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Cellular and Molecular Guidance of GABAergic Neuronal Migration from an Extracortical Origin to the Neocortex

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Summary

Formation of the normal mammalian cerebral cortex requires the migration of GABAergic inhibitory interneurons from an extracortical origin, the lateral ganglionic eminence (LGE). Mechanisms guiding the migratory direction of these neurons, or other neurons in the neocortex, are not well understood. We have used an explant assay to study GABAergic neuronal migration and found that the ventricular zone (VZ) of the LGE is repulsive to GABAergic neurons. Furthermore, the secreted protein Slit is a chemorepellent guiding the migratory direction of GABAergic neurons, and blockade of endogenous Slit signaling inhibits the repulsive activity in the VZ. These results have revealed a cellular source of guidance for GABAergic neurons, demonstrated a molecular cue important for cortical development, and suggested a guidance mechanism for the migration of extracortical neurons into the neocortex.

Introduction

It has long been assumed that neurons in the neocortex originate within the ventricular zone (VZ) of the neocortex, from which precursor cells migrate radially to their final positions in other layers of the cortex. Work in the past decade has challenged this assumption and revealed an extraneocortical origin for a major type of neocortical neurons, interneurons containing the neurotransmitter γ-aminobutyric acid (GABA). Both inhibitory and excitatory neurons play crucial roles in information processing in the nervous system as well as in signaling between the nervous system and other systems. The major type of inhibitory neurons in the mammalian neocortex are the GABAergic interneurons.

It is now known that the majority, if not all, of GABAergic neurons have their origins in the lateral ganglionic eminence (LGE), the primordium of the striatum, and that precursor cells for GABAergic neurons migrate tangentially from the LGE to the neocortex in the striatal-neocortical pathway (reviewed in Lumsden and Gulisano, 1997). This conclusion is supported by results from several experiments. Immunohistochemical examinations of the developing cortex suggest possible involvement of tangential migration in the distribution of GABAergic neurons in the neocortex (Van Eden et al., 1989; DeDiego et al., 1994). Tracing with tritiated thymidine and the lipophilic dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine (DiI) has provided direct evidence for the migration of GABAergic neurons from the LGE to the neocortex (De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997). The absence of neocortical GABAergic neurons in mutant mice with genetic deletions of *Dlx-1* and *Dlx-2*, two homeobox genes expressed in the ventricular and subventricular zones of the LGE (Anderson et al., 1997), indicates a striatal origin for the majority of neocortical GABAergic neurons (Anderson et al., 1997). Experimental manipulations show that prevention of neuronal migration from the LGE to the neocortex results in a dramatic decrease in the number of GABAergic neurons in the neocortex (Anderson et al., 1997; Tamamaki et al., 1997). These studies have therefore demonstrated the importance of the migration of GABAergic interneurons in the striatal-neocortical pathway (reviewed in Lumsden and Gulisano, 1997). However, cellular and molecular mechanisms underlying neuronal migration from the LGE to the neocortex are not known.

Here, we have used an in vitro explant assay to study neuronal migration from the subventricular zone of the LGE. This assay allowed us to study cellular and molecular mechanisms guiding the migration of GABAergic neurons. We have found that the VZ of the LGE contains a repulsive activity for migrating neurons. This region was previously known to express mouse *Slit1* (*mSlit1*) (Nguyen Ba-Charvet et al., 1999; Yuan et al., 1999), which was known to promote axon branching and guide axon projection and neuronal migration (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Wang et al., 1999; Wu et al., 1999; Yuan et al., 1999). We have now found that Slit proteins can provide directional guidance for neurons migrating from the LGE to the neocortex. Our studies have thus revealed the cellular source and molecular identity of guidance cues for neurons migrating from an extracortical origin into the neocortex, which should contribute to furthering our understanding of neocortical development in mammalian embryos.

Results

An In Vitro Assay for Studying Neuronal Migration from the LGE

In order to study the guidance of GABAergic neurons migrating from the LGE, we have established an in vitro assay involving the culture of LGE explants, an assay similar to those used previously for studies of neurons migrating in the olfactory system in the postnatal brain (Hu and Rutishauser, 1996; Wichterle et al., 1997; Wu et al., 1999). Explants of the subventricular zone of LGE (Figure 1A) were isolated and cultured either in collagen gel matrix (Guthrie and Lumsden, 1994) or in a mixture of collagen gel and matrigel, a three-dimensional extracellular matrix gel of collagen IV, laminin, heparan sulfate proteoglycans, and entactin-nidogen (Kleinman et al.,

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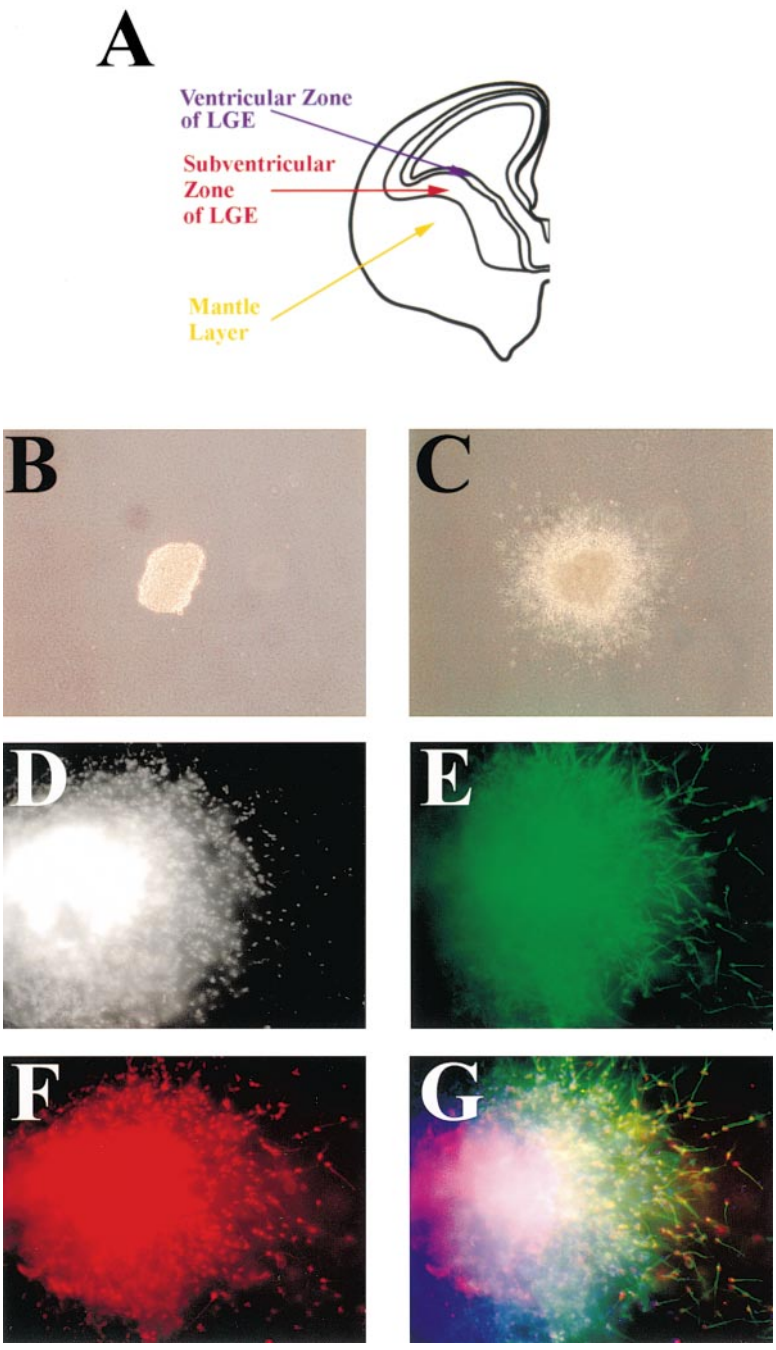

Figure 1. Migration of GABAergic Neurons from LGE Explants

(A) A diagram of regions in the telencephalon, showing the subventricular zone in the LGE from which explants were isolated and cultured, the ventricular zone, and the mantle layer of LGE.

