

neuronal migration, radial migration from corresponding neuroepithelium and tangential migration of cells of separate other origin. The combination of the two types of neuronal migration elaborates the complex and well-organized cortical structures, such as the cortical structure of the neocortex (Rakic, 1972; Anderson et al., 1997; Marin and Rubenstein, 2003), hippocampus (Altman and Bayer, 1990a–c), cerebellum (Altman and Bayer, 1985; Hatten and Heintz, 1995), and olfactory bulb (Hinds, 1968; Kishi, 1987).

Previous studies of hippocampal development have shown that two separate sites of origin generate the neurons for its two different cortical structures, Ammon's horn and the dentate gyrus, via different migration routes. The pyramidal neurons in CA1-CA3 are generated by an extensive area of neuroepithelium and migrate radially to the Ammon's horn, while dentate granule cells are generated by a narrow area of neuroepithelium adjacent to the fimbria (FI) and migrate tangentially through the subpial area to form the C-shaped cortical structure (Altman and Bayer, 1990a–c; Bagri et al., 2002). The radial glial arrangement in the developing hippocampus has also been investigated as a crucial substrate for neuronal migration (Rickmann et al., 1987), and the cell migration involved in the hippocampal cortical layer formation has been analyzed by a variety of methods, such as Golgi staining (Stensaas, 1967a–e; Nowakowski and Rakic, 1979; Eckenhoff and Rakic, 1984), labeling the nuclei of migrating cells with [³H] thymidine (Stanfield and Cowan, 1979; Nowakowski and Rakic, 1981; Altman and Bayer, 1990a–c; Reznikov, 1991), and retrovirus vector labeling (Bagri et al., 2002). However, the precise morphology of the migratory neurons and their interrelation with the radial glial arrangement are not adequately understood because there was no method of labeling neuronal precursors at specified times and sites and concomitantly visualizing the morphology of the labeled cells.

The in utero electroporation method enables highly efficient locally and temporally defined introduction of a marker gene into ventricular surface cells in order to characterize the morphology of various phases of migration and the phenotype of the migrating cells (Inoue and Krumlauf, 2001; Tabata and Nakajima, 2001; Saito and Nakatsuji, 2001), and it has been successfully applied to the study of neuronal generation and migration during the development of the cerebral neocortex (Tabata and Nakajima, 2001, 2003; Bai et al., 2003; Kawauchi et al., 2003). Neuronal precursor cells on the ventricular surface should

be intensely labeled by transfer of the marker gene in the final mitotic period. After electroporation, the embryos continue to develop normally, and it is possible to analyze the subsequent process of development in vivo. In the present study, the morphology of the migratory neurons and their interrelations with the radial glial arrangement in the mouse hippocampal primordium were investigated by labeling the neuronal precursor cells with EGFP-expression vector at specified stages of development.

MATERIALS AND METHODS

Animals

ICR strain mice were purchased from CLEA Japan (Tokyo, Japan). The day of confirmation of vaginal plug was defined as embryonic day zero (E0), and the day of birth was defined as postnatal day zero (P0). At least five animals were used in each experiment. All animal experiments were conducted according to the *Guide for Care and Use of Laboratory Animals* (1996, National Academy of Sciences, USA). All procedures in the animal experiments in this study were approved by the Animal Care Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry.

Plasmids

Expression vector pCX-EGFP (Niwa et al., 1991), which contains EGFP cDNA under the control of the CMV enhancer and chick β -actin promoter, was provided by Dr. J. Miyazaki (Division of Stem Cell Regulation Research, Osaka University Medical School, Osaka, Japan).

In utero DNA transfer by electroporation

Plasmid DNA was purified with a CONCERT plasmid maxi kit (Invitrogen, Carlsbad, CA) and dissolved in 1 mM Tris-HCl and 0.1 mM EDTA (pH 8.0) to a concentration of 3–4 μ g/ μ l. The DNA solution also contained 0.05% Fast Green to monitor the injection. Pregnant mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg body weight, Dainippon Pharmaceutical, Osaka, Japan). The uterine horns were exposed and ~2–4 μ l of DNA solution was injected through the uterus into the lateral ventricle of the embryonic forebrain with a glass micropipette (type G-1, Narishige, Tokyo, Japan). After the injection the embryo in the uterus was placed between the electrodes of an electroporator (CUY21, NEPA GENE, Chiba, Japan), and six 50-msec pulses of 35–38 volts were delivered at 75-msec intervals. The uterus was placed back into the abdominal cavity to allow embryonic development to continue. When pCX-EGFP was transfected at E14, and the examination was carried out at E16, this set of conditions was recorded as EGFP/E14:E16 in this study.

Observation of EGFP labeling and immunostaining

The pregnant mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital and the embryos were removed by cesarean section. The embryos were fixed by transcardiac perfusion with 4% paraformaldehyde (PFA) dissolved in 0.1 M sodium phosphate buffer (PB, pH 7.4), and postfixed overnight at 4°C with the same fixative. Brains were dissected out, embedded in 3% agar in phosphate-buffered saline (PBS), and sliced coronally

Abbreviations

AH	Ammon's horn
CP	cortical plate
DG	dentate gyrus
DGE	external limb of dentate gyrus
DGI	internal limb of dentate gyrus
DGM	dentate migration
FI	fimbria
HP	hippocampal plate
IMZ	intermediate zone
LV	lateral ventricle
MZ	marginal zone
NC	neocortex
PS	pial surface
SVZ	subventricular zone
VZ	ventricular zone

with a Microslicer (DTK-3000, DOSAKA EM, Kyoto, Japan) into 200- μ m sections. The sections were coverslipped with 80% glycerol and fluorescence images were obtained directly with a confocal laser-scanning microscope (LSM5 Pascal, Zeiss, Oberkochen, Germany).

For immunostaining, the free-floating sections were incubated with the following primary antibodies: mouse anti-MAP-2 monoclonal antibody (HM-2, 1:2,000 dilution, Sigma, St. Louis, MO), mouse anti-neuronal class III β -tubulin monoclonal antibody (TuJ1, 1:2,000 dilution, COVANCE, Berkeley, CA), rabbit anti-gial fibrillary acidic protein (GFAP) polyclonal antibody (1:500 dilution, DAKO, Glostrup, Denmark), mouse anti-nestin monoclonal antibody (Rat-401; Hockfield and McKay, 1985; 1:200 dilution, Developmental Studies Hybridoma Bank, Iowa City, IA), Alexa Fluor-labeled secondary antibodies (1:500 dilution, Molecular Probes, Eugene, OR) were used to detect antigen localization. For double immunostaining for NeuroD and green fluorescent protein (GFP), the sections were treated with 2N-HCl in 0.9% NaCl at room temperature for 1 hour and then washed with PBS five times. Next, the sections were incubated at 4°C overnight with goat polyclonal anti-NeuroD (N-19, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-GFP (1:200 dilution, Molecular Probes). After rinsing with PBS, the sections were incubated at room temperature for 2 hours with Alexa 488-conjugated donkey anti-rabbit IgG (1:500 dilution, Molecular Probes) and Alexa 594-conjugated donkey anti-goat IgG (1:500 dilution, Molecular Probes). The stained sections were mounted on glass slides with 80% glycerol and the sections were examined with a confocal laser-scanning microscope (LSM5 Pascal).

