Slit-2 repels the migration of olfactory ensheathing cells by triggering Ca\textsuperscript{2+}-dependent cofilin activation and RhoA inhibition

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Summary

Olfactory ensheathing cells (OECs) migrate from the olfactory epithelium towards the olfactory bulb during development. However, the guidance mechanism for OEC migration remains a mystery. Here we show that migrating OECs expressed the receptor of the repulsive guidance factor Slit-2. A gradient of Slit-2 in front of cultured OECs first caused the collapse of the leading front, then the reversal of cell migration. These Slit-2 effects depended on the Ca\textsuperscript{2+} release from internal stores through inositol (1,4,5)-triphosphate receptor channels. Interestingly, in response to Slit-2 stimulation, collapse of the leading front required the activation of the F-actin severing protein cofilin in a Ca\textsuperscript{2+}-dependent manner, whereas the subsequent reversal of the soma migration depended on the reversal of RhoA activity across the cell. Finally, the Slit-2-induced repulsion of cell migration was fully mimicked by co-application of inhibitors of F-actin polymerization and RhoA kinase. Our findings revealed Slit-2 as a repulsive guidance factor for OEC migration and an unexpected link between Ca\textsuperscript{2+} and cofilin signaling during Slit-2-triggered repulsion.

Key words: Ca\textsuperscript{2+}, Cofilin, Migration, Olfactory ensheathing cells, RhoA, Slit-2

Introduction

Olfactory ensheathing cells (OECs) are a unique type of glial cells in the olfactory system, and have been discovered to promote the growth of olfactory sensory axons during development and the regeneration of injured axons after being transplanted into nerve injury sites (Cao et al., 2004; Li et al., 1998; Raisman and Li, 2007; Ramon-Cueto et al., 1998; Vincent et al., 2005). Derived from the olfactory placode, OECs migrate out of the olfactory epithelium (OE) together with growing olfactory sensory axons from the lamina propria (LP) and accumulate as a superficial mass upon reaching the telencephalic vesicle at embryonic day (E) E13–E18 in rat, contributing to the formation of the presumptive olfactory nerve layer (Chuah and West, 2002; Valverde et al., 1992). However, how the migration of OECs is guided during development remains unclear.

Slit and Robo are a pair of conserved repulsive ligands and receptors for axon pathfinding (Dickson and Gilestro, 2006). Slit was first identified in Drosophila as a molecule secreted by midline cells, and was later shown to repel the extension of axons expressing Robo receptors (Brose et al., 1999; Kidd et al., 1999; Seeger et al., 1993) and to regulate the migration of cells such as neuronal precursors (Wu et al., 1999). In the olfactory system, members of the Slit and Robo families are expressed in a specific spatio-temporal pattern and play important roles in the guidance of olfactory sensory axons (Cho et al., 2007; Marillat et al., 2002; Nguyen-Ba-Charvet et al., 2008; Yuan et al., 1999). Whether OECs, which have the same developmental origin as olfactory sensory neurons, are also responsive to Slits is unknown.

The signal transduction underlying the guidance of axon pathfinding and neuronal migration has been studied in cell culture. In dissociated culture of cerebellar granule cells, a frontal gradient of Slit-2 triggers the elevation of the intracellular concentration of Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}) in the leading growth cone, and the subsequent propagation of a Ca\textsuperscript{2+} wave from the growth cone to the soma mediates the reversal of soma translocation by inhibiting the activity of the small GTPase RhoA (Guan et al., 2007; Xu et al., 2004). However, the molecular mechanism underlying the Slit-2-induced collapse of neuronal growth cones is unclear.

In the present study, we tested the guidance effect of Slit on the migration of cultured OECs. We found that a Slit-2 gradient in front of migrating OECs triggered a Ca\textsuperscript{2+}-dependent collapse and reversal of OEC migration. Furthermore, Slit-2 triggered the activation of the F-actin severing protein cofilin and the inhibition of RhoA, leading to the collapse of the leading front and the subsequent reversal of the polarity of OEC migration.