(B) The typical appearance of an LGE explant soon after placed into the culture.

(C) Another LGE explant after 20 hr of culturing; note that cells had migrated out of the explant and were symmetrically distributed around the circumference of the explant.

(D) An explant after 20 hr of culturing, showing Hoechst dye staining. The original color was blue, but the panel was shown in black and white here to facilitate visualization of the printed picture.

(E) The same explant as that in (D), showing staining with TuJ1.

(F) The same explant as those in (D) and (F), showing staining with anti-GABA.

(G) Superimposition of (D), (E), and (F). (D) was used in its original blue color.

1982). At the beginning of culturing, cells stay within the explants, and the edges of explants were clear (Figure 1B). After culturing, cells migrated out of LGE explants and were symmetrically distributed around the circumference of each explant (Figure 1C). To determine whether these cells were neurons, we carried out immunocytochemistry with the TuJ1 antibody, which recognizes the neuron-specific β-tubulin (Figure 1E). When counted against nuclear staining of all cells by Hoechst dye (Figure 1D), 81% of the cells that had migrated out of the LGE explants were found to be TuJ1-positive neurons. When an anti-GABA antibody was used to examine GABAergic neurons (Cobas et al., 1991; Del Rio et al., 1992; Yan et al., 1992; Lauder et al., 1986), about 73% of all cells were positive for GABA-like immunoreactivity (Figure 1F). Double staining with TuJ1 and anti-GABA showed that 91% of TuJ1-positive neurons were GABAergic. When antibodies to the glial fibrillary acidic protein (GFAP) was used to stain glial cells and their fibers, the glial cells were observed within the explants and did not migrate out of them (data not shown). These results indicate that GABAergic neurons could migrate out of LGE explants, suggesting that cultures of LGE explants can serve as an in vitro model for studying mechanisms guiding the migration of GABAergic neurons from the subventricular zone of the LGE.

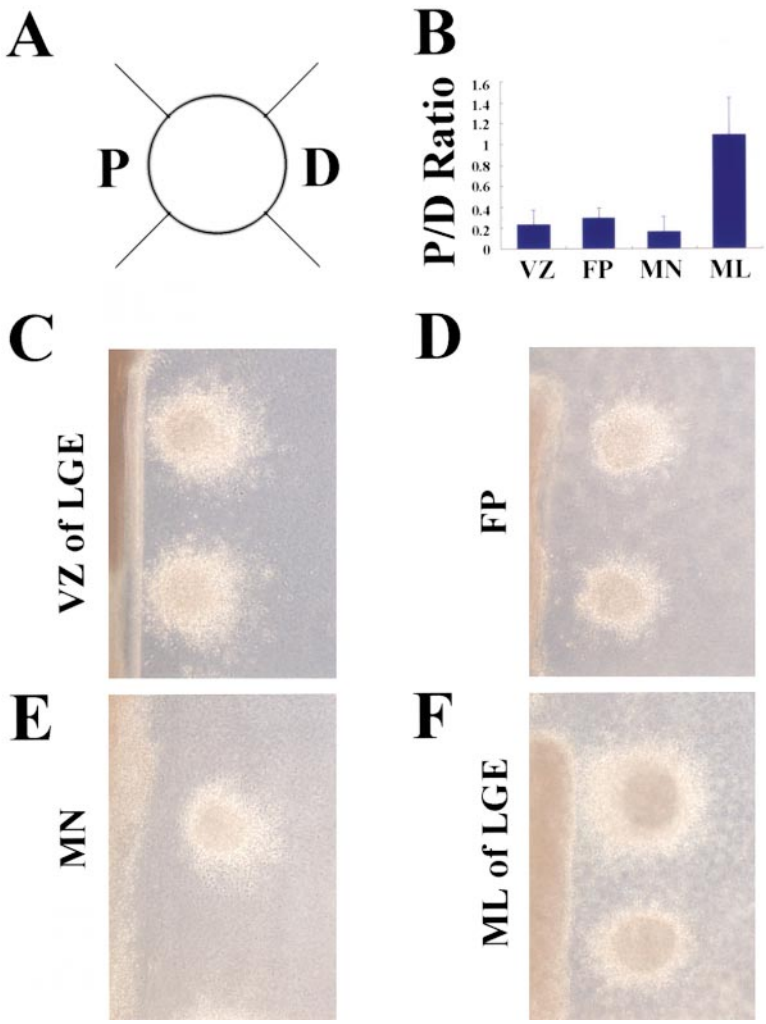

Figure 2. Cellular Sources of Guidance Cues for LGE Neurons

(A) A diagram showing the division of quadrants around each LGE explant, P being the proximal quadrant and D being the distal quadrant.

(B) Effects of different regions of the CNS on the distribution of cells migrating out of explants of the subventricular zone of the LGE. P/D ratios were calculated from the numbers of cells in the proximal quadrants divided by those in the distal quadrants. VZ indicates the ventricular zone of the LGE, ML the mantle layer of the LGE, FP the floor plate of the spinal cord, and MN the motoneurons of the spinal cord. The cell numbers were counted from 18 pieces of VZ explants, 10 FP explants, 2 MN explants, and 18 ML explants.

(C) Effect of the ventricular zone (VZ) of LGE on the migration of cells from explants of the subventricular zone of the LGE.

(D) Effect of the floor plate (FP) of the spinal cord on cells from LGE explants.

(E) Effect of the motoneurons (MN) of the spinal cord on cells from LGE explants.

(F) Effect of the mantle layer (ML) of the LGE on cells migrating from explants of the subventricular zone of the LGE.

Determination of Guidance Cues in Different Regions of the CNS

To search for cellular sources of potential guidance cues for migratory LGE neurons, we dissected different regions of the central nervous system (CNS) and cocultured them with LGE explants. The VZ and the mantle layer of the LGE were chosen because we were interested in testing whether regions close to the origin of migrating cells in the subventricular zone of the LGE could provide guidance cues (Figure 1A). The floor plate and motoneurons of the spinal cord were chosen because they might provide clues about molecules common in the spinal cord and the brain.

To quantitate the distribution of migrating cells, the area surrounding each explant was divided into four quadrants (Figure 2A); the number of cells in the quadrant proximal (P) to a potential source of guidance was compared to that in the distal (D) quadrant (P/D ratio in Figure 2B). When LGE explants were cocultured with a piece of the VZ of the LGE, the distribution of migrating cells was asymmetric with a higher number of cells in the distal quadrant than that in proximal quadrant (Figures 2C and 2B, column VZ), suggesting that the ventricular zone contained a repulsive cue for neurons migrating from the subventricular zone of the LGE. On the other hand, after coculturing with the mantle layer of the LGE, cells migrating out of explants of the subventricular zone of the LGE were symmetrically distributed (Figures 2F and 2B, column ML). Interestingly, LGE neurons were also repelled by the floor plate (Figures 2D and 2B, column FP) and the motoneurons of the spinal cord (Figures 2E and 2B, column MN), suggesting that the molecular cue(s) in the VZ of the LGE is similar to those in the floor plate and the motoneurons of the spinal cord.