Photodocumentation

Z-series of confocal images of the sections were assembled as a single image with the LSM5 software program. All digital files were imported into Adobe PhotoShop 5.5 (San Jose, CA). After adjustment of contrast and brightness, montages of images were constructed.

Cell counts

The number of labeled cells tended to vary widely from animal to animal, probably because of variability in gene transfection efficiency in the ventricular zone (VZ). All EGFP-labeled cells from the VZ to the cortical plate (CP) were counted to obtain the total cell number. At least 200 EGFP-labeled cells per brain were counted as the total cell number. This approach yielded reproducible percentages of labeled multipolar cells and pyramidal neurons in each animal under the same conditions.

RESULTS

Ammonic pyramidal cell migration

Since previous studies by [3 H] thymidine labeling had shown that most pyramidal cells in Ammon's horn are generated around E14 (Angevine, 1965), pCX-EGFP was transfected into the neuroepithelium corresponding to Ammon's horn at E14 in the lateromedial direction, as shown in Figure 1a, and the subsequent migration and differentiation of labeled cells were observed. In 2 days, at E16, EGFP-labeled cells were observed in the VZ, subventricular zone (SVZ), and intermediate zone (IMZ) of the

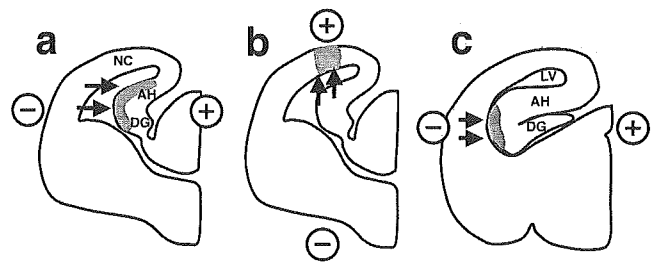


Fig. 1. The relationship between the region of vector transfection by in utero electroporation and the position of the electrodes. The arrows show the direction of the electric current. Plasmid DNA was introduced into the restricted neuroepithelium shown in gray. **a:** The Ammonic neuroepithelium was labeled at E14 in the lateral-to-medial direction. **b:** The neocortical neuroepithelium was labeled at E14 in the ventral-to-dorsal direction. **c:** The primary dentate matrix was labeled at E16 in the lateral-to-medial direction. In all photomicrographs of coronal sections, top is dorsal and left is lateral.

Ammonic primordium (Fig. 2a,e). In the VZ, the cells were elliptic in shape and one end was exposed to the lateral ventricle (Fig. 2e). Most of the labeled cells that had detached from the VZ displayed multipolar morphology with fine processes; that is, had become so-called "multipolar cells" (Tabata and Nakajima, 2003) (Fig. 2e). At E17, some of the multipolar cells in the IMZ had extended processes and had started to differentiate into pyramidal cells (Fig. 2b,f). At E18, spindle-shaped pyramidal cells with branched apical dendrites began to form the incipient CA1 cortical layer, while considerable numbers of multipolar cells remained in the IMZ (Fig. 2c,g). At postnatal day 2 (P2), all the EGFP-labeled cells had long apical dendrites and were aligned in the pyramidal layer, and only small numbers of labeled cells were seen in the VZ (Fig. 2d,h).

To compare corticogenesis in Ammon's horn with corticogenesis in the neocortex, plasmid DNA was introduced into the neocortex in the ventrodorsal direction, as shown in Figure 1b. EGFP/E14:E16 brain is shown in Figure 2i. As reported by Tabata and Nakajima (2003), some of the EGFP-labeled pyramidal cells reached the CP within 2 days after labeling, and large numbers of multipolar cells remained in the SVZ and IMZ (Fig. 2i). In the EGFP/E14:E18 brain, most of the pyramidal cells were aligned in the CP and very few multipolar cells were seen in the SVZ and IMZ (Fig. 2j). By contrast, no EGFP-labeled cells were found within the CP of Ammon's horn at E16 (Fig. 2a,e), and considerable numbers of multipolar cells remained in the SVZ at E18 (Fig. 2g). It took 4 days for Ammonic pyramidal cells to reach the CP (Fig. 2g), in striking contrast to the ~2 days taken for neocortical pyramidal cells to reach the cortex (Fig. 2i).

The percentages of EGFP-labeled cells that were multipolar and pyramidal cells were calculated to analyze the course of morphological differentiation in the CA1 primordium labeled at E14 (Fig. 3). EGFP-positive multipolar cells gradually decreased as a percentage of EGFP-positive cells, and the percentage of EGFP-positive pyramidal cells increased inversely during the period from E16 to P2 (Fig. 3).

To examine the neuronal differentiation of EGFP-labeled cells in Ammon's horn, the hippocampal primordium of EGFP/E14:E18 brain was immunostained with the early-neuron marker TuJ1 or differentiated-neuron