Results

Robo1 is expressed in OECs both in vitro and in vivo

To explore the potential effects of Slits on OEC migration, we examined the expression of Robos in cultured OECs that exhibit active cell migration (Huang et al., 2008). In purified OEC culture, RT-PCR experiments revealed that mRNAs of both Robo1 and Robo2 were highly expressed. However, mRNA of Robo3 was undetectable (Fig. 1A). Robo1 protein could also be detected in cultured OECs by western blotting, and its expression could be
Slit-2 repels OEC migration

To test whether OECs are responsive to Slit-2, single-cell migration assays were first performed in a low-density culture of OECs obtained from olfactory bulb (OB) tissue. A gradient of Slit-2 was produced in front of isolated migrating OECs by using a micropipette loaded with Slit-2 with repetitive injection by air pressure (Huang et al., 2008). As shown in Fig. 2C,F and supplementary material Movie 1, after the application of a Slit-2 gradient, the leading front of migrating OECs with elaborated lamellipodia was inhibited in their motility and showed collapse and retraction within 20 minutes. For 23 out of 26 of tested cells, the soma later reversed their direction of translocation, with the original trailing tail becoming a new leading front. Interestingly, after OEC migration had been reversed by the Slit-2 gradient, when the Slit-2 gradient was applied to the same OEC from the reverse direction, OEC migration was reversed again (Fig. 2C). By contrast, OECs appeared to have enhanced motility toward the gradient of lysophosphatic acid (LPA), a known attractant for OECs (Huang et al., 2008; Yan et al., 2003) (Fig. 2B,F); whereas OEC migration was not affected by the gradient of another axon guidance molecule Netrin-1, or by phosphate-buffered saline (PBS) (Fig. 2A,F).

To quantify the effect of tested factors on OEC migration, we measured migration rates of each cell during the period before and after the application of factors and calculated their ratio (after/before). A ratio >1 implies accelerated cell migration in response to the treatment. Conversely, a ratio <1 implies inhibition of migration, and a ratio <0 implies a reversal of migration (Huang et al., 2008). As shown by the cumulative distribution graph (Fig. 2D), ratios of migration rate for most OECs under Slit-2 gradient were <0, as indicated by a significant left shift in the cumulative distribution curve of migration ratios compared with that of the PBS-treated group (P<0.001, Kolmogorov–Smirnov test), with the average migration ratio under Slit-2 gradient markedly decreased (Fig. 2E). By contrast, ratios of migration rate for most OECs under LPA gradient were >1, and there was a significant right shift in the distribution curve of migration ratios under LPA treatment compared with that of the PBS-treated group (P<0.001, Kolmogorov–Smirnov test) (Fig. 2D). The average migration ratio was markedly increased under LPA gradient, but not changed under Netrin-1 gradient (Fig. 2E). Furthermore, in the presence of anti-Slit-2 antibody or the soluble protein of the Robo1 ectodomain (RoboN), a competitive inhibitor for Slit–Robo interaction (Wu et al., 1999), spontaneous migration of OECs was not affected. However, the Slit-2-induced collapse and reversal of migration of OECs were both blocked, compared with treatment with a normal IgG or control media, respectively (Fig. 2E,F). These results strongly suggest that Slit-2 repels the migration of these cultured OECs.
Similar collapse and reversal of migration of OECs in response to a Slit-2 gradient were observed in cultured OECs obtained from postnatal day 0 (P0) OE tissue (supplementary material Fig. S2A–D). Moreover, in the high-density culture, when Slit-2 gradient was applied toward a group of OECs, cells exhibited the obvious collapse and retraction and escaped from the tip of the micropipette within 35 minutes in a distance-dependent manner (supplementary material Fig. S2E). Taken together, these results show that Slit-2 is a repulsive guidance factor for the migration of cultured OECs.

Ca²⁺ release from Ins(1,4,5)P₃ receptor channels is essential for the effect of Slit-2

We next examined whether Ca²⁺ signaling was involved in Slit-2-induced repulsion of OECs. In OECs loaded with the Ca²⁺-sensitive fluorescent dyes Fluo-4 and Fura-Red, we observed a transient elevation in the ratio of the fluorescent intensity (Fluo-4/Fura-Red) both in the leading process and in the soma in response to the Slit-2 gradient, indicating that Slit-2 triggers the elevation of [Ca²⁺]ᵢ in these cultured OECs (Fig. 3A,B). By contrast, when a PBS gradient was applied at a similar distance from the OEC, no [Ca²⁺]ᵢ elevation was observed in either the leading process or the soma (Fig. 3C,D). The elevation of [Ca²⁺]ᵢ was abolished by pretreatment with thapsigargin, a drug that depletes the intracellular Ca²⁺ stores, or with 2-APB, an inhibitor of inositol (1,4,5)-trisphosphate [Ins(1,4,5)P₃] receptors at the internal stores, but not by removing the extracellular Ca²⁺ (Fig. 3C,D). Furthermore, the repulsive effect of Slit-2 on OEC migration was totally blocked by bath application of thapsigargin or 2-APB, but not by removing the extracellular Ca²⁺ (Fig. 3E,F). Thus, Ca²⁺ release from internal stores through Ins(1,4,5)P₃ channels is essential for both Slit-2-induced collapse and reversal of migration of OECs.