Effect of Slit on Neurons Migrating out of LGE Explants

We have previously noticed the expression of vertebrate *slit* genes in the ventricular zone of the LGE (Yuan et al., 1999) as well as in the floor plate and motoneurons of the spinal cord (Brose et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Wang et al., 1999; Yuan et al., 1999). Because regions with *slit* expression correlate with those possessing a repulsive activity for the LGE, we wondered whether Slit could regulate the migration of GABAergic neurons.

To directly test the function of Slit proteins, we transfected cDNAs expressing full-length mouse Slit1 (mSlit1)

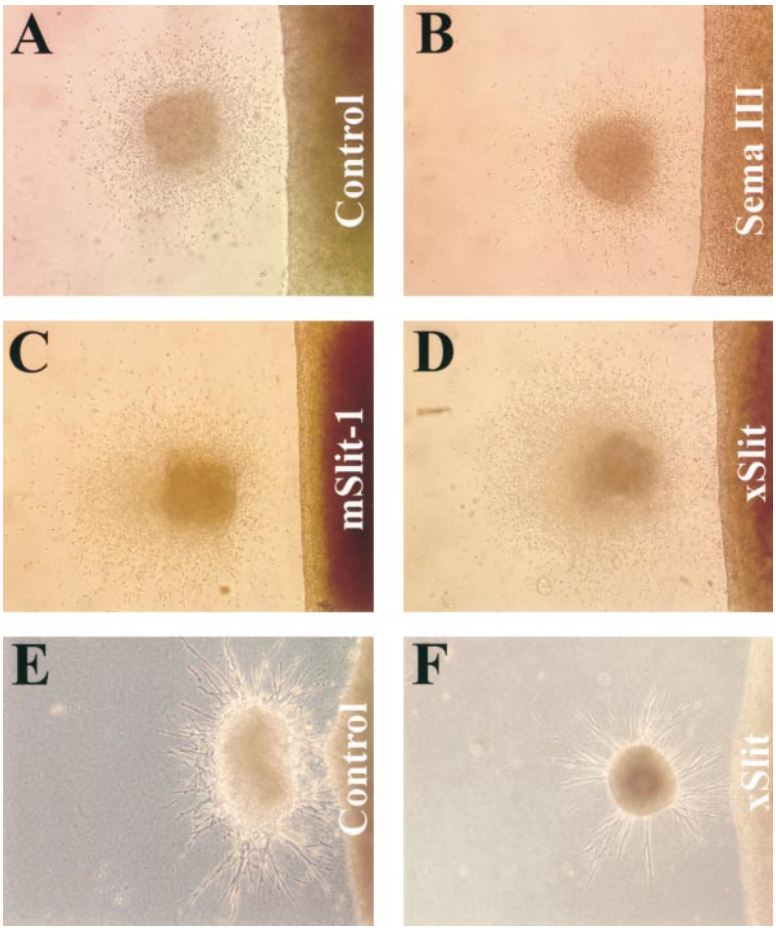

Figure 3. Effect of Slit on Cells Migrating from LGE Explants

(A) Symmetric distribution of cells around the LGE explant cocultured with an aggregate of cells transfected with the vector (n = 87).
(B) Symmetric distribution of migrating LGE cells when cocultured with an aggregate of human Sema III–expressing cells (n = 25).
(C) Asymmetric distribution of migrating LGE cells when cocultured with an aggregate of mSlit1-expressing cells (n = 74).
(D) Asymmetric distribution of migrating LGE cells when cocultured with an aggregate of xSlit-expressing cells (n = 89).
(E) Symmetric migration of endothelial cells from an aorta explant in the presence of control HEK cells.
(F) Symmetric migration of endothelial cells from an aorta explant in the presence of HEK cells expressing xSlit.

and *Xenopus laevis* Slit (xSlit) proteins into human embryonic kidney (HEK) 293 cells and obtained cells either transiently expressing mSlit1 or stably expressing xSlit. Aggregates of control or Slit-expressing cells were cocultured with explants of the subventricular zone of the LGE. Migrating LGE cells were symmetrically distributed around LGE explants when placed next to HEK cells transfected with the vector plasmid (Figure 3A) (84/87 explants with symmetric distribution). In contrast, migrating LGE cells were asymmetrically distributed around the explants when cocultured with aggregates of Slit-expressing cells (Figures 3C and 3D); there were more cells in the distal quadrant than those in the proximal quadrant (Figures 3C and 3D). Both HEK cells transiently expressing mSlit1 and those stably expressing xSlit were repulsive to LGE neurons (Figures 3C and 3D) (89/89 explants with asymmetric distribution for xSlit and 73/74 asymmetric explants for mSlit1). These results indicated that Slit proteins were chemorepellents to cells migrating out of LGE explants. To test whether other axon repellents were also repulsive to LGE cells, we transfected HEK cells with a cDNA for Semaphorin III (Sema III), another major repulsive molecule for axons. Aggregates of Sema III–expressing HEK cells did not affect the migration of LGE cells (Figure 3B) (21/25 explants being symmetric). To examine whether Slit repels all migrating cells, we used the aorta ring assay to investigate whether Slit repels endothelial cells (Malinda et al., 1999). It was found that Slit did not repel or attract cells migrating out of the aorta (Figure 3F). When an endothelial cell line was tested, Slit also could not affect the direction of cell migration (data not shown). Although these results do not rule out possible involvement of Slit in angiogenesis, they indicate that Slit could not guide all migrating cells such as the endothelial cells that we have tested here.

The effect of Slit on migrating LGE neurons has been quantified both spatially and numerically (Figure 4). Slit aggregates could change the distribution of cells in the proximal versus distal quadrants; in the presence of Slit aggregates, more cells migrated further away from the explants in the distal quadrants than those did in the proximal quadrants (Figure 4B). These results demonstrate that Slit is repulsive to cells migrating from explants of LGE. Staining with TuJ1 confirmed that cells repelled by Slit were neurons (Figures 5D–5F). Because earlier results have shown that a fraction of, but not all, migrating neurons were GABAergic (Figure 1F), it was important to determine whether Slit repelled GABAergic neurons. Immunocytochemistry with the anti-GABA antibody showed that GABAergic neurons were indeed repelled by Slit (Figures 5G–5I). The effects were similar either when total cells were counted or when only GABAergic neurons were counted (Figures 4B–4F).

To examine whether Slit act indirectly on GABAergic neurons by influencing the distribution of glial cells and