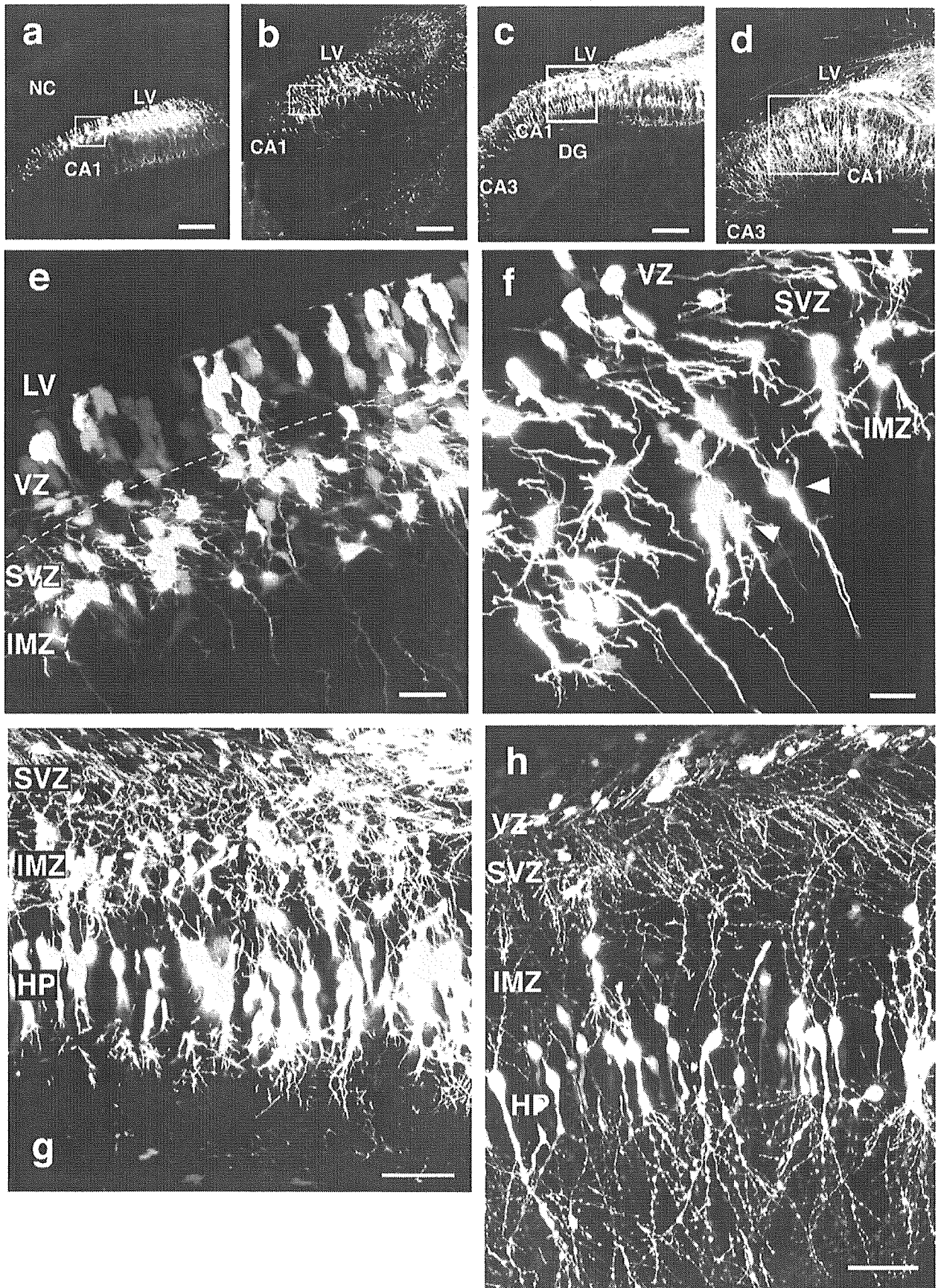


Figure 2 (Continued)

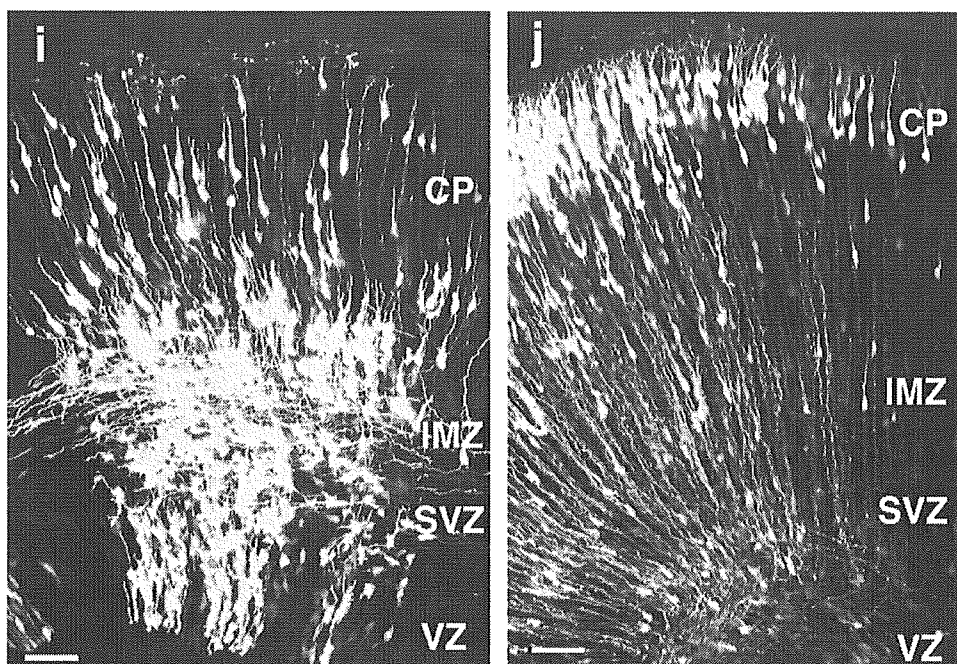


Fig. 2. Migration and differentiation of EGFP-labeled cells in the Ammonic CA1 and neocortical primordia. Coronal sections of the cerebrum transfected with pCX-EGFP at E14. Ammonic primordium (a-h); neocortical primordium (i,j). **a:** EGFP/E14:E16. Most of the EGFP-expressing cells were found in the VZ and SVZ. **b:** EGFP/E14:E17. The labeled cells were found as far as the IMZ. **c:** EGFP/E14:E18. CA1 cortical structure is labeled along with the subcortical layer. **d:** EGFP/E14:P2. Most of the EGFP-labeled cells have settled in the CA1 cortical plate. **e:** Higher magnification of the boxed area in (a). Both the ventricular neuroepithelium and the multipolar cells in the SVZ are labeled. The broken line represents the border between the VZ and SVZ. **f:** Higher magnification of the SVZ and IMZ in the boxed

area in (b). Elongated spindle shaped-cells (indicated by arrowheads) have appeared among the multipolar cells in the SVZ and IMZ. **g:** Higher magnification of IMZ and HP in the boxed area in (c). Pyramidal cells are arranged in the HP, while many multipolar cells remain in the IMZ. **h:** Higher magnification of the boxed area in (d). EGFP-labeled pyramidal cells are aligned in the CA1 cortex. **i:** EGFP/E14:E16. Some EGFP-labeled cells have already reached the neocortical plate 2 days after labeling. Multipolar cells are seen in the SVZ and IMZ. **j:** EGFP/E14:E18. Most of the EGFP-labeled cells have reached and became aligned in the neocortical plate. Scale bars = 200 μ m in a-d; 20 μ m in e,f; 50 μ m in g-j.

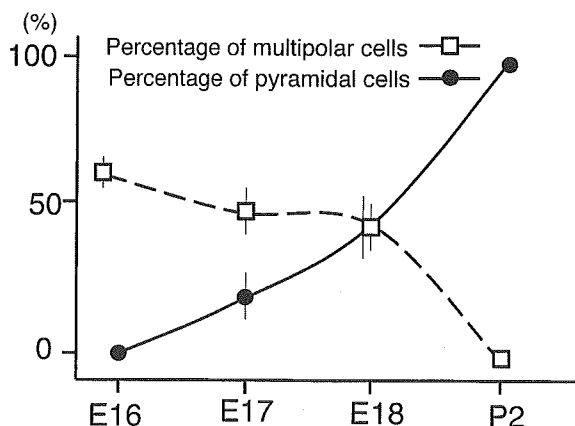


Fig. 3. Multipolar cells and pyramidal cells as a percentage of EGFP-labeled cells during the development of Ammonic CA1 primordium. The vector was transfected at E14 and subsequent changes in each subset as a percentage of all labeled cells were examined. The percentage of multipolar cells (open squares) decreased as the percentage of pyramidal cells (closed circles) increased. The number of cells in each subset is shown as a percentage of all EGFP-labeled cells along the ordinate, and the stage of development is indicated along the abscissa. Each value is a mean \pm SEM ($n = 4$).

marker MAP2. The apical dendrites of EGFP-labeled cells in the densely stratified pyramidal layer were positively immunostained with TuJ1 (Fig. 4a) and MAP2 (Fig. 4b), and the numerous processes of the multipolar cells were also positively immunostained with TuJ1 (Fig. 4c).

