Netrin 1 regulates ventral tangential migration of guidepost neurons in the lateral olfactory tract

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In the developing nervous system, functional neural networks are constructed with intricate coordination of neuronal migrations and axonal projections. We have previously reported a ventral tangential migration of a special type of cortical neurons, lot cells, in the mouse embryo. These neurons originate from the ventricular zone of the entire neocortex, tangentially migrate in the surface layer of the neocortex into the ventral direction, align in the future pathway of the lateral olfactory tract (LOT) and eventually guide the projection of LOT axons. In this study, we developed an organotypic culture system to investigate the regulation of this cell migration in the developing telencephalon. Our data show that the neocortex contains the signals that direct lot cells ventrally, that the ganglionic eminence excludes lot cells by repelling the migration and that lot cells are attracted to netrin 1, an axon guidance factor. Furthermore, we demonstrate that mutations in the genes encoding netrin 1 and its functional receptor Dcc lead to inappropriate distribution of lot cells and subsequent partial disruption of LOT projection. These results suggest that netrin 1 regulates the migration of lot cells and LOT projections, possibly by ensuring the correct distribution of these guidepost neurons.

KEY WORDS: Netrin 1, Ventral tangential migration, Lateral olfactory tract, Guidepost neuron, Lot cells, Mouse

INTRODUCTION

In the developing telencephalon, neurons vigorously migrate towards their final destination (Marin and Rubenstein, 2003). Among the various modes of migration, recent studies have emphasized the roles of tangential migration in the supply of constituent neurons to their distant targets (Marin and Rubenstein, 2003). For example, the majority of cortical GABAergic interneurons originates from the ventral telencephalon and is supplied to the neocortex through dorsal tangential migration (O’Rourke et al., 1992; de Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997; Anderson et al., 2001; Wichterle et al., 2001; Jimenez et al., 2002). Given that these neurons are essential for cortical function, this migration is considered to be precisely controlled during development by multiple guidance molecules (Marin and Rubenstein, 2003).

We discovered a unique ventral tangential migration stream of early-generated neurons in the mouse telencephalon (Sato et al., 1998). These neurons, designated as lot cells, are characterized by staining with the monoclonal antibody (mAb) lot1, and serve a specific function as guideposts for olfactory bulb axons into the lateral olfactory tract (LOT) (Sato et al., 1998). Although lot cells specifically populate the LOT area during the guiding process, the cells originate from the widespread area of the neocortex in an earlier developmental stage and subsequently migrate ventrally and tangentially towards the future LOT area, which is located on the boundary between the neocortex and the ganglionic eminence (GEM) (Tomioka et al., 2000). In the LOT area, lot cells terminate their ventral tangential migration, abruptly changing their orientation from the dorsoventral to rostrocaudal axis, and assemble into a compact cellular array (Tomioka et al., 2000), which thereafter serves as the scaffold of growing olfactory bulb axons. A similar neuronal migration is observed in the telencephalon of chick embryos (Striedter et al., 1998), suggesting evolutionary conservation of this tangential migration stream. Nevertheless, the precise mechanism of this migration remains to be elucidated.

The present study is aimed at determining the mechanisms that regulate the ventral tangential migration in the developing telencephalon. We reproduced this migration in an organotypic culture system, and found that signals in the neocortex direct lot cells ventrally, while other mechanisms exclude lot cells from the GE. The screening for candidate modifiers in the migration identified netrin 1, a prominent axon guidance molecule (Kennedy et al., 1994; Serafini et al., 1994), as an attractant for lot cells. In mice mutant for genes encoding netrin 1 and its functional receptor Dcc, lot cells were not appropriately distributed in the LOT area and there was a slight but specific disruption in the projection of olfactory bulb axons. The results indicate that netrin 1 regulates the migration of lot cells and LOT projection in vivo.

