Multimodal tangential migration of neocortical GABAergic neurons independent of GPI-anchored proteins

Daisuke Tanaka[1,*], Yohei Nakaya[1,*, †], Yuchio Yanagawa[2,3,4], Kunihiko Obata[2,3,4,†] and Fujio Murakami[1,4,5,‡]

[1]Graduate School of Frontier Biosciences, Osaka University, Machikaneyama 1-3, Toyonaka, Osaka 560-8531, Japan
[2]Laboratory of Neurochemistry, National Institute for Physiological Sciences, Myodaiji, Okazaki, 444-8585, Japan
[3]The Graduate University for Advanced Studies, Hayama, Kanagawa 240-0193, Japan
[4]SORST, Japan Science and Technology Corporation, Kawaguchi, 332-0012, Japan
[5]Division of Behavior and Neurobiology, Department of Regulation Biology, National Institute for Basic Biology, Myodaiji-cho, Okazaki 444-8585, Japan
*These author contributed equally to this work
†Present address: YN, Nippon Shinyaku. Co., Ltd, 14,Nishinosho-Monguchi-cho, Kisshoin, Minami-ku, Kyoto 601-8550, Japan; KO, BSI, Riken, Hirosawa 2-1, Wako, 351-0198, Japan
‡Author for correspondence (e-mail: murakami@fbs.osaka-u.ac.jp)

Summary

Neuronal migration is crucial for the construction of neuronal architecture such as layers and nuclei. Most inhibitory interneurons in the neocortex derive from the basal forebrain and migrate tangentially; however, little is known about the mode of migration of these neurons in the cortex. We used glutamate decarboxylase (Gad)67-green fluorescent protein (GFP) knock-in embryonic mice with expression of GFP in gamma-aminobutyric acid (GABA)-ergic neurons and performed time-lapse analysis. In coronal slices, many GFP-positive neurons in the lower intermediate zone (IZ) and subventricular zone (SVZ) showed robust tangential migration from lateral to medial cortex, while others showed radial and non-radial migration mostly towards the pial surface. In flat-mount preparations, GFP-positive neurons of the marginal zone (MZ) showed multidirectional tangential migration. Some of these neurons descended toward the cortical plate (CP). Intracortical migration of these neurons was largely unaffected by a treatment that cleaves glycosylphosphatidylinositol (GPI) anchors. These findings suggest that tangential migration of cortical interneurons from lateral to medial cortex predominantly occurs in the IZ/SVZ and raise the possibility that a part of the pial surface-directed neurons in the IZ/SVZ reach the MZ, whereby they spread into the whole area of the cortex. At least a part of these neurons may descend toward the CP. Our results also suggest that intracortical migration of GABAergic neurons occurs independent of GPI-anchored proteins.

Movies and supplemental data available online

Key words: Neuronal migration, GABAergic interneuron, Neocortex, Time-lapse analysis, PI-PLC, Gad67, GPI-anchored protein, Tag1

Introduction

During the development of the nervous system, neurons migrate from their sites of origin to their final destinations. This is crucial not only for the establishment of architecture such as layers and nuclei, but also for the development of neuronal heterogeneity. In the cerebral cortex, neurons derived from the ventricular zone (VZ) migrate radially to give rise to cortical projection neurons (Noctor et al., 2001; Hatanaka and Murakami, 2003). Recent experiments demonstrated that cells originating from the basal forebrain structure called ganglionic eminences (GEs) give rise to forebrain interneurons; those mainly from the medial ganglionic eminence (MGE) migrate tangentially towards the cortex to give rise to gamma-aminobutyric acid (GABA)-ergic interneurons (Anderson et al., 1999; Parnavelas, 2000; Corbin et al., 2001; Lambert de Rouvroit and Goffinet, 2001; Marín and Rubenstein, 2001; Nadarajah and Parnavelas, 2002). The lateral and caudal ganglionic eminences have also shown to be origins of cortical interneurons (Anderson et al., 2001; Nery et al., 2002).

A number of studies have attempted to provide a phenomenological description and to elucidate the mechanisms of the migration of GABAergic neurons from the GEs to the cortex. These include labeling of GE neurons with fluorescent dyes followed by in vitro culture (de Carlos et al., 1996; Tamamaki et al., 1997; Lavdas et al., 1999; Anderson et al., 2001; Jimenez et al., 2002; Nadarajah et al., 2002), analysis of transgenic animals lacking genes that affect GEs (Anderson et al., 1997; Casarosa et al., 1999; Sussel et al., 1999) and transplantation of tagged tissues (Anderson et al., 2001; Wichterle et al., 2001; Nery et al., 2002; Polleux et al., 2002). In vitro experiments suggested the possible involvement of repellent molecules such as slits (Zhu et al., 1999; Wichterle et al., 2003) and semaphorins (Marín et al., 2001; Tamamaki et al., 2003). Recently, an axonal surface molecule, Tag1 (previously TAG-1) expressed by corticofugal axons, has been suggested to serve as a substrate for the migration of GE-derived cortical neurons (Denaxa et al., 2001). Moreover, it has also been shown that hepatocyte growth factor (HGF) can act

as a motogen for these neurons (Powell et al., 2001). It appears that cues in the cortex also contribute to the migration of these neurons (Marín et al., 2003).

After entering the neocortex, GE-derived GABAergic neurons are thought to distribute over the entire cortex, while the remainder continue migration to the hippocampus (Pleasure et al., 2000). However, little is known about the migration of these neurons within the cortex. To understand how the GE-derived neurons are sorted in the cortex to distribute as cortical interneurons, we visualized intracortical migration of GE-derived neurons, using glutamate decarboxylase (GAD) 67-green fluorescent protein (GFP) knock-in mice, in which GFP is specifically expressed in GABAergic neurons. This transgenic animal has allowed us to analyze systematically the distribution of cortical GABAergic neurons in fixed sections and the dynamics of the migration of these neurons in acute slices as well as flat-mount preparations of the cortex, respectively. We report that cortical GABAergic neurons exhibit several modes of migration: ordered migration in a ventrolateral-to-dorsomedial direction along the lower IZ and the subventricular zone (SVZ); radial and non-radial migration towards the pial surface; multidirectional migration in the tangential plane of the marginal zone (MZ); and radial migration from the MZ to the cortical plate (CP). Intracortical migration of GABAergic neurons was not affected after treatment by an enzyme that cleaves glycosylphosphatidylinositol (GPI) anchors, suggesting that substrates other than GPI-anchored proteins regulate intracortical migration of these neurons. We propose a model that would explain the significance of multimodal migration of cortical GABAergic neurons.

Materials and methods

The generation of Gad67-GFP knock-in mice will be described elsewhere (K. Kaneko et al., unpublished). In brief, a cDNA encoding enhanced GFP (EGFP; Clonetech, Palo Alto, CA) was targeted to the locus encoding the gene *Gad67* (*Gad1* – Mouse Genome Informatics) using homologous recombination. Gad67 is a rate-limiting enzyme of GABA biosynthesis and is known to be a marker for GABAergic neurons. We focused on Gad67, because it predominantly regulates the production of GABA at embryonic stages (Asada et al., 1997). Timed pregnant mice were used [noon of the day of vaginal plug detection was termed as embryonic day (E) 0.5]. Mice heterozygous for the Gad67-GFP allele were mated with ICR wild-type mice or intercrossed to heterozygous mice to obtain heterozygous (Gad67$^{\text{gfp/+}}$) or homozygous (Gad67$^{\text{gfp/gfp}}$) strains, respectively. GFP transgenic mice could be recognized by their fluorescence under a dissecting microscope with epifluorescence illumination. Polymerase chain reaction (PCR) amplification was carried out to discriminate homozygous from heterozygous individuals.

All studies were carried out in accordance with the guidelines of the Animal Studies Committee of Osaka University.