To examine the interrelationships between migrating neuronal precursors and radial glial fibers, the hippocampal primordium of EGFP/E14:E16 brain was immunostained with the radial glial marker nestin at E16. Although the processes of the multipolar cell extended independently of the radial fibers, the spindle-shaped cells in the VZ-IMZ were found to be arranged along the radial glial processes (Fig. 4d).

Granule cell migration to the dentate gyrus

Since previous studies have reported that [3 H] thymidine uptake by dentate granule cells of the mouse starts to become intense on E16 (Angevine, 1965), the generation and migration of dentate granule cells was investigated by transfecting pCX-EGFP into the embryonic brain in the lateromedial direction at E16, as shown in Figure 1c.

In the EGFP/E16:E18 brain, the migratory stream of the labeled cells was observed from the restricted area of the VZ adjacent to the fimbria (FI), e.g., the primary

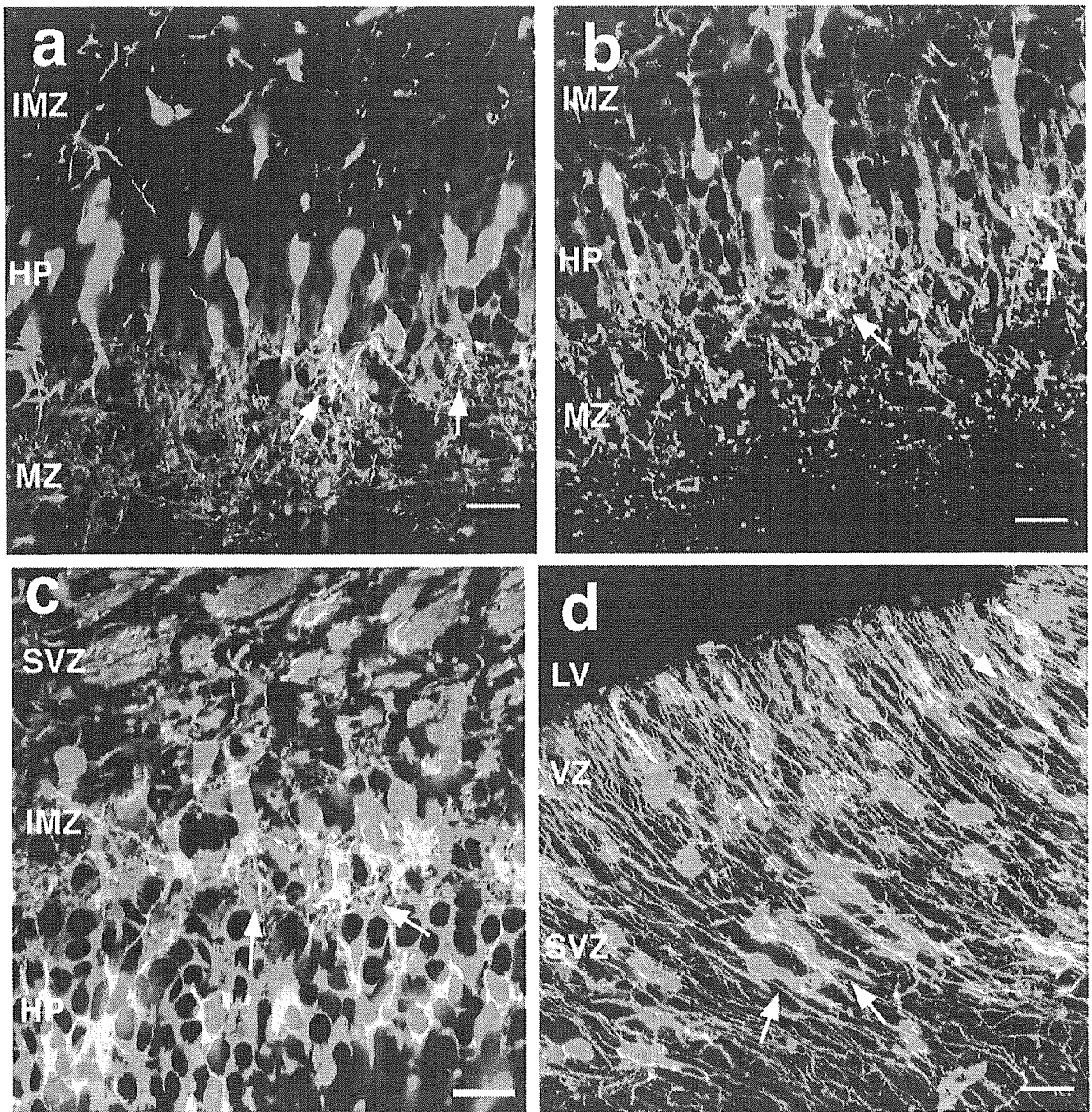


Fig. 4. Neuronal differentiation and neuron-glia interrelation in the Ammonic CA1 primordium. **a:** EGFP/E14:E18. The EGFP-labeled cells are aligned in the HP and exhibit pyramidal-cell morphology, and the neuronal marker TuJ1 (red) is colocalized with the EGFP-positive dendrites (green). **b:** EGFP/E14:E18. MAP2-immunoreactivity (red) is also colocalized with the EGFP-positive dendrites (green) in the pyramidal cells of HP. The arrows in a,b point to TuJ1-positive dendrites and MAP2-positive den-

drites, respectively. **c:** EGFP/E14:E18. EGFP-labeled multipolar cells (green) in the IMZ are also immunopositive for TuJ1 (red). The arrows point to TuJ1-positive processes. **d:** EGFP/E14:E16. The EGFP-labeled elongated bipolar cells (green) are arranged along the nestin-immunopositive radial fibers (red). By contrast, the processes of the EGFP-labeled multipolar cell show little association with radial glial processes. The arrows point to elongated bipolar cells. Scale bars = 20 μ m.

dentate matrix (Altman and Bayer, 1990c), and labeled cells were found on the migratory route through the subpial space (Fig. 5a). They migrated a long distance. The front of the stream had reached the vicinity of the dentate

gyrus at E18, but the labeled cells had not yet stratified (Fig. 5a). At this stage, Ammonic neuroepithelium was also labeled, but there were very few radially migrating cells from VZ to the cortex. Most of the labeled somata

