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Biased selection of leading process branches mediates chemotaxis during tangential neuronal migration

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Current models of chemotaxis during neuronal migration and axon guidance propose that directional sensing relies on growth cone dynamics. According to this view, migrating neurons and growing axons are guided to their correct targets by steering the growth cone in response to attractive and repulsive cues. Here, we have performed a detailed analysis of the dynamic behavior of individual neurons migrating tangentially in telencephalic slices using high-resolution time-lapse videomicroscopy. We found that cortical interneurons consistently display branched leading processes as part of their migratory cycle, a feature that seems to be common to many other populations of GABAergic neurons in the brain and spinal cord. Analysis of the migratory behavior of individual cells suggests that interneurons respond to chemoattractant signals by generating new leading process branches that are better aligned with the source of the gradient, and not by reorienting previously existing branches. Moreover, experimental evidence revealed that guidance cues influence the angle at which new branches emerge. This model is further supported by pharmacological experiments in which inhibition of branching blocked chemotaxis, suggesting that this process is an essential component of the mechanism controlling directional guidance. These results reveal a novel guidance mechanism during neuronal migration that might be extensively used in brain development.

KEY WORDS: Cellular dynamics, Chemotaxis, Neuronal migration, Mouse

INTRODUCTION

Neuronal migration and axon guidance play central roles in the assembly of neuronal circuits. During axon guidance, directional movement appears to rely on growth cone dynamics (Dickson, 2002; Lin and Holt, 2007; Round and Stein, 2007). According to this view, growing axons are guided to their correct targets by steering the growth cone in response to attractive and repulsive cues (Kalil and Dent, 2005). Given the typical bipolar morphology of many migrating neurons, with a leading process extending in the way of their migration and a trailing process in the opposite direction, this model of directional guidance have also been extensively used to explain the chemotaxis of migrating neurons (Miyata and Ogawa, 2007; Noctor et al., 2001; Rakic, 1990; Yee et al., 1999). However, not all migrating neurons have the same morphology. In the developing cerebral cortex, for example, some migrating interneurons have been described to exhibit a bipolar morphology with a single leading process (Jiménez et al., 2002; Polleux et al., 2002), whereas many others have been reported to display branched leading processes (Anderson et al., 1997; Bellion et al., 2005; Friocourt et al., 2007; Kappeler et al., 2006; Lavdas et al., 1999; Marín and Rubenstein, 2001; Nasrallah et al., 2006; Polleux et al., 2002). The existence of branched leading processes is by no means specific to cortical interneurons, as other types of tangentially migrating neurons also adopt this morphology (López-Bendito et al., 2006; Marín and Rubenstein, 2001; Ward et al., 2005).

The issue that arises in relation to the branched morphology of some migrating neurons is how they navigate using two leading processes to explore a wide prospective territory. In the case of cortical interneurons, work over the past few years has revealed that they originate in the subpallium and migrate over long distances using multiple tangential pathways (Corbin et al., 2001; Marín and Rubenstein, 2001). Moreover, we now know that interneuron migration is controlled by a complex combination of long-range attractive and repulsive signals, short-range instructive molecules, cell-adhesion complexes and motogenic factors (Marín and Rubenstein, 2003; Métin et al., 2006). With the exception of nucleokinesis, for which the underlying principles are just beginning to be elucidated (Bellion et al., 2005; Kappeler et al., 2006; Nasrallah et al., 2006), very little is known about the cellular mechanisms that control the directional movement of cortical interneurons in response to all these multiple guidance cues. Our results suggest that dynamic regulation of leading process branching is an essential mechanism for directional guidance in tangentially migrating interneurons.

MATERIALS AND METHODS

Mice

We used wild-type (CD1 and C57/b6), *Gfp* (CD1) and *Gad65-Gfp* (C57/b6) transgenic mice (Hadjantonakis et al., 2002; López-Bendito et al., 2004). The day of vaginal plug was considered to be embryonic day (E) 0.5. Animals were maintained under Spanish and EU regulation.

Slice and explant culture experiments

Organotypic slice cultures of the embryonic mouse telencephalon were prepared as previously described (Anderson et al., 1997). In some experiments, vehicle solution (H_2O) or Y27632 (30 μm) was added to the medium 12 hours after culture and replaced after 12 hours. Slices were then cultured for another 12/24 hours. For immunohistochemistry, slices were re-sectioned to 60 μm and incubated with rabbit-anti GFP (1:2000, Invitrogen) overnight at 4°C followed by 488 Alexa donkey anti-rabbit (1:500, Invitrogen) for 2 hours at room temperature. Slices were mounted using Mowiol-Dabco with Bisbenzamide (1:1000, Sigma).

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Medial ganglionic eminence (MGE) explants were dissected out from E13.5 slices and confronted with COS cell aggregates (expressing *dsRed* alone, or *dsRed* and *Ig-Nrg1*) in Matrigel matrices. Explants were incubated for 12 hours at 37°C in Neurobasal (Invitrogen, San Diego, CA) before the addition of vehicle or Y-27632 (30 μm). Vehicle and inhibitor were replaced after 12 hours and explants incubated for a total of 48 hours. In other experiments, small pieces of the MGE from E13.5 *Gfp* transgenic mice were transplanted into wild-type host slices, as described elsewhere (Marín et al., 2003).

For acute treatment of individual neurons, micropipettes (tip diameter ~1 μm) were placed above (~10 μm) and at a distance of ~20 μm from superficial dsRed-expressing neurons. Pressure pulses (0.1-0.4 bar; duration, 20 ms) were applied at a frequency of 2 Hz for 1 hour. Pipette solution contained Y27632 (3 mM) or the recombinant EGF domain of Nrg1 (NRG1-β1, Peprotech; 13 nM), and 0.4 mM Alexa Fluor 488 sodium salt (Invitrogen) dissolved in PBS or H_2O.

In vitro focal electroporation

pCAGGS-based *Gfp* and *dsRed* expression vectors were pressure injected focally into the MGE of coronal slice cultures and focally electroporated as described before (Flames et al., 2004).

Time-lapse videomicroscopy

Slices were transferred to the stage of an upright Leica DMLFSA or inverted Leica DMIRE2 microscope coupled to a confocal spectral scanning head (Leica TCS SL) and viewed through 10–60× water immersion or 20× oil objectives. Slices were continuously superfused with warmed (32°C) artificial cerebrospinal fluid at a rate of 1 ml/minute or maintained in supplemented neurobasal medium.

Quantification

Canvas (ACD Systems) and ImageJ (NIH, http://rsb.info.nih.gov/ij/) software were used for image analyses. For single pair-wise comparisons, a two-tailed *t*-test was used. Chi-square analysis was used to compare frequency distributions.

