An antagonistic interaction between PlexinB2 and Rnd3 controls RhoA activity and cortical neuron migration

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A transcriptional programme initiated by the proneural factors Neurog2 and Ascl1 controls successive steps of neurogenesis in the embryonic cerebral cortex. Previous work has shown that proneural factors also confer a migratory behaviour to cortical neurons by inducing the expression of the small GTP-binding proteins such as Rnd2 and Rnd3. However, the directionality of radial migration suggests that migrating neurons also respond to extracellular signal-regulated pathways. Here we show that the Plexin B2 receptor interacts physically and functionally with Rnd3 and stimulates RhoA activity in migrating cortical neurons. Plexin B2 competes with p190RhoGAP for binding to Rnd3, thus blocking the Rnd3-mediated inhibition of RhoA and also recruits RhoGEFs to directly stimulate RhoA activity. Thus, an interaction between the cell-extrinsic Plexin signalling pathway and the cell-intrinsic Ascl1-Rnd3 pathway determines the level of RhoA activity appropriate for cortical neuron migration.

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In humans, the cerebral cortex constitutes the site of higher cognitive functions, including memory, attention, perceptual awareness, thought and language. The elaboration of these sophisticated functions relies on appropriate neuronal positioning and connectivity that are predominantly established during embryonic development. In particular, the formation of the mammalian cerebral cortex requires the sequential generation of different neuronal populations and extensive migration of neurons from their germinal zones to their final destination. The importance of neuronal migration during fetal stages for normal functioning of the mature brain is underscored by the fact that multiple neurological disorders, including mental retardation and epilepsy are caused by defects in this specific developmental step\(^1\). Understanding how neuronal migration is controlled during cerebral cortex development is therefore crucial to provide insights into the pathogenesis of many neurodevelopmental cognitive disorders.

During the development of the cerebral cortex, newborn pyramidal neurons undergo radial migration to reach their final position within the cortical plate (CP). Radial migration is a multi-phasic process starting with the detachment of the new neurons from the apical surface of the ventricular zone (VZ)\(^4\). Neurons then move to the intermediate zone (IZ) where they acquire a multipolar shape and actively extend and retract dynamic processes. After sojourning in the IZ, neurons enter the CP where they become bipolar, extending a leading process towards the pial surface and a trailing process in the opposite direction. During this phase, cells migrate towards the upper part of the CP using glia-guided locomotion, a mode of migration characterized by repetitive migratory cycles of extension of the leading process, translocation of the nucleus and retraction of the trailing process\(^5\). As neurons approach their destination, they change again their mode of migration from locomotion to terminal translocation\(^6\) and finally settle in a specific cortical layer.

The precise orchestration of radial migration of cortical neurons depends on both intracellular and extracellular cues\(^7\). We have shown previously that two small GTP-binding proteins, Rnd2 and Rnd3, have crucial roles in different phases of cortical neuron migration through inhibition of RhoA signalling in different subcellular locations. Rnd2 controls the transition from the multipolar to the bipolar stage and the extension of the leading process while Rnd3 regulates locomotion\(^9\). Rnd2 and Rnd3 proteins are regulated at the transcriptional level by the proneural factors Neurog2 and Ascl1, respectively. In addition to such intrinsic regulators of neuronal motility, a huge variety of secreted and membrane-bound extracellular molecules have been shown to orchestrate cortical neuron migration, by regulating the motility or the guidance of neurons\(^11\). Although the function of guidance molecules has been mostly characterized in tangentially migrating cortical interneurons, recently the Semaphorin family member Sema3A and the Netrin receptor Uncl5D have been reported to direct radial migration of pyramidal neurons\(^2,13\). Moreover, the Semaphorin receptor, Plexin B2, has been implicated in various aspects of cortical development, although its specific contribution to cortical neuron migration has been difficult to address because of earlier defects in Plexin B2 mutant cortices\(^14\).

In spite of these advances, little is known about the signalling machinery that mediates the response of radially migrating neurons to extrinsic cues. Although intrinsic and extrinsic mechanisms regulating neuronal migration have been extensively studied separately, it remains unknown how these two types of pathways are integrated to precisely control the migratory behaviour of cortical neurons. To address this issue, we have examined whether Rnd proteins interact with pathways initiated by extracellular signals. We chose to focus on the interaction between Rnds and Plexin B receptors because of the previous description of Rnd1 involvement in Plexin B1 signalling in cultured cells\(^5,16\). However, functional interaction between Plexin B molecules and Rnd members other than Rnd1 has not been addressed yet.