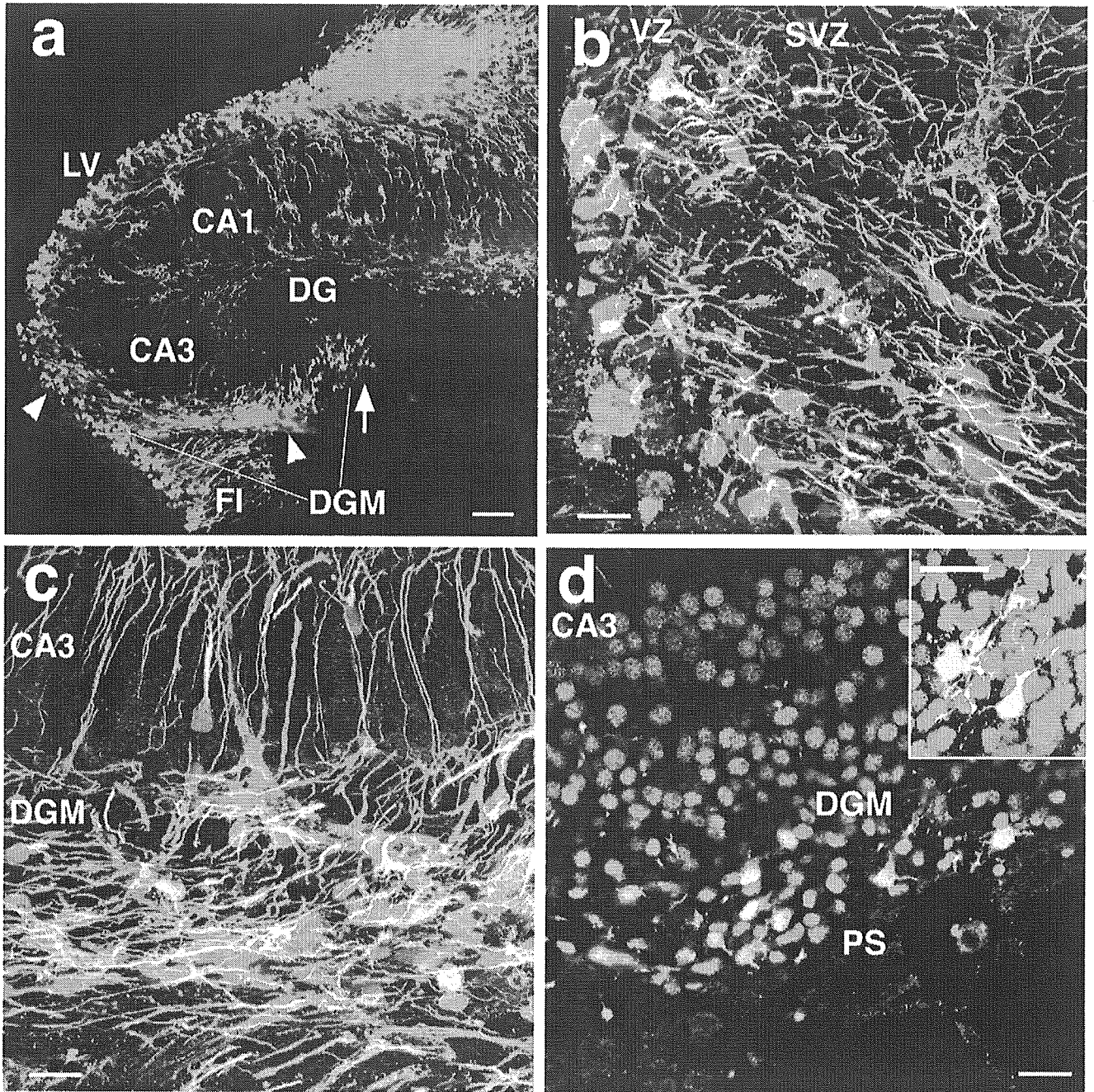


Fig. 5. Migration and differentiation of the granule cell precursors of the dentate gyrus. EGFP/E16:E18 brains were examined histologically. **a:** The stream of EGFP-labeled cells towards the dentate gyrus originated in the primary dentate matrix. The arrow indicates the front of the stream of migrating cells. **b:** Higher magnification of the primary dentate matrix corresponding to the region indicated by the white arrowhead in (a). In the VZ and SVZ, there was little association between the EGFP-labeled cells (green) and nestin-positive radial glial processes (red). **c:** Higher magnifi-

cation of the subpial stream corresponding to the region indicated by the yellow arrowhead in (a). EGFP-labeled migrating cells (green) are oriented along the tangentially arranged nestin-positive fibers (red). **d:** Double labeling of the subpial migratory stream with anti-EGFP antibody (green) and anti-NeuroD antibody (red). The nuclei of most of the EGFP-positive cells in the subpial migration were immunoreactive for NeuroD. They extended many processes as seen in the projection view of the confocal image in the inset. Scale bars = 100 μ m in a; 20 μ m in b-d, inset.

were found in the VZ and had extended long radial processes (Fig. 5a). By 5–6 days later (P1–2), dentate migration (DGM) had become stratified in the dentate gyrus (Fig. 6a).

The characteristics of dentate granule cell migration were examined at each step along the path of migration. In the initial stage of migration, most of the labeled cells destined to form the dentate gyrus rapidly detached from