MATERIALS AND METHODS

Mice

Time-pregnant ICR wild-type mice were purchased from SLC (Shizuoka, Japan). Mice expressing green fluorescent protein (GFP; green mice) were provided by Okabe et al. (Okabe et al., 1997). Xr/Xr mutant embryos, which have deletions in the Gli3 gene, were generated as described previously (Tomioka et al., 2000). When the Xr mutation was introduced into green mice, green mice were crossed with Xr/+ mice and the resulting Xr/+ mice expressing GFP were intercrossed to obtain Xr/Xr green mouse embryos. Heterozygous netrin 1 mutant mice (Serafini et al., 1996) and Dcc mutant mice (Fazeli et al., 1997) were crossed to obtain homozygous embryos. The day on which a vaginal plug was found was designated embryonic day 0.5 (E0.5). All experimental protocols were approved by the Animal Committee of National Institute of Genetics.

Organotypic culture of telencephalon strips

Each telencephalic hemisphere of the E10.5 mouse embryo was dissected along the dorsoventral axis into a strip ~1.5 mm wide, including the neocortex, the presumptive LOT area and the lateral and medial ganglionic eminences (GE) (see Fig. 1A). In the strip, the presumptive LOT area was located around the middle along the dorsoventral axis. In a standard culture, the strip was labeled by injection of dextran tetramethylrhodamine...
Development 133 (5)

(D-3308, Molecular Probes, Eugene, OR) or 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) into a small area of the dorsal neocortex, unfolded on a collagen-coated membrane filter (Transwell-COL inserts #3492, Corning, Acton, MA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Sigma-Aldrich, St Louis, MO) containing 10% fetal bovine serum (Cansaers, Rexdale, ONT, Canada) and 5% horse serum (Invitrogen, Carlsbad, CA) for 2 days. In co-cultures, explants for combination were prepared from littermates and cultured as described above. In candidate screenings, HEK293T cells were transfected with expression plasmids for candidate molecules using FuGENE 6 transfection regent (Roche, Mannheim, Germany) and prepared as cell aggregates by hanging drop culture (Kennedy et al., 1994). Each design of culture was repeated at least five times.

Histochemistry
Immunofluorescent labeling was performed as described (Tomiioka et al., 2000; Hirata et al., 2002; Tozaki et al., 2002). Whole-mount immunostaining of telencephalons with mAb lot1 and anti-neuropilin 1 antibody was described previously (Sato et al., 1998; Tomiioka et al., 2000). β-Galactosidase staining followed a method described previously (Saga et al., 1992). In situ hybridization was performed as described previously (Schaeren-Wiemers and Gerfin-Moser, 1993) with mouse Dcc cRNA probe (Cooper et al., 1995) and mouse netrin 1 cRNA probe (bp 1-477). In DiI labeling of olfactory bulb axons, a small crystal of DiI was inserted into the medial part of the olfactory bulb as described previously (Hirata and Cooper et al., 1995) and mouse netrin 1 cRNA probe (bp 1-477). In DiI labeling of olfactory bulb axons, a small crystal of DiI was inserted into the medial part of the olfactory bulb as described previously (Hirata and Fujisawa, 1999). For TUNEL assay, In Situ Cell Death Detection Kit AP (Roche) was used according to the manufacturer’s instructions.

RESULTS
Ventral tangential migration of lot cells is reproducible in organotypic culture

Although the ventral tangential migration of lot cells has been demonstrated by cell tracing in whole-embryonic culture (Tomiioka et al., 2000), this culture system involves complex procedures and is inadequate for experimental manipulations. Thus, to analyze the mechanism of the migration in detail, we developed an organotypic culture system that is easy to manipulate and applicable for experimental assays. Briefly, a strip including the neocortex and the GE was prepared from the E10.5 mouse telencephalon along the dorsoventral axis (Fig. 1A). The dorsal neocortex of the strip was labeled with fluorescent dye rhodamine (asterisk). (B) The strip 3 hours after preparation. Rhodamine-labeled cells (magenta) do not migrate yet from the labeled position (asterisk). Boundary between the neocortex and the GE is visualized by immunostaining against reticulin 1 (green). (C) The strip after 36 hours in culture. Merged image of rhodamine (magenta) and immunostaining with mAb lot1 (green). One of the rhodamine-labeled cells migrating ventrally reacts with mAb lot1 (arrowhead). (D,E) The strip 2 days after culture. Many rhodamine-labeled cells (magenta in D, white in E) migrate in the ventral direction from the dye injection point (asterisk) and stop before entering the GE. Reticulin 1 is immunostained in green in D. (F,G) High-magnification views of the upper (F) and lower (G) boxes in E. The processes of many rhodamine-labeled cells are directed in the ventral direction in F, but reoriented in the rostrocaudal direction at the presumptive LOT area in G. Left, rostral aspect; top, dorsal aspect; dcx, dorsal neocortex; vcx, ventral neocortex; GE, ganglionic eminence. Scale bars: 500 μm in B,D; 50 μm in C,F,G.

