Regulation of neural progenitor cell state by ephrin-B

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Maintaining a balance between self-renewal and differentiation in neural progenitor cells during development is important to ensure that correct numbers of neural cells are generated. We report that the ephrin-B–PDZ-RGS3 signaling pathway functions to regulate this balance in the developing mammalian cerebral cortex. During cortical neurogenesis, expression of ephrin-B1 and PDZ-RGS3 is specifically seen in progenitor cells and is turned off at the onset of neuronal differentiation. Persistent expression of ephrin-B1 and PDZ-RGS3 prevents differentiation of neural progenitor cells. Blocking RGS-mediated ephrin-B1 signaling in progenitor cells through RNA interference or expression of dominant-negative mutants results in differentiation. Genetic knockout of ephrin-B1 causes early cell cycle exit and leads to a concomitant loss of neural progenitor cells. Our results indicate that ephrin-B function is critical for the maintenance of the neural progenitor cell state and that this role of ephrin-B is mediated by PDZ-RGS3, likely via interacting with the noncanonical G protein signaling pathway, which is essential in neural progenitor asymmetrical cell division.

Introduction

In the developing mammalian cerebral cortex, the majority of projection neurons originate from neural progenitor cells located in the ventricular zone (VZ), which lines the lateral ventricles. Initially, neural progenitor cells proliferate to expand their population. As development proceeds, they progressively enter asymmetrical cell divisions and give rise to neurons and glia. Newborn cortical neurons embark on migration away from the VZ into the cortical plate (CP), where they form highly organized connections within the cortex or with subcortical targets. During development, the balance between self-renewal and differentiation of neural progenitor cells is tightly regulated to ensure that correct numbers of neural cells are generated to form a functional cortex. The molecular basis of this critical developmental regulation is, however, not well understood (McConnell, 1995; Monuki and Walsh, 2001; Temple, 2001; Gupta et al., 2002; Fishell and Kriegstein, 2003; Gotz and Huttner, 2005; Rakic, 2006; Molyneaux et al., 2007).

The ephrin/Eph family of molecules is known to function in tissue segmentation, axon guidance, neuronal migration, and dendritic and synaptic modulation during neural development (Flanagan and Vanderhaeghen, 1998; Drescher, 2002; Poliakov et al., 2004; Davy and Soriano, 2005; Egea and Klein, 2007; Arvanitis and Davy, 2008; Pasquale, 2008). Recently, ephrin/Eph molecules have also been suggested to regulate proliferation, differentiation, and survival of neural progenitor/stem cells (Conover et al., 2000; Aoki et al., 2004; Depaepe et al., 2005; Holmberg et al., 2005; Katakowski et al., 2005; Ricard et al., 2006). The B subfamily of ephrins and Ephs is prominently associated with neural progenitor/stem cells both in embryonic brains (Lu et al., 2001; Stuckmann et al., 2001) and in the adult subventricular zone (SVZ; Conover et al., 2000; Ricard et al., 2006). Ephrin-Bs are type I membrane proteins containing one membrane-spanning region and a short cytoplasmic domain. After their original identification as ligands for Eph receptors, ephrin-Bs were found to be capable of "reverse signaling" into

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Abbreviations used in this paper: BrdU, bromodeoxyuridine; CDS, coding sequence; CP, cortical plate; DCX, doublecortin; E, embryonic day; IZ, intermediate zone; p-H3$^+$, phospho–Histone H3$^+$; RGS, Regulator of G protein Signaling; shRNA, short hairpin RNA; SVZ, subventricular zone; VZ, ventricular zone.

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the bearing cells (Holland et al., 1996; Bruckner et al., 1997). This led to a model for bidirectional signaling in contact-mediated cell–cell communication via Eph–ephrin interaction: ephrin-B can both activate "forward signaling" via the Eph receptor and initiate reverse signaling through its own cytoplasmic domain. In one reverse signaling pathway (Lu et al., 2001), signaling of ephrin-B is mediated through PDZ-RGS3, a regulator of G protein signaling (RGS) protein known to function as an inhibitor of G protein signaling (De Vries et al., 2000; Ross and Wilkie, 2000; Siderovski and Willard, 2005).