In co-culture experiments (Fig. 4), the migration angle was measured between the virtual vertical lines positioned at 0 or 90° (for quadrants 1 or 4, respectively) and the leading process closer to the COS aggregate.

For the analysis of the orientation of migrating cells at the pallial/subpallial boundary in slice cultures (Fig. 5), a virtual box (366 $\mu m^2 \times 225\ \mu m^2$) was defined at the place in which interneurons arrive to the cut in each slice. Cells within the box and in the adjacent 150 μm on both sides were classified into one of three groups depending on the orientation of their leading process: type 1 (leading process parallel to the cut), type 2 (process away from the cut) and type 3 (process towards the cut) cells. In cells displaying more than one leading process branch, the one with the swelling was selected. In few cases in which this was not evident, we consistently selected the widest and/or longest of the branches. To maximize the resolution of the cell morphology, cells were identified in 15-25 μm *z*-stacks.

For the analysis of the orientation of migrating cells in the cortex of slice cultures (Fig. 6), we identified Gad65-Gfp/BrdU double-positive cells in each slice and their main leading process was drawn using Canvas software. The main process was defined as that containing the organelle swelling in front of the nucleus; in a few cases in which this was not evident, we consistently selected the widest and/or longest of the branches. For each slice, we draw a grid of virtual radial lines (lines perpendicular to the ventricular zone and the pia) and orientated each cell in relation to the most adjacent 'radial line'. Cells that deviated less than 25° from radial lines were considered as radially oriented; those that deviate more than 25° were designated as tangentially oriented (Fig. 6F). We systematically exclude from this analysis those cells located in the more lateral or medial regions of the cortex, so that the curvature of the slice in those regions would not interfere with our analysis.

RESULTS

Leading process branching characterizes many tangentially migrating neurons in the CNS

Detailed analysis of *Gad65-Gfp* embryos, in which most γ-aminobutyric acid-containing (GABAergic) neurons express *Gfp* (López-Bendito et al., 2004), revealed that a branched leading process is a common feature of many tangentially oriented neurons in different regions of the brain and spinal cord (see Fig. S1 in the supplementary material) (Ward et al., 2005). To study the dynamic behavior of leading process branching in migrating interneurons, we used confocal time-lapse videomicroscopy in organotypic slice cultures. In this preparation, migrating interneurons display morphologies that closely resemble those described in vivo (Fig. 1A,B) (Ang et al., 2003). In a first series of experiments, we carried out focal electroporation of a plasmid encoding for the red fluorescence protein dsRed in the MGE of E13.5 telencephalic slices to label putative GABAergic interneurons and performed time-lapse videomicroscopy after 12-36 hours in culture (Fig. 1C,D). Using this approach, we labeled a reduced number of relatively scattered interneurons per slice, which allowed maximal resolution of the individual processes in each migrating cell. Every tangentially migrating interneuron observed displayed stereotyped dynamics (n>200 cells from at least 30 independent experiments), including nuclear movements in register with constant remodeling of the leading process. A representative case is presented in the sequence of photographs shown in Fig. 1E. At some point during their migratory cycle, all interneurons display a branched leading process, with the soma and the nucleus located at a specific distance behind the bifurcation (Fig. 1E) (t=0:00 h). Forward movement of the nucleus occurs in a two-step sequence, as previously described (Bellion et al., 2005; Schaar and McConnell, 2005) (see Fig. S2 in the supplementary material). First, a dilatation of the cell soma located in front of the nucleus moves forward towards the bifurcation (Fig. 1E) (t=0:10 h). This movement is subsequently followed by the forward displacement of the nucleus until it reaches the bifurcation (Fig. 1E) (t=0:30 h). After reaching the branching point, which is maintained stationary during nucleokinesis, the nucleus continues moving forward along one of the branches (Fig. 1E) (t=0:52 h). Furthermore, when the nucleus enters one of the primary branches, the other branch is already retracting and eventually becomes integrated in the trailing process (Fig. 1E) (t=0:52 h).

Observation of migrating neurons for extended periods of time revealed that the sequence of steps described above represents a migratory cycle that is consistently repeated by tangentially migrating interneurons. A typical example is presented in the sequence of photographs shown in Fig. 1F, which illustrate the dynamic behavior of a tangentially migrating interneuron (see also Fig. 1F′; see Movie 1 in the supplementary material). The movement of this cell can be described by successive translocations of the nucleus along one of the branches of the leading process from one bifurcation point to the next one, in a sequence that is continuously repeated. Thus, with the exception of small and transitory branches (Fig. 1F, small arrows), the bifurcation point of the leading process consistently marks the future position of the nucleus at the end of each nucleokinesis (Fig. 1F, broken lines). In many cases, the non-selected branch retracts before the nucleus reaches the branching point (Fig. 1F) (t=6:45 h; n=47 out of 83 retracting branches, from 38 different cells). In the remaining cases, the branch that is not selected by the nucleus becomes the trailing process (Fig. 1F) (t=2:45 h; n=36 out of 83 retracting branches, from 38 different cells), and eventually disappears. Branch selection correlated with rapid changes in the morphology of the growth cones tipping the leading process branches: selected branches displayed elaborate growth cones, whereas the growth cone of non-selected branches showed a collapsed morphology prior to retraction (see Fig. S3 in the supplementary material). We also performed time-lapse videomicroscopy in acute E15.5 telencephalic slices obtained