Observation of fixed brains

To visualize GFP$^+$ cells in fixed tissues, embryos at E12.5, 13, 13.5 or 15.5 either heterozygous or homozygous for the Gad67-GFP allele were killed, decapitated and the brains were dissected out. The brains were fixed in 4% paraformaldehyde from 6 hours to overnight at 4°C and transferred to phosphate buffer (0.1 M, pH7.4) or phosphate-buffered saline. They were then mounted onto a stage of a vibrating microtome (VT-1000, Leica Microsystems, Tokyo, Japan) and sectioned coronally at 50 μm. The sections were transferred to slides

and coverslipped. Images were then captured from either side of the cortex using a CCD camera (Axiocam, Zeiss, Jena, Germany) attached to an epifluorescence microscope (BX-60, Olympus, Tokyo, Japan) and stored in a hard disk. Mirror images of captured files were created using Adobe PhotoShop software (ver. 6.0) as necessary.

To determine the zone of neuronal migration, Nissl staining with Methylene Blue was performed on adjacent sections.

In order to immunostain for GABA, anti-GABA rabbit polyclonal antibody (1/2000 dilution, Sigma, St Louis, MO) was applied, for 2 hours at room temperature, to 10 μm cryostat sections obtained from heterozygous mice embryos fixed in a fixative containing 0.1% glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid in phosphate buffer (0.1 M, pH 7.4). Thereafter, Cy3-conjugated anti rabbit IgG (1/300 dilution, Jackson ImmunoRes, West Grove, PA) was applied for 1 hour at room temperature. For Tag1 staining, supernatant of 4D7 monoclonal antibody (a gift from Dr Miyuki Yamamoto) was applied to 20 μm cryostat sections followed by Cy3-conjugated anti mouse IgM (1/500 dilution, Jackson ImmunoRes). To immunostain for microtubule-associated protein 2 (MAP2), an anti-MAP2 mouse monoclonal antibody (HM-2, Sigma) was applied at 1:500 dilution for 3 hours at RT to 12 μm cryostat sections obtained. This was followed by incubation in Cy3-conjugated anti rabbit IgG (1/500 dilution, Jackson ImmunoRes) for 1 hour at room temperature. Heterozygous mice embryos fixed in 4% paraformaldehyde were used for Tag1 and MAP2 staining.

Time-lapse imaging in coronal slices

Brains of E13.5 or E15.5 mouse embryos either heterozygous or homozygous for the Gad67-GFP allele were embedded in 4% low melting-point agarose. Coronal slices were then cut at 250 μm, using a vibrating microtome. Slices were selected from the anterior half of the cerebral hemispheres, at the level that includes the lateral and medial ganglionic eminences (LGE and MGE, respectively), and mounted on membrane inserts (Millicell-CM Low Height Culture Plate Inserts, Millipore, Bedford, MA). The membrane was coated with poly-L-lysine (1 μg/ml) prior to tissue mounting. Brain slices were covered with a thin layer of collagen gel and soaked in 5% or 10% fetal bovine serum in Hanks' solution (Nissui, Tokyo) supplemented with D-glucose (6 mg/ml) and streptomycin (Sigma, 20 mg/l). In some cases, insulin (10 μg/ml) and transferrin (100 μg/ml) were added to the solution. A coverslip was placed over the Millicell insert to prevent evaporation, and the preparation was transferred to a temperature-controlled (36-38°C) plastic chamber fitted onto a confocal microscope stage (MRC-1024, Biorad, Hercules, CA). GFP$^+$ cells were viewed through an objective (×20, N.A.=0.4 each) of an upright light microscope (BX-50, Olympus, Tokyo). Images were collected using 488 nm excitation and 522/35 nm emission filters from the dorsolateral cortex, at a depth of 40-80 μm below the cut surface of the slice. To follow the movement of cells continuously, images were taken every 5 minutes for up to 3 hours. Cell motility did not decrease systematically with time, suggesting that the cells were healthy. The health of migrating cells was further checked by carefully comparing migratory behavior of neurons with morphological features observed in fixed slices of corresponding developmental stages. In some slices, we observed aberrant migratory behavior not seen in fixed slices such as abundant migrating cells in the VZ. Such behavior was likely to be an artifact and was therefore interpreted with caution.

Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

Cortical slices prepared as above and embedded in collagen gels were kept for 20 minutes. Time-lapse imaging was started after incubation of the slices in 0.5 U/ml PI-PLC (Molecular Probes, Eugene, OR) for 2 hours at 37°C. Some of the slices were fixed immediately after the incubation to examine the effect of PI-PLC. After time-lapse imaging, which normally took 1.5 hours, the slices were fixed and immunostained for Tag1 (Yamamoto et al., 1986; Dodd et al., 1988).

Time-lapse imaging in flat-mount cortical preparation

The methods for observation were essentially the same as those for slice preparations. E13 or E13.5 mouse embryo neocortical tissue was dissected encompassing from the medial edge to the corticostriatal boundary, at the level of the MGE and the LGE. The cortical tissue was then flat mounted on Millicell inserts with the ventricular side down. The recording was carried out for 90-165 minutes at 5 minutes intervals. At every time point, stack of images were created from a series of 3-6 consecutive images taken along the z-axis at 5 or 10 μm intervals.

Quantitative analysis of migratory behaviors

For quantification of migratory behaviors, slice preparations in which the distribution of GFP+ cells closely resembled that in fixed preparations at the end of the time-lapse analysis were selected. Owing to the high density of GFP+ cells, it was not possible to trace all labeled cells. Thus, the analysis was carried out for all individually distinguishable GFP+ cells. From the position of each cell, a vertical line was drawn to the ventricular surface. Another line was then drawn connecting the initial position of the neuron and the position after 95 or 105 minutes observation. The direction of migration was defined by the angle of the line relative to the vertical line. The distance of migration was defined as the length of this line, and this was divided by observation time to calculate average speed of migration. Because migration of neurons away from the midline was also observed, deflection from the line oriented ventrolaterally was represented by negative angles.

Results

GABAergic neurons in fixed preparations

We first analyzed GFP+ cells in fixed slices from mice heterozygous for the Gad67-GFP allele. At E11.5 and earlier, GFP signal was detectable only in the basal forebrain (data not shown); however, at E12, GFP+ cells were first detected in the lateral cortex near the corticostriatum boundary (Fig. 1A).

At E13-13.5, the distribution of GFP+ cells expanded into a larger region of the lateral cortex (Fig. 1B). Two major streams of GFP+ cells were found, one in the MZ (or the primitive plexiform layer (Del Rio et al., 1992)) and the other in the lower IZ. In this zone, many cells with dorsomedially oriented leading processes were recognized (Fig. 1B, arrow 1, left inset). Occasionally, their leading processes were oriented obliquely to the tangential axis (Fig. 1B, arrow 2, right inset). By contrast, cells in the MZ extending leading processes were only occasionally observed. This may be because many of them are oriented perpendicular to the plane of section, although it is also possible that their high cell density have hampered recognition.