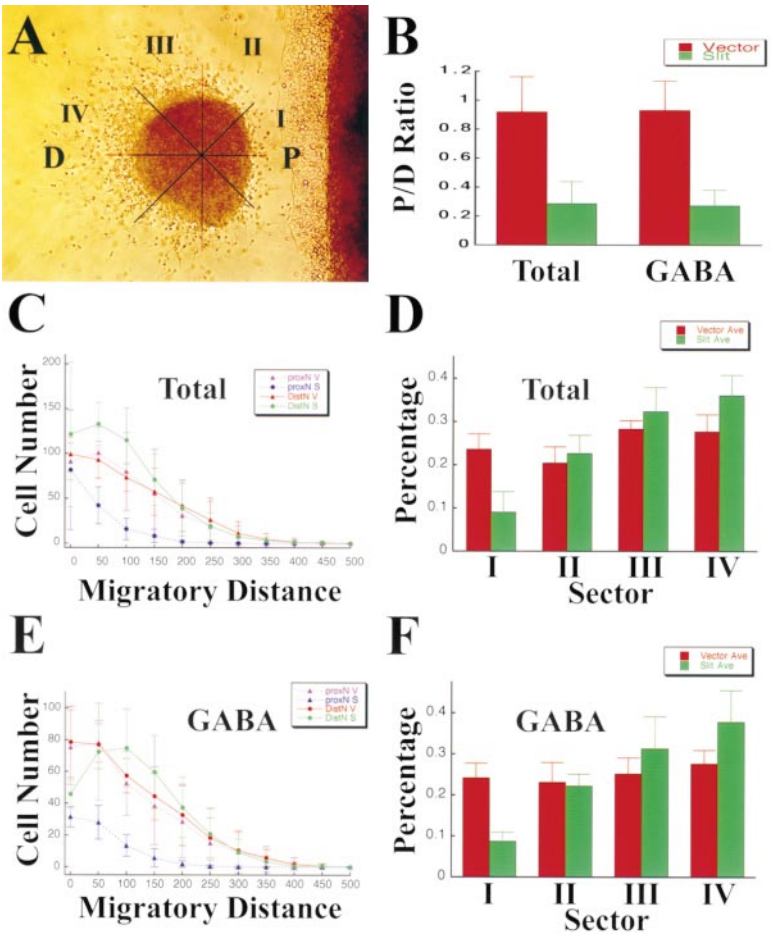

Figure 4. Quantification of the Effect of Slit

(A) A diagram of the areas surrounding an LGE explant; I–IV are 1/8 sectors while P and D are proximal and distal quadrants, respectively.

(B) The ratios of cell numbers in the proximal (P) and distal (D) in the presence of Slit (green) or control (red) aggregates. Total numbers of cells were shown in two columns on the left, while numbers of GABAergic neurons were counted in the two columns on the right. Seven explants were counted in each of these experiments. The differences between control and Slit cocultures were statistically very significant ($p < 0.001$) when counting either all cells or GABAergic neurons.

(C) Spatial distribution of migrating LGE cells in the presence of Slit or control aggregates. Cell numbers were counted from Hoechst staining of seven explants. On the y axis is the average number of cells migrating within a ring of 50 μm in width with the distance of the inner edge of the ring indicated on the x axis. Green circles indicate the number of cells in the distal quadrants in the presence of Slit aggregates; blue circles are those in the proximal quadrants in the presence of Slit aggregates; red triangles are those in the distal quadrants in the presence of control HEK aggregates; and rosy triangles are those in the proximal quadrants in the presence of control HEK aggregates.

(D) Percentages of LGE cells migrating in each sector from LGE explants in the presence of Slit (green) or control (red) HEK cells. Percentages were calculated from cell numbers counted from Hoechst staining of seven explants.

(E) Similar to (C), but the cell numbers were counted from anti-GABA staining of the same seven explants.

(F) Similar to (D), but ratios were calculated from anti-GABA staining of the same seven explants.

their fibers, anti-GFAP staining was carried out on LGE explants cocultured with aggregates of xSlit-expressing cells (Figure 6). Even though cells migrated out of LGE explants (Figures 5M and 5N), GFAP-positive cells stayed within LGE explants (Figure 5O) and did not migrate out. These results suggest that it is unlikely that Slit first act on glial cells that then guide the direction of GABAergic neurons. This suggestion is consistent with the previous knowledge that tangential migration is independent of glial cells.

Effect of Slit on Neurons Migrating from the LGE to the Neocortex

Results from the explant assays have shown that Slit is repulsive to LGE neurons migrating in vitro. However, they could not answer the question of whether Slit acts on LGE neurons in their normal migratory pathway, the striatal-cortical pathway. To investigate a possible role of Slit in directing neuronal migration from the LGE to the neocortex, we used the slice assay in which a coronal section of the rat embryonic brain containing the entire migratory pathway of LGE neurons was preserved in culture (Anderson et al., 1997; Tamamaki et al., 1997). Previous studies have established that DiI labeling in this system can trace GABAergic neurons migrating from the LGE to the neocortex (Anderson et al., 1997; Tamamaki et al., 1997). In our experiments, we placed Slit cells or control cells at the juncture of the LGE and the neocortex and examined their effects on cells migrating from the LGE to the neocortex (Figure 6).

Slices of coronal sections of rat brains were labeled with Hoechst dye to reveal the outlines of the sections (Figures 6A and 6E). Aggregates of Slit-expressing cells or control cells were labeled with 3, 3′dioctadecyloxacarbocyanine (DiO). In some experiments, we placed Slit or control cells at the junction of the LGE and the neocortex (Figures 6B and 6F, green cells). The migrating LGE neurons were traced by inserting a crystal of DiI in the subventricular zone of the LGE (Figures 6C and 6G, red cells). Superimposition of three color images of Hoechst dye, DiO, and DiI revealed the positions of LGE cells migrating into the neocortex relative to the aggregates of Slit or control cells (Figures 6D and 6H). In 53 explants, we placed Slit aggregates on the cortical-striatal junction on the left side of the brain and control aggregates on the right junction. Among them, neurons