In addition to lot cells, other cell populations are suggested to migrate in the ventral tangential stream (Tomiioka et al., 2000). In organotypic culture, some rhodamine-labeled cells in the deeper part of the LOT area were actually found to be lot1-negative (data not shown), while lot1-positive cells were always detected on the surface layer. However, the late expression of lot1-antigen made it impossible to strictly define lot1-positive and -negative cells in the culture. Therefore, the following labeling study, we handled all ventrally migrating cells together as a whole, which contained labeled cells in organotypic culture was in accordance with that of lot cells in whole-embryonic culture and in vivo, thus demonstrating that the behavior of lot cells was reproducible in the organotypic culture system.
Unidirectional ventral migration characterizes the telencephalon in the early developmental stage

The dorsal neocortex was isolated from the E10.5 green mouse embryo, in which all cells expressed green fluorescent protein (GFP), and substituted for the same dorsal part in the wild-type strip, maintaining the orientation. Consequently, we obtained a more dramatic image of extensive cell migration; a great number of green cells migrated from the green dorsal neocortex into the entire area of the wild-type ventral neocortex, crossing the combination boundary, although these green cells still respected the neocortex-GE boundary by terminating the migration (Fig. 2A). By contrast, when the dorsal neocortex isolated from the wild-type embryo was combined with the ventral neocortex of the green mouse strip, the green cells did not penetrate into the dorsal neocortex (Fig. 2B). Therefore, the cells migrate only ventrally but not dorsally in the 2-day culture of the E10.5 telencephalon.

Previous studies have reported dorsal tangential migration of GABAAergic interneurons from the GE to the neocortex (Marin and Rubenstein, 2003). However, dorsal tangential migration was never detected in the above organotypic culture. This appears to reflect differences in the stages of the two tangential migration streams; the ventral tangential migration stream is only transiently seen at the early stage (Tomioaka et al., 2000), whereas the dorsal migration stream starts at a little later stage (Tamamaki et al., 1997; Anderson et al., 2001).

The neocortex contains sufficient directional signals for ventral cell migration

One possible model for ventral tangential migration is its regulation by a long-range signal secreted by the adjacent region. For example, cells may be repelled by the dorsomedial margin of the telencephalon called the cortical hem, or attracted by ventral regions such as the presumptive LOT area and the GE. To ascertain whether these adjacent regions of the neocortex were responsible for the ventral tangential migration, the cortical hem, the presumptive LOT area and the GE were all removed from strips and their respective contributions to migration were evaluated in culture. Even in the absence of these adjacent regions, rhodamine-labeled cells still migrated in the ventral direction within the isolated neocortex down to the ventral edge (Fig. 2C). The ventral migration was similarly marked in any region of the neocortex when isolated and cultured (data not shown). These results suggest that the entire region of the E10.5 neocortex contains sufficient signals to direct ventral cell migration (Fig. 2I).

We also co-cultured two dorsal neocortices combined in the dorsoventrally reversed mirror image, ventrally facing each other, after labeling only one of the cortices. The labeled cells migrated in the ventral direction down to the combination boundary of the two cortices, but never crossed the boundary nor penetrated into the other cortex combined in the reversed orientation (Fig. 2D). These results suggest that the directional force for the ventral migration is relatively stable and probably created by cues that are displayed over the neocortex in a spatially immobilized manner.