At E15.5, the cerebral wall was thicker and numerous GFP+ cells appeared in it (Fig. 1C). The increase in cell density was remarkable in the MZ and the SVZ. The majority of IZ GFP+ cells had mediodorsally oriented leading processes, roughly parallel to the pial surface (Fig. 1C, arrow 1, left inset). As was the case at E13.5, high cell density seems to be hampered recognition of leading processes of the MZ GFP+ cells. In the SVZ, cell density was also high, but tangentially oriented leading processes were frequently observed (data not shown). We also observed that a substantial number of cells extended processes in non-tangential directions. This included pial surface and ventricle-oriented cells, but the former appeared to be predominant (Fig. 1C, arrows 2 and 3, middle and right insets). Ventrolaterally oriented cells (towards the basal
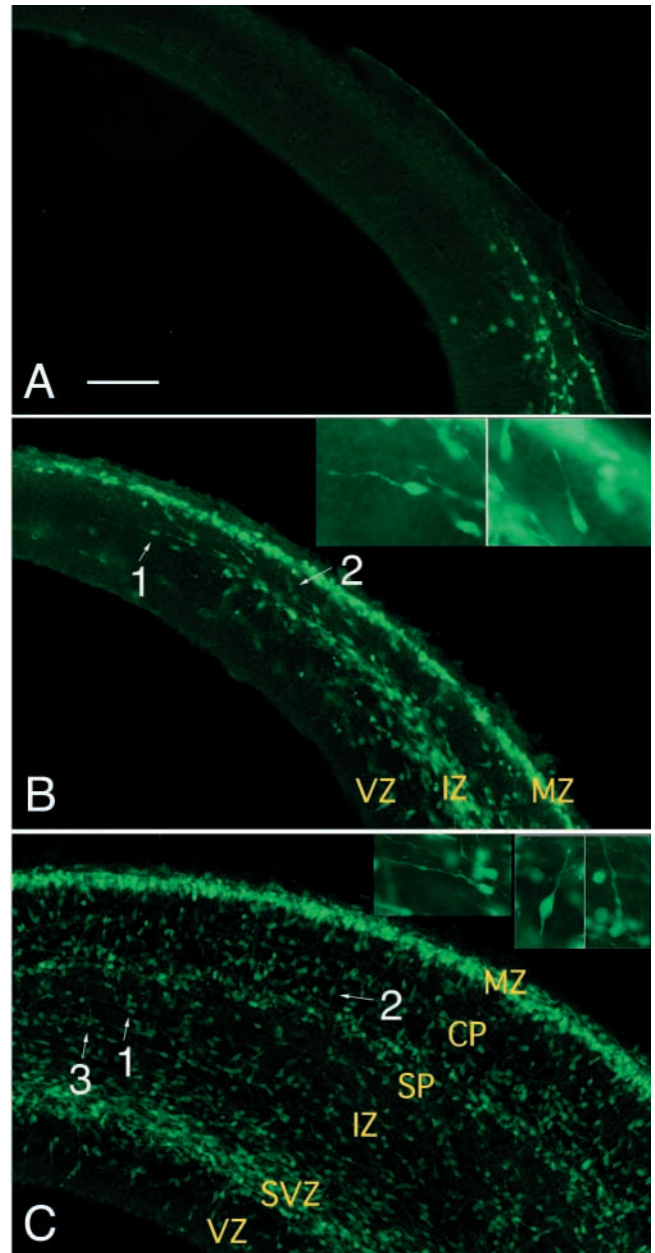

Fig. 1. GFP+ neurons in the dorsal cortex of heterozygous (Gad67[gfp/+]) mice. Insets are high-magnification views of migrating cells identified by numbered arrows. Corresponding insets are arranged from left to right. Note that there are many cells that appear to be migrating tangentially (B-1, C-1) or obliquely to the tangential axis (B-2). Some cells are oriented radially (C-2,3). GFP+ neurons can also be found in the VZ. (A) E12; (B) E13.5; (C) E15.5. GFP+ neurons penetrated into the lateral cortex at E12 and are distributed in more medial parts at later stages. VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; SP, subplate; CP, cortical plate; MZ, marginal zone. Scale bar: 100 μm.

forebrain) were also observed, albeit few in number (data not shown).

As development proceeds further, the cerebral wall became thicker and a larger number of radially oriented GABAergic neurons were observed in the cortical plate (data not shown).

E13.5
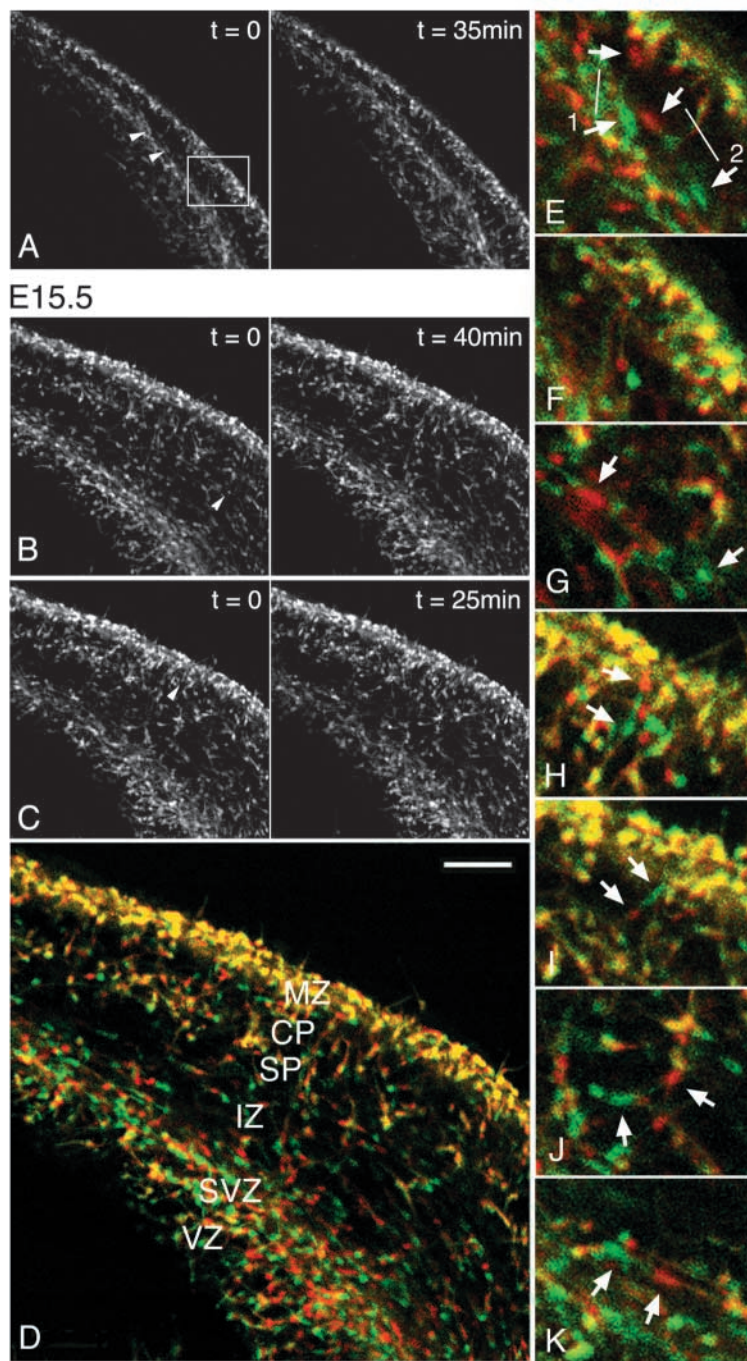
E15.5

Fig. 2. Migration of GFP$^+$ neurons in cortical slices. (A) The time sequence of migrating neurons in a coronal slice taken over a time interval of 35 minutes in an E13.5 mouse embryo. (B,C) Similar to A but from an E15.5 mouse and taken at time intervals of 40 and 25 minutes, respectively. (D) A merged view of a time sequence taken at a 30 minutes interval from an E15.5 mouse. The image taken at an earlier time of observation was converted to green and later captured to red. Thus, cells colored yellow mostly represent cells that stayed in the same position during the intervals, although part of them could represent two different cells, one of which migrated to original position of the other. Those in red or green represent cells that had migrated during the interval. Note that most MZ neurons are yellow, suggesting no migration. (E,F) Enlarged view of an image generated by merging the two panels of A after color conversion described above. (E) Two tangentially migrating cells indicated by arrowheads in A are shown by a pair of arrows. The neuron labeled as '1' is deflected towards the MZ, whereas neuron '2' migrated tangentially. (F) Enlarged view of the area shown by a rectangle in A. Note that there are many yellow dots near the surface, suggesting that many cells in this region may be stationary. (G) A tangentially migrating neuron in the IZ. (H) A radially migrating neuron in the cortical plate towards the MZ. (G,H) Enlarged views of a merged image generated from B and C, respectively and the positions of these cells are indicated by arrowheads. (I) Radially migrating neuron away from the MZ. (J) A radially migrating neuron, with some angle, in the IZ over a 25 minutes interval. (K) A tangentially migrating neuron in the SVZ but in a reversed direction over a 30 minutes interval. (I-K) Merged view from an E15.5 mouse embryo. Medial is towards the left and dorsal is upwards. Scale bars: 100 µm in A–C; 50 µm in D; 25 µm in E-J. For animated versions of the complete time-lapse sequence, see Movies 1 and 2 at http://dev.biologists.org/supplemental/

directly, we observed GFP$^+$ cells in coronal slices in time-lapse sequences. As expected, GFP$^+$ cells were highly motile in E13.5 lower IZ (Fig. 2A,E; see Movie 1 at http://dev.biologists.org/supplemental/). However, most MZ GFP$^+$ neurons showed limited motility (Fig. 2F, yellow dots), although some showed tangential migration (see Movie 1 at http:// dev.biologists.org/supplemental/). In deeper zones of the cortex, many neurons were motile, migrating medially and occasionally deflected toward the MZ. Similar behavior of GFP$^+$ neurons was observed in 12 slices. These observations suggest that intracortical tangential migration takes place mainly in the lower IZ at this stage and raise the possibility that some neurons in the lower IZ translocate to the MZ.