In the present study, we combine genetic, biochemical and molecular approaches to investigate whether the Ascl1-Rnd3 axis promotes cortical neuron migration via regulation of Plexin B signalling. We provide evidence for a Plexin B2-Rnd3 antagonistic interaction that promotes radial migration of cortical neurons through fine regulation of RhoA activity. We propose therefore that Rnd3 may constitute a hub where an intrinsic programme, driven by the proneural factor Ascl1, and an extrinsic signal, represented by the Plexin-Semaphorin pathway, meet to coordinate neuronal migration.

**Results**

**Plexin B2 is required for cortical neuron migration.** To determine whether Rnd proteins functionally interact with Plexin B receptors in migrating cortical neurons, we began by examining the expression and function of members of the Plexin B family in the murine embryonic cortex (Fig. 1a). RNA in situ hybridization of sections of cerebral cortex at different developmental stages with probes for members of the Plexin B family revealed that *Plexin B1* and *Plexin B2* had very similar expression patterns in the VZ, subventricular zone (SVZ) and CP, whereas *Plexin B3* was not expressed in this tissue. *Plexin B1* and B2 expression also resembled that of *Rnd3* (but not that of *Rnd2*, Supplementary Fig. 1a), suggesting a possible functional relationship between these genes.

To determine whether *Plexin B1* and *Plexin B2* regulate neuronal migration, such as *Rnd3*, we silenced each gene by *in utero* electroporation of short hairpin RNA (shRNA) vectors\(^10,17\). Constructs that efficiently reduced the expression of endogenous *Plexin B1* and *Plexin B2* in P19 cells (Supplementary Fig. 1b,c) were electroporated in the cortex of E14.5 mouse embryos. Analysis of electroporated cortices 3 days later (E17.5) revealed that silencing *Plexin B2* profoundly affected the migration of electroporated neurons, whereas silencing *Plexin B1* had no detectable effect (Fig. 1b). Quantification of the distribution of electroporated cells in the different zones of the cortex showed that more neurons remained in the VZ/SVZ and the IZ, and fewer reached the CP when *Plexin B2* was silenced than in control experiments (Fig. 1b). Moreover, many *Plexin B2*-silenced neurons that had reached the CP had abnormal morphologies, including a forked leading process and/or multiple processes sprouting from the cell body (42.1 ± 3.7%; \(n > 150\) cells from three different experiments; Fig. 1c), whereas the vast majority of CP neurons in control experiments were bipolar with a characteristic thick leading process and thin trailing process (81.0 ± 3.0%; \(n > 150\) cells from three different experiments; Fig. 1c). Interestingly, similar morphological anomalies were seen in *Rnd3*-silenced neurons that had reached the CP\(^10\). Co-electroporation of a knockdown-resistant version of *Plexin B2* (marked *Plexin B2*; Supplementary Figs 1d and 6) together with the *Plexin B2* shRNA fully rescued the migratory defect of *Plexin B2*-silenced cells, thus demonstrating the specificity of this phenotype (Fig. 1d).