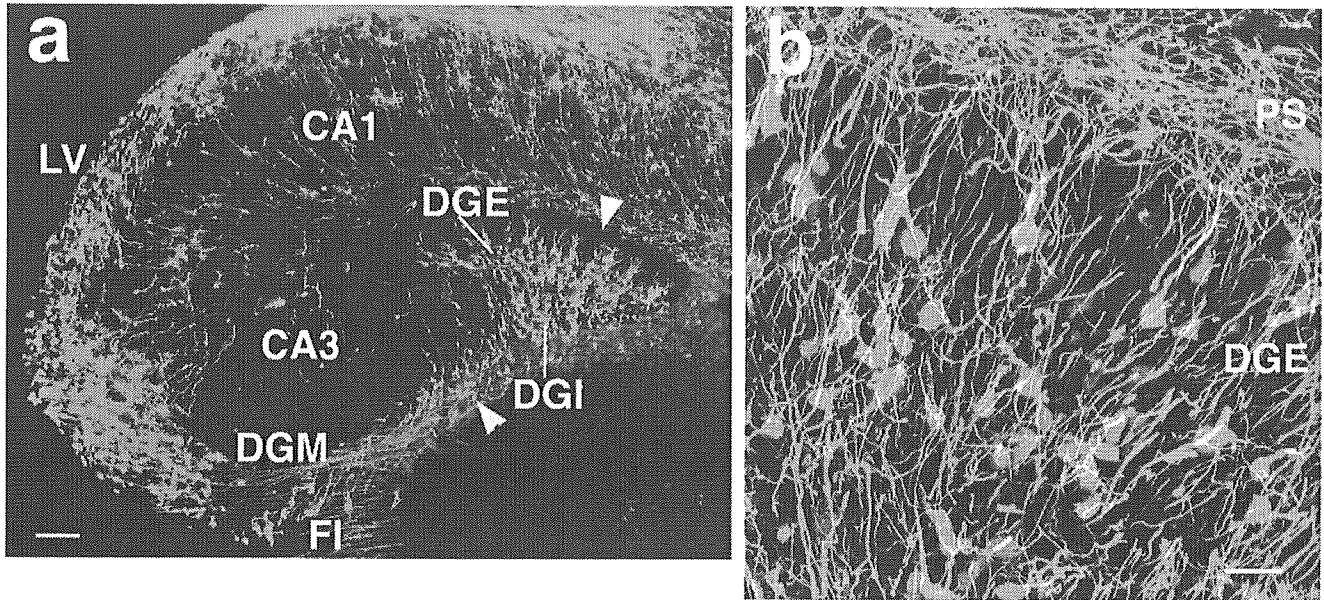


Fig. 6. Granule cell migration and stratification in the dentate gyrus. The EGFP/E16:P2 sections were analyzed. **a:** The migratory stream of EGFP-labeled cells in the subpial region is still evident (yellow arrowhead), and a large number of labeled cells have begun to stratify in the DGE (white arrowhead). **b:** Higher magnifica-

tion of the DGE indicated by the white arrowhead in (a). EGFP-labeled granule cells (green) migrating towards the dentate cortex are arranged radially and are closely associated with the GFAP-positive radial fibers (red). Scale bars = 100 μm in a; 20 μm in b.

the VZ and SVZ (Fig. 5a,b). They then entered into the tangentially migrating stream in the subpial space and they extended many processes (Fig. 5c,d). To examine the relationship between the migrating granule cells and radial glial fibers, the hippocampal primordium of EGFP/E16:E18 brain was immunostained with an anti-nestin antibody, a marker for radial glia. The arrangement of the glial processes in the VZ of the primary dentate matrix was not radial, and few of the glial process extended in the same direction as the path of the initial cell migration (Fig. 5b).

In the dentate-granule-cell subpial migration stage at E18, the stream was composed of EGFP-labeled cells, and its front had reached the forming dentate gyrus (Fig. 5a). The tangentially migrating cells entering the subpial area had fewer processes and formed a compact stream (Fig. 5c). At the entrance to the subpial region, the EGFP-labeled migratory cells were apposed to tangentially arranged glial processes that were immunopositive for nestin (Fig. 5c). Since NeuroD has been reported to be expressed in immature granule cells (Miyata et al., 1999; Pleasure et al., 2000), double immunostaining with anti-GFP antibody and anti-NeuroD antibody was performed to identify the tangentially migrating cells as immature granule cells. Most of the tangentially migrating EGFP-labeled cells were positive for NeuroD (Fig. 5d), and the tangentially migrating cells that were positive for EGFP and NeuroD had many thin processes (Fig. 5d, inset). Although we also performed immunostaining for NeuN and calretinin, which are also markers of differentiated granule cells, only a few migrating cells in the subpial area were positive at E18 (data not shown).

In the final stage of dentate granule cell migration, EGFP-labeled cells reached dentate gyrus in 5 days and became arranged in the cortical layer in EGFP/E16:P2 brain (Fig. 6a,b). Some of the labeled cells were still migrating tangentially in the subpial space (Fig. 6a). The relationship between the EGFP-labeled granule cells and radial fibers was investigated by immunostaining the dentate gyrus of EGFP/E16:P2 brain with anti-GFAP antibody as a marker of late-stage radial glia, and the result showed that the EGFP-labeled unipolar cells in the external limb of the dentate gyrus (DGE) were radially arranged in close apposition to the radial processes of the dentate unipolar astroglia (Fig. 6b).

Cell migration to CA3

Since adult CA3 neurons of the mouse were labeled when [^3H] thymidine was injected around E14 (Angevine, 1965), cell migration to the CA3 was studied by introducing the EGFP-expression vector into the hippocampal primordium at E14 in the lateromedial direction (Fig. 1a), and the embryos were dissected at E18. A large number of EGFP-labeled cells were found in the neuroepithelium between the CA1 primordium and the fimbria (FI) (Fig. 7a), and most of the cells migrating to the CA3 region exhibited multipolar morphology (Fig. 7b). Some of the CA3-forming cells had originated elsewhere. Examination of EGFP/E16:E18 brain showed that most of the EGFP-labeled cells had migrated tangentially in the subpial space (Fig. 7c), but that a small proportion of labeled cells with a single process had detached from the compact stream (Fig. 7c,d) and migrated along the radially arranged nestin-positive glial processes oriented toward CA3 (Fig. 7d).

