Regulated formation and selection of neuronal processes underlie directional guidance of neuronal migration

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Axon guidance and neuronal migration are critical features of neural development, and it is believed that extracellular gradients of secreted guidance cues play important roles in pathfinding. It has been well documented that the growth cones of extending axons respond to such extracellular gradients by growing toward or away from the source of the secreted cue via asymmetrical extension of a single growth cone. However, it is unclear whether migrating neurons change direction in response to guidance molecules using the same mode of turning as extending axons. In this study, we demonstrate that migrating neurons turn away from the chemorepellent Slit through repeated rounds of process extension and retraction and do not turn through the reorientation of a single growth cone. We further show that Slit increases the rate of somal process formation and that these processes form preferentially on the side of the cell body furthest away from the Slit source. In addition, Slit causes cell turning through asymmetric process selection. Finally, we show that multiple types of migrating neurons employ this mode of cell turning in response to a variety of guidance cues. These results show that migrating neurons employ a unique type of turning when faced with secreted guidance cues that is distinct from the type employed by axons.

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Introduction

Neuronal migration plays a critical role in the development of the nervous system, and defects in neuronal migration cause a number of human diseases, such as lissencephalies and some types of epilepsy (Rakic, 1971; Hatten and Heinz, 1998; Hatten and Mason, 1990; Kornack and Rakic, 2001; Rice and Curran, 2001; Ross and Walsh, 2001). It is thus imperative to elucidate the cellular and molecular mechanisms that control neuronal migration to further our understanding of neural development and the etiology of neurological diseases.

Secreted guidance molecules such as netrins, ephrins, semaphorins, Slits, and the chemokine SDF have been implicated in controlling neuronal migration (Colamarino and Tessier-Lavigne, 1995; Hu and Rutishauser, 1996; Flanagan and Vanderhaeghen, 1998; Wu et al., 1999; Zhu et al., 1999; Raper, 2000; Klein, 2001; Guan and Rao, 2003; Nguyen-Ba-Charvet et al., 2004). Many of these molecules also play important and well-characterized roles in axon guidance. During development, gradients of these guidance cues are established throughout the nervous system. These gradients are crucial components in long distance pathfinding of migrating neurons and extending axons as they move from their place of origin to their final destination. In an extending axon, the growth cone acts in both a sensory and protrusive manner, while the cell body remains static. Depending on whether the growth cone interprets this cue as an attractant or repellent, the growth cone will gradually turn toward or away from the source of the cue, respectively (Zheng et al., 1996; de la Torre et al., 1997; Ming et al., 1997; Hong et al., 1999; Buck and Zheng, 2002; Ming, 2002). Such turning occurs through asymmetric protrusion of the growth cone during neurite extension, wherein one side of the growth cone extends at a faster rate than the other side, resulting in gradual reorientation of the growth cone and axon shaft. This asymmetric extension of the growth cone in the presence of guidance cues is accomplished through asymmetric formation, stabilization, and invasion of filopodia (Gundersen and Barrett; Zheng et al., 1996). Recent studies have shown that exposure of a growth cone to a guidance cue gradient results in polarization of activated guidance cue receptors and downstream signaling machinery (Hong et al., 2000; Guirland et al., 2004), which is believed to cause changes in the underlying cell cytoskeleton that results in growth cone turning (Gallo and Letourneau, 2003).

Because migrating neurons possess growth cones and respond to the same guidance cues as extending axons, it is possible that migrating neurons sense and respond to gradients of guidance cues in manners similar to extending axons, i.e. with single growth cones turning toward or away from secreted cues. However, in preliminary studies of migrating neurons in brain slices, it has been observed that migrating neurons can turn by sending out new processes...
leading processes from either existing leading processes or directly from the cell body (Nadarajah et al., 2001; Murase and Horwitz, 2002; Nadarajah et al., 2002; Polleux et al., 2002; Yacubova and Komuro, 2002; Nadarajah et al., 2003; Tabata and Nakajima, 2003; Tanaka et al., 2003). Similar observations of migrating GABAergic interneurons have been made in vivo (Ang et al., 2003). Although these observations raise the possibility that the turning behaviors of migrating neurons and extending axons differ, their relevance in the context of directed cell migration remains unclear. An alternative possibility is that that the use of neuronal processes in turning is employed by migrating neurons only in specific and/or limited circumstances and that single growth cone reorientation is the predominant mode of turning employed by migrating neurons when turning in response to secreted guidance cues. In addition, prior studies do not address how neuronal processes employed during turning are regulated.