Co-electroporation of the related receptor *Plexin B1* with the *Plexin B2* shRNA, resulted in a cell migration phenotype indistinguishable from that of *Plexin B2* shRNA alone (Fig. 1d and Supplementary Fig. 1e) showing that *Plexin B2* shRNA does not silence *Plexin B1*, demonstrating that *Plexin B1* cannot compensate for the reduction of *Plexin B2* function, and therefore the two molecules have divergent activities in migrating neurons.
Plexin B2 and Rnd3 functionally interact in migrating neurons. The involvement of both Rnd3 (ref. 10) and Plexin B2 (Fig. 1) in the migration of cortical neurons raised the possibility that the two molecules act together to promote neuronal migration. To examine this idea, we first asked whether the two proteins interact physically by performing co-immunoprecipitation experiments. By immunoprecipitating FLAG-tagged Rnd3 with a FLAG antibody, we were able to detect VSV-tagged Plexin B2 in the co-immunoprecipitated material, demonstrating that the two proteins are able to form a complex (Fig. 2a). The specificity of this interaction is demonstrated by the fact that the related receptor Plexin B1 does not interact with Rnd3 (Supplementary Fig. 1f). Moreover, endogenous Rnd3 and Plexin B2 co-localize at the plasma membrane in acutely dissociated embryonic cortical neurons, suggesting that the two molecules might also interact in cortical cells in vivo (Fig. 2b).
Next we asked whether the two genes cooperate in cortical neurons to promote the acquisition of proper morphology and cell migration. To this end, we silenced both genes simultaneously by co-electroporating the two shRNA constructs in the murine embryonic cortex. If the two genes function synergistically, combining the two shRNAs should exacerbate the migratory phenotypes produced by each shRNA separately. However, the double knockdown resulted instead in a significant improvement of the migration defects of single knockdown cells, with 13.7 ± 0.5% double knockdown cells reaching the upper part of the CP compared with 7.5 ± 1.2% and 9.0 ± 1.2% single Plexin B2- and Rnd3-silenced cells, respectively, and 18.1 ± 1.5% cells in the control experiment (Fig. 3a–c), indicating that Rnd3 and Plexin B2 antagonize each other during cell migration. The antagonistic interaction with Plexin B2 was specific to Rnd3, as co-electroporation of shRNAs for Plexin B2 and Rnd2 did not produce any improvement of the individual migration phenotypes (Supplementary Fig. 2), even if Rnd2 is capable of antagonizing with PlexinB2 in a different context (Supplementary Fig. 1D).

Interestingly, simultaneous knockdown of Plexin B2 and Rnd3 rescued the migration defects of single knockdown cells only in the CP, as double knockdown cells accumulated in the VZ/SVZ and IZ to the same extent as single knockdown cells (Fig. 3a,b).

This suggests that Plexin B2 and Rnd3 display antagonistic activities specifically in neurons undergoing locomotion in the CP, while they function independently of one another before the onset of neuronal migration in the VZ/SVZ and/or during early phases of migration in the IZ. In support of the idea that Plexin B2 and Rnd3 antagonize each other in the CP, the aberrant morphological features observed in the CP in Plexin B2-silenced neurons and in Rnd3-silenced neurons were fully corrected by silencing both genes (Fig. 3d,e). Thus, functional antagonism between Plexin B2 and Rnd3 has an important role in regulating the activity of the two molecules in neurons that have reached the CP (Fig. 3f).

In contrast, the accumulation of Rnd3-silenced cells in the VZ/SVZ and IZ could be attributed mostly to the recently reported role of Rnd3 of suppressing the proliferation of basal cortical progenitors through inhibition of the translation of the cell cycle regulator Ccnd1 (ref. 19). As already shown, silencing Rnd3 resulted in excessive proliferation of basal progenitors, leading to an accumulation of proliferating cells in the VZ/SVZ and IZ and a delay in cell cycle exit and subsequent migration of post-mitotic cells to the CP (Supplementary Fig. 3e,g). Silencing Plexin B2 alone had no significant effect on progenitor proliferation (Supplementary Fig. 3a,b,d,g), thus explaining the lack of rescue of the accumulation of Rnd3-silenced cells in the...
VZ/SVZ and IZ by co-electroporation of *Plexin B2* shRNA (Supplementary Fig. 3).

**Plexin B2 activates RhoA in part by inhibiting Rnd3.** The foregoing results showed that *Plexin B2* and *Rnd3* are both required for the migration of CP neurons but that their activities are mutually antagonistic. To determine the possible cause of this apparent paradox, we examined the mechanism underlying the interaction between these two molecules. Similar to *Rnd3* (ref. 10), Plexin B receptors have been shown to act in cultured cells through regulation of RhoA signalling, with different studies finding that these receptors either increase\(^{16,20-23}\) or reduce\(^{24}\).
RhoA activity. We therefore asked whether Plexin B2 acts in migrating cortical neurons by regulating activation of RhoA, either alone or in concert with Rnd3. Active RhoA levels at the cell membrane were measured in intact cells in vivo by co-electroporating shRNA and a RhoA fluorescence resonance energy transfer (FRET) biosensor in the cortex of E14.5 embryos. FRET analysis was subsequently performed in fixed brain slices 1 day after electroporation (Fig. 4a,b; refs 10,26). RhoA activity was detected in cells in the upper IZ and lower CP, and this activity was enhanced by Rnd3 silencing, as previously reported (Fig. 4c,d; ref. 10). In contrast, RhoA activity was significantly decreased when Plexin B2 was silenced, indicating that Plexin B2 positively regulates RhoA activity in migrating cortical neurons (Fig. 4c,d).