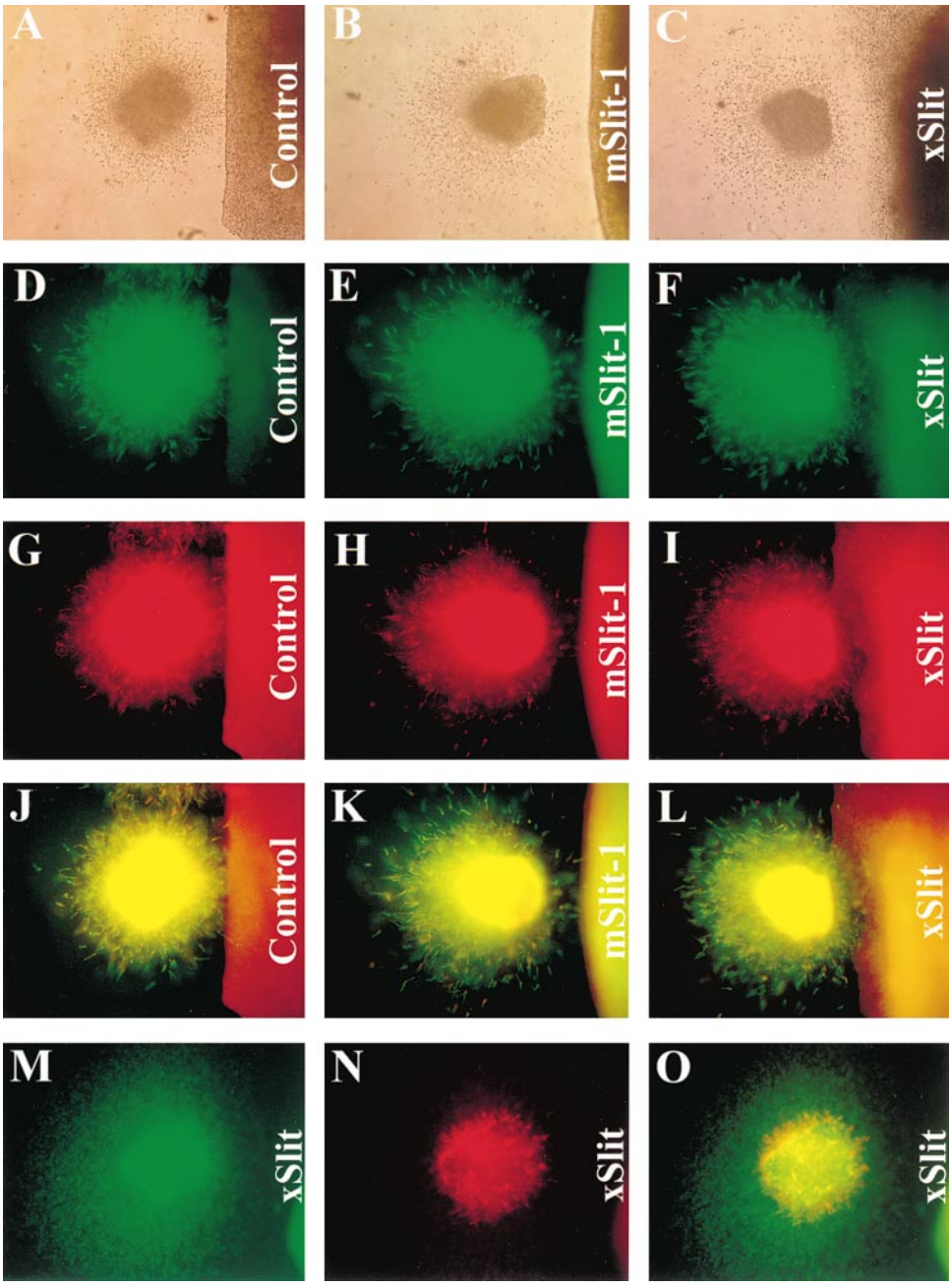

Figure 5. Neuronal and GABAergic Nature of LGE Cells Repelled by Slit

(A–C) Bright-field views of cells migrating out of LGE explants.

(D–F) Fluorescent views showing TuJ1-positive cells.

(G–I) Fluorescent views showing GABAergic cells; a significant fraction but not all of the cells are positive for anti-GABA staining.

(M–O) Double staining with the Hoechst dye and anti-GFAP antibodies of an LGE explant cocultured with an aggregate of cells expressing xSlit.

(A), (D), (G), and (J) are the same explant with (J) being the superimposition of (A), (D), and (G); similarly, (B), (E), (H), and (K) are the same explant with (K) being the superimposition of (B), (E), and (H); (C), (F), (I), and (L) are the same explant with (L) being the superimposition of (C), (F), and (I).

(M) Distribution of cells stained by the Hoechst dye in the presence of an xSlit aggregate; the staining was converted on the computer from its original blue color to a green color that is easier for visualization against the black background.

(N) Distribution of GFAP-positive cells in the same explant as that shown in (M).

(O) Superimposition of (M) and (N); again the Hoechst dye staining is shown in the artificial green color for better visualization.

migrated from the LGE past the region overlaid with an aggregate of control cells into the neocortex in 52 out of 53 slices. In 50 out of the same 53 slices but on the side with a Slit aggregate, no LGE neurons migrated past the region overlaid with Slit aggregates into the neocortex. In the three explants in which LGE neurons

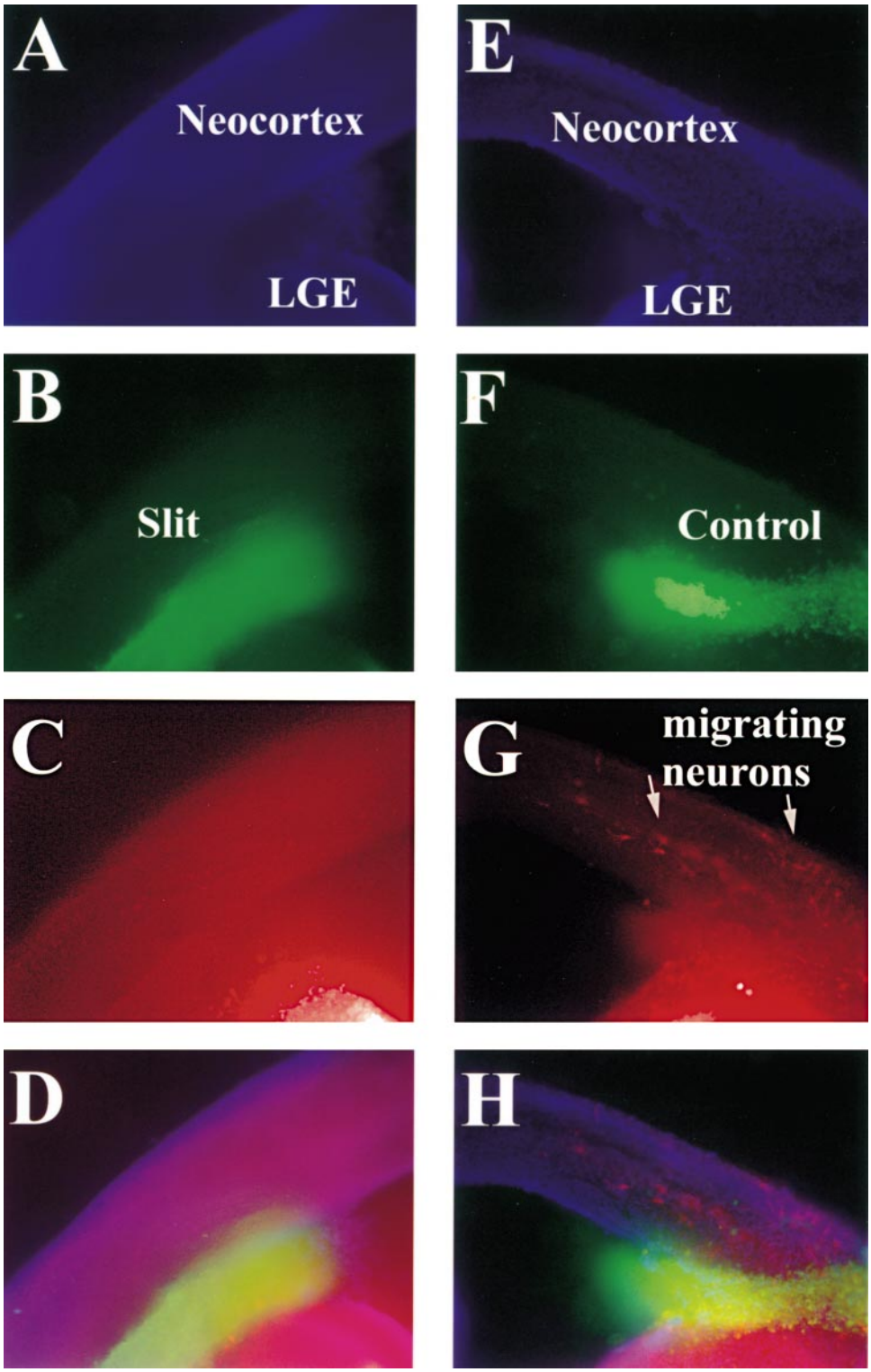

Figure 6. Effect of Slit on Migration of Cells from the LGE to the Neocortex

Results from E16.5 slices are shown here. Similar results have been obtained with E15.5 and E17.5 slices.

(A–D) Different views of the same explant on which a strip of Slit cells (green) has been placed on the junction between the neocortex and the LGE.

(E–H) Different views of the same explant on which a strip of control cells (green) has been placed on the junction between the neocortex and the LGE.

(A) and (E) are Hoechst dye staining to show outlines of the brain slices. (B) and (F) show DiO-labeled aggregates of Slit (B) or control (F) cells; the green bands are the aggregates.

(C) and (G) show DiI-labeled LGE neurons; note that, in the neocortex, there were migrating cells in (G) but not in (C).

(D) is the superimposition of (A), (B), and (C), whereas (H) is the superimposition of (E), (F), and (G); note the relationship of HEK cells (green), the LGE (red) and migrating neurons in the neocortex (red), and the outlines of the slices (blue).

migrated past Slit cells, the migration of LGE neurons into the neocortex was significantly reduced by Slit aggregates when compared to control aggregates (data not shown). These results indicate that Slit is a repellent for LGE neurons migrating in their normal pathway to the neocortex.