Using time lapse microscopy to study Slit repulsion of primary migrating SVZa neurons, we found that Slit-induced turning did not occur through reorientation of individual growth cones away from the Slit source. Rather, migrating neurons turned through repeated rounds of process extension and retraction that resulted in selection of a dominant process and directional turning away from Slit. These results have broad implications regarding the manner in which migrating neurons sense and interpret guidance cues and may necessitate modification of current models concerning the translation of gradients of extracellular molecules into intracellular polarity during directed neuronal migration.

Results

SVZa cells change migratory direction exclusively through continuous process extension and retraction, not growth cone bending

In postnatal rodents, neuronal precursors migrate tangentially from the anterior subventricular zone (SVZa) toward the olfactory bulb via a prominent pathway known as the Rostral Migratory Stream (RMS) (Doetsch and Alvarez-Buylla, 1996; Kirschenbaum et al., 1999; Liu and Rao, 2003; Nguyen-Ba-Charvet et al., 2004). To visualize the migratory behavior of these SVZa cells in situ, time lapse fluorescent microscopy was used to document the behavior of these neurons as they migrated in RMS slices. It was observed that these neurons routinely changed directions while migrating in these tissue slices and alternated between pauses and bursts of fast

Fig. 1. SVZa cells change directions during migration through continuous process extension and retraction, not growth cone bending. (a) Saggital slice of a postnatal rat brain in which cells have been labeled with DiI crystal and followed using time lapse microscopy. Static fields of view are shown. Time in hours: minutes are in the upper right corner. Filled arrowheads identify new process formation; asterisk identifies formation of a new process from the soma. These modes of turning were observed in all SVZa cells migrating in RMS slices regardless of cell speed. (b,c) The different types of process formation by migrating SVZa neurons, visualized via time lapse imaging of SVZa cells migrating in collagen/matrigel. (b) New processes could form through branching of existing processes, which could occur at the growth cones of existing processes (open arrowhead), or through side/interstitial branching (closed arrowhead). (c) Extension of a new process from cell soma (arrowhead) and reversal of migration direction. (d) Relative rates of process formation via existing process branching or somal process formation, represented as mean events/cell/h. n = 17 cells in two independent experiments. Error bars = SEM. Scale bars = 10 μm.
migration, which is consistent with previously described behaviors of SVZa neurons migrating in slices (Murase and Horwitz, 2002; Liu and Rao, 2003; Suzuki and Goldman, 2003). However, SVZa cells did not turn in the same manner as an extending axon, which turns through asymmetric growth of its single growth cone followed by reorientation of the axon shaft. Rather, SVZa cells turned by continuously forming and retracting growth cone tipped, axon-like extensions, which we will refer to as ‘processes’ (Fig. 1a, Supplementary Fig. 2), leading to stepwise changes in migration direction that varied from a few degrees to complete reversals (Fig. 1a, asterisk). All cells imaged displayed this behavior.

To better characterize the turning behavior of isolated primary SVZa cells, independent of contact with other cells, we followed individual SVZa cells migrating freely in a three-dimensional collagen/termigel. We found that SVZa neurons formed new processes in two distinct manners. First, a new process could form from the growth cone or side of an existing process (Fig. 1b), which we will refer to as ‘branching’. Second, a new process could form directly from the cell soma (Fig. 1c), which we will refer to as ‘somal process formation’. Cells could possess more than two processes simultaneously (note Fig. 1b). Any individual cell also had the potential to form new processes using either of these methods (note Figs. 1a, b, c). The relative frequencies of these two types of process formation were then determined by counting all new processes formed per cell over a defined time period (Fig. 1d). The majority of new processes formed through the branching of existing processes (3.0 ± 0.27 new processes/h). The formation of new processes from the cell soma was relatively rare by comparison (0.30 ± 0.07 new processes/h).