As Plexin B2 and Rnd3 antagonize each other’s functions in migrating neurons, we reasoned that these molecules might mutually suppress each other’s activity of regulation of RhoA (Fig. 4e). To test this possibility, we co-electroporated the RhoA FRET probe with both Plexin B2 and Rnd3 shRNAs. When both genes were silenced, RhoA activity returned to a level not significantly different from that found in control experiments, suggesting that the improvement in migration of double knockdown neurons can be partially attributed to the correction of RhoA activity (Fig. 4d). Moreover, the restored level of active

![Figure 4](image-url)
RhoA seen in PlexinB2-silenced neurons when Rnd3 was also depleted suggests that Plexin B2 activates RhoA by inhibiting Rnd3. The predominant mechanism by which Rnd3 suppresses RhoA activity is by interacting with the RhoA GTPase-activating protein p190RhoGAP\textsuperscript{27}. Indeed, the inactivation of RhoA by Rnd3 overexpression is reverted in the absence of p190RhoGAP (Fig. 5a,b and Supplementary Fig. 4a). As expected, p190RhoGAP silencing in cultured neurons results in a strong increase of RhoA activity (Fig. 5a,b), and its silencing in vivo induces migration defects that can be attribute to RhoA hyperactivity (Fig. 5c).

**Figure 5 | p190RhoGAP mediates Rnd3 inhibitory function towards RhoA and competes with Plexin B2 for Rnd3 binding.** (a) *In vitro* FRET analysis of RhoA activity in dissociated cortical cells in culture, 2 days after the electroporation of the constructs indicated. Upper panels show the CFP signal from the FRET probe; the RFP signal (in insets) marks electroporated cells (scale bar in insets, 10 μm). Lower panels show FRET efficiency. Scale bar, 10 μm. (b) Mean ± s.e.m.; (n = 11 cells for each condition, from three independent experiments; t-test: *P < 0.05 and **P < 0.01 compared with control). (c) Mouse embryonic cortices electroporated in utero with control shRNA or p190RhoGAP shRNA at E14.5 and analysed 3 days later. Scale bar, 200 μm. The distribution of GFP\textsuperscript{+} cells in the different cortical compartments revealed defects in the migration of p190RhoGAP-depleted neurons. Mean ± s.e.m. from six sections prepared from three different experiments; t-test; **P < 0.01 and ****P < 0.0001. (d) Plexin B2 interacts with the same site as p190RhoGAP on Rnd3 protein. COS7 cells were co-transfected with HA-PlexinB2 (cytoplasmic domain) and wild-type and mutated (T55V) FLAG-Rnd3 constructs. The lysates were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted with anti-HA or anti-FLAG antibodies. The mutation of a single residue, Threonine 55 in the effector binding domain, of Rnd3 disrupted the interaction with Plexin B2, similar to p190RhoGAP\textsuperscript{4}. (e) Competition between Plexin B2 and p190RhoGAP for Rnd3 binding. Expression vectors encoding FLAG-Rnd3 and Myc-p190RhoGAP-B (middle domain) were co-transfected with increasing amounts of VSV-Plexin B2 into COS7 cells. Cell lysates were IP with anti-FLAG antibody, then immunoblotted with the indicated antibodies. For full blots see Supplementary Fig. 6.
We have previously shown that Rnd3 interacts with p190RhoGAP via its Thr55 residue\(^{10,27}\). Interestingly, mutation of Thr55 to a Val residue disrupts interactions not only with p190RhoGAP but also with Plexin B2 (Fig. 5d), suggesting that Plexin B2 and p190RhoGAP might bind Rnd3 in a mutually exclusive manner. To test this model, we performed competitive co-immunoprecipitation experiments with Plexin B2, p190RhoGAP and Rnd3 co-expressed at different levels. We found that increasing Plexin B2 concentrations resulted in a decrease in p190RhoGAP binding to Rnd3 (Fig. 5e), suggesting that Plexin B2 competes with p190RhoGAP for Rnd3 binding. The disruption of the Rnd3-p190RhoGAP interaction could account at least in part for the activation of RhoA by Plexin B2.