Role of Endogenous Slit Signaling in Repelling LGE Neurons

Experiments shown above indicate that Slit is capable of guiding the migration of LGE neurons. To test whether endogenous Slit contributes to the repulsive activity in the ventricular zone of the LGE, we have made use of RoboN, the extracellular portion of the Slit receptor Roundabout (Robo). RoboN can bind Slit (Wu et al., 1999) but cannot transduce the signal to intracellular compartments and is thus a competitive blocker of Slit.

Aggregates of HEK 293T cells transiently transfected with the control vector or with a plasmid expressing RoboN were made and placed at the bottom layer of the collagen gel matrix. Explants from the ventricular zone and those from the subventricular zone of the LGE were cocultured at the top layer. LGE neurons were repelled by the ventricular zone in the presence of control HEK 297T cells (Figures 7A and 7D), whereas the presence of RoboN-expressing cells inhibited the repulsive activity of the ventricular zone. These results indicate that endogenous Slit present in the ventricular zone of the LGE is repulsive to LGE neurons.

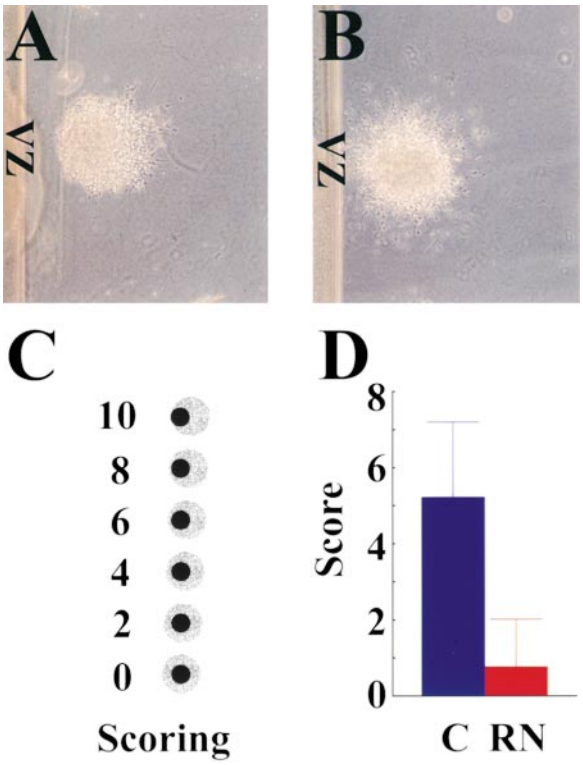

Figure 7. Inhibition of the Repulsive Activity in the Ventricular Zone of the LGE by RoboN

(A) The presence of control HEK 293T cells did not affect the asymmetric distribution of migrating cells from an explant of the subventricular zone of the LGE after coculturing with an explant of the ventricular zone (VZ).

(B) In the presence of HEK 293T cells expressing RoboN the repulsive activity of the VZ was inhibited.

(C) A diagram of the scheme for semiquantitation of cell migration. Each LGE explant was scored according to this scheme.

(D) Scores of repulsive activities in the presence of control HEK 293T cells (C) or RoboN-expressing cells (RN). Scores were tabulated from 222 explants in (C) and 319 in RN.

Discussion

We have established an explant assay for studying the migration of neurons from the subventricular zone of the LGE. Using this assay, we have found that the ventricular zone of the LGE is repulsive to LGE neurons and that the Slit proteins are diffusible chemorepellents for LGE neurons. Although these neurons are heterogeneous, it is clear that the majority of them are GABAergic, the major type of inhibitory interneurons in the mammalian neocortex. Results from the present studies have thus revealed that a cellular source of guidance has been found for guiding neurons migrating into the neocortex and shown directly a molecular guidance cue for neocortical neurons. Taken together with our studies in the olfactory system (Wu et al., 1999), these results suggest that Slit is of general importance in glia-independent migration of neurons.

Cellular Sources of Guidance Cues for GABAergic Neurons

The extracortical origin of GABAergic neurons has been established by previous studies (Walsh and Cepko, 1988; Van Eden et al., 1989; DeDiego et al., 1994; De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; reviewed in Lumsden and Gulisano, 1997). It was not clear, however, what mechanisms guide the migratory direction of the GABAergic precursor cells in the striatal-neocortical pathway. The use of an explant assay made it possible to study both the cellular and molecular bases underlying the guidance of GABAergic neurons.

We have shown that the VZ of the LGE contains a diffusible repulsive activity for the GABAergic neurons, while the mantle layer does not attract or repel GABAergic neurons (Figure 2). We have also obtained evidence that two regions medial to the subventricular zone of the LGE, the VZ of the medial ganglionic eminence (MGE) and the septum, are both repulsive to GABAergic neurons (data not shown). Interestingly, although the cortex also expresses *slit* (Yuan et al., 1999), so far a chemorepulsive activity in the neocortex has not been observed for GABAergic neurons migrating from the LGE either in slice assays or in explant assays. Perhaps differences in Slit protein isoforms or expression of Robo or other receptor or signal transduction components may account for such differences in responsiveness of GABAergic neurons from the LGE. Taken together, these results suggest that there could be a gradient of repulsive activity with the strongest repulsion at the medial side of the striatal primordium, which drives the GABAergic neurons to migrate laterally into the neocortex.

It is not clear at the present time what determines the final destination of each GABAergic neuron. Is it simply timing? Or is it due to the absence of a sufficiently steep

gradient near the end of the migratory pathway? Do individual GABAergic neurons gradually lose their responsiveness to the gradient? Are there signals in the neocortex that act to stop migrating neurons?

There are obviously other cells that originate in the LGE and the MGE and remain in the striatum. The repulsive activity guiding GABAergic neurons into the neocortex may not be sufficient to drive these cells out of the striatum. These cells may not respond to the repulsive cue simply because they lack the receptor or signal transduction mechanisms to mediate the response to the repulsive cue. Alternatively, these cells require multiple mechanisms for guiding their migration. For example, some neurons in the striatum may require glial fibers for their migration. For these cells, even if they can respond to the repulsive cue from the VZ and the medial regions, because they have to move along glial fibers arranged perpendicularly to the VZ the end results would be that these cells would migrate into the striatal proper but not laterally into the neocortex.

Molecular Identity of Guidance Cues for GABAergic Neurons

The *slit* genes, originally identified in *Drosophila* (Nüsslein-Volhard et al., 1984; Rothberg et al., 1988, 1990), have recently been shown to encode for secreted ligands with axon branching activity and with repulsive activities in axon pathfinding, and neuronal migration (Battye et al., 1999; Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Wang et al., 1999; Wu et al., 1999; Yuan et al., 1999). Results presented here indicate that Slit can directly guide the migration of LGE neurons. Since *mslit1* is expressed in the VZ of the LGE and the MGE, and *mslit1* and *mslit2* are expressed in the septum (Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Yuan et al., 1999), it is possible that mSlit proteins may form a concentration gradient that drives GABAergic neurons from the LGE to migrate into the neocortex. It is not clear whether Slit guides GABAergic neurons in the entire migratory pathway from the LGE to the neocortex. Nor is it clear whether the distribution of the Slit gradient determines where LGE neurons stop or that there are active stop signals in the neocortex that determine the final positions of neurons migrating from the LGE. In the latter case, the stop signal(s) can either be a form of Slit that is made and distributed locally in the cortex (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999; Wang et al., 1999; Yuan et al., 1999) or a molecule of other families.