Cytoskeletal reorganization induced by Slit-2

Because cytoskeletal reorganization is essential for cell motility (Suetsumg and Takenawa, 2003), we next examined the cytoskeletal reorganization in OECs after Slit-2 exposure by using immunofluorescent staining. As shown in Fig. 4, in untreated OECs, a thin layer of F-actin was evenly distributed in the peripheral region of the leading front, and F-actin bundles spanned the central region of the fan-like lamellipodia of the leading front and at the soma. By contrast, microtubules localized to the central domain of lamellipodia, parallel to the F-actin bundles, with a few microtubules penetrating the peripheral region of the leading front. After Slit-2 stimulation, F-actin in the peripheral region of the leading front disappeared rapidly (within 10 minutes), and F-actin density in the central domain of the lamellipodia reduced gradually. However, microtubules remained extending in the central and peripheral region of the lamellipodia before the full collapse of the
leading front, even though F-actin had disappeared in the peripheral region (Fig. 4B,C). Thus, the F-actin in the peripheral region is more sensitive to the Slit-2 stimulation than microtubules, and the disruption of F-actin in the peripheral region of the leading front is implicated in initiating the collapse of leading front in OECs.

Cofilin activation through Ca²⁺-calcineurin signaling is required for Slit-2-induced collapse of the leading front and reversal of migration

What causes the loss of F-actin in the peripheral region of the cell in response to Slit-2? Because cofilin is a key F-actin severing protein (Theriot, 1997), we therefore examined whether cofilin is involved in the Slit-2-induced loss of F-actin in the leading front of OECs. As shown in Fig. 5A, western blot analysis showed that stimulation of OECs with Slit-2 markedly decreased the level of phosphorylated cofilin (p-cofilin, the inactive form of cofilin) in a time-dependent manner, without affecting the total cofilin level. Immunofluorescent staining showed that in untreated OECs, the inactive p-cofilin was mainly localized at the soma and colocalized with F-actin at the peripheral region of the leading front. After Slit-2 incubation, the p-cofilin level in the whole cell dramatically decreased, and its signal in the peripheral region of the leading front disappeared, a process correlated with the loss of F-actin in the peripheral region (Fig. 5B). Furthermore, anti-p-cofilin and anti-cofilin double-staining showed that the p-cofilin signal rapidly decreased at the peripheral ruffles in Slit-2-stimulated OECs (10 minutes) before the full collapse of the leading front; however, cofilin signal still localized at the peripheral ruffles (Fig. 5C). These results suggest that Slit-2 activates cofilin by dephosphorylation at the peripheral region, which could be responsible for the disruption of F-actin at the peripheral region in response to Slit-2.

To further examine whether the activation of cofilin depends on the Ca²⁺ signal, we added the intracellular Ca²⁺ chelator BAPTA-AM, or cyclosporin A (CsA), a specific inhibitor of the Ca²⁺- and calmodulin-dependent phosphatase (calcineurin), to the bath before Slit-2 treatment. Interestingly, the Slit-2-induced dephosphorylation of cofilin in OECs was blocked by either BAPTA-AM or CsA (Fig. 5D). These results suggest that Slit-2 activates cofilin by dephosphorylation at the peripheral region, which could be responsible for the disruption of F-actin at the peripheral region in response to Slit-2.