Unlike extending axons, in which branching can lead to multiple persistent axons, SVZa cells did not accumulate processes (although multiple processes could be observed transiently; see above). In SVZa cells, formation of a new single process was always followed by the eventual retraction of either a pre-existing process or the newly formed process. Process retraction could occur rapidly after formation (Fig. 1b, open arrowhead) or persist until the cell soma moved to the branch point of two processes (Fig. 1b, 43 min). When this latter event occurred, one process always collapsed before resumption of migration. In a cell with more than one process, the one that persisted determined the direction of migration (which we will refer to as the ‘dominant process’). Transient branches or somal processes did not change the direction of cell migration. In the absence of guidance cues, changes in migration direction were often short-lived and later cancelled by additional process formation/retraction events (note Fig. 1b).

Fig. 2. Extracellular gradients of Slit cause repulsion of SVZa cells through sequential branch formation and new somal process formation, not growth cone bending. (a) Migrating SVZa cells were cultured in the absence of guidance cues then exposed to an aggregate of Slit secreting cells (bottom of images) and recorded using time lapse microscopy (see Experimental methods). A static field of view is shown. SVZa cells reversed their direction of migration when exposed to Slit using either new somal process formation (closed arrowhead) or sequential process formation via branching (open arrowhead). Representative cells migrating in a static field of view are shown. A single cell could utilize both turning modalities (closed arrowhead). (b) Percentage of cells that turn using new processes formed exclusively via branching vs. new processes formed via somal process formation. Individual cells on the proximal half of the SVZa explant were classified as having turned either through exclusive use of processes formed via branching or through at least one somal process formation event. Percentages represent the average percentages of 5 independent experiments (137 total cells, error bars = SEM). Scale bars = 50 μm.
SVZa cells turn in response to Slit using repeated rounds of process formation and retraction

We have previously shown that the secreted protein Slit acts as a direct chemorepellent of SVZa cells (Wu et al., 1999; Ward et al., 2003). To determine how cells turned in response to a Slit gradient, we exposed migrating SVZa cells to an aggregate of Slit secreting cells and followed their behavior via time lapse microscopy using a previously characterized turning assay (Ward et al., 2003). Interestingly, cells that turned in response to Slit did so through repeated rounds of process extension and retraction (Fig. 2a and Supplemental Movie), using the same two modes of turning observed above in the absence of guidance cues. Strikingly, cell turning in response to a Slit gradient through the reorientation of a single growth cone was never observed.

Some cells turned by forming a new process directly from the cell soma, which then became the dominant process and resulted in a rapid and dramatic change in migration direction away from the Slit source (Fig. 2a and Supplemental Movie). Other cells made gradual sweeping turns away from the Slit source through repeated rounds of new process formation via branching (Fig. 2a and Supplemental Movie). It is important to note that single cells often used a combination of these two modes of process formation to turn in response to Slit, suggesting that there are no multiple subclasses of SVZa cells wherein each utilizes a different mode of turning in response to Slit.

We then quantified the percentage of cells that turned using these two modes of process formation. Of cells that turned in response to Slit, 24% used processes formed exclusively through branching, while 76% of cells turned via somal process formation (Fig. 2b). Since process formation via branching occurred in all cells after exposure to Slit, process formation via branching potentially played a role in the final trajectory of cells that turned via somal process formation. These results suggest that Slit could regulate either process formation and/or dominant process selection to cause cell repulsion.

Slit regulates the rate and the polarity of somal process formation in SVZa cells

We found that the majority of cells turned in response to Slit by extending and selecting a new process from the cell soma (Fig. 2b). Since this type of turning behavior in collagen/matrigel was a relatively rare event in the absence of exogenous guidance cue addition (Fig. 1e, 0.2 events/h/cell), we speculated that Slit might influence the rate of somal process formation. To address this, we measured the rate of somal process formation in the same cells before and after exposure to a gradient of Slit. Slit addition caused a large and statistically significant increase in the rate of somal process formation (Fig. 3a). This increase in somal process formation did not occur at the expense of process formation via branching (Fig. 4a).