To examine the migration of GFP$^+$ neurons at a more developed stage when the cortical plate (CP) and the subplate (SP) are well developed, we observed time-lapse sequences of E15.5 slices. As with E13.5 mice, many of MZ neurons were stationary and only a fraction of them showed motility (Fig. 2B-D, yellow dots in D; see Movie 2 at http://dev.biologists.org/supplemental/). In the CP/SP region, many cells also appeared stationary (Fig. 2D). Occasionally, however, we encountered migrating neurons; these included cells moving radially, either towards (Fig. 2H) or away from the pial surface (Fig. 2I) (see Movie 2 at http://
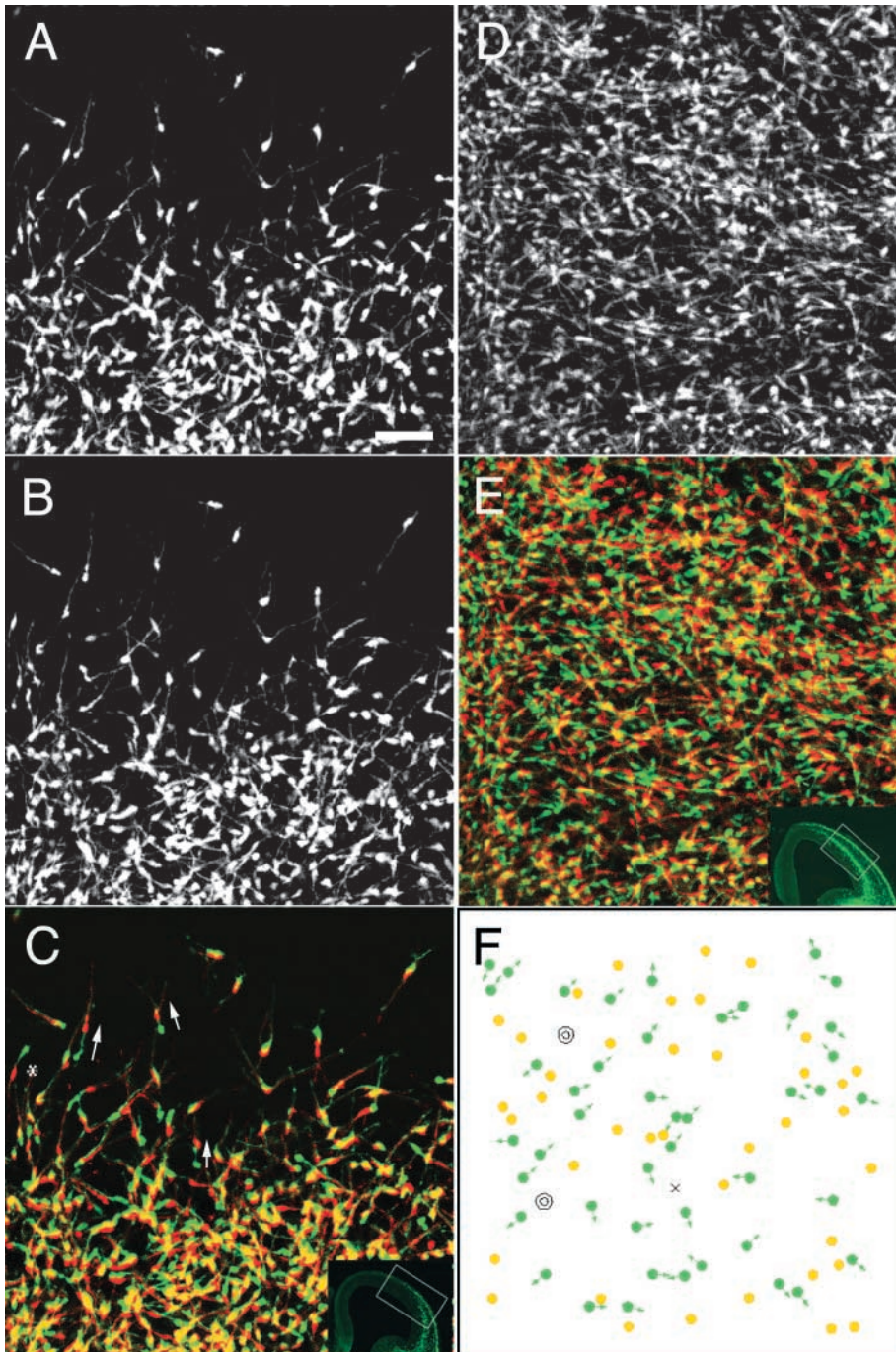
The distribution of GFP$^+$ cells in the cortex was consistent with that of GABA immunoreactive neurons (Del Rio et al., 1992; Jimenez et al., 2002), indicating that GFP$^+$ cells observed here are likely to be GABAergic neurons. Immunolabeling of GFP$^+$ cells with anti-GABA and anti-MAP2 antibodies confirmed that most of them indeed correspond to GABA immunopositive neurons (data not shown).

Dynamics of migration in acute coronal slices of the cortex

To examine intracortical migration of GABAergic neurons

Fig. 3. Migration of GFP⁺ neurons observed in flat-mount preparation of the neocortex. (A,B) A is an image from E13 mouse dorsal cortex and B is the same cortex taken 50 minutes later. (C) Merged view of images in A,B. The images were taken from the leading edge of migratory streams. Most cells stay almost in the same position (yellow). Although they show some motility, migration toward a specific direction was not observed. Occasionally, some cells near the leading edge showed medially directed migration (arrows). *A neuron moving laterally. The white rectangle in the inset illustrates approximate position of the image. (D) The same as A but from an E13.5 mouse dorsal cortex. (E) An image captured 50 minutes later (red) was merged with D (green) as in C. The white rectangle in the inset illustrates approximate position of the image. (F) The movement of 85 arbitrarily selected cells in D, taken during a 20 minutes interval, was plotted. Yellow dots indicate stationary cells and green dots with arrows indicate migrating cells and their direction of migration. X symbols and double circles indicate where cells disappeared or appeared, respectively. Medial is upwards, lateral is downwards and anterior is towards the left. For an animated version of the complete time-lapse sequence, see Movies 3 and 4 at http://dev.biologists.org/supplemental/. Scale bar: 50 μm.

direction of movement was noted. We obtained similar results from 12 slices. Thus, at E15.5 tangential migration of GABAergic neurons predominantly takes place in the lower IZ and the SVZ but non-tangential migration towards the MZ including radial migration become more pronounced compared with E13.5.