Molecules that determine the distribution of Slit gradient should be able to regulate neuronal migration. For example, the *Dlx-1* and *Dlx-2* genes may not only be involved in the generation of GABAergic precursor cells in the LGE (Anderson et al., 1997) but may also be required in setting up the Slit gradient. These homeobox genes are expressed in the ventricular and subventricular zones of the LGE and MGE, and their loss of function leads to the absence of GABAergic neurons in the neocortex (Anderson et al., 1997). Expression studies of *mslit* genes in *Dlx-1* and *Dlx-2* mutants and transplantation experiments should help in addressing the question of whether these genes play a role in guiding the migration of GABAergic neurons. If wild-type GABAergic neurons can migrate into the neocortex after transplantation into slices of wild-type LGE but cannot migrate into the neocortex after transplantation into the LGE of *Dlx-1* and *Dlx-2* mutants, a role for regulating migration in addition to generation of GABAergic neurons can be suggested for the *Dlx-1* and *Dlx-2* genes.

Guidance of Glia-Independent Tangential Neuronal Migration

There are two major types of neuronal migration in the CNS: the more classically established radial migration and the more recently discovered tangential migration. In addition to a difference in the direction of the migration relative to the surface of the CNS, only radial, but not tangential, migration depends on glial cells.

Tangential migration occurs in multiple regions of the CNS (Van Eden et al., 1989; Austin and Cepko, 1990; Gray et al., 1990; Walsh and Cepko, 1990; O'Rourke et al., 1992, 1995, 1997; Fishell et al., 1993; Luskin, 1993, 1998; Tan and Breen, 1993; Lois and Alvarez-Buylla, 1994; Menezes and Luskin, 1994; Tan et al., 1995; Reid et al., 1995; De Carlos et al., 1996; Doetsch and Alvarez-Buylla, 1996; Halliday and Cepko, 1992; Lois et al., 1996; Anderson et al., 1997; Goldman and Luskin, 1998; Pearlman et al., 1998). The relative prevalence of tangential versus radial migration has been estimated to be between 10% to 70%, depending perhaps on the regions of the CNS, the time of migration, and animal species studied (Kornack and Rakic, 1995; O'Rourke et al., 1995, 1997; Rakic, 1995; Reid et al., 1995; Soriano et al., 1995; Tan et al., 1995). There are estimates that, in the cerebral ventricular zone, tangential migration is equally as prevalent as radial migration (O'Rourke et al., 1995).

Taken together with the our finding of a role for Slit in guiding tangential migration in the olfactory system (Wu et al., 1999), the finding here of the functional activity of Slit in the striatal-neocortical pathway suggests that Slit is of general significance in glia-independent tangential migration and that similar repulsive or attractive mechanisms may function in other systems involving tangential migration. However, the expression pattern of the *mslit1* gene makes it unlikely that a single Slit could be the guidance molecule explaining tangential migration in all regions of the CNS. It is possible that either multiple members of the Slit family or molecules of other families may be involved in glia-independent tangential migration.

Roles of Slit in Cell Migration in *Drosophila*

The *slit* gene was first discovered in *Drosophila* (Nüsslein-Volhard et al., 1984; Rothberg et al., 1988, 1990), and there is strong evidence that *slit* play a role in guiding the projection of axons in *Drosophila* embryos (Battye et al., 1999; Kidd et al., 1999). While Slit has not been implicated so far in guiding neuronal migration in *Drosophila*, mesodermal phenotypes in *slit* mutant embryos provide genetic evidence suggesting that Slit is required for mesodermal cell migration.

Phenotypic analysis of *Drosophila slit* mutant embryos revealed that muscles stretched across the midline of the CNS (Battye et al., 1999; Kidd et al., 1999). Results from genetic studies indicate that the requirement for Slit in correct positioning of the muscle precursor cells (Kidd et al., 1999) is due to its role in muscle

cell migration away from the midline of *Drosophila* embryos (T. Kidd and C. Goodman, personal communication). Certain muscle precursor cells can migrate away from the midline source of Slit for a distance of several cells in wide-type but not in *slit* mutant embryos (Kidd and Goodman, personal communication). The migration of the muscle precursor cells away from the midline can be reversed and pulled back when the mesoderm cells transgenically express a chimeric Robo-Frazzled receptor that makes the Slit signal attractive rather than repulsive (Bashaw and Goodman, 1999). Work in *Drosophila* has also revealed genes that may function upstream of *slit*. For example, a muscle phenotype similar to that in *slit* mutants has been observed in single-minded (*sim*) mutant embryos (Lewis and Crews, 1994). It seems likely that the transcriptional factor encoded by *sim* may affect mesodermal migration by regulating *slit* expression.

The dorsal median (DM) cells are a set of mesodermal cells essential for the formation of transverse nerves. These cells are normally situated at the midline of the embryo. However, although they are originally found at the midline, the DM cells fail to stay there in *slit* mutant embryos (Zhou et al., 1997). It will be interesting to distinguish whether Slit is responsible for the maturation and survival of DM cells as suggested previously (Zhou et al., 1997) or that Slit is an attractant for DM cells and the loss of DM cells is secondary to abnormal positioning of these cells in the absence of an attractant.

Guidance of Glia-Dependent Radial Migration of Neurons

Radial migration is a major mode of neuronal migration (Rakic, 1971a, 1971b, 1972, 1988, and 1990). Although a guidance cue has now been found for glia-independent neuronal migration, the guidance cue(s) for glia-dependent radial migration remain unknown. We hypothesize that glia-dependent migration may also involve guidance cues that function similarly as Slit does in glia-independent migration.

In vivo, glia-dependent migration is unidirectional, whereas in vitro, previous studies with cerebellar granule cells in primary cultures have shown that dissociated granule cells migrate in both directions along the glial processes (Hatten and Liem, 1981; Hatten et al., 1984; Edmondson and Hatten, 1987; Gregory et al., 1988; Mason et al., 1988; Hatten and Mason, 1990; Hatten, 1993; Hatten and Heintz, 1995; Hatten, 1999). Based on analogy to the mechanism revealed for Slit in glia-independent tangential migration, it seems likely that there may also be guidance molecules in the ventricular or marginal zones for glia-dependent radial migration in the CNS.

Phenotypic analyses of mouse mutants and human patients have implicated multiple genes in cortical neuronal migration that is dependent on glial cells. However, the precise functional roles of those genes are not known. The cloning of these genes indicates that the products of most of these genes are nuclear or cytoplasmic proteins, suggesting that they could not serve as guidance cues. Of particular interest are potential roles in cortical guidance for extracellular or cell surface molecules such as Reelin and astrotactin (Falconer, 1951; D'Arcangelo et al., 1995; Hirotsune et al., 1995; Zheng et al., 1996), intracellular proteins such as filamin (Fox et al., 1998) and disabled (Howell et al., 1997; Sheldon et al., 1997; Ware et al., 1997; Yoneshima et al., 1997; Rice et al., 1998), or cdk5 or its regulator (Ohshima et al., 1996; Chae et al., 1997). The *reeler* gene is involved in cortical lamination, and Reelin is believed to control cell-cell interactions and cell positioning (D'Arcangelo and Curran, 1998; Rice et al., 1998). It would be interesting to obtain direct evidence to distinguish whether Reelin acts as a stop signal or an adhesive molecule for migrating neurons (D'Arcangelo and Curran, 1998; Pearlman et al., 1998). Because secreted molecules can affect cortical migration indirectly (e.g., Rio et al., 1997), it will also be interesting to determine whether the effect of Reelin is direct or indirect (D'Arcangelo and Curran, 1998; Frotscher, 1998; Pearlman et al., 1998; Rice et al, 1998). These experiments may help establish whether Reelin is a direct guidance cue for cortical neurons. Recent studies of mouse mutants suggest that transmembrane proteins including very low density lipoprotein (VLDL) receptor and ApoE receptor 2 (ApoER2) could directly or indirectly participate in the Reelin pathway (Trommsdorff et al., 1999).