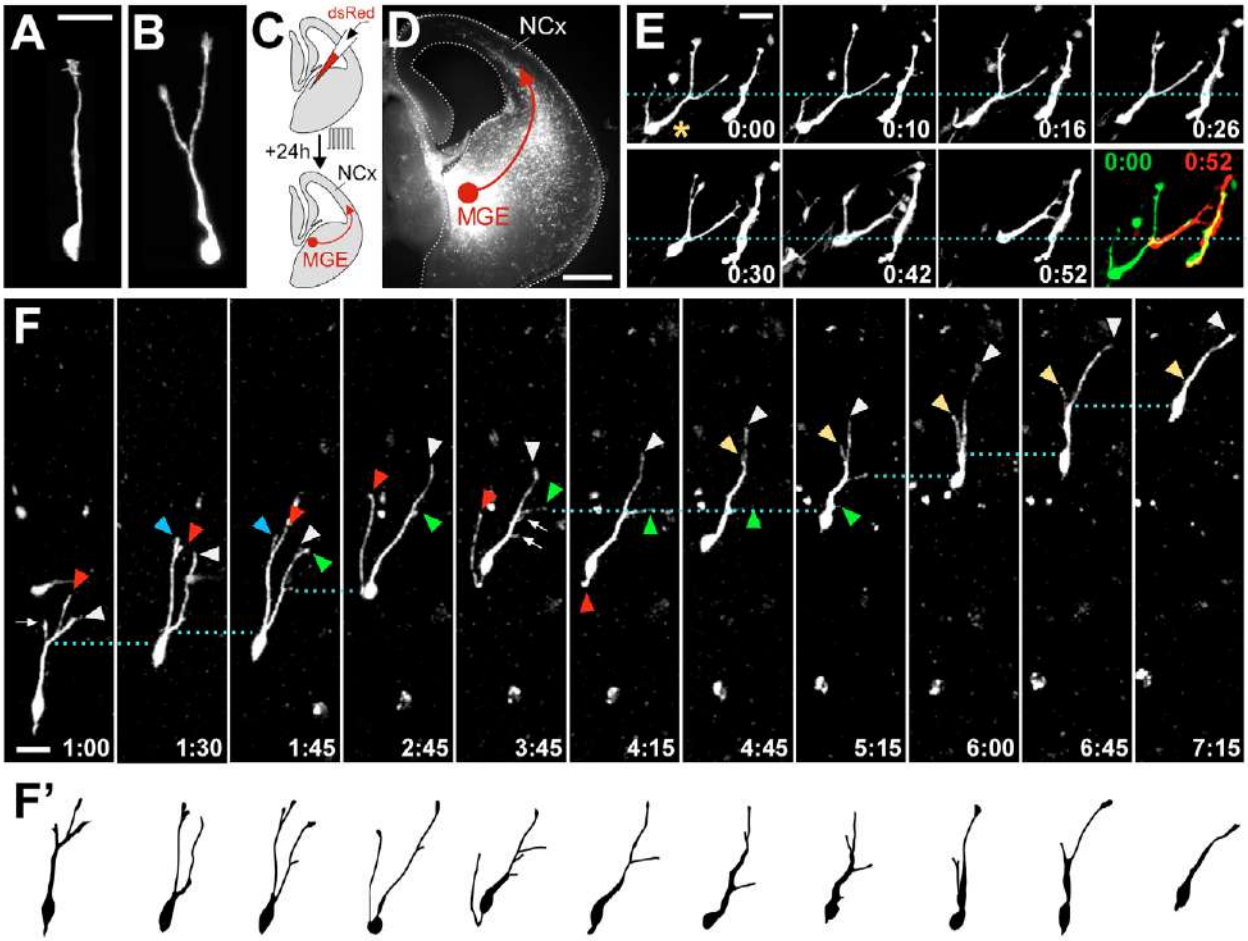


Fig. 1. Stereotyped dynamic behavior of tangentially migrating interneurons. (**A**,**B**) Different morphologies of interneurons derived from the medial ganglionic eminence (MGE). (**C**) Experimental paradigm. NCx, neocortex. (**D**) Migration of MGE-derived cells in E13.5 *dsRed*-electroporated slice after 36 hours in culture. (**E**) Time-lapse sequence of a *dsRed*-electroporated interneuron (asterisk) migrating from the subpallium to the cortex in a slice culture. Time is depicted in hours: minutes. The bifurcation point of the leading process is marked by a broken blue line. The last frame in the sequence shows superimposed images of the frame *t*=0:00 (green) and *t*=0:52 (red). (**F**,**F′**) Time-lapse sequence of a *dsRed*-electroporated interneuron migrating through the subpallium in a slice culture. This neuron was recorded for more than 7 hours to analyze several migratory cycles; only selected frames are displayed (see Movie 1 in the supplementary material for a complete movie version). The bifurcation points are marked by broken blue lines, and each leading process branch is coded with a colored arrowhead. Small arrows indicate small very transient processes. The drawings in F′ illustrate the morphology of this cell for each of the frames shown in F. Scale bars: 20 μm in A,B,E-F′; 300 μm in D.

from *Gad65-Gfp* transgenic mice. Simultaneous analysis of multiple GFP-expressing cells in the cortex of *Gad65-Gfp* embryos revealed that interneurons tangentially migrating through the subventricular zone (SVZ), intermediate zone (IZ) or marginal zone (MZ) navigate by alternative selection of leading process branches, independently of their speed of migration (see Movie S2 in the supplementary material; n>200 cells from at least 20 slices). In conclusion, bipolar morphologies such as those observed in static images (Fig. 1A) represent a phase in the migratory cycle of tangentially migrating neurons, in which the leading process remodels continuously as they move.

Directional guidance involves biased choices of leading process branches

How do migrating neurons with branched leading processes achieve directional guidance? Detailed observation of individual migrating interneurons revealed that leading process branches do not steer much as the cell moves (see Fig. S4 in the supplementary material). Instead, branches tend to grow or retract following the trajectory they initiate at the branch point, and so the geometry of the branches, and not the turning of their growth cones, appears to dictate the overall cell trajectory. In other words, the angle at which branches are formed determines the possible directions that can be followed by migrating cells. In cells that follow quasi-linear trajectories, such as those migrating in the subpallium (LGE in Fig. 2A,B) or in the cortical subventricular zone (SVZ) (Fig. 2A,C), the distribution of branch angles in the entire population follows a Gaussian distribution, with average angles relatively small (LGE: 45.32°±1.77°, n=109 cells) (Fig. 2B′); (SVZ: 35.76°±2.14°, n=64 cells) (Fig. 2C′). Dynamic observation of these cells also revealed that when interneurons navigate in a relatively constant direction the nucleus tends to transit alternatively through left and right branches in successive cycles (Fig. 2B″; see Movie 3 in the supplementary material). Analysis of the generation of new leading process branches revealed that neurons migrating in quasi-linear trajectories generate left or right branches with almost equal probability (50.9%