Migration of GABAergic neurons in flat-mount cortical preparations

GABAergic neurons in the MZ showed limited motility when observed in coronal

dev.biologists.org/supplemental/). Neurons of (lower) IZ showed robust tangential migration, moving dorsomedially in parallel with the pial surface (Fig. 2G) or obliquely to the tangential axis (Fig. 2J) deflecting towards the pial surface. Curiously, neurons that migrated ventrolaterally, opposite to the direction of most cells, were also found (Fig. 2K). Consistent with observations in fixed sections, many tangentially migrating neurons were also observed in the SVZ in time-lapse images (see Movie 2 at http://dev.biologists.org/supplemental/). However, there were also many stationary cells (Fig. 2D, yellow dots). Some neurons in the VZ also showed migratory behavior, but no preferred

slices. However, this does not preclude the possibility that, in vivo, they migrate in directions not detectable in coronal slices. The flat-mount preparations of the cortex enabled us to test this possibility. Figure 3A-C shows confocal images of the dorsal view of the cortex captured at appropriate time intervals in an E13 mouse. It is likely that these represent MZ neurons as they are located near the surface of the cortex. At this stage, the leading edge of GFP⁺ neurons was observed in the lateral cortex (Fig. 3C, inset). At the leading edge, it was possible to observe the morphology of individual GFP⁺ neurons because of their relatively low density. Time-lapse sequences indicated that some GFP⁺ neurons showed pronounced motility in the tangential

plane extending and retracting their processes (see Movie 3 at http://dev.biologists.org/supplemental/). Near the leading edge, some cells showed lateral-to-medial migration (Fig. 3C, arrows). In the region behind the leading edge, however, many GFP+ neurons appeared stationary (see yellow cells in Fig. 3C; see Movie 3 at http://dev.biologists.org/supplemental/) and medially directed migration of GFP+ neurons was not recognizable as a whole (*n*=7). At E13.5, the leading edge progressed medially (Fig. 3E, inset), enabling us to observe the motility of the cells behind the leading edge by observing the lateral cortex. In this region, a fraction of GFP+ neurons showed low motility (Fig. 3D-F). However, many of them quickly migrated among stationary cells (Fig. 3F). This migration of individual neurons occurred in many directions (*n*=17) (see Movie 4 at http://dev.biologists.org/supplemental/). It was difficult to observe migration of individual cells in flat-mount preparations of E15.5 mice because of high cell density, but the cells at this stage also appeared to exhibit multidirectional migration at this stage (data not shown). Thus, the observation in the flat-mount preparation has revealed that a substantial proportion of MZ neurons migrate in all directions. The virtual absence of lateral-to-medial migration of GFP+ cells as a whole is consistent with the observation of coronal slices and indicates that MZ neurons do not show obvious lateral-to-medial tangential migration.

Quantitative analysis of zone-specific migrating behavior

To quantify zone-specific migration of GABAergic neurons, we measured the speed and orientation of cell migrations in coronal slices. Fig. 4A,B show the average speed of migration plotted against the distance from the ventricular surface (represented as the percentage of the distance to the pial surface) at E13.5 (Fig. 4A) and E15.5 (Fig. 4B), respectively. It can be clearly seen that the cells at E13.5 are most vigorously moving in the lower IZ. Likewise, at E15.5, the motility of cells was most pronounced in the IZ/SVZ, although a small population of the cells migrated vigorously at the SP/CP. It can also be seen that there is a great variation in the speed of migration among individual neurons.

We also analyzed zone-specific direction of the migration of GFP+ cells. For this, the orientation of migration as measured from deflection from a line drawn vertical to the ventricular surface was measured. There was a remarkable variation in the angle of migration among these neurons, with almost any angle of migration being observed. In the IZ, most neurons migrated around the angle of 90°, i.e. roughly tangentially; however, a substantial fraction of the neurons migrated with an angle greater than 90° (Fig. 4C,D), indicating that they deflected toward the pial surface. Presence of neurons migrating with an angle around 0° in and just below the MZ suggests that they may be descending away from the pial surface. Thus, cortical GABAergic neurons show zone-specific and individual variations in migratory behavior. The fraction of neurons migrating with an angle greater than 120° was larger at E15.5 compared with E13.5, suggesting that more neurons are deflected towards the pial surface at a later stage.

The results of the quantification clearly demonstrated occurrence of multidirectional tangential migration, in the coronal plane, biased towards the pial surface. They also
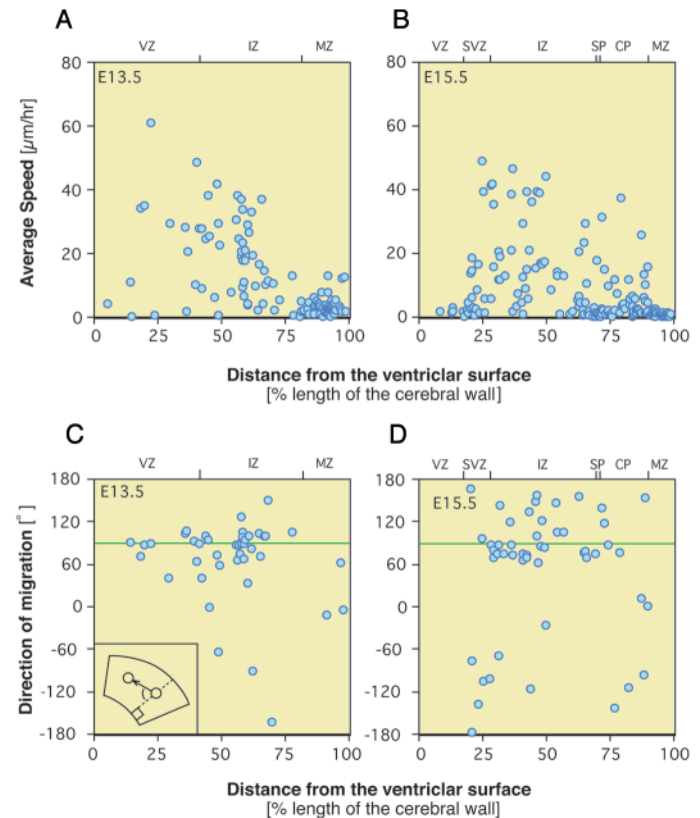

Fig. 4. The relationship between the position of GFP+ neurons in the cerebral cortex and their rate or orientation. (A,B) The average speed of migration for individual cells plotted against the distance from the ventricular surface. Each point represents a migrating neuron at (A) E13.5 and (B) E15.5 mouse. (C,D) The orientation of migration as measured from deflection from a line drawn vertical to the ventricular surface was plotted against the distance from the ventricular surface at (C) E13.5 and (D) E15.5. Inset illustrates the method of angle measurement. The green line represents 90°. Note that a substantial proportion of cells in the IZ, SP and CP migrate with angles of more than 90°, indicating that they deflect towards the pial surface. Negative angles indicate the occurrence of medial-to-lateral migration. All cells that could be traced were plotted. The high density of GFP+ neurons, particularly in the SVZ and the MZ, allowed measurement of only a fraction of the cells. The speed of migration is underestimated, because rapidly migrating cells in cell-dense regions could not be followed and curved trajectories of migrating cells were approximated by straight lines. Although most MZ neurons were stationary, some slight dislocations can be observed. This is largely due to a drift of the slice during observation. Thus, in C and D, cells that moved less than 10 μm per hour were excluded from the analysis.

showed that lateral-to-medial migration of these neurons mainly occurs in the IZ/SVZ.