In the adult SVZ, ephrin-B1 and ephrin-B2 are expressed by astrocytes, which function as neural stem cells (Conover et al., 2000; Ricard et al., 2006). Infusion of an ectodomain of EphB (EphB-Fc) or an ectodomain of ephrin-B (ephrin-B-Fc) into the SVZ leads to proliferation of neural stem cells (Conover et al., 2000). These results indicate that the activation or disruption of ephrin-B/EphB signaling can positively regulate stem cell proliferation in the SVZ. Ablation of ephrin-B3, the third member of the ephrin-B subfamily, results in increased neural stem cell proliferation and cell death in the SVZ (Ricard et al., 2006). This observed effect of ephrin-B3 is probably through an indirect process, as ephrin-B3 was reported to be expressed outside the SVZ where neural stem cells reside (Ricard et al., 2006). Hence, the function of ephrin-B in the neurogenic areas of the embryonic or adult brains remains largely unclear.

In the developing cerebral cortex, ephrin-B1 appears to be selectively expressed in the VZ, associating with neuroepithelial progenitor cells but not young neurons (Lu et al., 2001; Stuckmann et al., 2001). Interestingly, PDZ-RGS3, a downstream signaling mediator of ephrin-B reverse signaling, is coexpressed with ephrin-B1 in the cortical VZ (Lu et al., 2001). This suggests that ephrin-B signaling may be important for regulating the balance between self-renewal and differentiation during development. In this study, we present evidence in support of RGS-mediated ephrin-B reverse signaling in maintaining the cortical neural progenitor cells.

Results

Ephrin-B, PDZ-RGS3, and EphB are expressed in cortical neural progenitor cells

To investigate a potential role for ephrin-B signaling in the developing cerebral cortex, we began by examining expression patterns in more detail. RNAs of ephrin-B1 and PDZ-RGS3 were found selectively in the VZ during cortical neurogenesis (Lu et al. 2001). Ephrin-B2 RNA was expressed at a lower level in the VZ and more abundantly in the SVZ region (unpublished data). Ephrin-B3 RNA was not detected in the VZ and SVZ areas (unpublished data). Cortical neural progenitor cells are marked by expression of nestin (Lendahl et al., 1990) and the radial glia marker RC2 (Fishell and Kriegstein, 2003; Gotz and Huttner, 2005). We found coexpression in the VZ of ephrin-B proteins with nestin and RC2 (Fig. 1 A) or PDZ-RGS3 with nestin (Fig. 1 B). Furthermore, pulse labeling of bromodeoxyuridine (BrdU) showed that ephrin-B staining colocalized with BrdU-positive cells in the VZ (Fig. 1 C). RNA in situ of several EphB receptors showed that although EphB1 was not detected, EphB2, 3, and 4 were expressed in the VZ to various degrees (Fig. 1 D): EphB2 was uniformly expressed in the radial cortical dimension, whereas EphB3 and 4 were preferentially expressed in the VZ. Using EphB2-Fc and ephrin-B1-Fc (a fusion protein between the extracellular domain of EphB2 or ephrin-B1 and human Fc), we further assessed the functional expression of ephrin-B and EphB in neural progenitor cells in acutely dissociated cultures (Hartfuss et al., 2001). Fc control did not give detectable staining on the dissociated neural progenitor cells (unpublished data). EphB2 and Ephrin-B1 binding sites were both found on the surface of nestin-positive progenitor cells (Fig. 1 E). EphB2-Fc was also seen to cause patches of staining, indicative of the clustering of ephrin-B (Bruckner et al., 1999; Himanen et al., 2001), on the surface of dissociated neural progenitor cells. Collectively, these expression analyses indicate that ephrin-B1, ephrin-B2, PDZ-RGS3, and some EphB receptors are expressed in the cortical VZ by neural progenitor cells.