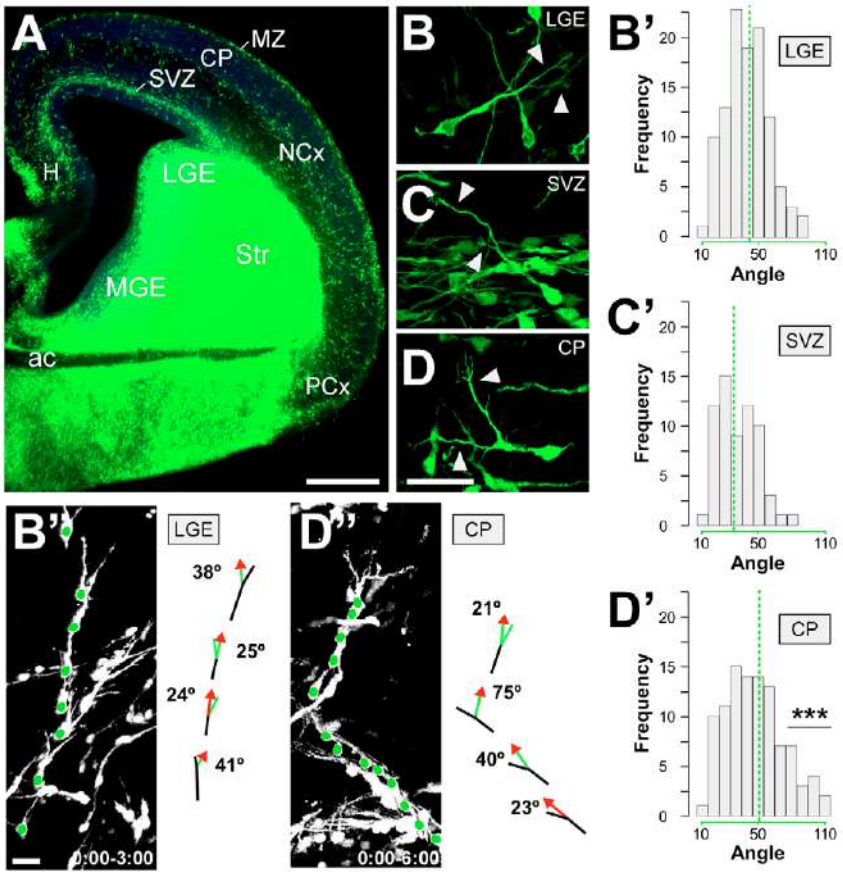


Fig. 2. Branch dynamics during tangential migration. (**A**) A telencephalic slice from an E16.5 *Gad65-Gfp* embryo. GABAergic cells are observed in green in the different regions in which migration was studied: subpallium (lateral ganglionic eminence, LGE), cortical subventricular zone (SVZ) and cortical plate (CP). Owing to the massive accumulation of migrating neurons in the subpallium, analysis of neurons migrating through the LGE was performed in slice cultures in which the MGE was electroporated with *Gfp* or *dsRed* (as in Fig. 1). (**B-D**) Representative images of the morphology of cells migrating through the LGE (B), SVZ (C) or CP (D). (**B′-D′**) Quantification of the frequency of angles form between leading process branches in cells migrating through the LGE (B′), SVZ (C′) or CP (D′). Mean angles are 45.32° (LGE), 35.76° (SVZ) and 51.83° (CP), respectively. Cells inside the highly packed SVZ generated very small branching angles with high frequency (C′), whereas neurons in the CP branched at large angles with much more frequency (D′). The frequency of angles greater than 70° is significantly higher in the CP than in the LGE or SVZ (χ^2 test, ***P<0.001). (**B″**,**D″**) Superimposed images of movie frames recreate the path followed by migrating neurons in the LGE (B″) and CP (D″). Green dots indicate the position of the nucleus in each nucleokinesis. Diagrams depict directional changes in these cells. New branches are shown in green, and the chosen branch is marked with a red arrowhead. Numbers indicate the angle formed by the branches. ac, anterior commissure; H, hippocampus; MZ, marginal zone; NCx, neocortex; PCx, piriform cortex; Str, striatum. Scale bars: 500 μm in A; 25 μm in B-D; 20 μm in B″,D″.

left branches, 49.1% right branches; n=104 branches from 20 different cells). Moreover, the selection of a certain branch did not seem to determine the generation of the next one (left and right branches were formed in 52.6% and 47.4% of the cases, respectively, following the selection of a left branch; left and right branches were formed in 47.8% and 52.2% of the cases, respectively, following the selection of a right branch; n=84 branches from 20 different cells).

A different picture emerged from the analysis of cells migrating close to the cortical plate (Fig. 2A,D), where interneurons tend to change direction very frequently (Ang et al., 2003; Polleux et al., 2002). Thus, although the average angle between branches was only slightly larger in cells migrating close to the cortical plate than in cells migrating through the LGE or SVZ (51.74°±2.52°, n=102 cells) (Fig. 2D′), we observed a prominent increase in the frequency of cells that displayed relatively large angles (Fig. 2D′, asterisks). There were two possible interpretations of these results. One alternative is that the region of the cortical plate contains two distinct populations of migrating interneurons, which branch at different angles. Another possibility is that the same cells can make relatively small or large branch angles depending on the environment. In agreement with this second hypothesis, dynamic analysis of interneurons migrating around the cortical plate revealed that rapid changes in direction are consistently preceded by the formation of leading process branches at relatively large angles (n=7 cells) (Fig. 2D″; see Movie 4 in the supplementary material).

Consistent with prior observations in LGE-derived SVZ neuroblasts (Ward et al., 2005), our previous results suggest that tangentially migrating interneurons change direction by biased choices of leading process branches. In addition, the differences observed in the net angle formed by leading process branches between cells following quasi-linear trajectories and those rapidly changing direction suggest that the amplitude of the angle at which new branches form could be influenced by guidance cues present in the cortex. To test this hypothesis, we performed experiments in which we forced interneurons migrating in a