Effect of GABA content on the migration of GABAergic neurons

It is possible that individual cell GABA content is reduced in Gad67-GFP knock-in mice. This might cause a decrease in GABA concentration in the cortex. Neuronal migration in Gad67-GFP mice might be distorted, because there is in vitro evidence that GABA affects neuronal migration (Behar et al.,
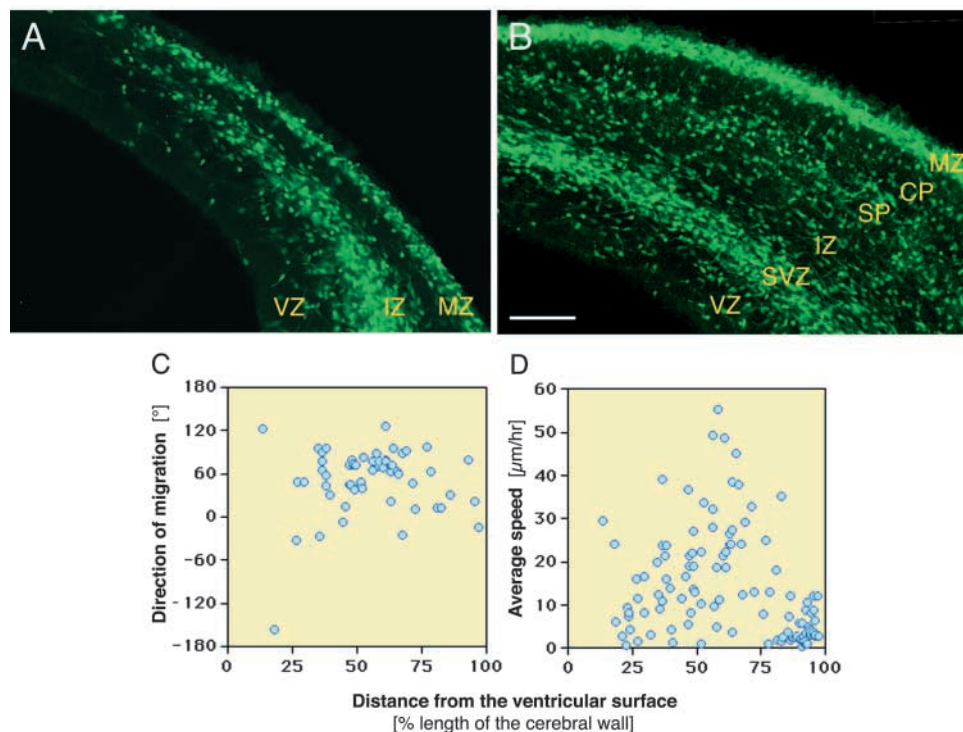
Fig. 5. Distribution and morphology of GFP$^+$ neurons in a transgenic mouse homozygous for the Gad67-GFP allele (Gad67$^{gfp/gfp}$). (A,B) GFP$^+$ neurons in fixed sections of the dorsal cortex from an E13.5 (A) or an E15.5 (B) homozygous (Gad67$^{gfp/gfp}$) mouse embryo. There is no notable difference in distribution and morphology of GFP$^+$ neurons between heterozygous (Gad67$^{gfp/+}$) and homozygous (Gad67$^{gfp/gfp}$) mice (compare with Fig. 1B,C). As in heterozygous mice, many neurons with a leading process are found in the lower IZ. (C,D) Quantitative representation of GFP$^+$ migrating cells from an E13.5 homozygous mouse. There is no noticeable difference in direction or average speed between heterozygous and homozygous mice (compare with Fig. 4A,C). Scale bar: 50 μm.

2001). To test this possibility, we observed the morphology and migratory behavior of GFP$^+$ neurons using mice homozygous for the Gad67-GFP allele (Gad67$^{gfp/gfp}$), comparing it with that of heterozygous mice (Gad67$^{gfp/+}$). Remarkable reduction in GABA content in *Gad67$^{-/-}$* mice that had been generated using a similar construct (Asada et al., 1997) suggests that GABA content may also be greatly reduced in Gad67$^{gfp/gfp}$ animals. In fixed slices, no differences were noted between heterozygous and homozygous mice in the distribution, zone-specific features or cellular morphology of GFP$^+$ neurons either in E13.5 (*n*=5: Fig. 5A) or E15.5 (*n*=5: Fig. 5B) embryos. Similarly, all features of migratory behaviors as described above were observed in homozygous mice (Fig. 5C,D). We also did not find notable differences in the rate and orientation of migrating GFP$^+$ neurons (compare Fig. 5C,D with Fig. 4A,C). These results suggest that a reduction of GABA content does not significantly affect the migratory behavior of GABAergic neurons, at least within the time window of our observation. This notion is further supported by the observation that gross structure of the brain appears normal in newborn *Gad67$^{-/-}$* mice (Asada et al., 1997).

Substrate for GABAergic neuron migration

DiI-labeled GE-derived neurons migrate in close apposition with Tag1$^+$ cortical fibers in vitro and their invasion into the cortex is markedly reduced by application of a Tag1 antibody (Denaxa et al., 2001), suggesting that Tag1 plays an important role in the migration of these neurons from GEs to the cortex. By contrast, MGE neurons transplanted to embryonic brains migrate tangentially in the cortex apparently avoiding axon-rich upper IZ (Wichterle et al., 2001). Likewise, cell expressing dorsal forebrain makers do not migrate along axon-rich areas (Nery et al., 2002). These findings raise the possibility that Tag1$^+$ cortical fibers are not involved in the

intracortical migration of GABAergic neurons, although it may play an important role for their migration from GEs to the cortex. We took advantage of the fact that virtually all GABAergic neurons can be visualized in our preparations to examine the role of Tag1 molecule in intracortical migration of these neurons. We first explored this hypothesis by comparing the distribution of Tag1 axons and GFP$^+$ neurons in cortical sections. Fig. 6 shows Tag1 immunostaining of coronal sections of Gad67-GFP mouse cortex. It can clearly be seen that the major stream of GFP$^+$ neurons (green) do not coincide with the bundle of Tag1$^+$ fibers running tangentially (red)(Fig. 6A,B). In the CP/SP, radially oriented Tag1$^+$ fibers can be recognized (Fig. 6A,C, arrows in C), but most GFP$^+$ neurons in this region did not appear to be associated with Tag1$^+$ fibers (Fig. 6C). These findings suggest that Tag1$^+$ axons play, if any, a limited role in the migration of GABAergic neurons in the cortex. We also observed that the majority of GFP$^+$ neurons did not associate with Tag1$^+$ axons in the lateral cortex (see Fig. S1 at http://dev.biologists.org/supplemental/).

To test this idea directly, we treated cortical slices with PI-PLC, an enzyme that cleaves glycosylphosphatidylinositol (GPI) anchors, because Tag1 is a GPI anchored protein. After treatment of cortical slices for 2 hours, Tag1 staining was almost completely abolished (Fig. 7A,B). However, GFP$^+$ neurons in treated slices showed migratory behavior indistinguishable from untreated preparations; this includes tangential migration occurring predominantly in the lower IZ/SVZ and multidirectional migration in other zones (Fig. 7C). Quantitative analysis supported this view (compare Fig. 7D,E with Fig. 4B,D). These findings are consistent with our immunohistochemical study and suggest that yet unidentified substrate molecules are involved in the migration of GABAergic neurons in the cortex.
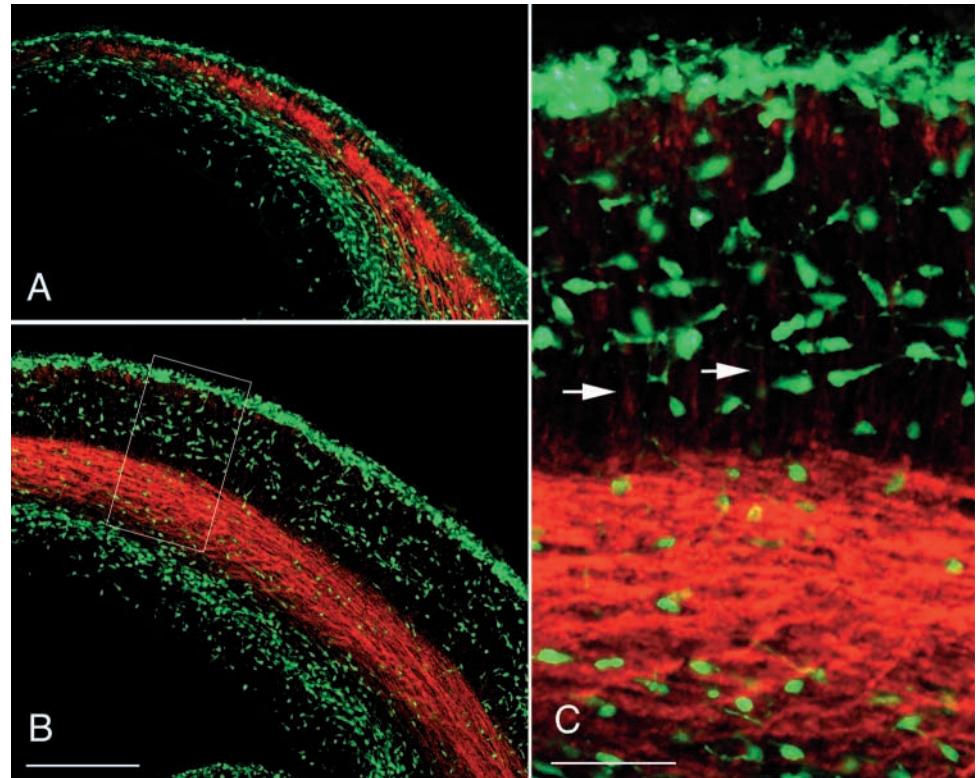
Fig. 6. Expression of Tag1 in the cortex and the distribution of GFP⁺ neurons. Coronal sections of an E13.5 (A) or E15.5 (B) mouse cortex were immunostained for Tag1 (red). Only a small fraction of GFP⁺ cells migrate in the region where Tag1 immunoreactivity can be observed. (C) High-magnification view of the area shown by a rectangle in B. Arrows indicate radially oriented Tag1⁺ fibers. Note while the fibers are strictly oriented radially (see Fig. 6A also) in the cortical plate, only a fraction of GFP⁺ neurons are. Scale bar: 50 μm.