Continued coexpression of ephrin-B1 and PDZ-RGS3 prevents differentiation of neural progenitor cells

To explore the function of ephrin-B signaling in neural progenitor cells, we used in utero electroporation-mediated DNA transfection (Fukuchi-Shimogori and Grove, 2001; Ohtsuka et al., 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001) to achieve gain of function and loss of function of ephrin-B in the cerebral cortex. This electroporation technique has proven to be useful for in vivo functional analyses of molecular organization of the developing cerebral cortex (Fukuchi-Shimogori and Grove, 2001; Bai et al., 2003; Elias et al., 2007; Mizutani et al., 2007). We first tested gain of function of ephrin-B and PDZ-RGS3 by using bicistronic expression under the control of a ubiquitous promoter (elongation factor 2 promoter). To perturb normal mouse cortical neurogenesis that spans the period from embryonic day (E) 12 to 18, we introduced expression plasmids of Ephrin-B1 and PDZ-RGS3 or the control EGFP into the cortices at E13.5 and analyzed the electroporated brains at later stages between E15.5 and 17.5 (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200708091/DC1). Control cells expressing EGFP alone showed a progressive accumulation in the CP from E15.5 to 17.5, which is consistent with normal outward migration of cortical neurons born in the VZ. In contrast, cells expressing EGFP-PDZ-RGS3-IRES-HA-ephrin-B1 were mostly found in the region surrounding the SVZ. For example, at E17.5, 63% of green cells were found in the area encompassing the VZ, SVZ, and IZ (collectively called hereafter the non-CP area) versus 27% in the control. Immunohistochemistry on cells coexpressing ephrin-B1 and PDZ-RGS3 showed that they were positive for nestin but negative for β-III tubulin (Fig. S1 B) and some of the cells could incorporate BrdU with a short pulse of labeling (Fig. S1 C). These results indicate that prolonged expression of ephrin-B1 and PDZ-RGS3 could block normal differentiation of the affected progenitor cells.

The EF2 promoter mentioned in the previous paragraph could cause an overexpression of ephrin-B1 and PDZ-RGS3 in progenitor cells, which would have both endogenously and exogenously expressed proteins. In this situation, it would be hard