An interaction between PlexinB2 and Rnd3 has been shown to regulate R-Ras signalling during axon guidance\(^{15}\). To determine whether the PlexinB2-Rnd3 interaction in cortical neurons also controls R-Ras activity, we performed FRET measurements with an intra-molecular FRET probe for R-Ras (pRaichu 205x) (ref. 28). Silencing PlexinB2 resulted in an increase in R-Ras activity but no change was observed when Rnd3 was silenced (Supplementary Fig. 4b). This result indicates that the inhibition of R-Ras activation by the intrinsic R-Ras GAP activity of Plexin B2 is independent of Rnd3, further supporting the conclusion that PlexinB2 and Rnd3 antagonistic functions in migrating cortical neurons predominantly fine-tune the activity level of RhoA.

**Plexin B2 activates RhoA in part by recruiting RhoGEF.** The correction by Rnd3 knockdown of both the migration defect and the low level of RhoA activity of Plexin B2-silenced neurons suggested that this migratory phenotype is due to reduced RhoA activity. However, there is currently little in vivo evidence, demonstrating that cortical neurons require a minimum level of RhoA activity to migrate. To determine whether the cell

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**Figure 6 | Plexin B2 activates RhoA in the cortex in part by recruiting RhoGEFs.** (a) The migration defect induced by Plexin B2 shRNA electroporation was rescued by co-electroporation of a RhoA expression vector. The graph shows the distribution of electroporated GFP-positive cells per cortical compartment in the different conditions. Mean ± s.e.m. from six sections prepared from three different experiments; one-way ANOVA followed by the Bonferroni post hoc test; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. Scale bar, 200 μm. (b) Schematic representation of the different domains of the Plexin B2 protein. The PDZ-binding domain (PDZ-BD) at the C terminus of wild-type Plexin B2 has been removed in the Plexin B2 C-terminal deletion mutant (PlexinB2ΔC). Sema: Sema domain; PSI: plexin, semaphorin and integrin domain; IPT: Ig-like, plexin and transcription factor domain; CC: convertase cleavage site; TM: transmembrane domain; GAP C1/C2: segmented GTPase activating protein (GAP) domain; G-BD: GTPase binding domain; PDZ-BD: PDZ-binding domain. (c) Images of electroporated cortices and quantification graph show that PlexinB2ΔC* ameliorates the defects induced by Plexin B2 knockdown, although not as efficiently as wild type PlexinB2*. The star (*) indicates that the constructs carry mutations conferring RNAi resistance. Mean ± s.e.m. from six sections prepared from three different experiments; one-way ANOVA followed by the Bonferroni post hoc test; *P<0.05, **P<0.01 and ***P<0.0001. Scale bar, 200 μm. (d) Model of how the Ascl1-Rnd3 and Semaphorin-PlexinB2 interact to regulate RhoA activity in cortical neuronal migration. On the one hand, Ascl1-Rnd3 maintains low background levels of RhoA activity by interacting with p190RhoGAP. On the other hand, upon extracellular activation, Plexin B2 promotes RhoA activation by two mechanisms, blocking Rnd3 interaction with p190RhoGAP and directly recruiting RhoGEFs.
migration of cortical neurons by recruiting RhoGEFs (Fig. 6d).