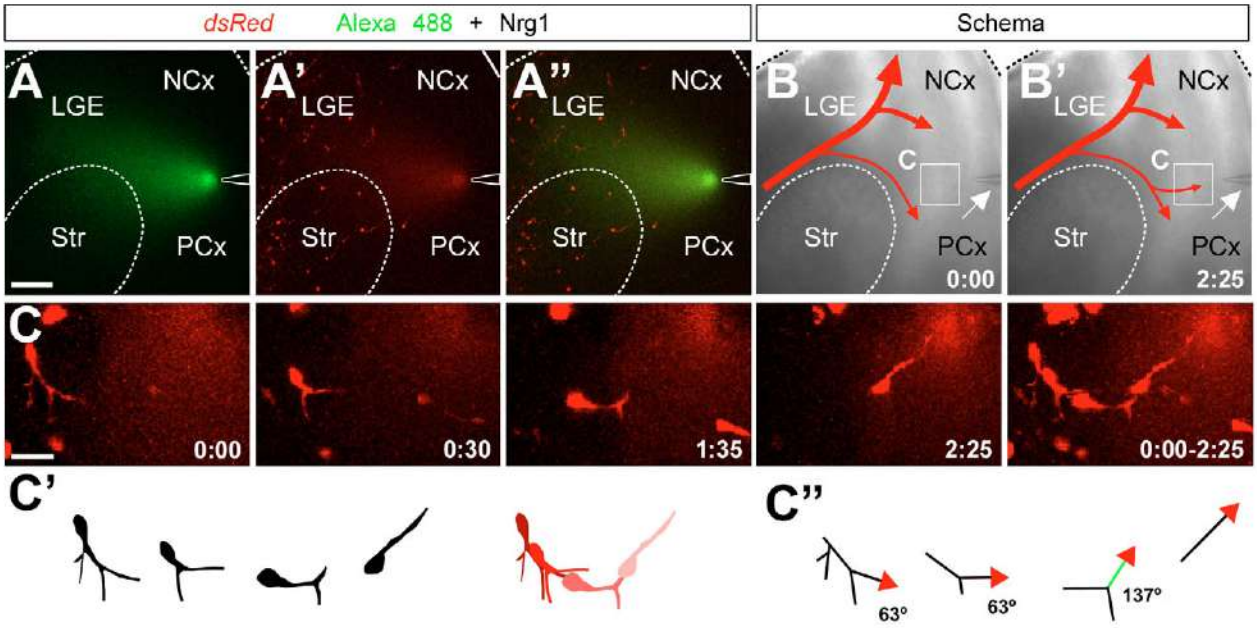


Fig. 3. Branch dynamics during Nrg1-induced chemotaxis. (**A-A″**) Images of a *dsRed*-electroporated slice perfused with a micropipette containing recombinant EGF domain from Nrg1 (13 nM) and Alexa 488 (green channel). To induce drastic changes in direction, cortical interneurons (red channel) migrating through the LGE were confronted with the micropipette at an angle that is perpendicular to their normal trajectory. (**B-B′**) Schematic representation of the trajectory change followed by the cell shown in C. White arrow indicates the micropipette. (**C**) Representative time-lapse sequences of a migrating cell that developed drastic trajectory changes in response to the chemoattractant. The cell generates a new leading process toward the pipette immediately before changing its trajectory (*t*=1:35). The angle generated before the most significant change in direction is the largest made by the neuron during this sequence (C). The cell chose the branch oriented towards the chemoattractant to continue migration. The gradient is also visualized in red owing to laser cross-contamination. (**C′-C″**) Drawings illustrate the morphology of the cell shown in C. Diagrams in C″ depict the movement of this cell. New branches are shown in green; chosen branch is tipped with a red arrowhead. The numbers indicate the angle formed by the branches. Scale bars: 100 μm in A-A″,B,B′; 25 μm in C,C′.

steady path to rapidly change their trajectory. For this, we focally electroporated the MGE of embryonic slices with *dsRed* and perfused the vicinity of individual migrating cells with a glass micropipette containing the cortical interneuron chemoattractant neuregulin 1 (Nrg1) (13 nM) (Flames et al., 2004). In these experiments, we consistently placed the micropipette perpendicular to the route followed by individual cells to stimulate a swift turn (Fig. 3A-A″). Observation of the migratory behavior of individual cells acutely attracted to a source of Nrg1 revealed that direction change was always preceded by the generation of a new leading process branch at a large angle (>60° in nine out of nine cells) (Fig. 3B,B′,C; see Movie 5 in the supplementary material). This new leading process branch was consistently oriented towards the source of the attractant, and was far more likely to be maintained than those extending in other directions (nine out of nine cells). This suggested that interneurons orient up intense chemoattractant gradients by branching the leading process at relatively larger angles than when they navigate in a constant direction, and by consistently choosing the new, better aligned branch, in their subsequent nuclear movement.

Chemotaxis in cortical interneurons requires leading process branching

Our previous results suggest that leading process branching is the mechanism that mediates directional sensing in cortical interneurons. To confirm this hypothesis, we performed experiments in which we pharmacologically perturbed the generation of new leading process branches in migrating interneurons. Previous studies have shown that Rho/ROCK inhibition causes the elongation of the leading process in pontine neurons through a mechanism that is likely to involve Rac activation (Causeret et al., 2004). When applied to telencephalic slices, the ROCK1/2 inhibitor Y-27632 (30 μM) also decreased the migration of cortical interneurons (*n*=19 slices) (see Fig. S5 in the supplementary material). As expected (Causeret et al., 2004), decreased migration was in part due to a reduction in the frequency of nucleokinesis in tangentially migrating neurons (*n*=6 cells) (see Fig. S6 in the supplementary material). However, we also found that bath application (*n*=35 cells) (see Fig. S5 in the supplementary material) or direct pipette perfusion with Y27632 (*n*=5 cells) (see Fig. S7 and Movie 6 in the supplementary material) decreased the frequency of leading process branching in cortical interneurons.

To evaluate the chemotaxis behavior of MGE-derived interneurons when leading process branching is perturbed, we performed explant co-culture experiments with a source of Nrg1 (Flames et al., 2004). In brief, we co-cultured E13.5 MGE explants with aggregates of COS cells expressing the secretable isoform of *Nrg1* (*Ig-Nrg1*) in matrigel three-dimensional matrices (Fig. 4A). In control experiments, cells derived from an E13.5 MGE explant confronted with COS cells transfected with a mock plasmid migrated uniformly in all directions (Fig. 4B). To quantify the effect of Nrg1 on the trajectory of the neurons, we subdivided the circle enclosing the MGE explant into eight sectors of equal size and measured the maximum angle at which migrating cells deviate from the vertical (migration angle, α) (Fig. 4E). In control experiments,