The expression of *slit* genes in regions with radial migration makes it tempting to speculate that Slit may contribute to directional guidance of glia-dependent neuronal migration, although it will also be interesting to investigate other molecular cues that may guide radial migration. Recent studies with cerebellar explants indicate that Netrin-1 exerts a direct attractive action on pontine neurons (K. Lee, H. Simon, M. Tessier-Lavigne, and D. O'Leary, personal communication).

Guidance of Cell Migration in Other Processes or Systems

In development, cell migration plays important roles in multiple processes (Montell, 1999). For example, the migration of neural crest precursor cells is crucial for the formation of the peripheral nervous system. During gastrulation, cells migrate around the primitive streak in avian and mammalian embryos. In vertebrate heart formation, precursor cells migrate from their dorsal origin to their eventual ventral destination. In *Drosophila* heart formation, precursor cells migrate from a ventrolateral position to the dorsal-most location to form the dorsal tube. During vertebrate muscle development, cells migrate from the somites to their final position. In almost all of these situations, our understanding of the directional guidance of migrating cells is limited. In some of the cases, members of the Slit family are expressed in the right region as candidate repulsive cues, although it would not be surprising that other molecules could be involved.

In normal angiogenesis or angiogenesis in response to pathological stimuli, attractive and inhibitory cues are known to play important roles for the migration of endothelial cells. It would be interesting to ask whether regulation of repulsive cues is involved in angiogenesis and promotion or inhibition of the repulsive activities could be effective ways to enhance or inhibit angiogenesis when desirable. Although the aorta ring assay showed that Slit does not attract or repel these cells, it remains possible that Slit family may regulate angiogenesis. Chemotaxis of cells in the blood is involved in

inflammatory and immune responses. So far, attractive molecules such as chemokines are the major known directional guidance cues for chemotaxis. If repulsive cues acting similar to Slit are involved in regulating chemotaxis, it would broaden our understanding of these processes and may also provide a new type of reagents to control chemotaxis for therapeutic applications.

In tumor metastasis, if some tumor cells inside or outside the nervous system respond to Slit or other repulsive cues, it is conceivable to use these molecules to control tumor invasion or metastasis. In cell-based therapies of neural and nonneural diseases, the availability of guidance cues for cell migration could be useful in directing cells to the target region. If necessary, introduction of receptors such as Robo may make usually unresponsive cells respond to guidance cues such as Slit in any of the therapeutic applications.

Experimental Procedures

In Vitro Migration Assay for LGE Neurons

Brains from E17-18 Sprague-Dawley rats (Charles River) were dissected out, and coronal sections of 200–300 μm were made with a vibratome. Tissues from the subventricular zone of LGE were isolated using a tungsten needle knife and further trimmed into blocks of 100–300 μm. The trimmed explants were embedded in the rat tail collagen gel or the 2:1 mixture of collagen and matrigel (Collaborative Research) and cultured with 10% fetal calf serum (FCS) and 100 μg/ml of penicillin and streptomycin Dulbecco's minimal essential medium (DMEM, Gibco) at 37°C in an incubator with 5% CO_2 for 12–20 hr.

Assays for Cellular and Molecular Guidance Cues for LGE Neurons

Multiple regions of the CNS were isolated and cocultured with explants of the subventricular zone of the LGE in the collagen gel matrices. In the coculture with the ventricular zone of the LGE (VZ), VZ explants were wrapped in a membrane filter (0.45 μm, Millipore) to prevent cells migrating from VZ that interfered with the observation of cells migrating from the subventricular zone of the LGE. There was no such interference with the mantle layer of the LGE or the floor plate and motoneuron explants of the spinal cord.

The stable xSlit and control HEK 293 lines have been described previously (Li et al., 1999). mSlit1 was expressed transiently in HEK 293T cells and behaved essentially the same as the xSlit. Sema III was also transiently expressed in HEK 293T cells. Results shown for Slit were from xSlit cells unless indicated otherwise. Aggregates of Slit or control cells were made by the hanging-drop method (Fan and Tessier-Lavigne, 1994). Cell aggregates were trimmed and cocultured with LGE explants embedded into a 2:1 mixture of collagen gel:matrigel for 12 to 20 hr. The distance of the cell aggregates to the explants was between 50 and 200 μm.

In the blocking experiments shown in Figure 7, a control vector or a plasmid expressing the extracellular portion of Robo (RoboN) was transiently transfected into HEK 293T cells. Transfected cells were incubated at 37°C in a humidified incubator with 5% CO_2 for 48 hr. Cell aggregates were embedded into rat tail collagen gel. After collagen gel solidified, explants of the ventricular zone and the subventricular zone of the LGE were overlaid onto the collagen gel and covered with another layer of collagen gel matrix. Semiquantitation of the effect of RoboN was scored in a scheme outlined in Figure 7C.

Aorta Ring Assay

Aorta ring assay was essentially carried out as described by Malinda et al. (1999). In brief, aortas were isolated from E17 rats, removed of the surrounding tissue, and cut into rings of 150–200 μm in thickness. Aorta rings were placed at a distance of 200–300 μm from Slit or control cell aggregates and embedded in rat tail collagen gel matrices. They were cultured in a humidified incubator at 37°C, 5% CO_2 with 10% FCS and DMEM for 17–24 hr.

Brain Slice Culture

Coronal sections of 300 μm were made by a vibratome and transferred onto a piece of Millipore filter (HABG01300, 0.45 μm pore size, 13 mm in diameter). A small piece of DiI crystal was inserted into the subventricular zone of the LGE. The slices were cultured with 10% FCS and 100 μg/ml of penicillin and streptomycin in DMEM medium for 36–48 hr. When testing the effects of Slit or control cell aggregates, a thin layer of cell aggregate was trimmed to 100–200 μm in length and then overlaid onto the neocortico-striatal sulcus region.

Quantitative Analysis of Migratory Cells

Explants were fixed with 4% paraformaldehyde and stained with Hoechst 33258 (Sigma). Images of Hoechst-stained explants were taken with a Spot digital camera and saved as TIFF files. Migratory cells on the images were counted by IPLab Spectrum (Version 3.1.1 for Macintosh, Signal Analysis Corporation). The coordinates of the migrating cells and the outlines of the explants were exported as text files. A C language program was written and used to calculate the positions of migrating cells relative to the edge of the explants and to group cells in different subdivisions or quadrants.

Immunohistochemical staining was carried out as described previously (Li et al., 1999) with a monoclonal anti-TuJ1 antibody and rabbit polyclonal anti-GABA antibodies and polyclonal anti-GFAP antibodies. To determine the percentage of cells that are GABAergic neurons, triple labeling with the Hoechst dye, anti-TuJ1, and anti-GABAergic staining was performed. Cells migrating out of seven explants were counted, with a total of 1901 Hoechst-stained cells, 1530 being TuJ1-positive and 1380 being GABAergic. The ration of TuJ1 versus Hoechst-positive cells (TuJ1/Hoecst) is 81.46% (± 13.53%), GABA/Hoechst is 73.81% (± 9.53%), and GABA/TuJ1 is 91.39% (± 10.53%).

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