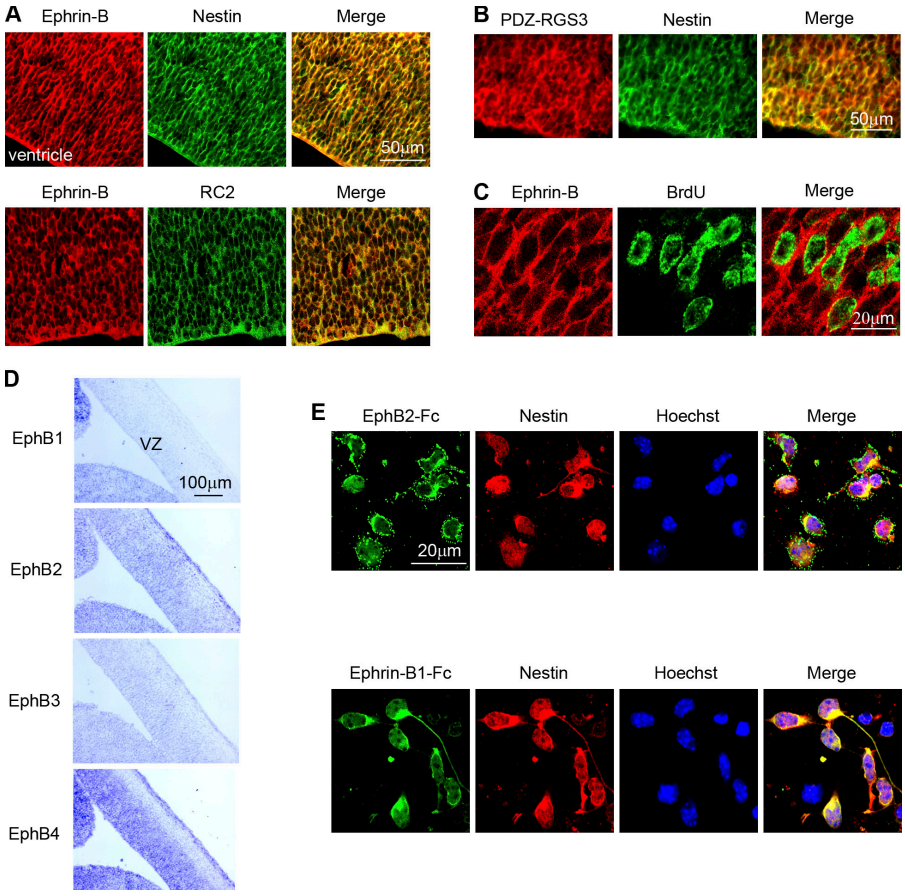

Figure 1. **Ephrin-B, PDZ-RGS3, and EphBs are expressed in neural progenitor cells in the developing cerebral cortex.** (A) Costaining of ephrin-B with neural progenitor cell marker nestin and RC2. (B) Costaining of PDZ-RGS3 with nestin. (C) Costaining of ephrin-B with pulse-labeled BrdU. (D) RNA in situ of EphBs. (E) Costaining using clustered EphB2-Fc or ephrin-B1-Fc with nestin in acutely dissociated neural progenitor cells.

to know whether a phenotype was a result of a direct effect of gain of function or simply because of an abnormal expression at the progenitor cell stage. To avoid this situation, we further tested coupling-continued expression of ephrin-B1 and PDZ-RGS3 to the onset of neuronal differentiation to prevent their normal down-regulation during cortical neurogenesis. To this end, ephrin-B1 and PDZ-RGS3, either alone or in combination, were expressed under the control of the promoter of the double-cortin (DCX) gene, an early marker for migrating cortical neurons (Francis et al., 1999; Gleeson et al., 1999). The DCX promoter used here was found to direct reporter gene expression, which is consistent with the endogenous DCX gene both in electroporation and in transgenic mice (Wang et al., 2007). Control cells expressing EGFP alone showed a progressive outward migration pattern, as was seen in cells carrying EGFP driven by the EF2 promoter (Fig. 2 A and Fig. S1 A). When ephrin-B1 or PDZ-RGS3 was electroporated alone, the transfected cells exhibited a largely similar migratory pattern to that of the control cells. In contrast, cells carrying EGFP-PDZ-RGS3-IRES-HA-ephrin-B1 showed a significant delay in migration or differentiation: at E15.5, while the control cells were in the intermediate zone (IZ) underneath the CP, cells with continued expression of ephrin-B1 and PDZ-RGS3 appeared to be mostly in the region surrounding the SVZ, with some cells even remaining deep in the VZ. In general, most green cells co-expressing ephrin-B1 and PDZ-RGS3 stayed in the SVZ area at all three stages analyzed, with significantly fewer cells entering the CP at E16.5 and 17.5. Between the two promoters used in these experiments, there were a smaller percentage of cells expressing DCX-EGFP in the non-CP area (53%; Fig. 2 A) than that of cells expressing EF-EGFP (92%; Fig. S1 A) at E15.5. This difference was likely because of the different specificity of the two promoters. DCX-EGFP would be specifically enriched in migrating neurons, which might represent the pool of progenitor cells that were differentiating or poised to differentiate at the time of electroporation, whereas EF-EGFP would be expected to be expressed in all transfected progenitor cells (progenitors that were differentiating or were at different stages of cell cycle) and perhaps in other cell types in the VZ area. Nonetheless, use of the two different promoters to coexpress ephrin-B1 and PDZ-RGS3 led to similar outcomes.

To address whether the cells that were stuck in the SVZ were not healthy or had not changed their location because of some generalized inability to migrate, we used a Transwell analysis (Lu et al., 2001) to assess their motility. Because neural progenitor cells are basic FGF responsive (Gage, 2000), we tested for basic FGF–induced migration. We found that cells expressing both ephrin-B1 and PDZ-RGS3 showed a similar migration profile toward basic FGF to the control cells expressing EGFP only (Fig. 2 B), indicating that continued expression of ephrin-B1 and PDZ-RGS3 did not render them unable to migrate.