Ventrally migrating cells are excluded from the GE

The ventral migration stream abruptly stopped at the boundary between the neocortex and the GE, and hardly penetrated into the GE in organotypic culture and in vivo (Fig. 1D,E), suggesting some mechanism that prevent ventrally migrating cells from invading the GE. To test this hypothesis, we combined the dorsal neocortex isolated from the green mouse embryo with the lateral side of the wild-type strip. In this type of culture, many green cells migrated into the presumptive LOT area and the GE, but very few invaded the GE (Fig. 2E). When the green dorsal neocortex was combined with the ventral side of the GE in the wild-type strip in the dorsoventrally reversed orientation, the green cells did not invade the adjoining GE at all (Fig. 2F). Thus, the...
whole area of the GE was inaccessible to ventrally migrating cells from any direction, suggesting some mechanisms to prevent the migration (Fig. 2I).

In the next step, we analyzed the behavior and morphology of individual cells when they encountered the GE by rhodamine-labeling of the dorsal neocortex combined with the ventral side of the GE in the reversed orientation (Fig. 2G). Although rhodamine-labeled cells did not cross the combination boundary with the GE, the cells migrated up to the immediate vicinity of the boundary, suggesting that the GE does not have a long-range repulsive action on ventrally migrating cells. Interestingly, some rhodamine-labeled cells at the boundary changed their orientation from the dorsoventral to rostrocaudal axis and were aligned parallel to the artificial combination boundary (Fig. 2H). Thus, encounter with the GE may be sufficient for ventrally migrating cells to change their orientation and make a special cellular arrangement.

Transcription factor Gli3 is cell-autonomously required for the ventral tangential migration

We have previously reported that ventral tangential migration is severely disrupted in homozygous embryos for the XtG mutant locus, which carry a deletion in the gene encoding Gli3, a zinc-finger type transcription factor (Schimmang et al., 1992; Hui and Joyner, 1993). In XtG/XtG mouse embryos, lot cells are produced in the neocortex as wild-type embryos, but migrate in various directions only for a short distance, creating ectopic clusters, and fail to reach the presumptive LOT area (Tomiioka et al., 2000). We prepared telencephalon strips from E10.5 Xtx/Xtx embryos and cultured them after rhodamine-labeling of the dorsal neocortex. The labeled cells hardly migrated towards the presumptive LOT area, making ectopic clusters over the neocortex (Fig. 3A,B), which reproduces the behavior of lot cells in Xtx/Xtx mouse embryos.

To examine cell-autonomy of the migration defect in the Xtx/Xtx telencephalon, we next co-cultured strips of wild-type and Xtx/Xtx mutant embryos. When the dorsal neocortex from the green mouse was combined with the ventral neocortex of the Xtx/Xtx strip, many green cells that had the wild-type Gli3 gene penetrated ventrally into the combined Xtx/Xtx ventral neocortex and stopped around the presumptive LOT area in the Xtx/Xtx strip (Fig. 3C). By contrast, when the dorsal neocortex isolated from the Xtx/Xtx green mouse was combined with the ventral neocortex in the wild-type strip, Xtx/Xtx green cells barely penetrated into the wild-type neocortex (Fig. 3D). These results indicate that cell-autonomous defects in Xtx/Xtx cells underlie the disturbance of ventral tangential migration in the Xtx/Xtx mouse embryo.

Gli3 functions as a mediator of Shh signaling in the dorsoventral patterning of the central nervous system (Jacob and Briscoe, 2003). Therefore, it is possible that Shh signaling is involved in the ventral migration. However, the treatment of strips with cycloponamine, an inhibitor of Shh signaling, did not affect the ventral tangential migration (data not shown).