Discussion

Technical consideration

Slice preparations were exposed to strong light excitation in addition to being subjected to cutting procedures. Thus, in vitro artifacts are inevitable. In general, it is difficult to distinguish aberrant migratory behavior caused by in vitro artifacts from bona fide migratory behavior, because the in vivo data of migration are not available. However, because the features observed in our fixed slice preparations can be regarded as a representation of in vivo migratory behavior of GFP⁺ neurons, the present study attempted to exclude artifacts by carefully comparing time-lapse images with those from fixed slices; in vitro slices preparations that show features substantially diverged from the observation in fixed sections were excluded from the present analysis.

Using Gad67-GFP mice allowed us to overcome some technical limitations associated with previous studies. First, we could observe the migration of virtually all GABAergic neurons, while the use of DiI (1,1-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate) labeling (Tamamaki et al., 1997; Jimenez et al., 2002) and tissue transplantation allows the observation of only a subpopulation of migrating cells of unknown identity, possibly including glial cells or their progenitors (Spassky et al., 1998; Olivier et al., 2001). This has made it possible to precisely assess the role of candidate molecules that regulate migration. Second, omission of staining procedures together with strong fluorescence emissions enabled us to observe migrating cells in flat-mount preparations in an in vivo-like environment, which has provided important information of neuronal migration.

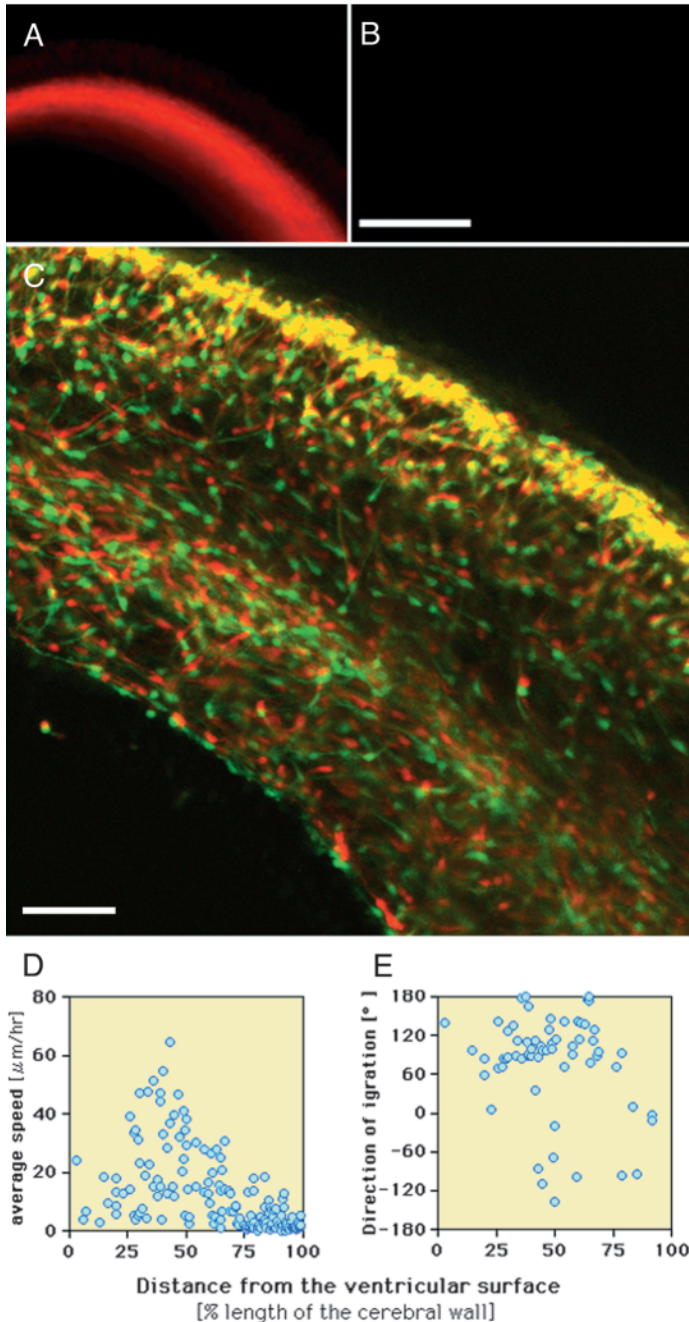It is noteworthy that there were neurons that extended leading processes but did not migrate within the window of our time lapse analysis. This indicates that observation of fixed tissues does not necessarily provide accurate information on the dynamic behavior of these neurons.

Zone-specific migration of GABAergic neurons

Robust lateral-to-medial tangential migration observed in the lower IZ/SVZ of the dorsal cortex implies that a part of GABAergic neurons migrating in these zones further migrate away from the cortex. This is consistent with the notion that GE-derived neurons migrate further to the hippocampus (Pleasure et al., 2000).

GFP⁺ neurons were also abundant in the MZ. Previous studies using in vitro slices labeled with fluorescent dyes provided evidence that GE-derived cells migrate along the MZ (de Carlos et al., 1996; Lavdas et al., 1999; Jimenez et al., 2002; Polleux et al., 2002) as well as the IZ. Our findings that in fixed preparations the dorsal leading edge of the migratory stream elongated dorsomedially and that the density of GFP⁺ neurons in the MZ increased as development proceeded (Fig. 1) are consistent with these observations. Unexpectedly, however, MZ GFP⁺ neurons as a whole did not show obvious directed tangential migration both in flat-mount preparations and coronal slices. One possible explanation for this apparent discrepancy is that medial extension of the MZ that includes GFP⁺ neurons resulted from supply of neurons from deeper zones of the neocortex. Indeed, in fixed tissues, we found many migrating neurons between the IZ and the MZ oriented towards the pial surface. Consistent with this, a substantial number of neurons did migrate from the lower IZ/SVZ towards the MZ in our in vitro coronal slice preparations. An interesting scenario, therefore, is that a part of the tangentially migrating neurons in the lower IZ/SVZ deflect towards the MZ (Fig. 8),

the transplanted cells were found in all layers throughout the dorsal cortex (Wichterle et al., 2001). Similarly, by co-culture of an explant of the GEs from a GFP mouse with wild-type mice cortical tissues, Polleux et al. observed initial migration of GFP+ neurons in the MZ and IZ but later also in the SP and CP (Polleux et al., 2002). These findings are consistent with our view that GABAergic neurons that have arrived at the MZ from deeper zones of the cortex migrate back to the CP (Fig. 8). After submission of this paper, a report appeared that also revealed descent of MZ neurons (Ang et al., 2003).