To further examine the direct role of cofilin in Slit-2-induced collapse of the leading front, we transfected GFP-tagged wild-type cofilin (GFP-cofilin-WT) and a non-phosphorylatable and constitutively active mutant of cofilin (GFP-cofilin-S3A) (Arber et al., 1998) into OECs and analyzed the morphology and motility of OECs transfected with these constructs. After transfection of
What causes the reversal of migration of OECs in response to Slit-2? Because RhoGTPases (RhoA, Cdc42, Rac1) play a key role in directional cell migration (Fukata et al., 2003) and the signal transduction of Slit in axon guidance (Wong et al., 2001), we next tested whether RhoGTPases are involved in the Slit-2-induced reversal of migration of OECs. In the presence of a general RhoGTPase inhibitor, toxin-B (10 ng/ml), the spontaneous migration of OECs was not affected (Fig. 6B). Frontal application of the Slit-2 gradient on these toxin-B-treated OECs still triggered the collapse of the leading front, but failed to reverse the migration of the soma, which stopped their forward migration after the full collapse of the leading front (Fig. 6B–D). By contrast, pretreatment with the specific phosphoinositide 3-kinase (PI3K) inhibitor LY-294002 (20 μM) did not affect Slit-2-induced repulsion of OECs (Fig. 6C–D). These results suggest that RhoGTPase activity is essential for the reversal of soma migration of OECs, but not for the collapse of leading process triggered by Slit-2.

Further experiments were carried out in OECs transfected with constructs expressing fusion proteins of GFP and various dominant-negative (DN) forms of RhoGTPases. We selected single OECs of similar level of GFP fluorescence intensity for cell migration assays (supplementary material Fig. S4A,B). We found that in cultured OECs expressing DN-RhoA–GFP, frontal application of Slit-2 gradient failed to induce the reversal of migration, although the leading process still exhibited collapse and retraction (Fig. 6C,D; supplementary material Fig. S4E). By contrast, Slit-2-induced collapse and reversal were not affected in cells expressing DN-Rac1–GFP or DN-Cdc42–GFP (Fig. 6C,D; supplementary material Fig. S4C,D). Thus, RhoA is specifically required for the soma reversal. In support of this notion, we found that Slit-2-induced reversal of migration was abolished by bath incubation with Y-27632 (20 μM), a specific inhibitor of RhoA-dependent kinase (Rho kinase) (Fig. 6C,D), with the spontaneous migration of these OECs being unaffected.

To further elucidate the role of RhoA in the reversal of migration, we used pull-down assays to examine the activity of RhoA in cultured OECs. We found that treatment with either serum (10%) or the RhoA activating agent LPA (10 μM) (Yan et al., 2003) resulted in marked elevation of RhoA activity in these cultured OECs (Fig. 7A,B). Interestingly, bath application of Slit-2 caused a reduction of RhoA activity in a time-dependent manner (Fig. 7C,D), suggesting that the reversal in the direction of soma translocation might be related to the inhibition, rather than the activation, of RhoA. This notion was further supported by photometric analysis of the active RhoA in migrating OECs by using a FRET (fluorescence resonance energy transfer)-based biosensor pRichu-RhoA, which was constructed by linking CFP-conjugated RhoA and the YFP-conjugated RhoA-binding domain (RBD) of Rhotekin, a configuration sensitive to RhoA activation by guanine nucleotide exchange factor (Yoshizaki et al., 2003). As shown in Fig. 7E, the FRET signal for the active RhoA displayed a polarized distribution in a migrating OEC, with the leading front exhibiting higher activity than the soma and the trailing end. Application of a Slit-2 gradient in front of migrating OECs transfected with pRichu-RhoA led to a reduction in the FRET signal of RhoA activity in the leading front and a reversal of the polarity of active RhoA in this OEC (Fig. 7E,F). Taken together, these results suggest that downregulation of RhoA signaling is specifically required for the soma reversal of OECs triggered by Slit-2.

**Collapse of the leading front with simultaneous inhibition of RhoA triggers the reversal of soma translocation**

To further examine how the coordination between the collapse of the leading front and the reversal of soma translocation in response...
to Slit-2 gradient is achieved, we applied a gradient of latrunculin A (LA) or cytochalasin D (CD), to mimic the Slit-2-triggered collapse of the leading front. After the application of a gradient of LA or CD (50 μM and 5 mM, respectively, in the pipette), the leading front of most OECs (15 out of 16 in CD; 14 out of 16 in LA) was inhibited in their motility and showed obvious collapse within 20 minutes. Only a few of these cells (4 out of 16 in 5 mM CD; 4 out of 16 in 50 μM LA) later reversed their soma translocation (Fig. 8B,E,F). Thus the collapse of the leading front does not seem to be sufficient to trigger the reversal of soma translocation, and other signals triggered by Slit-2 should be required.