Netrin 1 attracts tangentially migrating cells in organotypic culture

We next focused on the molecular mechanism that controls the ventral tangential migration. Various types of tangentially migrating neurons respond to axon guidance molecules (Tessier-Lavigne and Goodman, 1996). Therefore, we selected 11 molecules (netrin 1, netrin 4/5-netrin, Shh, Sema3a, Wnt1, Wnt5a, Wnt6, Wnt7a, Wnt7b, Slit1 and Slit2) as candidates and investigated their effects on the ventral tangential migration by culturing telencephalon strips with cell aggregates expressing these molecules. Among these candidates, netrin 1, a long-range diffusible molecule that guides various axons and cells migration (Kennedy et al., 1994; Serafini et al., 1994; Serafini et al., 1996; Alcantara et al., 2000; Hamasaki et al., 2001) exerted obvious attraction for the rhodamine-labeled cells. Many rhodamine-labeled cells migrated towards the cells secreting netrin 1, ignoring the LOT area (Fig. 4A,B), but not toward the mock-transfected control cells (Fig. 4C,D,H).

Expression of netrin 1 and its receptor Dcc in the embryonic telencephalon

Attractive signal of netrin 1 is transduced through the functional receptor Dcc (Keino-Masu et al., 1996). From E10.5 to E12.5, while the ventral tangential migration is most marked (Tomiioka et al., 2000), netrin 1 mRNA was strongly expressed in the neuroepithelium of the GE, whereas Dcc mRNA was expressed on the surface layer of the neocortex, which contained migrating lot cells (Fig. 5A-C,E-G) in agreement with previous reports (Cooper et al., 1995; Schwarting et al., 2004). The expression of Dcc protein in lot cells was confirmed by double immunostaining with anti-Dcc and lot1 antibodies (Fig. 5I-M). In the Xtx/Xtx telencephalon, the expression patterns of netrin 1 and Dcc seemed essentially conserved in spite of severe dysplasia (Fig. 5D,H).

We used the netrin 1-lacZ knock-in mutants to mark the expression of the netrin 1 gene in whole-telencephalon preparations. In the heterozygous knock-in telencephalon, the neuroepithelium of...
the GE was \textit{lacZ}-positive from E10.5 onwards (Fig. 6C), which well reflected the endogenous expression patterns of netrin 1 shown by in situ hybridization (Fig. 5A-C). In addition, after E13.0, the strong expression of \textit{lacZ} manifested in the rostroventrally restricted area in the telencephalon beneath the olfactory bulb, corresponding to the rostral part of the olfactory tubercle (Fig. 6F,G). In situ hybridization also detected the expression of netrin 1 mRNA in cells that occupied the surface area of this part at E12.5 (Fig. 5C). Thus, in later embryonic stages, netrin 1 is expressed on the surface area of the olfactory tubercle, in addition to the neuroepithelium of the GE.

Defects in the distribution of lot cells in netrin 1 and Dcc mutant mice

To assess the physiological requirements for netrin 1 in migration of lot cells, we investigated distribution of lot cells in netrin 1-\textit{lacZ} knock-in mutants whose netrin 1 allele is severely hypomorphic (Serafini et al., 1996). By E12.5, differences in the distribution of lot cells between wild-type and homozygous mutant embryos were not evident; lot cells were dorsally restricted in the presumptive LOT area, shaping a narrow cellular array, in both of the genotypes (Fig. 6A,B). From E13.0 onwards, the distribution of lot cells ventrally expanded in wild-type and heterozygous embryos; lot cells covered the ventral part of the LOT area and particularly widely spread the rostral part that continued from the olfactory bulb (Fig. 6D,G). Therefore, the ventrolateral side of the olfactory bulb was now surrounded by lot cells, broadening the rostral part of the cellular array. The front of this ventral expansion of lot cells abutted the surface area of this part at E12.5 (Fig. 5C). Thus, in later embryonic stages, netrin 1 is expressed on the surface area of the olfactory tubercle, in addition to the neuroepithelium of the GE.
netrin 1-expressing domain in the rostral olfactory tubercle (Fig. 6G, I). In the homozygous mutant embryos, the distribution of lot cells did not expand ventrally even after E13.0, leaving the ventral part of the LOT area unoccupied by lot cells (Fig. 6E, H, J). The dense pack of lot cells in the ventral margin of the cellular array delineated the edge more sharply (Fig. 6E, H, L) compared with wild-type and heterozygous embryos (Fig. 6D, G, K). Dcc mutant embryos (Fazeli et al., 1997) phenocopied the above abnormal distribution of lot cells (data not shown). These results suggest that netrin 1-Dcc signaling controls the late phase of ventral migration of lot cells in vivo, specifically when the cells migrate further ventrally from the LOT, where the late expression of netrin 1 in the rostral tubercle seems functional.