Besides interacting with Rnd3, Plexin B2 has previously been shown to promote axon guidance and growth cone collapse by activating RhoA through direct recruitment of PDZ domain-containing Rho GTP exchange factors (RhoGEFs)16,23. We thus asked whether the role of Plexin B2 in cortical neuron migration also involves interactions with RhoGEFs. We tested the capacity of a C-terminal deletion mutant of Plexin B2 (PlexinB2 ΔC), which has lost its capacity to interact with RhoGEFs52,53, to rescue the migratory phenotype of Plexin B2-silenced neurons (Fig. 6b,c). Although co-electroporation of the Plexin B2 shRNA and the knockdown-resistant form of Plexin B2 fully reverted the silencing phenotype, co-electroporation of a knockdown-resistant form of the C-terminal deletion mutant ameliorated the phenotype only partially (42.7 ± 3.5% neurons reached the CP when Plexin B2 shRNA alone was electroporated, 61.2 ± 1.0% with Plexin B2 shRNA + Plexin B2* and 51.9 ± 2.1% with Plexin B2 shRNA + Plexin B2 ΔC; n = 6 sections from three different experiments) (Fig. 6c). When we tested the ability of Plexin B2 ΔC to activate RhoA in vivo or in cultured neurons exposed to the Plexin B2 extracellular ligand Semaphorin 4D, we found that Plexin B2 ΔC no longer promoted RhoA activation, whereas full-length Plexin B2 did (Supplementary Fig. 5a–d). Semaphorin-mediated Plexin B2 recruitment of RhoGEFs is therefore essential for local activation of RhoA and correct neuronal migration. Altogether, our data suggest that, as well as suppressing Rnd3 activity, PlexinB2 also stimulates RhoA activation and promotes the migration of cortical neurons by recruiting RhoGEFs (Fig. 6d).

Discussion

In this study, we have investigated how different pro-migratory signals are integrated at a molecular level to coordinate the multistep process of radial migration of cortical projection neurons. Recent studies have highlighted the importance of transcriptional mechanisms in the regulation of neuronal migration9,30. In particular, transcriptional control of Rnd proteins is crucial to regulate cytoskeleton dynamics in radially migrating cortical neurons9,10,19,31. However, how this cell-intrinsic control of the cytoskeleton interfaces with extracellular signal-regulated pathways that orient the migration of neurons has remained unclear. Here we show that the Ascl1 target Rnd3 and the axon guidance receptor Plexin B2 antagonize each other’s activity to regulate RhoA activity and promote cortical neuron migration.

Rnd1 has previously been implicated in the signal transduction pathway activated downstream of the Plexin B1 receptor during growth cone collapse and inhibition of axon extension15. Our results extend this finding by showing that the interaction between Plexin B2 and Rnd3 has a major role in the control of cortical neuron migration in vivo. However, we have identified a major difference from previous models. Although the binding of Rnd1 to Plexin B1 intracellular domain has been shown to open the conformation of the receptor and to allow the transmission of the downstream signal in a synergistic manner32,33, our genetic and molecular data indicate instead that Plexin B2 and Rnd3 antagonize each other’s activity. This is reminiscent of other situations where pairs of genes oppose each other when regulating cell migration and the cytoskeleton. For example, the genes Dcx and Mark2 exhibit antagonistic activities in the control of microtubule stability in migrating neurons, so that cortical neurons fail to migrate properly when either gene is inactivated, whereas simultaneous inactivation of both genes corrects the migration defects19. Rnd3 controls actin polymerization in migrating neurons in part via regulation of coflin10. The opposite regulation of RhoA activity by PlexinB2 and Rnd3 is therefore likely to influence the organization of the actin cytoskeleton, which undergoes rapid remodelling during neuronal migration34,35. Our results thus emphasize the importance of the balanced activity of pathways that regulate cytoskeleton dynamics in migrating cells. In agreement with our model of a pro-migratory function of Plexin B2 in cortical neurons, abnormal migration phenotypes have also been observed in the cerebellum and the rostral migratory stream of Plexin B2 knockout animals36–38.

By using FRET imaging, we show that Plexin B2 stimulates RhoA activity in migrating cortical neurons, and that this regulation is essential for the migration of these cells, as the migratory defect of Plexin B2-silenced neurons is rescued when RhoA is overexpressed. Similar results have been reported for endothelial and breast cancer cells, where Plexin B1 induces cell migration via a RhoA-ROCK pathway39, suggesting that RhoA activation may represent a general mechanism by which Plexin B receptors promote the migration of various cell types. The recruitment of RhoA GEFs is the predominant mechanism by which Plexins activate RhoA in navigating growth cones16. A similar mechanism also operates in migrating cortical neurons, as preventing RhoGEF recruitment partially abolishes the pro-migratory activity of Plexin B2 and prevents RhoA activation upon Semaphorin stimulation. However, we show that RhoGEF recruitment is not the sole mechanism by which Plexin B2 activates RhoA. Plexin B2 also disrupts the interaction between Rnd3 and p190RhoGAP by competing with p190RhoGAP for Rnd3 binding, resulting in the activation of RhoA. In fact, Plexin B2 must both inactivate Rnd3 and recruit RhoGEFs to promote RhoA activity, as preventing Plexin B2 recruitment of RhoGEFs without interfering with its interaction with Rnd3 is not sufficient to stimulate RhoA activity.