We further assessed the expression of cellular markers in cells carrying both ephrin-B1 and PDZ-RGS3 driven by the

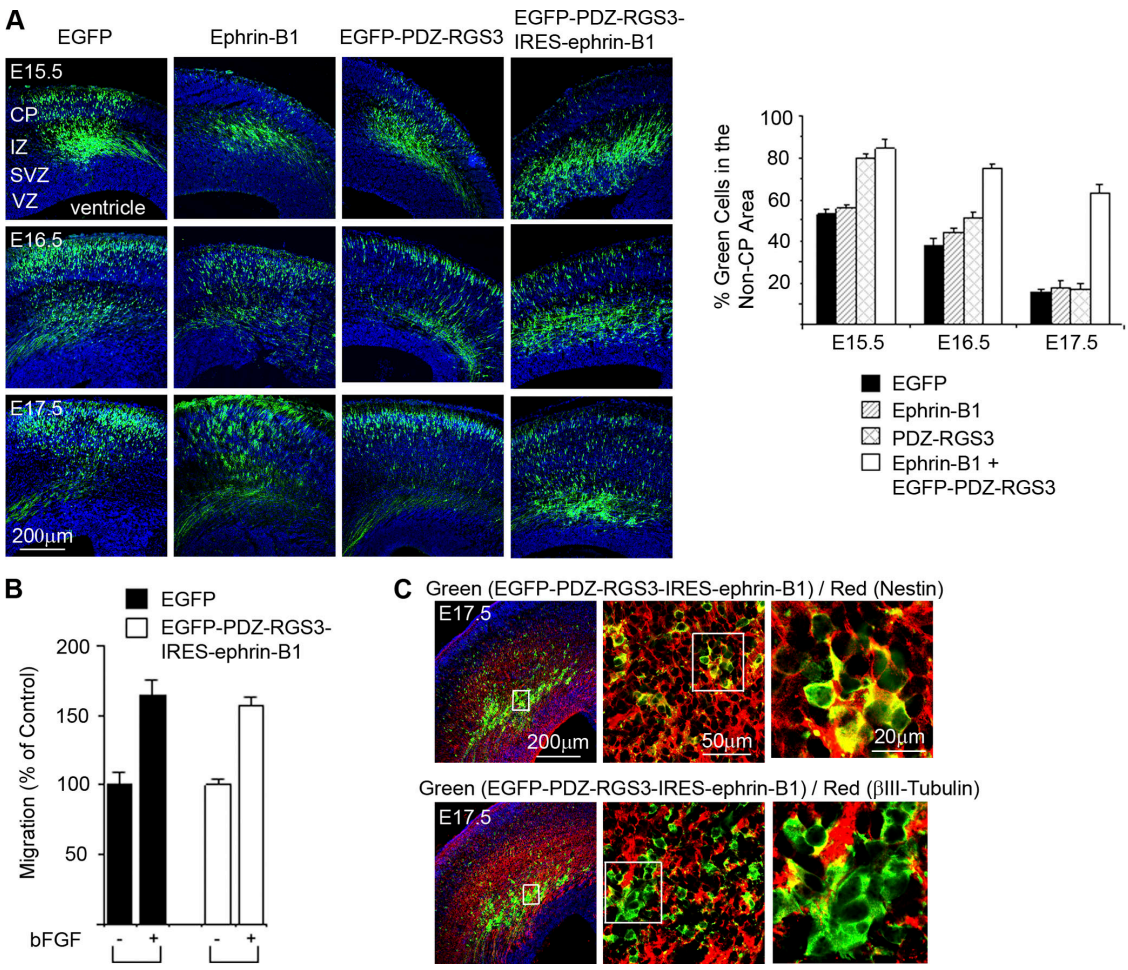

Figure 2. **Persistent coexpression of ephrin-B1 and PDZ-RGS3 in the cerebral cortex prevents differentiation.** (A) Ephrin-B1 and PDZ-RGS3 alone or in combination are expressed under the control of the DCX promoter/enhancer. The percentage of green cells in the non-CP area (VZ, SVZ, and IZ) versus the total green cells through the entire thickness of the cortex is plotted (averaged from five to seven brains per construct). EGFP: E15, 53% (3,468/6,543); E16, 38% (2,369/6,234); E17, 15% (895/5,968). ephrin-B1: E15, 56% (3,037/5,424); E16, 44% (2,255/5,125); E17, 18% (876/4,867). EGFP-PDZ-RGS3: E15, 80% (4,987/6,234); E16, 51% (3,066/6,012); E17, 17% (997/5,866). EGFP-PDZ-RGS3-IRES-ephrin-B1: E15, 84% (4,777/5,687); E16, 75% (4,049/5,399); E17, 63% (3,268/5,187). (B) Transwell migration analysis on cells electroporated with either EGFP or EGFP-PDZ-RGS3-IRES-HA-ephrin-B1. (C) Coimmunostaining of the cells having continued coexpression of ephrin-B1 and PDZ-RGS3 with nestin or βIII-tubulin. Boxed area are shown in enlarged images. Error bars show SD.