We have shown that the Slit-2 gradient inhibits RhoA activity in OECs. To further examine whether the downregulation of RhoA activity in the leading front is sufficient to trigger the reversal of soma translocation, we applied a gradient of the Rho kinase inhibitor Y-27632 (10 mM in the pipette) in front of migrating OECs to mimic a gradient of RhoA inhibition across the cell. As shown in Fig. 8C,E,F, the Y-27632 gradient did not cause the collapse of the leading front nor the reversal of soma migration, but dramatically reduced the soma motility. Surprisingly, when we applied a gradient of the mixture of Y-27632 and LA in front of migrating OECs, most cells (14 out of 16) quickly reversed their migration after the collapse of the leading front (Fig. 8D–F), a process reminiscent of the collapse and reversal of migration triggered by Slit-2 (Fig. 2C). These results suggest that Slit-2 triggers a coordinated collapse of the leading front along with a gradient of RhoA inhibition across the cell to reverse OEC migration.

**Discussion**

It has been shown that the OB secretes some soluble factors that might attract OEC migration from the OE towards the OB (Liu et al., 1995). Another potential guidance mechanism for OEC migration is that the OE might secrete some repellants to promote OEC migration toward the OB during early development. Recent studies have shown that glial cell migration can also be directed by some axon guidance molecules (Tsai and Miller, 2002). Because OECs share a common origin (olfactory placode) with olfactory sensory neurons and migrate out from the OE to the OB together...
with olfactory sensory axons during the same developing period (Valverde et al., 1992), it is likely that guidance cues for olfactory sensory axons, like Slit (Cho et al., 2007; Nguyen-Ba-Charvet et al., 2008), might also guide the migration and distribution of OECs.

In the present study, we have shown that Robo proteins are expressed in cultured OECs and exhibit enriched distribution at the leading edge. A Slit-2 gradient indeed strongly repelled the migration of these cultured OECs. To our knowledge, this is the first guidance factor discovered to repel OEC migration. Because Slit-2 is highly expressed in the apical cells of OE, it is likely that it might help Robo-expressing OECs and olfactory axons migrate out of the OE through chemorepulsion during early development. Slits expressing in the OB might also regulate the stop and scattering of OECs that have arrived at the surface of the OB. OECs have been reported to pioneer the olfactory sensory nerves and provide a conductive substrate for the growth of olfactory sensory axons during development (Tennent and Chuah, 1996; Tisay and Key, 1999). An intriguing possibility is that the guidance of OECs by Slits might contribute to the guidance of axons because of the close interaction between neurons and glia. Extensive future studies using Slit and Robo mutant mice are needed to address these possibilities. Because multiple Slit and Robo family members are expressed in the developing olfactory system, double or even triple mutants of Slits and Robos might be required to clarify whether and how Slits guide the migration and distribution of OECs in developing olfactory system. Conditional knockout mutants with OEC-specific deletion of Robos are required to address whether Slit proteins could directly guide the migration of OECs in vivo and whether this guidance of OEC migration by Slit contributes to the pathfinding of olfactory sensory axons.

The intracellular signal transduction for Slit-2-induced collapse of the leading front of migrating cells has been largely unclear. Cultured OECs provide a good experimental system for addressing this issue because of their large cell size, active migration and high responsiveness to Slit-2. In the present work, we found that cofilin is a major downstream target of Slit-2 in triggering the collapse of the leading front of migrating OECs. ADF/cofilin family members are key regulators of F-actin dynamics and mediate the rapid turnover of F-actin by severing F-actin near the pointed ends (Bamburg, 1999; Moon and Drubin, 1995; Theriot, 1997). Recent studies have shown that cofilin has complicated regulation of F-actin dynamics and diverse effects on F-actin polymerization (Van Troys et al., 2008). Whether cofilin promotes F-actin assembly or disassembly depends upon the concentration of cofilin relative to actin and the relative concentrations of other actin-binding proteins. A low cofilin to actin ratio promotes actin disassembly, whereas a high ratio promotes actin assembly in vitro (Andrianantoandro and Pollard, 2006; Chan et al., 2009; Van Troys et al., 2008). During chemotaxis, spatial and temporal regulation of cofilin activity is required for cell directional migration (Mouneimne et al., 2006; Nishita et al., 2005). A previous study has shown that Slit-2 could induce the local synthesis of cofilin in Xenopus retinal growth cones (Piper et al., 2006). In our studies, we observed that stimulation of OECs with Slit-2 significantly decreased the p-cofilin level, without affecting the total cofilin level. These results suggest that Slit-2-induced collapse of OECs is mediated by changes of the ratio of p-cofilin to non-p-cofilin, but not by an increase of total cofilin proteins in these migratory glial cells. We also found that cofilin proteins were mainly the phosphorylated inactive form at the peripheral ruffle of normal migratory OECs. Upon Slit-2 stimulation, cofilin was quickly activated at cell peripheral ruffles, a process that might be responsible for the disruption of F-actin in this region and the subsequent collapse of leading front. In support of this notion, F-actin at cell peripheral ruffles was lost upon Slit-2 stimulation (Fig. 4) and a gradient of CD or LA, which promotes F-actin de-polymerization, fully
mimicked the Slit-2-induced collapse of the leading front (Fig. 8).