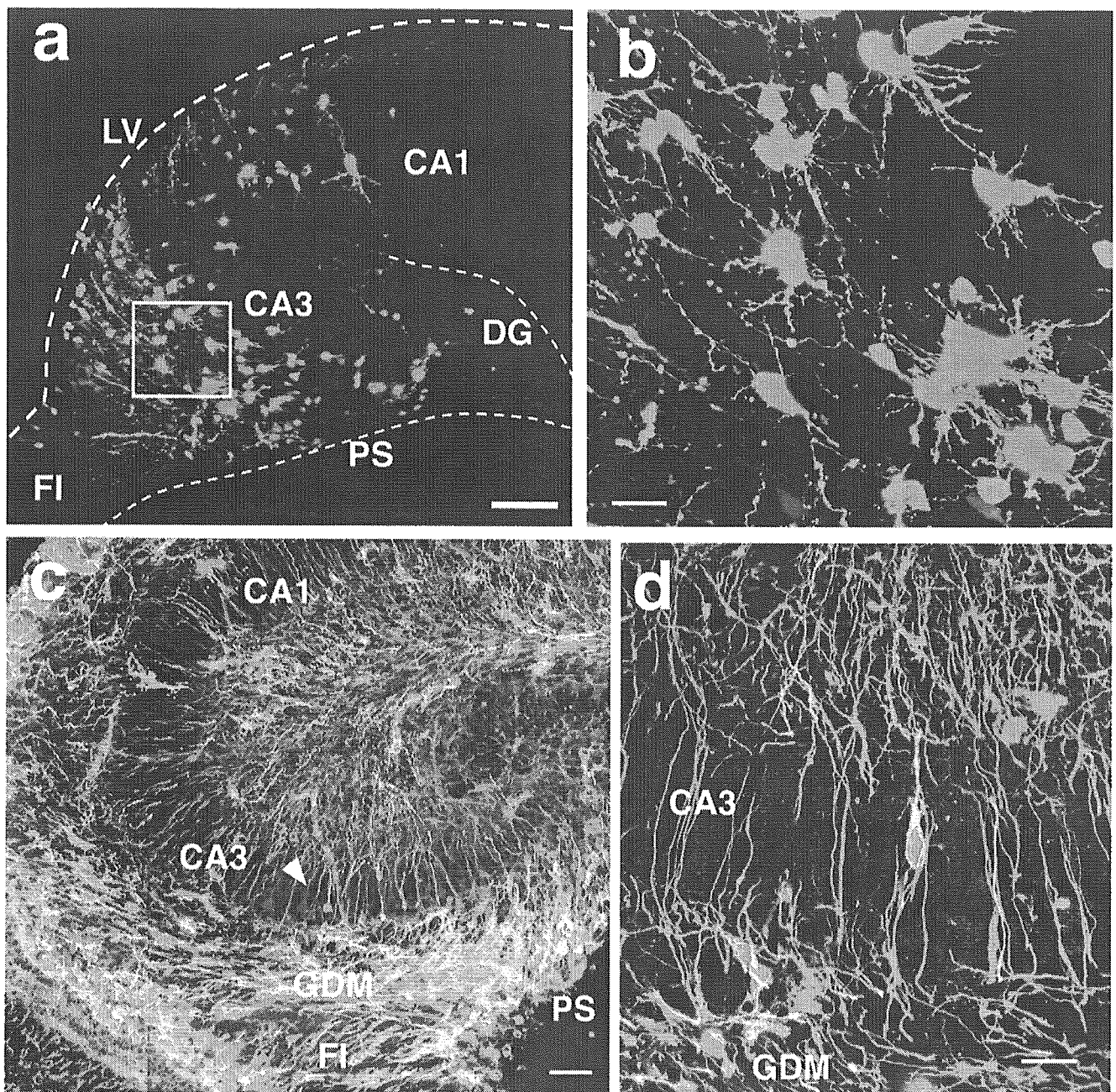


Fig. 7. Neuronal migration to the CA3. **a:** EGFP/E14:E18. Large numbers of EGFP-labeled cells were distributed from the VZ to the forming CA3. **b:** Higher magnification of the boxed area in (a). The labeled cells exhibit multipolar cell morphology. **c:** EGFP/E16:E18. The section was immunostained with anti-*nestin* antibody. EGFP-labeled cells (green) are seen arranged along the *nestin*-immunopositive radial

processes (red) in the CA3 cortical plate. Small numbers of EGFP-labeled cells are migrating radially towards the CA3 (arrowhead) from a large stream of EGFP-labeled cells tangentially migrating in the subpial region. **d:** Higher magnification of the region indicated by the arrowhead in (c). EGFP-labeled unipolar cells are closely associated with *nestin*-positive radial fibers. Scale bars = 100 μm in a; 20 μm in b,d; 50 μm in c.

DISCUSSION

The results of this study demonstrate the morphological basis of the migration and differentiation of hippocampal neurons during corticogenesis, as summarized in Figure 8. The neuronal precursors of Ammonic pyramidal cells and dentate granule cells were labeled with EGFP-expression vector at specified developmen-

tal stages. The formation of each cortical structure consisted of multistep mechanisms of migration and differentiation and different neuron-glia interrelationships. To our knowledge, this is the first report of a study in which hippocampal migratory neurons were morphologically analyzed by foreign gene transfer by means of in utero electroporation.

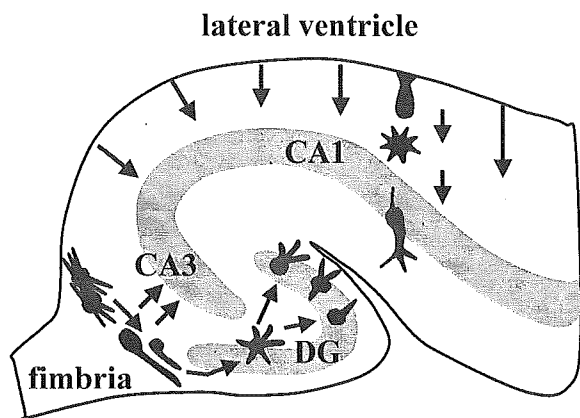


Fig. 8. Diagram summarizing the neuronal migration in the hippocampal primordium demonstrated in the present study. The pyramidal cells of CA1 and CA3 arose from an extensive area of neuroepithelium at E14 and changed the morphology during radial migration, as shown in the CA1 region. Dentate granule cells arose from the restricted VZ adjacent to the fimbria, migrated tangentially in the subpial area, and stratified in the dentate gyrus; their morphology also changed during migration. Some of the tangentially migrating cells changed their route and migrated toward CA3.

Migration and differentiation of pyramidal cells in the development of cortical structure of CA1

Previous studies by [^3H] thymidine labeling demonstrated the prolonged sojourn of immature pyramidal neurons along the migratory pathway towards the Ammonic cortex in the rat (Altman and Bayer, 1990b), and it took 4 days for the migratory pyramidal cells to reach the Ammonic cortical plate after the final mitosis in the VZ. Similar results were obtained in the mouse Ammonic primordium when the pyramidal cell precursors were labeled with EGFP-expression vector at E14 in the present study. The labeled neurons reached the Ammonic plate at E18 and exhibited the morphology of differentiated pyramidal cells. Since the final mitosis of CA1 pyramidal cells in the mouse occurs during E13–15 (Angevine, 1965), the intensely labeled population should have completed their final mitosis around the time of labeling.

The remarkable finding in the present study was the transient appearance of large numbers of EGFP-labeled multipolar cells in the SVZ and IMZ during E16–18 (Fig. 2e–g). Their morphology has been described in the hippocampal primordium of the rabbit (Stensaas, 1967a,b) and monkey (Nowakowski and Rakic, 1979) by the Golgi method, but their significance has not been adequately discussed. The presence of the multipolar cells in the SVZ and IMZ has also been reported in neocortical development (Noctor et al., 2001, 2004; Tabata and Nakajima, 2003). In our study, the multipolar cells accounted for 30.1% of the EGFP-labeled cells in the neocortical primordium of the mouse at E16 (Fig. 2i). Their migratory pattern within the subcortical zone is nonradial and has been described as “multipolar migration” (Tabata and Nakajima, 2003), as opposed to radial locomotion or somal translocation. The multipolar cells migrate slowly, at about one-fifth the rate of the bipolar cells in the neocortex (Tabata and Nakajima, 2003; also Fig. 2i,j).

The multipolar cells in the hippocampal CA1 primordium displayed morphology very similar to that of the multipolar cells in the neocortical primordium. During E16–18, large numbers of multipolar cells remained in the SVZ and IMZ, and there was little correlation between the processes of those multipolar cells and the radial glia arrangement (Fig. 4d), suggesting nonradial migration, the same as in the neocortex. By contrast, at E16 small numbers of elongated bipolar cells were found in the subcortical zone and were arranged in parallel with nestin-immunopositive radial glial processes (Fig. 4d). The number of multipolar cells had decreased significantly by E18, and they had disappeared by P2. During the same period, pyramidal cells appeared and increased in the Ammonic plate of CA1 (Fig. 3). These findings suggest that multipolar cells transdifferentiate into pyramidal neurons during migration to the Ammonic plate, the same as in the neocortex (Noctor et al., 2004).