DCX promoter. Immunohistochemistry revealed that these cells were positive for nestin but negative for βIII-tubulin (Fig. 2 C). Because DCX expression did not seem to be affected in these cells (unpublished data), these results suggest that coexpression of ephrin-B1 and PDZ-RGS3 under the control of the DCX promoter was able to partially block or reverse the progression of differentiation in the affected cells.

Collectively, the results from experiments using both a ubiquitous promoter and a migrating neuron-specific promoter suggest a potential role of PDZ-RGS3–mediated ephrin-B signaling in promoting the maintenance of neural progenitor cells in the cerebral cortex.

Knockdown of ephrin-B1 causes differentiation of neural progenitor cells

We next used RNAi to specifically knock down ephrin-B1 expression in the cortex. The short hairpin RNAs (shRNAs) used in our experiments, shRNAB1.3 (sequence within the coding region of ephrin-B1) or shRNAB1-3UTR (sequence within the 3′ untranslated region of ephrin-B1 gene), specifically inhibit the expression of ephrin-B1 without affecting the expression of ephrin-B2 or B3 in transfected cells (Fig. S2, A and C, available at http://www.jcb.org/cgi/content/full/jcb.200708091/DC1) or in cortical neural progenitor cells (Fig. S2, B and D). As a control, we also used a mutant-type shRNA (shRNAB1.3mt) that does not affect the expression of ephrin-B1. All shRNAs were expressed by pNUTS, a mouse U6 promoter–containing plasmid which also carries EGFP under the control of the ubiquitin promoter, and were introduced into E13.5 cerebral cortices through in utero electroporation. Electroporated brains were dissected out and sectioned for analyses at E15.5. Although green cells containing shRNAB1.3mt were mostly found in the VZ at E15.5, many green cells carrying the wild-type shRNAB1.3 had already moved into the CP at that stage (Fig. 3 A). In general, there was a trend that more cells affected by the wild-type shRNA moved away from the apical part of the VZ than those cells carrying the mutant-type shRNA at E15.5 (Fig. 3 A). Electroporation of shRNAB1-3UTR caused a similar shift of cells away from