Several studies have demonstrated that excessive RhoA activity (achieved through loss of the proneural gene Neurog2 or loss of Rnd genes) arrests cortical neurons at different stages in their migration route, and that cell migration can be restored by reducing RhoA expression or blocking its activity40–42. Conversely, we have shown in this study that a reduced activity of RhoA also interferes with neuronal migration, as Plexin B2-silenced neurons that have reduced RhoA activity are stalled and overexpressing RhoA rescues their migration. Surprisingly, however, a recent study has shown that RhoA-deficient neurons migrate correctly in a wild-type environment43, suggesting that RhoA activity is not required cell autonomously for radial migration in the developing cerebral cortex. However, it is likely that other molecules can substitute for RhoA when the RhoA gene is deleted in neurons. Both RhoB and RhoC are expressed in the developing CP44, and RhoB has been shown to be strongly upregulated in the absence of RhoA45. The fact that both inhibition and activation of RhoA are necessary for radial migration in the cortex (this work and refs 10,46) is a striking illustration of the need for Rho GTPase activity to be tightly regulated. The multipronged regulation of RhoA may allow it to be simultaneously active in some parts of the cell and suppressed in other parts. The two RhoA inhibitors Rnd2 and Rnd3 are distributed in distinct subcellular compartments (endosomes and plasma membrane, respectively19), and are therefore likely to contribute to the
differential regulation of RhoA within the cell. Similarly, Plexin B2 may stimulate RhoA activity only locally, such as in the proximal region of the leading process and at the rear of the cell, where active RhoA has been shown to be localized and essential for actin-based cellular contractility and nucleokinesis35. Localized activity of Plexin B2 may result from a restricted subcellular distribution of the receptor itself or from an extracellular gradient of a Semaphorin ligand. Several class 4 Semaphorins are expressed in the embryonic CP (Supplementary Fig S e) and may activate the Plexin B2-RhoA pathway in the leading process of cortical neurons to promote their directed migration.

In conclusion, we propose that the main role of the Ascl1-Rnd3 pathway is to maintain RhoA activity at a low level to provide the actin cytoskeleton with the plasticity required for efficient migration. Conversely, local Semaphorin-Plexin B2 signalling in the leading process or at the cell rear may activate RhoA and stimulate contractility by releasing Rnd3-mediated RhoA inhibition, and also by promoting Rho GEF-mediated RhoA activation. This work identifies a mechanism that fine-tunes RhoA activity in migrating neurons and will help better understand the pathogenesis of human neurodevelopmental disorders.

Methods

Plasmid constructs. PlexinB1 shRNA (ID-3: 5'-GGAGTCTATATTTCTGCTTA TCT-3') and PlexinB2 shRNA (ID-3: 5'-GGAGTCTATATTTCTGCTTA TCT-3') plasmids were purchased from SABiosciences. The control shRNA plasmid for PlexinB2 and PlexinB1 was provided by SABiosciences and contains the non-targeting sequence 5'-GGAATCTTCTGATCATGATC-3'. For FRET experiments, the shRNA for PlexinB2 and the relative control shRNA were co-transfected into Neuro2A cells with the expression vectors containing with N-ecdysone (Invitrogen) at the concentration of 30 ng per gram of animal. Mixed sex embryos (E14.5) were purified using Amersham column. Non-radioactive nucleotides (Roche) under standard reaction conditions and the probes were subjected to gel purification. RNA probes for Rnd2 and Rnd3 were previously described47. RNA probes for Sema4D, Sema4A, Sema2B, Sema4C, Sema4D, Sema4F and Sema4G were previously described47.