Because the organotypic culture of telencephalon strips only reproduces the early phase of ventral tangential migration, the migration defects of Dcc or netrin 1 mutant embryos were undetectable, and the cells migrated seemingly normally when the mutant strips were cultured (Fig. 4E), even though the cells in homozygous Dcc mutant strips completely lost the reactivity to exogenous source of netrin 1 (Fig. 4F, H). Therefore, the initial stage of the ventral migration can progress without netrin 1-Dcc signaling, which presents a contrast to the early migration defect in Xr/Xr mutant embryos. During ventral attraction of commissural axons in the spinal cord, netrin 1 and Shh have redundant functions, and Shh partially compensates for the axonal defect resulting from the loss of netrin 1-Dcc signaling (Charron et al., 2003). We therefore suspected compensatory action of Shh in the mutants and blocked the Shh signaling by cyclopamine in homozygous Dcc mutant strips. However, the ventral migration was still normally observed in the strips (Fig. 4G), suggesting that neither Shh nor netrin 1-Dcc signaling is required for the early phase of ventral tangential migration.

**Pathfinding error in olfactory bulb axons of netrin 1 and Dcc mutant mice**

Because lot cells guide olfactory bulb axons (Sato et al., 1998), the aberrant distribution of lot cells in netrin 1 and Dcc mutant embryos may cause a defect in the projection of these axons. Thus, we investigated the trajectory of olfactory bulb axons in whole-mount immunostaining for neuropilin 1, a marker of these axons. The axons, originating from the medial part of the olfactory bulb, encircled the bulb via either the ventral or dorsal side before converging onto to the LOT. Similar numbers of axons were normally found to take the ventral and dorsal pathways (Fig. 7A, C). In netrin 1 and Dcc homozygous mutant embryos, the axons projecting through the ventral pathway were markedly reduced compared with those in the wild-type and heterozygous embryos. The mutations, however, did not seem to influence the axons in the dorsal pathway nor the overall trajectory of LOT axons after convergence of the two pathways (Fig. 7A, B). This axonal defect in the ventral pathway was detected from E13.5 and was clearly visible after E14.5 by immunostaining.

Fluorescent dye labeling of axons from the medial olfactory bulb specified the defect in the ventral pathway in more detail. In wild-type and heterozygous embryos, the axons taking the ventral pathway smoothly projected over the rostroventral part of the LOT area, which had received the late invasion of lot cells, and adjoined the ventral margin of the LOT bundle, keeping the orderly arrangement among the neighboring axons (Fig. 7E, G). In the homozygous embryos, the axons through the ventral pathway were tangled and often formed an off-course bundle that strayed from the LOT. These aberrant bundles usually turned back into the bulb, forming a focused terminus on the dorsolateral position of the olfactory bulb (Fig. 7F, H).

**DISCUSSION**

The ventral tangential migration was reproducible by culturing the isolated neocortex without other tissues surrounding the neocortex. Therefore, the neocortex at E10.5 contains sufficient signals to direct tangentially migrating cells into the ventral direction, although the results do not exclude the possibility that the molecules secreted from the adjacent tissue before the initiation of culture are retained and function in the neocortex. Because ventral migration exhibited similar levels of robustness in all regions of the neocortex, the directional signals appear to be widespread over the entire neocortex. Such extensive two-dimensional signals could be created by a graded distribution of guidance molecules; either a dorsal-high repulsive or ventral-high attractive factor could fulfill this task as
NETRIN 1 and neuronal migration