We speculated that Slit might regulate cell turning via somal process formation by polarizing the formation of new somal processes across the cell body. Assuming that each new somal process has an equal chance of becoming dominant, then...
increasing the number of processes forming on the distal side of the soma (i.e. furthest from the Slit source) compared to processes forming on the proximal side would bias the chances that the dominant somal process would be pointed away from the Slit source. When measured, we found that somal processes formation was indeed biased to the distal half of the soma. Processes that formed in the presence of a Slit gradient were 1.5 times more likely to form on the distal half of the cell soma than on the proximal half of the cell soma (Figs. 3b and c). We then measured the rates of transient process formation/retraction on the proximal and distal halves of the cell soma. We found that the rate of transient process formation/retraction was not statistically different between the proximal and distal halves of the soma (Fig. 3d). However, while 62% of all processes (transient plus final processes) formed on the distal half of the cell soma, 90% of the final dominant processes that resulted in directional migration were on the distal half of the cell soma. This suggested that, in addition to regulated process formation, the selection of a dominant process may be involved in Slit regulated cell turning since distally generated processes were less likely to retract than proximally generated processes. Nonetheless, these results indicate that Slit regulates both the rate and the polarity of somal process formation in migrating SVZa cells.

Slit does not change the rate or polarize the formation of processes generated through branching in SVZa cells, but it does regulate the selection of a dominant process

Because 24% of cells that turned away from a Slit source did so through the exclusive use of processes formed via branching (Fig. 2b), we sought to determine how Slit regulated this type of cell turning. Since Slit influenced the rate of somal process formation in SVZa cells, we asked whether Slit also affected the rate of processes formation via branching. As above, we measured the rate of process formation generated through the branching of existing processes in the same cells before and after Slit gradient application. Unlike somal process formation, however, Slit had no effect on the rate of processes formation via branching (Fig. 4a).

Because Slit affected the polarity of somal process formation (Fig. 3), we speculated that Slit might also regulate the polarity of processes formed via branching. However, when we analyzed Slit-distal versus Slit-proximal process formation events that occurred via branching, we found that there were equal numbers of proximal and distal process formation events (Fig. 4b). These results indicated that Slit does not regulate cell turning by affecting the rate or polarity of processes generated through branching.
Since Slit can cause the collapse of axons in non-migrating neurons when applied uniformly (Ba-Charvet et al., 1999), we asked whether Slit could influence SVZa neuron turning by regulating the selection of a dominant process. This model would assume that dominant process selection is polarized, in that the process furthest away from the Slit source would be the most likely to become dominant and thus cause a change in the direction of cell migration. To test this hypothesis, dissociated SVZa cells were plated on a thin layer of collagen/matriigel and allowed to form processes in the absence of exogenous guidance cues. Then, cells having two processes of equal length were selected, and a micropipette loaded with control or Slit conditioned medium was placed near the growth cone of one of the processes. This cell was then recorded via timelapse microscopy (Figs. 4c, d). When exposed to control conditioned medium, most SVZa cells retained both processes at the end of the imaging session (62%). Of the remaining cells, there was a relatively equal occurrence of proximal and distal process collapse (20% and 18%, respectively). In contrast, when cells were exposed to Slit conditioned medium, most cells had preferentially retracted their proximal processes (60% proximal-only retraction, 15% distal-only retraction; 25% no process retraction). It is important to note that, while dissociated SVZa cells plated on collagen/matriigel actively formed and retracted processes in the absence of exogenous guidance cues, they failed to migrate. Since dissociated SVZa cells that are re-aggregated into explants are able to migrate (Ward et al., 2003), it is possible that an undefined factor (e.g. chemorepellent) present when cells are cultured in explants is necessary for migration in three-dimensional collagen/matriigel.