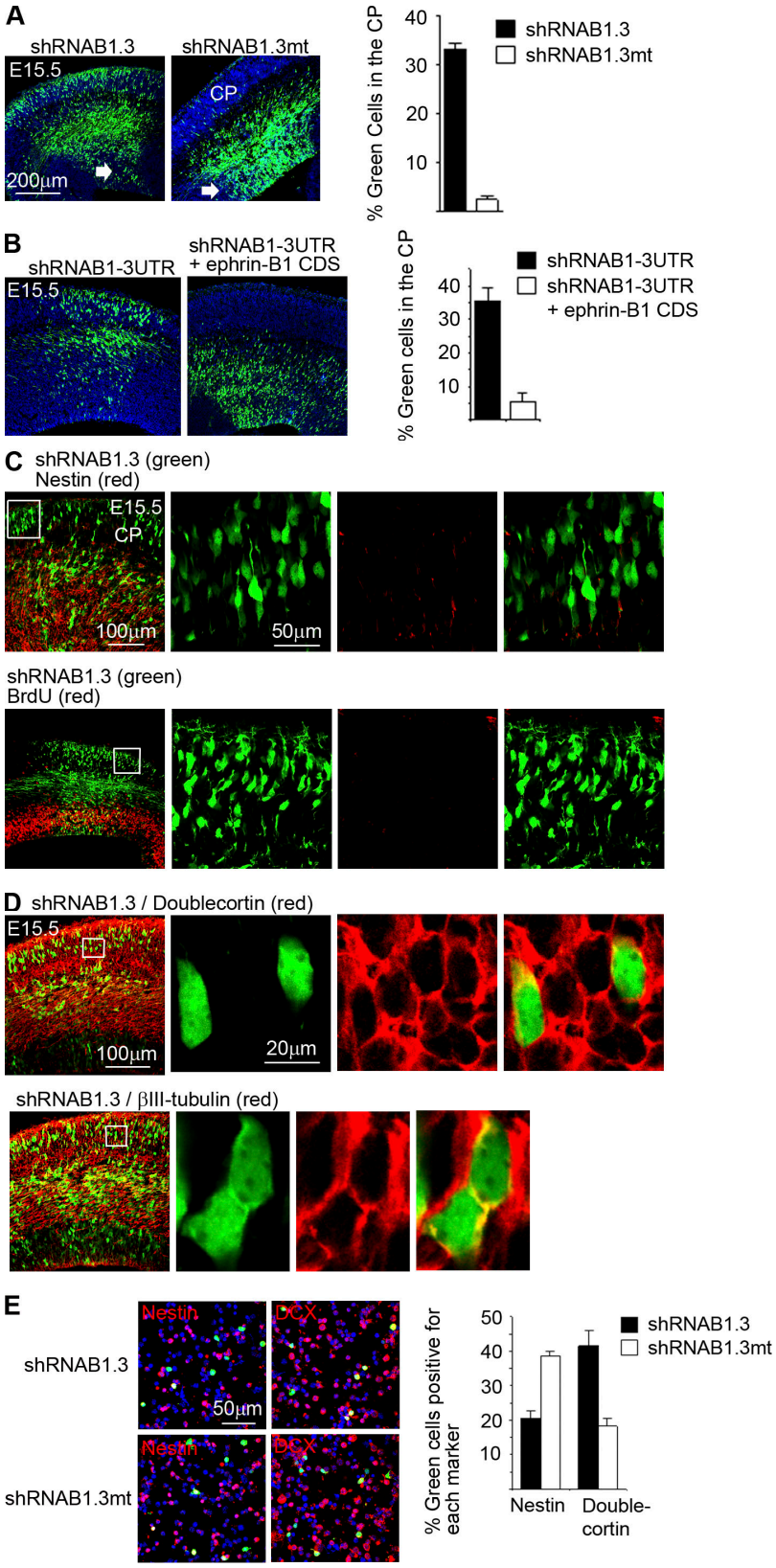

Figure 3. **Blocking ephrin-B1 expression causes differentiation of neural progenitor cells.** (A) Embryonic brains are electroporated with shRNAs at E13.5 and sectioned for imaging at E15.5. The percentage of green cells in the CP versus the total green cells through the entire thickness of the cortex is plotted (averaged from five to seven brains per shRNA). shRNAB1.3, 33% (2,294/6,895); shRNAB1.3mt, 2.3% (158/6,816). Arrows indicate that more cells affected by the wild-type shRNA have moved away from the apical side of the VZ. (B) Electroporation of an shRNA against the 3′UTR of ephrin-B1 (shRNAB1-3UTR) causes a similar effect to that of shRNAB1.3. This effect can be suppressed by coexpression of the CDS of ephrin-B1. shRNAB1-3UTR, 35% (1,634/4,651); shRNAB1-3UTR + CDS, 5.4% (221/4,036). (C and D) Electroporated brains are stained for nestin and pulse-labeled BrdU (C) or βIII-tubulin and DCX (D). Green cells that have migrated into the CP are positive for neuronal markers. (E) Quantification of green cells (expressing shRNA plasmids) that are positive for each marker using an acutely dissociated cell culture derived from electroporated brains. Nestin: shRNAB1.3, 21% (42/201); shRNAB1.3mt, 39% (83/215). DCX: shRNAB1.3, 42% (108/259); shRNAB1.3mt, 18% (34/188). Error bars show SD.

the ventricle, and this effect could be suppressed by coexpression of the coding sequence (CDS) of ephrin-B1, which is resistant to the shRNA (Fig. 3 B and Fig. S2 C), suggesting that the effect of RNAi of ephrin-B1 was not likely because of an off-target effect. The up-shift pattern might indicate that the progenitor cells affected by RNAi of ephrin-B1 had differentiated.