DEVELOPMENT

DCC +/−  DCC −/−

Fig. 7. Pathfinding of olfactory bulb axons in the telencephalon of Dcc mutant embryo. (A–D) Projection of olfactory bulb axons in E14.5 heterozygous (A,C) and homozygous (B,D) Dcc mutant embryos visualized with anti-neuropilin 1 antibody. The number of axons elongating from the ventral side of the olfactory bulb in the homozygous mutant (arrow in D) is much lower than that in the heterozygous mutant. The phenotype was apparent in all of the 20 homozygous mutant telencephalons. Left, rostral aspect; top, dorsal aspect. (E–H) Fluorescent dye labeling of medial olfactory bulb axons in E16.5 heterozygous (E,G) and homozygous (F,H) Dcc mutant embryos. (E,F) Lateral views of left hemispheres. Left, rostral aspect; top, dorsal aspect. (G,H) Ventral views of left hemispheres showing the dye-labeled ventral pathways; left, rostral aspect; top, lateral aspect. Dye-labeled axons split into the dorsal (white arrowheads in E,F) and ventral (arrows in E-H) pathways to enter into the LOT. In the homozygous telencephalon, axons projecting in the ventral pathway are tangled and misdirected back to the olfactory bulb. This aberrant projection of olfactory bulb axons was detected in all of 15 homozygous Dcc mutants and three out of five homozygous netrin 1 mutants. Scale bars: 1 mm in A,B; 500 μm in C–H.

...demonstrated in other nervous systems (Drescher et al., 1997). Indeed, several molecules are expressed in the embryonic neocortex making a dorsoventral gradient (Medina et al., 2004; Hasegawa et al., 2004).

The present study identified netrin 1 as a component of the physiological mechanism that controls the migration of lot cells. In the absence of netrin 1-Dcc signaling, lot cells reached the dorsal part of the LOT area but did not spread further ventrally from there. Although our study does not completely rule out the possibility that the lack of netrin 1-Dcc signal slowed down the general speed of ventral tangential migration, which eventually prohibits the cells from arriving in the ventral-most area, the physiological function of netrin 1 is more likely to be local attraction of the cell into the ventrally restricted LOT area during the late phase of the migration, because the initial arrival of the cells at the LOT area was not delayed in homozygous netrin 1 or Dcc mutants. Furthermore, netrin 1 is expressed in the rostral tubercle from the late developmental stage in a manner perfectly matched with the role for local attractants, and the early phase of ventral migration reproduced in culture was indistinguishable between the mutant and wild-type strips. Another concern about the mutant phenotypes may be excessive cell death because netrin 1 and Dcc are involved in apoptosis (Mehlen et al., 1998; Arakawa, 2004). However, we confirmed that apoptosis is only enhanced in the netrin 1 mutant, but not the Dcc mutant telencephalon (see Fig. S1 in the supplementary material), as expected by the dependence receptor hypothesis (Bredesen et al., 2005). Therefore, the phenotypes observed in the mutant telencephalon can not be attributed to the excessive death of lot cells or their surrounding tissues, but appear to be direct consequences of the impaired reception of netrin 1-Dcc signaling in cells of the ventral migration stream. In conclusion, netrin 1 is suggested to function as a local attractant in the ventral area that controls the late phase of ventral migration.

What, then, controls the early phase of ventral migration? In Xi/ mutant telencephalon, the early phase of the migration was severely disrupted, even though netrin 1 and Dcc were expressed in the appropriate time and place. The responsible gene for the Xi/ mutation, Gli3, was cell-autonomously required in the ventrally migrating cells, probably for their actual movement or reception of guidance signals. Gli3 is a downstream component of Shh signaling (Jacob and Briscoe, 2003), and Shh acts directly as a chemoattractant on axons in the spinal cord, collaborating with netrin 1 (Charron et al., 2003). However, the present study did not support the direct involvement of Shh in guidance of the ventral migration stream, because inhibition of Shh signaling by cycloheximide did not affect the ventral migration, even under conditions lacking netrin 1-Dcc signaling. The candidate screening of guidance molecules also failed to detect apparent activity of Shh for the ventral migration stream, although this ectopic supply of Shh severely affected morphogenesis and patterning of telencephalon strips in culture and hampered the high sensitive analysis (data not shown). Because Gli3 is more than just a Shh mediator (Liu et al., 1998; Mullor et al., 2001), this transcription factor may mediate other signals in the cells during the ventral migration.