In order to sense extracellular Slit, one would expect that SVZa cell processes would express the transmembrane receptor for Slit, Robo. Indeed, we found that Robo expression was enriched in the growth cones of extending processes (Fig. 4e, arrowheads), suggesting that the growth cone is the major Slit sensory apparatus of SVZa cells. Taken together, these results indicate that, in addition to regulating soma process formation and polarity, Slit can also influence process selection such that the process furthest from the Slit source is statistically most likely to become dominant.

Cell turning via process selection occurs in response to diverse guidance cues and in multiple cell types

To determine whether this mode of turning is an isolated phenomenon or is utilized more broadly, we examined the response of SVZa cells to the secreted guidance cue Netrin and cerebellar

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Fig. 5. Regulated turning via process selection is a widely utilized mode of directed cell migration. (a) SVZa cells turned using process selection in response to Netrin. Cells were cultured as in Fig. 2 followed by placement of a Netrin-secreting cell aggregate and time lapse microscopy. (b) Cerebellar granule cells could turn via branch selection in the absence of guidance cues. Granule cells were plated on PLL/laminin-coated coverslips and observed using phase contrast time lapse microscopy. In these cells, the formation of new processes occurs exclusively through growth cone bifurcation (arrowheads) or conversion of the trailing process into a leading process. (c) Slit could cause the repulsion of granule cells via process selection. Cells were cultured as described in panel (b), and Slit was pulsed from a micropipette facing the cell. A cell that turned by converting its trailing process (arrowhead) into a leading process is shown. (d) Sema could cause the repulsion of granule cells via process selection. Cells were cultured as described in panel (b), and Sema was pulsed from a micropipette facing the cell. A cell that turned via growth cone bifurcation and subsequent process selection (arrowhead) is shown. Scale bars = 10 μm.
granule neurons to the guidance cues Slit and Sema. We have previously shown that Netrin acts as a chemorepellent of SVZa cells (Liu and Rao, 2003). The same repulsion assay utilized in Fig. 2 was used, except that a Netrin-secreting cell aggregate was employed instead of a Slit-secreting cell aggregate. We found that SVZa cells displayed similar morphological turning responses in the face of a Netrin gradient as they did when faced with Slit gradients; cells turned using repeated rounds of process extension and retraction to turn away from the Netrin source (Fig. 5a).

Unlike SVZa cells, which migrate tangentially toward their destination in the developing organism, cerebellar granule neurons migrate radially within the cerebellum (Lois and Alvarez-Buylla, 1994; Zhu, 2002). Using time lapse microscopy of dissociated granule neurons, we found that these cells could turn using repeated rounds of process extension and retraction in the absence of guidance cues (Fig. 5b). To determine whether these cells used similar turning modes when faced with gradients of guidance cues, Sema3a or Slit was pulsed from a micropipette 50 μm from the tip of the leading processes of cells. Similar to SVZa cells, granule cells turned away from the Sema- or Slit-filled micropipettes via process selection (Figs. 5c, d). However, two differences between granule neuron turning and SVZa cell turning were observed: First, while SVZa cells do not have trailing processes, granule cells sometimes reversed direction in the face of a chemorepellent by converting their trailing process into a leading process (Fig. 5b). Second, while we never observed cell turning via growth cone bending in migrating SVZa cells, we sometimes observed growth cones bending away from Slit- or Sema-filled micropipettes in the case of granule neurons. Despite such variations in migration behavior, these results suggest that process selection may be a broadly utilized mode of cell turning employed by diverse types of migrating neurons.

Discussion

These results provide a mechanism for the regulation of SVZa turning in response to Slit, which will provide a framework for analyzing signal transduction during molecular guidance of neuronal migration. Whereas an axon senses extracellular gradients of guidance cues and turns using a single growth cone, our results indicate that a migrating neuron utilizes multiple growth cones, each enriched in guidance cue receptors, to sense and turn in the presence of such gradients. Furthermore, our results suggest that the molecular mechanisms underlying directional growth also differ between extending axons and migrating neurons: In extending axons, it is currently held that extracellular gradients polarize intracellular growth cone signaling machinery and the cytoskeleton, resulting in growth cone bending (Guan and Rao, 2003). Since growth cones of SVZa cell processes cells do not bend in response to guidance cues, it is therefore unlikely that intracellular polarization across an SVZa growth cone is a critical mechanistic feature of guided neuronal migration. We propose that, during directed neuronal migration, intracellular polarization takes place between morphologically distinct processes, not inside of individual processes. Such molecular differences between processes may result in changes in the stability of individual processes, which would lead to cell turning. In fact, differences in cell signaling between individual processes have been implicated in the establishment of axon/dendrite polarity in developing hippocampal neurons (Shi et al., 2003).