Furthermore, overexpression of the constitutive active cofilin-S3A mutant could block the Slit-2-triggered collapse of OECs. The global presence of cofilin-S3A might set a new balance of F-actin turnover at a very high speed, override any local regulation of cofilin activity triggered by Slit-2 gradient, and thus abolish the Slit-2-induced collapse of the leading front of OECs. It is likely that similar signaling mechanisms might happen during Slit-triggered collapse and repulsion of neuronal growth cones. Interestingly, other repulsive guidance molecules, such as Semaphorin-3A, Semaphorin-3F, bone morphogenetic protein (BMP) and myelin-associated inhibitors including Nogo-66, have been shown to trigger the collapse and repulsion through cofilin activation (Aizawa et al., 2001; Hsieh et al., 2006; Shimizu et al., 2008; Wen et al., 2007). Thus, activation of cofilin and the consequent fast severing of F-actin at the leading front might be a general molecular mechanism for the collapse of the leading front in response to different repulsive factors.

The F-actin-severing activity of cofilin can be regulated by reversible phosphorylation at the Ser3 residue, with the loss of activity upon phosphorylation. It has been shown that LIM kinases (LIMK) specifically phosphorylate cofilin at Ser3, whereas phosphatases Slingshot and calcineurin are capable of dephosphorylating it (Arber et al., 1998; Huang et al., 2006). Previous studies have shown that regulation of cofilin activity by LIMK and Slingshot is involved in regulating growth cone motility and morphology in response to extracellular cues, including Semaphorin-3A, BMP, brain-derived neurotrophic factor, and myelin-associated inhibitors (Aizawa et al., 2001; Gehler et al., 2004; Hsieh et al., 2006; Meberg and Bamburg, 2000; Wen et al., 2007). It is also shown that the Ca2+-calcineurin signaling pathway can dephosphorylate cofilin and mediate BMP-triggered repulsion of OECs.
In the present study, we found that RhoA was inhibited by Slit-2 in cultured OECs. Because inhibition of RhoA activity was specifically required for Slit-2-induced reversal of migration, but not for the collapse of leading front, the collapse of the leading front and the reversal of soma migration depends on the coordination of a reversal of migration of another cell type, cultured Schwann cells. Frontal application of Y-27632 together with LA also reliably triggered the collapse and reversal of migration of another cell type, cultured Schwann cells (supplementary material Fig. S5).

Materials and Methods
Primary culture and OEC purification
Primary OEC cultures were prepared from the OB of adult male Sprague-Dawley rats and purified by differential cell adhesiveness as described previously (Huang et al., 2008). Briefly, the olfactory nerve layer was peeled away from the rest of OB,
dissociated with 0.25% trypsin (Sigma, St Louis, MO) at 37°C for 15 minutes. Tissue was triturated with a Pasteur pipette and plated on an uncoated 25 cm² cell culture flask twice; each sample was then incubated for 36 hours at 37°C in 5% CO₂. Non-adhesive cell suspension was collected, seeded onto 35 mm dishes (Corning, NY) coated with poly-L-lysine (0.1 mg/ml, Sigma), and incubated with DMEM/F-12 (1:1, vol/vol; DF12, Gibco, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 2 μM forskolin (Sigma) and 10 ng/ml NGF (Sigma). The overall purity of OECs was around 95%. Primary cultures of OECs from OE of P0 Sprague-Dawley rats were prepared as described previously (Au and Roskams, 2003).