The combination culture demonstrated that the GE rigidly excludes the ventral migration stream. However, the GE is not a complete obstacle to all cell migrations, because other neuron types migrate through the GE. For example, a fraction of reelin-positive cells tangentially migrate onto the surface of the GE at the embryonic stage similar to that for lot cells (Takiguchi-Hayashi et al., 2004), and progenitors of cortical interneurons vigorously migrate through the deeper region of the GE at a later stage (Marin and Rubenstein, 2003). Thus, not the simple physical structure of the GE but target cell-specific mechanisms appear to operate for the exclusion of ventrally migrating cells. Because the GE did not repel the migrating cells from a distance, short-range guidance molecules appear to be responsible for this exclusion.

Lot cells construct the cellular array between the neocortex and the GE, where the two types of guidance signals, the ventrally directed driving force in the neocortex and the short-range repulsion by the GE, are confronting each other. This encounter between these two signals may be sufficient for lot cells to alter their cellular arrangement, because the cells changed their orientation and aligned along the artificial boundary on which the neocortex and the GE were directly combined. In fact, the presumptive LOT area between the two compartments may not be essential for formation of the cellular array. A previous study reported that in Pax6 mutant.
embryos, although many characteristics of the neocortex-GE boundary are missing (Stoykova et al., 1997), lot cells were still capable of forming a continuous cellular array on the area adjacent to the GE (Hirata et al., 2002).

We consider two possibilities for the pathfinding error in olfactory bulb axons of netrin 1 and Dcc mutants. First, because olfactory bulb neurons transiently express Dcc on their axons (Shu et al., 2000; Inaki et al., 2004), it is possible that olfactory bulb axons are directly guided by netrin 1, so that the axons in the mutant embryos are misdirected as a direct consequence of the loss of netrin 1-Dcc signaling. However, netrin 1 does not exert any apparent effects on olfactory bulb axons in culture (Li et al., 1999; de Castro et al., 1999), and therefore we could not address this possibility any further. Secondly, the pathfinding error in the mutants might be indirectly induced by the aberrant distribution of lot cells. The route for the ventral pathway of olfactory bulb axons involves the rostroventral part of the LOT area, which is normally populated with abundant lot cells. In the absence of netrin 1-Dcc signaling, the distribution of lot cells into this area was abandoned and, at the same time, the axons couring through this area showed the most severe defects (Fig. 8). Thus, considering that lot cells are required for the guidance of olfactory bulb axons (Sato et al., 1998), the loss of lot cells around the area could have serious consequences for the axons in the ventral pathway.

The results of the present and previous studies (Tomioka et al., 2000) suggest that, in addition to lot cells, other cell populations tangentially migrate in the ventral direction in the developing neocortex. Reelin-positive marginal zone cells are generated from the caudomedial wall of the telencephalon and distributed almost over the entire neocortex and part of the GE by tangential migration (Takiguchi-Hayashi et al., 2004). As lot cells do not express reelin (Sato et al., 1988), these two types of cells appear to belong to different cell populations but migrate in the routes overlapping to some extent. Another recent study has revealed a ventral tangential migration stream of olfactory cortical neurons that is seemingly very similar to that of lot cells but governed by a distinct stop signal (T. Nomura and N. Osumi, personal communication). These observations taken together support the view that multiple neuronal populations migrate simultaneously in the ventral direction on the early embryonic neocortex. Although this migration mode had been somehow overlooked until recently, the present study clearly shows that the ventral tangential migration is the most common migration mode in the early telencephalon when neurogenesis has just started, and therefore has immeasurable impact on all the following processes. Hence, it is of great interest to determine the precise constituents, migration mechanisms and physiological functions of these processes in the future.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/5/845/DC1

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