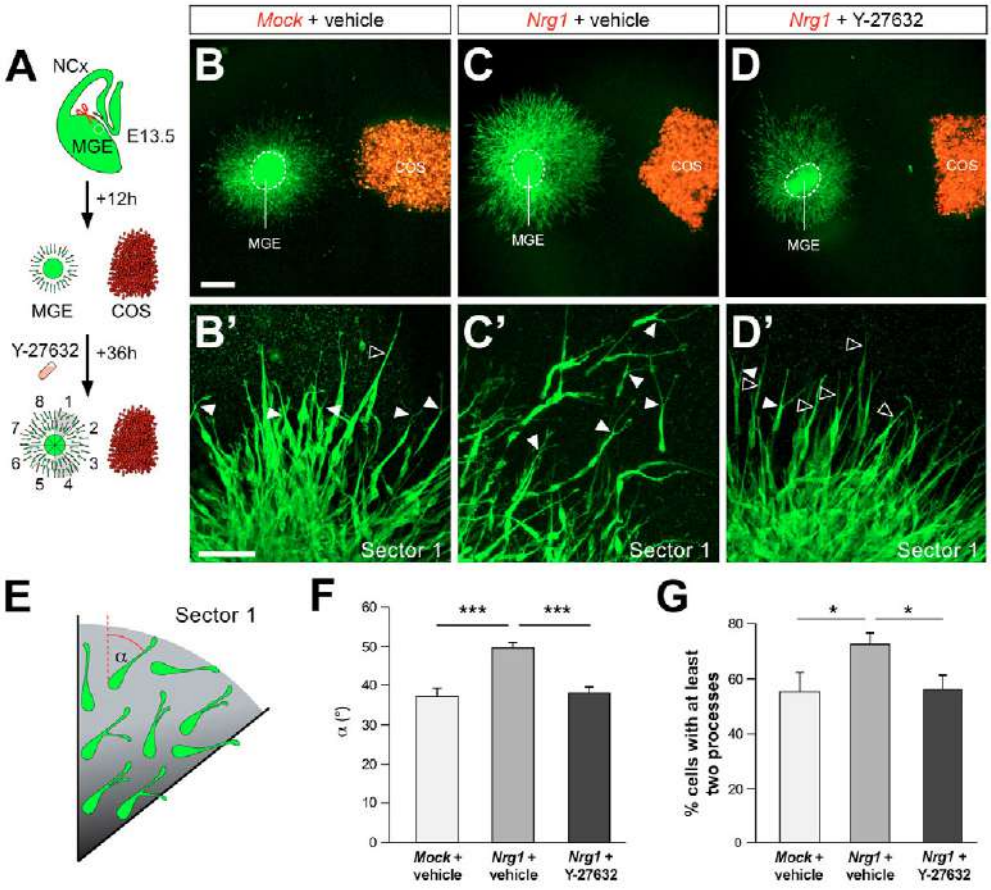


Fig. 4. Chemotaxis in MGE-derived cells requires leading process branching. (**A**) Schematic diagram of experimental design. (**B-D**) Migration of MGE-derived cells in response to mock-transfected (B) or *Nrg1*-transfected (C,D) COS cells aggregates cultured in matrigel matrices for 36 hours in the presence of vehicle solution (B,C) or the ROCK inhibitor Y27632 (30 μM) (D). COS cells were also transfected with *dsRed* to aid their visualization. Broken lines indicate the limits of the explants before culture. (**B′-D′**) Confocal images of cells migrating through Sector 1, as defined in the schematic shown in A, in control (B′), Nrg1 (C′) and in Nrg1+Y27632 (30 μM) (D′)-treated explants. Solid and open arrowheads indicate branched and non-branched interneurons, respectively. (**E**) Schematic view of the method used to quantify the orientation of cells (α angle). (**F**) Quantification of α angle in Sector 1. Bars show mean±s.e.m. 37.02±2.14° (control, *n*=167 cells from three independent experiments), 49.65±1.29° (Nrg1, *n*=520 cells from three independent experiments) and 38.10±1.41° (Nrg1 +Y27631, *n*=381 cells from three independent experiments). *t*-test, ***P<0.001. (**G**) Quantification of percentage of neurons located in Sector 1 with at least two leading process branches. Bars show mean±s.e.m. 54.85±7.41% (Ctrl, *n*=161 cells from three independent experiments), 72.21±4.49% (Nrg1, *n*=528 cells from three independent experiments) and 55.73±5.34% (Nrg1+Y27631, *n*=317 cells from three independent experiments). *t*-test, *P<0.05. Scale bars: 100 μm in B,C,D; 40 μm B′,C′,D′.

this angle was roughly 45°, with a mean value of ~35° (37.02±2.14°, *n*=167 cells from three independent experiments) (Fig. 4B′,E,F). By contrast, when MGE explants were co-cultured along COS cells expressing Nrg1, the maximum migration angle observed within sectors 1 and 4 was roughly 90°, with a mean value of ~50° (49.65±1.29°, *n*=520 cells from three independent experiments) (Fig. 4C,C′,E,F). These values reflect that many neurons derived from the MGE were attracted towards a source of Nrg1 (Flames et al., 2004), and therefore they reoriented their migratory path towards the COS cell aggregate. As in slices, addition of Y27632 to the explant culture medium modified the morphology of migrating neurons, decreasing the number of processes generated by interneurons (*n*=317 cells from three independent experiments) (Fig. 4G). In this scenario, migrating interneurons failed to reorient their migration towards Nrg1. The migration angle of MGE-derived neurons in response to Nrg1 in the presence of Y27632 was similar to that of explants confronted with control COS cells (38.10±1.41°, *n*=381 cells from three independent experiments) (Fig. 4D,D′,E,F), demonstrating that interneurons failed to reorient when leading process branching is perturbed.

The previous experiments suggest that leading process branching is required for interneurons to appropriately orient up intense chemoattractant gradients. A caveat of these experiments, however, is that blocking ROCK could directly interfere with Nrg1 signaling. To rule out this possibility, we performed another set of experiments in which we physically – rather than chemically – forced interneurons to change their migratory direction. In brief, we performed homotypic and isochronic transplants of small MGE pieces from E13.5 *Gfp*-transgenic mice into host slices from wild-type embryos and then follow the migration of MGE-derived cells (Fig. 5A). After a few hours, we cut off the pallium from organotypic slice cultures, therefore blocking the normal migratory pathway of cortical interneurons (Fig. 5A,B). In the absence of a cortex, migrating cells continue to migrate dorsally (Marín et al.,