We further propose that, in the absence of guidance cues, the selection of a dominant process is unregulated, resulting in random and unproductive changes in the direction of migration. However, the presence of a Slit source causes differences in the extracellular concentration of Slit between processes, which results in different levels of Slat/Robo signaling between processes. These different levels of receptor activation between processes cause different levels of downstream cell signaling, leading to the destabilization of the proximal branch and/or stabilization of the distal branch. We have previously shown that Slit repulsion of SVZa cells requires the activation the Rho-GAP protein srGAP, which results in inactivation of the Rho-GTPase Cdc42 (Wong et al., 2001). In light of our recent findings, it will be interesting to determine whether differences in srGAP and Cdc42 activities/localization are present between processes of SVZa cells that are exposed to a Slit gradient.

Interestingly, Slit appears to have both positive and negative functions in SVZa cells, through stimulating asymmetric somal process formation as well as inducing the asymmetric collapse of leading processes. It has been previously reported that Slit causes the collapse of axonal growth cones in certain neurons (Ba-Charvet et al., 1999) but causes the stimulation of axon branching in other neurons (Wang et al., 1999; Ozdiner and Erzumulu, 2002); however, positive and negative effects of Slit have never been observed in a single cell type. It is interesting to note that, when SVZa cells were exposed to a Slit gradient, fewer processes formed on the proximal sides of cell bodies than the distal sides of cell bodies (Fig. 3c), suggesting that the cell somas of an SVZa cells are involved in gradient sensing as well as cellular processes. It is possible that, in SVZa cells, Slit inhibits the initiation of process formation across the cell soma in a concentration-dependent fashion. Alternatively, the rate of initiation of process formation may be equal on both sides of the cell soma, but Slit inhibits the maturation/stability of filopodia/lamellapodia, which were not observable in our studies, into processes. In this manner, filopodia/lamellapodia that are formed on the distal half of the cell soma are more likely to mature into somal processes than those formed on the proximal half of the cell soma. Taken as a whole, our findings suggest that the combination of positive and negative effects of Slit work together causes repulsion in SVZa cells. In this manner, Slit both increases the chance that a newly formed process is oriented away from the Slit source and biases the selection of a dominant process (whether formed via somal process formation or branching) so that distal processes are more likely to become dominant than proximal processes.

Recently, transport of intracellular material between the growth cones of separate axonal branches has been observed in non-migrating neurons (Denburg et al., 2005). It possible that such molecular communication also occurs between processes during directed neuronal migration, which may result in feedback loops similar to those postulated to occur across the growth cone of an axon and the leading edge of a leukocyte (Rickert et al., 2000; Xu et al., 2003). Such communication may result in coordinated stabilization of the distal process and destabilization of the proximal process of migrating neurons in a Slit gradient. Alternatively, Slit may cause process collapse when present above a certain concentration, such that the concentration of Slit at the proximal process causes collapse while the concentration of Slit at the distal process does not. In this model, the level of Slit-induced signaling that causes
process collapse would be expected to change as a cell moves further away from the Slit source. Such adaptation has been observed in extending axons as they extend toward increasing concentrations of attractants (Ming et al., 2002).