The average migration rate of the pyramidal precursors during CA1 development (4–5 days) was much slower than that during neocortical development (1–2 days). In the hippocampal primordium, multipolar cells make up most of the population of subcortical precursors of the Ammonic plate ($57.1 \pm 3.3\%$; Fig. 3). The prolonged sojourn of pyramidal cells is thought to at least partly be due to their differentiation through the slowly migrating multipolar cells. The molecular basis that underlies the difference in migratory rate between bipolar cells and multipolar cells is not known at present. Reelin, a large extracellular protein that controls neuronal migration (D’Arcangelo et al., 1995; Gilmore and Herrup, 2000; Rice and Curran, 2001; Luque et al., 2003) and exhibits an inhibitory action on the radial migration of cortical neurons (Dulabon et al., 2000), may be one of the molecules responsible for the retention of CA1 neurons. Since the number of Cajal-Retzius cells secreting Reelin in the marginal zone is significantly larger in the hippocampus than in the neocortex (Soriano et al., 1994), the higher production of Reelin (Nakajima et al., 1997; Alcantara et al., 1998) may diminish the migratory speed of CA1 neurons to a larger extent than the migratory speed of neocortical neurons. The retention of CA1 neurons may be significant in terms of the specification of axonal patterns. The later growth of afferent axons into the hippocampal primordium at E17–18 (Super and Soriano, 1994; Soriano et al., 1994) is well coordinated with the late stratification and dendrite growth of their target CA1 pyramidal neurons. The finding that afferent fiber segregation in the CA1 depends on the position of the postsynaptic target neurons supports the above idea (Deller et al., 1999).

Granule cell migration and differentiation

The granule cells generated by the dentate neuroepithelium (primary dentate matrix) in the embryonic stage are thought to migrate out and form the secondary dentate matrix as they migrate tangentially. The tertiary dentate matrix subsequently forms the deep layer of the dentate gyrus, and neurogenesis continues to adulthood in the hilus (Altman and Bayer, 1990c). Although dentate granule cells continue to proliferate in adulthood, heavy labeling of dentate granule cells in the adult hippocampus can be obtained by [^3H] thymidine administration from E16 onward in the mouse (Angevine, 1965). These previous findings indicate that a large portion of dentate granule cells complete their final mitosis at sites ranging from the primary dentate matrix to the tertiary matrix on E16 or thereafter. In the present study, labeling of migratory

granule cells by the transfection of EGFP-expression vector into the VZ was also possible on E16 onward. Labeling the VZ on E16 should have labeled a cohort of granule cells that completed their final mitosis just around the time of transfection. A previous study demonstrated that the granule cells generated during the prenatal period form the outside shell of the granular layer in an outside-in pattern (Altman and Bayer, 1990c), a finding that is highly consistent with the finding in our own study that only the outer layer of the dentate gyrus was labeled by transfection of EGFP-expression vector at E16 (Fig. 6a,b).

Little information has ever been obtained about the morphology of the granule cells that migrate to the dentate gyrus, especially in the embryonic stage. The EGFP labeling by in utero electroporation allowed at least a subset of the granule cells generated in the prenatal period to be morphologically analyzed. The process of migration observed in the present study could be divided into three steps: 1) initial migration from the primary dentate matrix, 2) subpial migration through the secondary dentate matrix, and 3) stratification into the cortical structure of the dentate gyrus.

In the primary dentate matrix, labeled immature granule cells detach from the VZ and migrate without apparent guidance by glial processes (Fig. 5b). These immature neurons extend many short processes in various directions. When they enter the subpial migratory stream, they display various morphologies, including bipolar cell (Fig. 5c) and multipolar cell morphology (Fig. 5d). Since NeuroD has been reported to be expressed in the dentate granule cells as early as the stage of detachment from the VZ (Pleasure et al., 2000), the subpial cells double-labeled by EGFP and NeuroD were considered to exhibit the morphology of migratory granule cells. The entire subpial stream of dentate granule cells followed the nestin-positive glial processes, which extend tangentially in the subpial space.

In the final stage of granule cell migration to the dentate gyrus, the arrangement of the EGFP-labeled granule cells was closely correlated with arrangement of the radial glial processes in the forming cortical structure of the dentate gyrus, especially in the outer cortical region (Fig. 6b). The migration of dentate granule cells was directed outward toward the pia mater, in contrast to the inward migration of cerebellar granule cells along the radial processes of Bergmann glia (Rakic, 1971; Komuro and Rakic, 1998). Thus, the final step of granule cell migration in the hippocampal dentate gyrus and cerebellar cortex is morphologically similar and the similarity is also corroborated by the morphological similarity between the unipolar astroglia in the dentate gyrus and the Bergmann glia in the cerebellum (Edwards et al., 1990). Moreover, the observation on guided migration of dentate granule cells along Bergmann glial processes in a culture system also suggested the involvement of common molecular mechanisms in cortical formation by cerebellar and dentate granule cells (Gasser and Hatten, 1990a,b).

Mutations in the genes involved in the Reelin signaling pathway (Stanfield and Cowan, 1979; Sheldon et al., 1997; Trommsdorf et al., 1999) induce the malformation of the unipolar astrocytes and disarrangement of granule cells in the dentate gyrus (Frotscher et al., 2003; Weiss et al., 2003). However, subpial migration is not very severely impaired, because it follows glial fibers but may not be very dependent on glial guidance. These findings are consistent with the different neuron-radial glia interrelations during granule cell migration observed in the present study.

Development of CA3 cortical structure

CA3 pyramidal neurons in the mouse have been reported to be generated during the E13–15 period, the same as CA1 neurons (Angevine, 1965). In the present study, a subset of migratory cells labeled with EGFP at E14 was found to originate from the neuroepithelium between CA1 and the dentate primordium, and a large proportion of the cells that migrated to CA3 also exhibited the morphology of multipolar cells in the SVZ and IMZ (Fig. 7b). The basic mechanism of CA3 corticogenesis is therefore thought to be similar to the mechanism in CA1.

However, a remarkable finding in CA3 corticogenesis was that another migratory stream that was labeled later, at E16, was also directed into CA3 during the period of dentate granule cell migration (Fig. 7c). Small numbers of EGFP-labeled cells detached from the migratory stream in the subpial area and migrated into the cortical layer of CA3 along the radial glia, which were arranged perpendicular to the subpial glial processes (Fig. 7d). These cells migrating into CA3 are thought to belong to the late-generated subpopulation of precursors of CA3 neurons.

The significance of the migratory population from the subpial stream to CA3 is unknown. The formation of CA3 is delayed, consistent with the delayed formation of the dentate gyrus (Altman and Bayer, 1990b), and the development of the mossy fiber projection from the dentate gyrus to CA3 may also be temporally coordinated in the neonatal stage to form the main intrahippocampal circuit (Gaarskjaer, 1985). It is tempting to think that the late-generated CA3 neurons migrate along the migratory route of the granule cells by using the same guiding cues.

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