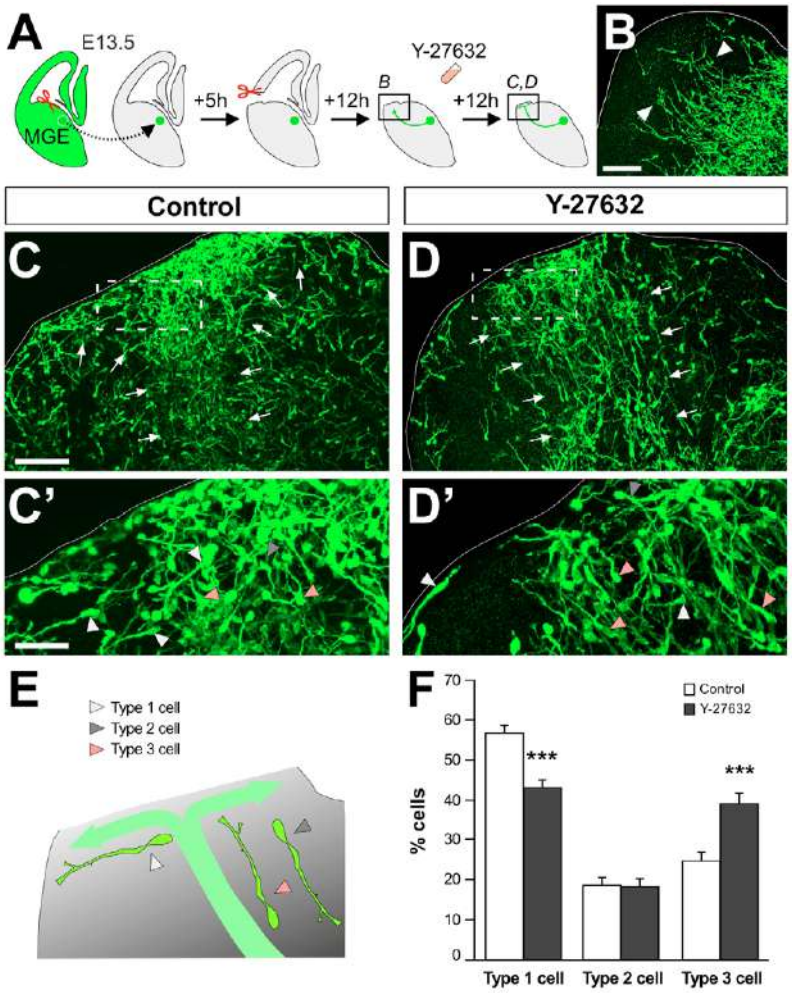


Fig. 5. Physically induced turning of interneurons requires leading process branching. (**A**) Schematic diagram of experimental design. After dissection of an E13.5 GFP MGE into small pieces, microtransplants were placed on a host E13.5 wild-type MGE and 5 hours later the pallium was removed from slices by cutting at the pallial-subpallial boundary. After 12 hours of incubation, GFP interneurons have migrated just a short distance from the cut (**B**). At this time, vehicle or Y27632 (30 μM) was added to the medium and slices were incubated for another 12 hours. (**C-D′**) Images of GFP-expressing interneurons migrating in slices in the presence of vehicle (C,C′) or Y27632 (D,D′). (C′,D′) High-magnification images of the boxed areas shown in C,D, respectively. Note that interneurons reach the cut in a fairly delineated stream in both control and experimental slices (arrows in C,D). In control slices, many interneurons turn 90° to continue migrating parallel to the incision (C), while turning is diminished in the presence of Y27632 (D). (**E**) Light-gray, dark-gray and pink arrowheads indicate type 1 (leading process parallel to the cut), type 2 (leading process away from the cut) and type 3 (leading process towards the cut) cells, respectively. (**F**) Quantification of the relative proportion of type 1-3 cells in control and experimental slices. Histograms show averages ±s.e.m. Control: 56.56±1.79%, 18.68±1.86%, 24.76±2.09% for cell type 1, 2 and 3 respectively; 1151 cells from eight slices in three independent experiments. Y27632: 42.80±1.85%, 18.17±1.98%, 39.03±2.36% for cell type 1, 2 and 3 respectively; 743 cells from eight slices in three independent experiments. χ^2-test: ***P<0.001. Scale bars: 100 μm in B,C,D; 25 μm in C′,D′.

2003), but they are forced to turn 90° laterally when they reach the incision (Fig. 5A). Prior to the arrival of interneurons to the incision (Fig. 5A,B), we added vehicle solution or Y27632 (30 μM) to the culture medium to test whether leading process branching was required for this rapid change of direction. After 12 additional hours in culture, we examined the orientation of GFP-expressing cells close to the incision (Fig. 5A). In control experiments, most migrating cells where oriented parallel to the incision (Type 1 cells) (Fig. 5C,C′,E,F), suggesting they have turned 90° after reaching the cut. In the presence Y27632, which decreases leading process branching (see Fig. S7 in the supplementary material), the percentage of migrating cells parallel to the incision was significantly reduced compared with controls (Fig. 5D,D′,E,F). Conversely, many more cells remained oriented towards the incision in Y27632-treated slices than controls (Type 3 cells) (Fig. 5D,D′,E,F). These experiments reinforced the view that leading process branching is required by interneurons to perform considerable changes in their migratory direction.

To explore whether turning also depends on branch dynamics in a physiologically relevant context, we next examined the behavior of interneurons in an environment in which they normally make profound changes in their migratory direction. For this, we analyzed the embryonic cortex at E15.5, when interneurons begin to reach their final position by rapidly deviating from their tangential routes towards the cortical plate (Ang et al., 2003; Polleux et al., 2002) (Fig. 6A). Although the molecules involved in this process are currently unknown (López-Bendito et al., 2008), we hypothesized that leading process branching would also be required for interneurons to perform this rapid change in direction (as in Fig. 2D″; see also Movie 5 in the supplementary material). To test this idea, we prepared slices from E15.5 *Gad65-Gfp* embryos that had received a BrdU pulse at E13.5 to unequivocally identify a cohort of synchronically born interneurons. At E15.5, interneurons have not yet begun to change their migration from tangential to radial, and most cells display a tangential orientation (94.56±1.61 %, n=74 cells from three slices of two independent experiments) (Fig. 6B). After 2 DIV, however, almost half of the entire population of interneurons has changed their orientation to radial (41.99±5.37% radially oriented, 58.00±6.02 % tangentially oriented; n=231 cells from four slices of two independent experiments) (Fig. 6C,E-G). By contrast, incubation of slices with Y27632 (30 μM) greatly reduced the transition of migrating interneurons from tangential to radial orientation (23.21±2.42% radially oriented, 76.79±2.42% tangentially oriented, n=280 cells from four slices of two independent experiments) (Fig. 6D-F,H). Altogether, our experiments strongly suggest that leading process branching is required for the chemotaxis of cortical interneurons.

DISCUSSION

By using cortical interneurons as a model, we have found that neuronal migration do not always involve growth cone turning as the mechanism for chemotaxis. In these cells, and probably in many other tangentially migrating neurons in the brain and spinal cord, directional