It is important to note that different methods of gradient generation were employed in this study (cell-aggregate delivery, pulsatile micropipette delivery, and passive micropipette delivery) and that the shape and concentration of gradients may be different between these delivery methods. Due to technical limitations, we were only able to observe SVZa cell turning in response to gradients generated by cell aggregates, and the shape of these gradients may be shallower than those generated through micropipettes. However, since the modes of cell turning employed by both SVZa cells and granule neurons were identical in the presence and absence of exogenous gradients (Figs. 1, 2, and 6), we speculate that the turning behaviors we observed in this study were not artifacts caused by the method of gradient generation. It would be interesting to determine the precise shape and concentration of endogenous Slit gradients within the RMS to see whether turning behavior correlates with gradients of Slit in vivo; however, at this time, the technology for measuring gradients in vivo has not been developed.

The use of multiple processes instead of a single growth cone as the sensory apparatus of migrating neurons may offer several advantages. Since the distance between separate processes is greater than the distance across a single growth cone, the differences in guidance cue concentration would also be greater across processes than across a single growth cone. This may enable migrating neurons to sense shallower gradients than extending axons or may result in greater accuracy in gradient sensing. This mode of turning also allows migrating neurons to change direction much more rapidly than if they used a single growth cone, which may be an important requirement in certain physiological circumstances within the developing brain.

Experimental methods

Dissection and culture of SVZa explants

Time-delayed co-culture of SVZa cells with Slit aggregates was described previously (Ward and Rao, 2004; Ward et al., 2003). Briefly, SVZa explants were dissected from the RMS of P2–P5 rat brains as previously described (Ward and Rao, 2004). Briefly, coronal sections of the caudal half of the olfactory bulb were made with a tungsten needle, and the RMS was identified by its translucent appearance and dissected out. This tissue was used to make small explants (200–400 µm in diameter), which were suspended in a 2:2:1 matrigel:collagen:DMEM mixture. Explants were then cultured in DMEM + 10% heat-inactivated FCS at 37°C in a 5% CO2 incubator for 24 h.

Brain slice time lapse

P2–P5 rat brains were embedded in 4% LMP agarose, and 200-µm-thick sagittal sections were made at 4°C using a vibratome. These slices were placed on filters (0.45 µm, Millipore) in a minimal amount of DMEM + 10% FCS, and a single DiI crystal was placed at the caudal end of the RMS. Slices were cultured for 12 h and imaged using epifluorescent time lapse microscopy, as described below.

Time-delayed co-culture of HEK aggregates with SVZa explants

The generation of HEK-293 cells expressing mSlit-2 was described previously (Wu et al., 1999). Aggregates of Slit secreting cells or HEK control cells were prepared using a hanging drop method. After the initial 24-h incubation of SVZa explants, media was removed from the matrigel/collagen pad, and a 400- to 600-µm-wide cell aggregate was placed next to the explant. Additional matrigel/collagen was added to the top of the cell aggregate/explant and allowed to harden followed by addition of L15 + 10% FCS.

Time lapse imaging of migrating SVZa neurons

Explant culture and cell aggregate placement were carried out as described above, except for the following modifications: 1–2 h prior to cell aggregate placement, DMEM culture medium was replaced with L-15 + 10% FCS (overlayed with light mineral oil to prevent evaporation). Time lapse microscopy was then performed using an inverted Nikon Eclipse microscope and CoolSnap ES camera equipped with a heated stage or custom-built microscope incubator. The same SVZa neurons were tracked both before and after cell aggregate placement.

Quantification

Somal process formation

SVZa cells were imaged before and after co-culture with a Slit-secreting cell aggregate. Due to the fact that cells migrated in a three-dimensional matrix, cells sometimes went into and out of focus throughout imaging. All cells that remained in focus throughout the experiment were used for quantification of somal process formation rates. A somal process was defined as any protrusion from the cell body that had a growth cone. Transient protrusions such as lamellapodia/filopodia were not counted as somal processes since they were rarely observed using the 10× DIC lenses utilized in this study.

Somal process angle

Angles were defined as the point of eminence of the process from the cell body as it related to the Slit source, not the angle that the process extended after it left the cell body. For each cell, the initial process was defined as the process present immediately prior to Slit aggregate addition. The final process was defined as the process that a cell migrated toward after Slit addition at the end of the recording session. Transient angles were defined as any new somal process that formed after Slit addition but before the cell changed its direction of migration. After Slit addition, process angles for a single cell were recorded until that cell changed directions and migrated away from the Slit source. Cells were selected for quantification if the point of eminence of their initial process was ±90° from the Slit source.