In vitro transcription was performed in the presence of digoxigenin (DIG)-marked nucleotides (Roche) under standard reaction conditions and the probes were purified using a MetaPhor nucleic acid purification kit (Invitrogen). Statistical analysis was performed with an Excel-based statistical software. An unpaired two-tailed Student's t-test was performed on 14-mum cryostat sections mounted on super frost slides (Fisher). The sections were hybridized overnight at 70 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetramethyl tetrazolium 5-bromo 4-chloro 3-indolyl phosphate (NBT/BCIP, Roche). The substrate of alkaline phosphatase (AP), to the sections was incubated overnight at 4 °C with DIG-labelled RNA probes complementary to target mRNAs. To detect the riboprobes bound to the tissue, sections were incubated overnight at 4 °C with an anti-DIG antibody conjugated with a tetra...
acceptor channels in pixels above threshold of an acceptor only labelled sample, before and after photobleaching.

Cell culture. Mouse embryonic teratocarcina P19 and monkey kidney COS7 cells were cultured in high-glucose Dulbecco’s modified eagle medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin, and they were incubated at 37 °C under 5% CO2 atmosphere. P19 cells were plated in 24-well plates and transfected with Lipofectamine 2000 reagent according to the manufacturer’s protocol (Invitrogen). Twenty-four hours post transfection (for PlexinB1 and PlexinB2 analysis) or 48 h post transfection (for p190RhoGAP analysis), cells were collected for protein or RNA extraction. COS7 cells were electrophorized with expression constructs and analysed 24 h after transfection.

Semaphorin production. Recombinant Semaphorin 4D was produced as a secreted protein fused with AP. To produce recombinant Semaphorin, human embryonic kidney HEK293T cells were transfected with Sem4D-AP in pcDNA vector36, using FuGene 6 reagent according to the manufacturer’s protocol (Promega). The supernatant of transfected cells was collected, filtered and concentrated 10-fold by Spin-X UF concentrators with a 100kDa cutoff (Millipore). Protein concentration was normalized to a range of 3–6 nM by measurement of the AP-activity using Alkaline Phosphatase Colorimetric Assay Kit (Abcam).

Real time PCR. PlexinB1, PlexinB2 and p190RhoGAP mRNA expression was quantified by quantitative real-time PCR in control and shRNA-treated samples. RNAs were extracted from P19 cells using TRIzol reagent (Invitrogen), followed by a classical phenol/chloroform separation. Purified RNAs were then reverse-transcribed into cDNA with the Applied Biosystems kit. Real time PCR was performed with Taqman probes according to the manufacturer’s protocol (TaqMan Gene Expression Assays, catalogue number 4331182, Applied Biosystems). The 7500 system (Applied Biosystems) was used to run the PCR and analyse the data. β-actin was used as an endogenous reference gene control and the values were normalized to control levels. Relative quantification was determined according to the ΔΔCt method. Data are presented as means ± s.e.m. from three independent experiments. Statistical analysis was performed with the Prism software using an unpaired two-tailed Student’s t-test between control and knockdown condition; **P < 0.01 and ***P < 0.001.

In vitro co-immunoprecipitation and western blotting. Biochemical interactions between Rnds and Semaphorin was tested in P19 cells and COS7 cells. P19 cells were co-transfected with pCMV5-FLAG-Rnd3, pCMV5-FLAG-Rnd2, pcDNA-PSV-PlexinB1 and pcDNA-PSV-PlexinB2. Twenty-four hours after transfection, cells were lysed with RIPA-like lysis buffer (50 mM Tris–HCl, pH 8; 150 mM NaCl; 0.5% NP40 Igepal; 10% glycerol; protease and phosphatase inhibitor cocktails (Roche; Calbiochem)) and the insoluble material was removed by centrifugation at 13,000 g for 4 °C for 10 min. Anti-FLAG (cross-linked) agarose beads (M2 from Sigma) were first blocked in lysis buffer containing 0.2 mg ml−1 chicken egg albumin and 0.1 mg ml−1 insulin for 1 h at room temperature. Then the soluble cell lysate was incubated overnight at 4 °C with the beads. The day after, the beads were washed in lysis buffer and the proteins were eluted in Laemmli sample buffer, (Roche). Co-immunoprecipitation and western blotting

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Author contributions

R.A., E.P. and F.G. designed and performed this study, analysed the data and wrote the manuscript; M.P. contributed to the p190RhoGAP experiments; D.v.d.B. and P.G. contributed to the p190RhoGAP experiments; D.v.d.B. and P.G. contributed to the p190RhoGAP experiments; R.H.F. helped with the Semaphorin experiments. All authors discussed the results and commented on the manuscript.

Additional information

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