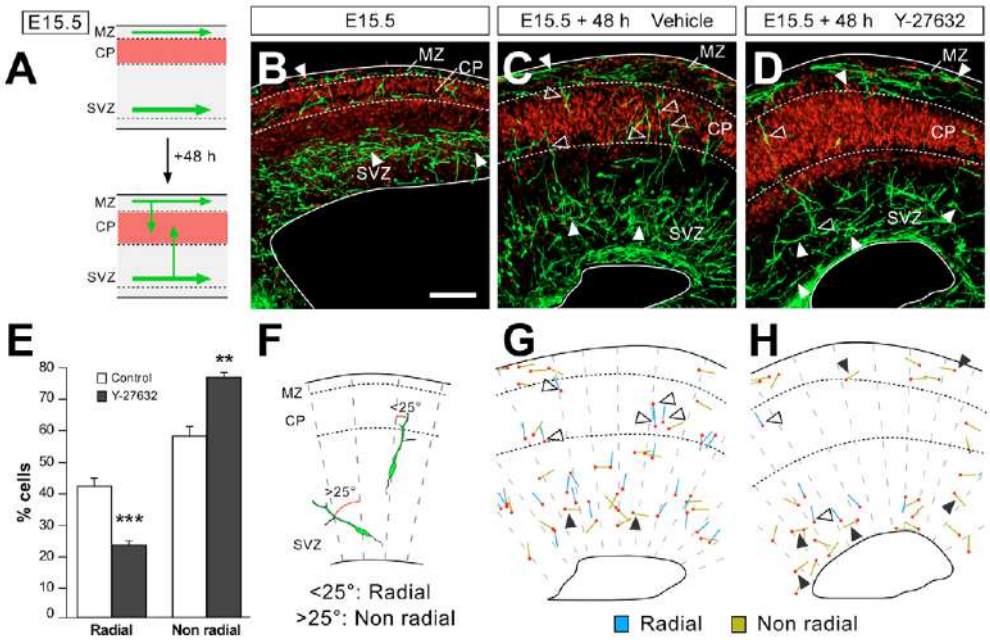


Fig. 6. Switch from tangential to radial position during CP invasion is perturbed after addition of Y27632. (**A**) Schematic showing the preferential orientation of interneurons in the cortex at E15.5 and after 48 hours in culture. Interneurons invade the CP performing a rapid change in direction (Ang et al., 2003; Polleux et al., 2002). (**B-D**) Images of cortical slices at E15.5 (B) or after 48 hours in culture (C,D) showing the distribution of GFP/BrdU+ interneurons, treated with vehicle (C) or Y27632 (D). Solid and open arrowheads indicate tangentially and radially oriented cells, respectively. (**E**) Quantification of percentage of GFP/BrdU+ radially and tangentially oriented neurons in control and Y27632-treated slices after 48 hours in culture. Bars show mean±s.e.m. Control: 41.99±5.37% (radially oriented), 58.00±6.02% (tangentially oriented), *n*=231 cells from four slices of two independent experiments; Y27632: 23.21±2.42% (radially oriented), 76.79±2.42% (tangentially oriented), *n*=280 cells from four slices of two independent experiments. χ^2-test, ***P<0.001, **P<0.01. (**F**) Schematic showing the criteria for the classification of the orientation of interneurons in cortical slices. (**G**,**H**) Schematic representation of the distribution of GFP/BrdU+ interneurons in C and D, respectively. Solid and open arrowheads indicate tangentially and radially oriented cells, respectively. Scale bar: 100 μm.

sensing depends on the generation and stabilization of branches in the leading process rather than growth cone steering. Consequently, the geometry of the leading process branches (i.e. the angle at which they branch) determines the possible directions to be followed by migrating neurons. Thus, chemotaxis in neurons with leading process branches is linked to the stabilization of the most suitable branch.

Leading process branching characterizes many tangentially migrating neurons

Radially migrating neurons are frequently described as bipolar in morphology, with a single leading process oriented in the direction of movement (Nadarajah and Parnavelas, 2002). By contrast, the observation of both bifurcated and non-bifurcated leading processes in tangentially migrating interneurons have led to the suggestion that different types of cortical interneurons may use different modes of migration (Nasrallah et al., 2006; Polleux et al., 2002). Our results demonstrate that the same cells adopt these two different morphologies as part of their migratory cycle, suggesting that leading process branching is part of the mechanism used by cortical interneurons to migrate. Interestingly, many other tangentially migrating neurons appear to branch their leading process as part of their migratory cycle (López-Bendito et al., 2006; Ward et al., 2005) (this study), suggesting that this might be a general feature for very distinct neuronal types. This, however, does not imply that all neuronal populations undergoing tangential migration have branched leading processes. For example, tangentially migrating precerebellar neurons display a single leading process during their entire trajectory (Bourrat and Sotelo, 1990).

The use of branches during tangential migration has been previously interpreted as a mechanism employed by the cell to explore a wide territory, in which guidance signals encountered by the tips of both branches, attractive or repulsive, are likely to be different (Ward et al., 2005). The differential activation of guidance receptors at both branches could lead to different signaling activities, which may then translate into distinct tendencies towards growth or collapse. Once the misbalance between signals at both branches reaches a crucial level, then one of the branches retracts and the other stabilizes. These observations suggest that the dynamic behavior of the two leading processes is highly interdependent, functioning in a continuous competing mode: a one-win/one-lose dichotomy. In that sense, the behavior of cortical interneurons appears to resemble more closely that of some unicellular organisms in which chemotaxis involves the generation of new protrusions by splitting the leading edge of the cell (Andrew and Insall, 2007). Whether similar mechanisms coordinate the generation of new pseudopods in *Dictyostelium* and leading process branches in cortical interneurons remains to be elucidated.

Leading process branching mediates directional guidance

For axons, directional growth in response to chemotactic cues is thought to involve a compass-like behavior in which axons are continuously instructed to the correct direction by differential actin polymerization across the growth cone (Wen and Zheng, 2006). In the case of tangentially migrating neurons, the dynamic behavior of the leading process, which continuously branch as part of the migratory cycle, suggests that these cells do not achieve directional

migration through a mechanism that involves leading process steering. In these cells, growth cone dynamics seem to be relevant only for the initial orientation of the branch. After that, they appear to serve exclusively for the elongation or retraction of the branches, the orientation of which remains stable during their entire lifetime. Consequently, directional movement is mediated by biased choices of nuclear movements into one of the leading process branches.

During quasi-linear migration, the nucleus alternates branches to maintain a defined course. Remarkably, the probability of selecting each of the two alternative branches is almost 50% during quasi-linear migration, which suggest that interneurons are set to follow a rather constant course when migrating in relatively shallow chemotactic gradients. By contrast, our observations suggest that interneurons achieve rapid changes of direction by modifying their basic behavior of leading process branching and selection. Under the influence of intense chemoattractant gradients, interneurons retract systematically those branches that are not oriented towards the source of the chemoattractant, branch at larger angles than they normally do when migrating in quasi-linear trajectories, and preferentially select the new branches, which are better aligned with the source of the chemoattractant. The importance of leading process branching in directional guidance is illustrated by our pharmacological manipulations, which suggest that a perturbation of the frequency at which interneurons branch their leading process alters their ability to react to guidance cues (e.g. Nrg1). A caveat of these experiments, however, is that blocking ROCK could not just prevent branching, but also directly interfere with Nrg1 signaling. We believe this is unlikely because inhibition of Rho-ROCK signaling appears to enhance rather than inhibit chemoattractive guidance in spinal commissural axons (Moore et al., 2008). Moreover, ROCK inhibition does not block Nrg1-mediated cell adhesion in B lymphoblasts (Kanakry et al., 2007), suggesting that Nrg1 signaling does not involve ROCK function. In any case, inhibition of leading process branching in slice cultures also makes rapid directional changes more difficult for interneurons under other circumstances. For example, inhibition of branch formation reduce the ability of interneurons to turn under mechanical constrains or during the process of cortical plate invasion. These experiments reinforce the view that the dynamic behavior of leading process branching is required for the chemotaxis of tangentially migrating interneurons.

In conclusion, our results suggest that directional migration can be achieved not only through growth cone steering, as it happens in neurons with a bipolar shape, but also by biased choices of leading process branches in those neurons that have a leading process with a more elaborate morphology.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/1/41/DC1

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