Process formation rate via existing process branching

Process formation rate was recorded for the same cell before and after Slit aggregate addition. SVZa cells were cultured and imaged before and after Slit aggregate addition as described above. All cells that remained in focus throughout the recording sessions were used for quantification.

Quantification of the side of process formation

After Slit aggregate addition, individual cells were followed, and process formation events were recorded as occurring through
bifurcation, through interstitial branching on the proximal side of the original process, or through interstitial branching on the distal side of the original process. The percentage of proximal, distal, and bifurcation events for each cell were then calculated. The mean percentages of 18 cells in 2 independent experiments were calculated. In order to classify proximal vs. distal formation events, cells were selected for recording if their process was ±30° in respect to the Slit aggregate, and the recording was stopped if their process was ±120° in respect to the Slit aggregate.

**Pipette assays**

**Conditioned media**

Conditioned media were obtained from Slit-secreting or HEK control cells. 80% confluent cells were cultured in DMEM + 10% FCS for 24 h, and their media was collected.

**Exposure of SVZa cells to micropipettes containing Slit or HEK conditioned medium**

SVZa cells were dissected as above but were then dissociated into single cells using Trypsin–EDTA and DNAseI. Thin layers of collagen-matrigel were obtained by spreading 5 μL of gel onto an 18 mm coverslip and allowed to polymerize, taking care not to let the gel dry. 150 μL of 20 × 10^4 cells/mL of SVZa cell suspension was then plated onto each coverslip. Cells were allowed to culture for 24–48 h prior to any experiments. Micropipettes were pulled with a vertical pipette puller (Narishigi, Japan), and pipette openings were approximately 1 μm in diameter. Micropipettes were back-filled with Slit or HEK conditioned medium and placed ~5 μm away from one of the branches. Cells that had two equal length processes branching from a single main process were selected for exposure to a Slit- or HEK CM-filled micropipette. Cells were recorded for 30 min, and the process behavior was scored as follows: proximal process retraction, distal process retraction, or no process retraction. Loss of both processes was never observed. Although similar results were obtained using a pulsing pipette placed 50 μm away from the cell, due to frequent micropipette clogging with conditioned medium, cells were exposed to passively diffusing micropipettes with Slit or HEK CM for purposes of quantification. Exposure of granule cells to Slit or Sema: For these experiments, Slit was partially purified as described previously (Xu et al., 2004). Sema 3A was purchased from TECHNE. Pulled micropipettes were backloaded with Slit or Sema 3A, placed 50 μm away from the growth cones of granule neurons (facing the growth cone) plated with Slit or HEK CM-filled micropipette. Cells were recorded for 30 min, and the recording was stopped if their process was through interstitial branching on the distal side of the original process. The percentage of proximal, distal, and bifurcation events for each cell were then calculated. The mean percentages of 18 cells in 2 independent experiments were calculated. In order to classify proximal vs. distal formation events, cells were selected for recording if their process was ±30° in respect to the Slit aggregate, and the recording was stopped if their process was ±120° in respect to the Slit aggregate.

**Immunofluorescence microscopy**

A polyclonal antibody generated against Robo 2 was described previously (Wong et al., 2001). Dissociated SVZa cells were cultured as above and fixed in fixation buffer (10 mM Pipes, pH 6.5, 127 mM NaCl–5 mM KCl, 1.1 mM NaH2PO4, 0.4 mM KH2PO4, 2 mM MgCl2, 5.5 mM glucose, 1 mM EGTA, 4% paraformaldehyde) for 5 min then washed 3× with PBS. Cells were then permeabilized with 0.5% Triton X in PBS for 10 min and blocked in 5% BSA + 0.2% Triton X in PBS for 1 h. Cells were incubated with primary antibody for 1 h, washed 3 × 10 min with PBS, incubated with anti-rabbit Cy2 for 1 h, washed 3 × 10 min with PBS, and mounted.

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**Appendix A. Supplementary data**


**References**


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