, in murine embryos, the pause of migration in the SVZ and IZ was studied extensively and the relatively quiescent cells were described as the population in the transitional field (Bayer and Altman, 1991) or as the slowly migrating subpopulation (Takahashi et al., 1996a). Multipolar cells that were found in the SVZ and stayed there for 3 days in the present study might correspond to the subpopulation. The present results further indicated that the cell population pausing in the SVZ is derived from radial glias, and that the cell migration continues after the pause to grow into neurons. Thus, it is an interesting idea that the neuronal precursors stays for a given period in the SVZ and take a multipolar form to look for the way where they project the axons.

Once we accept the idea that radial glias divide with a cleavage angle parallel to the ventricular surface to produce neuronal precursors (Fig. 4), we should reconsider the mechanism for the radial migration of neuronal precursors. The present study revealed that a long radial process connected to the pia mater is inherited by the daughter cell on the pia side during radial glial cell division. According to the study by Chenn and McConnell (1995), the daughter cell on the pia side becomes a postmitotic neuron and migrates radially. At this point, the most reasonable interpretation will be that all the postmitotic neurons have a radial process attached to the pia mater during their production. Such a retained radial process in cell division and in migration was reported earlier (Berry and Rogers, 1965; Morest, 1970; Book and Morest, 1990). However, recent studies investigating radially migrating neurons using retrovirus or organic dyes did not clarify this point. Noctor et al. (2001) put forth that the radial process is inherited by a daughter cell on the ventricular side in radial glial cell division. However, the figure they used to derive this conclusion was not clear

enough to judge which daughter cell inherited the radial process. Nadarajah et al. (2001) observed that radially migrating neurons have radial processes reaching the pia mater. They put forth that the radial process is made by extending a leading process from the SZ after the production of postmitotic neurons. However, they did not provide any evidence that the postmitotic neurons extend leading processes to span entire cortical layers.

The proximal part of the radial processes became thick as their somata approached the pia mater (Fig. 3F), suggesting some retraction mechanism of the processes. Even if migrating neurons use nearby radial processes as a scaffold for their migration (Rakic, 1972, 1988, 1990), we hereby propose that a significant number of radially migrating neurons use their own radial processes for guidance.

Using our recombinant adenovirus at different embryonic stages, many phenomena in the developing neocortex, such as the cell cycle of neural stem cells and migration of postmitotic neurons, will be further elucidated.

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