Multidirectional migration in the MZ

In this study, we have demonstrated multidirectional tangential migration of identified GABAergic neurons in the MZ for the first time using flat-mount preparations of the cortex. These neurons appeared to migrate in random in this plane, suggesting it unlikely that this migration contributes significantly to lateral-to-medial tangential expansion of GABAergic neurons in the cortex. Cortical GABAergic neurons originate from specific regions in the basal forebrain, primarily from the MGE, and migrate tangentially towards the cortex (see Introduction). However, it has remained unknown how they become widely distributed in the cortex. Multidirectional migration of GABAergic neurons in the MZ might explain the spread of GABAergic neurons over the entire cortex at least in part.

Implication to the mechanism of intracortical migration of GABAergic neurons

In vitro, GABA acts as a chemoattractant for cortical neurons (Behar et al., 2001) and inhibition of GABA$_B$ receptors modulates the migration of cortical interneurons (Lopez-Bendito et al., 2003). These findings raise the possibility that GABA may be involved in the migration of cortical interneurons. However, in this study no notable difference was found in the distribution, cellular morphology or migratory behaviors of GABAergic neurons between heterogygous (Gad67$^{gfp/+}$) and homozygous (Gad67$^{gfp/gfp}$) mice, in which GABA should be greatly diminished. These findings suggest that GABA does not play an important role in the intracortical migration of GABAergic neurons, although a low level of GABA could be sufficient to regulate their migration.

Recently, corticofugal axons have been suggested to provide a substrate for tangential migration in the IZ (Denaxa et al., 2001). However, this fails to explain the multidirectional

contributing neurons to the layer of GABAergic neurons in the MZ.

At all developmental stages examined, radial migration occurred both towards and away from the MZ. Although pial surface-directed migration was prevalent at E13.5 and 15.5, migration towards and away from the MZ appeared to be roughly balanced at later stages of development (S. Tada, D. T. and F. M., unpublished). Considering the possibility that GABAergic neurons in the MZ originate from deeper zones of the cortex, it would be interesting to speculate that these neurons in the MZ migrate back to deeper zones for settlement (Fig. 8). Wichterle et al. carried out homotypic transplantation of the tissue from the MGE and found migrated cells initially in the SVZ and later in the MZ of host embryos. Postnatally,
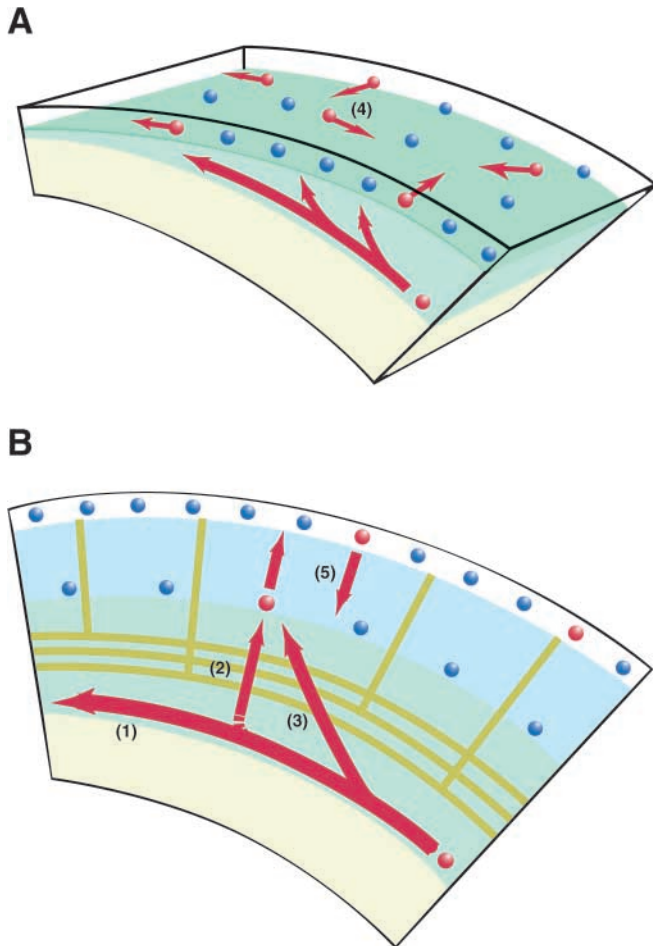
Fig. 8. Summary diagram of the present study and a model for intracortical migration of GABAergic neurons. (A) The migratory behavior of GABAergic neurons in E13.5 neocortex. Dorsomedially directed tangential migration was most prominent in the lower intermediate zone (IZ), but some of the neurons deflected towards the marginal zone (MZ). Neurons in the MZ tangentially migrated in many directions (4), although many of them are stationary (blue spheres). (B) The modes of migration of GABAergic neurons observed in the coronal plane of E15.5 neocortex. Tangentially migrating IZ/SVZ neurons continue to migrate towards the hippocampus (1), whereas those deflected towards the pial surface migrate radially (2) or with some angle to the radial axis (3), towards the MZ. In the MZ, a part of these neurons may migrate on the tangential plane in many directions and spread into the whole cortical areas (see neurons labeled by 4). A part of these neurons may descend from the MZ to be integrated into CP (5). Intracortical migration occurs independent of Tag1^{+} axons (yellow). Red spheres represent motile cells.

migration of GABAergic neurons observed in the coronal plane. Moreover, incomplete overlap of the migratory stream with axonal tracts (Wichterle et al., 2001) suggests that the axonal tract may only partially serve as a substrate. The present finding that the major stream of GFP^{+} migrating neurons did not coincide with Tag1^{+} fibers is consistent with this idea. Moreover, we showed that all features of the migration of GABAergic neurons within the cortex were unaffected by PI-PLC treatment. This indicates that GPI-anchored proteins including Tag1 are not required for intracortical migration of GABAergic neurons. Thus, unidentified factors may contribute to the establishment of the substrate of migration within the cortex. Although the present results do not preclude the possibility that Tag1 plays an important role in the migration of GABAergic neurons from the MGE to the cortex (Denaxa et al., 2001), the lack of changes in gross anatomy of the cerebral cortex in Tag1-deficient mice (Fukamauchi et al., 2001) further supports the notion that Tag1 plays a limited role in the development of the cerebral cortex.

There was a diversity of behavior of GABAergic neurons even in a given zone of the cortex. The ability to classify GABAergic neurons into several subtypes, depending on biochemical properties, morphology and electrophysiological properties (Kawaguchi and Kubota, 1997), raises the possibility that such diversity of migratory behavior reflects the inherent diversity of GABAergic neurons. Alternatively, even if these neurons uniformly respond to guidance cues, there might be some stochastic process in the machinery that mediates the signaling of guidance molecules. It is also possible that the intrinsic program of migrating GABAergic neurons contributes to their behavioral diversity (Yacubova and Komuro, 2002).

Recently, ventricle-directed migration of GABAergic neurons was shown to be prevalent in E16-18 embryonic rat cortical slices (Nadarajah et al., 2002). Such neurons may seek the cortical VZ to receive layer information. The proportion of such neurons was low in our preparations, however. Further studies should reveal whether this is due to differences in preparations or in the method of labeling neurons.

In conclusion, Gad67-GFP knock-in mice enabled us to analyze the in situ migration of GABAergic neurons in the neocortex. This has allowed us to compare the migratory behavior of GABAergic neurons in different zones and to assess the role of GPI-anchored proteins. Moreover, it has revealed the presence of several modes of intracortical migration of GABAergic neurons: directed tangential migration in the IZ/SVZ possibly towards the hippocampus, migration towards the MZ, multidirectional tangential migration in the MZ and migration away from the MZ. These modes of migration were not affected by PI-PLC treatment, suggesting that GPI-anchored proteins are not important for these migrations. We propose a model that cortical GABAergic neurons initially invade the MZ by departing from the major migratory stream in the IZ/SVZ and then disperse in the MZ. Some of these neurons may descend away from the MZ to be distributed in the CP.

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