RT–PCR and western blotting

For RT-PCR, total RNA was extracted from cultures of purified OECs with Trizol reagent (Invitrogen, Gaithersburg, MD), converted to cDNA using the Revert Aid First Strand cDNA Synthesis Kit (MBI Fermentas, Hanover, MD), and one twentieth of the product was used in 20 μl PCR reactions. Primers for Robo1 were designed and the specificity of primers was measured by Primer Premier 5.0 software. Robo1, forward, 5’–GACAGGCAGCAGATGATG-3’; reverse, 5’–ATTAAGGCCGCCTTCC–3’, 12% SDS-PAGE gel electrophoresis and transferred with a horseradish-peroxidase-conjugated antibody against rabbit IgG (1:10,000; Cell Signaling Technology, Beverly, MA) or anti-p-cofilin (Ser3, 1:1000;Santa Cruz Biotechnology). For visualization of F-actin, cells were incubated with rhodamine-conjugated phallolidin (1:60; Molecular Probes) at room temperature for 1 hour. Images were acquired on an Olympus FV-1000 confocal system using a multichannel setup and processed using Adobe Photoshop CS 8.0.

Calcium imaging

Calci u m imaging was performed as described previously (Guan et al., 2007). Briefly, OECs were rinsed three times before being loaded with Fluo-4 AM and Fura-Red AM (2 μM, Molecular Probes, Eugene, OR), with 0.1% dimethyl sulphoxide (DMSO) in the extracellular medium (ECM), for 30 minutes at 37°C, and then incubated for another 30 minutes after being rinsed three times with ECM. Imaging was performed to observe the fluorescent signal. The Ca²⁺ signals were measured as the change in the ratio of Fluo-4 to Fura-Red, relative to the baseline before applying Slit-2 (∆F/F₀), in selected regions using Leica SP-5 software. The leading process and soma in OECs were defined as described in a previous work (Huang et al., 2008). To quantify the Ca²⁺ signal at the leading process, the fluorescence signal in the whole leading process was measured (Fig. 3A). In some experiments, cells were incubated in ECM containing pharmacological agents for at least 30 minutes before measurement.

Immunostaining

For immunostaining, embryonic brains were directly fixed and fixed with 4% paraformaldehyde (PFA) at E16. Sagittal brain sections of 20 μm were cut on a freezing microtome and immediately processed for immunostaining by 1 hour blocking in 5% BSA plus 0.3% Triton X-100 at room temperature, overnight incubation with primary antibodies at 4°C, and for 1 hour at room temperature incubation with appropriate secondary antibodies (1:1000; Molecular Probes, Eugene, OR). The primary antibodies used were anti-p75 (1:500; Promega, Madison, WI), anti-Slit-2 (1:200; Chemicon) and anti-Robo1 (1:200; R&D) (Farmer et al., 2008; Marlow et al., 2008; Nguyen-By-Charvet et al., 2008). Sections were counterstained for Toto-3 (1:1000; Molecular Probes) to visualize the nuclei. For OEC staining, cultured OECs were fixed with fresh 4% PFA for 20 minutes. The primary antibodies used were anti-Slit-2 (1:200; Chemicon), p75 (1:500; Promega), cofilin (1:200; Santa Cruz Biotechnology), Slit-2 (1:200; Chemicon) or acetylated tubulin (1:1000; Sigma). For visualization of F-actin, cells were incubated with rhodamine-conjugated phallolidin (1:60; Molecular Probes) at room temperature for 1 hour. Images were acquired on an Olympus FV-1000 confocal system using a multichannel setup and processed using Adobe Photoshop CS 8.0.

Plasmids and cell transfection

GFP-tagged cofilin-WT and cofilin-S3A were constructed by subcloning the full-length cofilin or cofilin-S3A into pEGFP vector (Clontech) between BamHI and HincII sites. Robo2–GFP, DN-RhoA–GFP, DN-Rac–GFP, and DN-Cdc42–GFP plasmids were constructed previously (Guan et al., 2007). The Robo-specific siRNA sequences are 5’-AAGAGAACACAGGGATGAAGG-3’ (Guan et al., 2008). For OEC transfection, we used rat Astrocyte Nucleofactor Kit (Axima, Cologne, Germany) according to the manufacturer’s instructions (program T-20).
Review article:

### Roles of Rho-family GTPases in neuronal migration

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