To address whether knockdown of ephrin-B1 expression had either caused neural progenitor cells to differentiate into

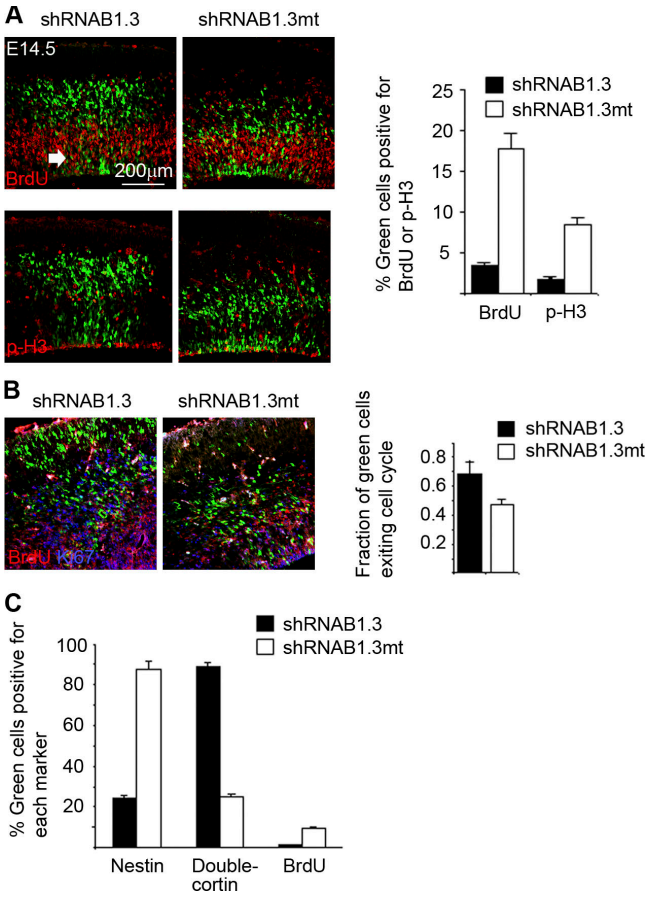

Figure 4. **Blocking ephrin-B1 expression causes differentiation of neural progenitor cells.** (A) Embryonic brains are electroporated with shRNAs at E13.5 and sectioned at E14.5 for immunohistochemistry of pulse-labeled BrdU and p-H3. Arrow indicates that more cells affected by the wild-type shRNA have moved away from the apical side of the VZ, where mitotic cells are enriched. Percentage of BrdU or p-H3–positive green cells is plotted. shRNAB1.3: BrdU, 3.5% (131/3,728); p-H3, 1.7% (68/4,019). shRNAB1.3mt: BrdU, 18% (943/5,316); p-H3, 8.4% (359/4,256). (B) Brains are electroporated at E13.5, labeled with BrdU at E14.5, and sectioned at E15.5 for immunohistochemistry of BrdU (red) and Ki67 (blue). Fraction of green cells that have exited cell cycle ($BrdU^+Ki67^-$) is quantified. shRNAB1.3, 69% (497/723); shRNAB1.3mt, 47% (198/418). (C) Cultured neural progenitor cells are transfected with shRNAB1.3 or control shRNA and are immunostained for nestin, DCX, or BrdU. The percentage of green cells positive for each individual marker versus the total green cells is plotted (averaged from three independent experiments). shRNAB1.3: nestin, 24% (82/342); DCX, 89% (298/336); BrdU, 1.4% (7/516). shRNAB1.3mt: nestin, 88% (276/314); DCX, 25% (89/355); BrdU, 9.5% (47/495). Error bars show SD.

neurons, which would normally migrate outwards, or alternatively had induced premature migration of progenitor cells into the CP, we performed immunohistochemistry on the green cells that had reached the CP. In the cortices electroporated with shRNAB1.3, green cells in the CP were negative for nestin and pulse-labeled BrdU (Fig. 3 C) but were positive for two markers for newborn neurons, βIII-tubulin and DCX (Fig. 3 D). Immunofluorescent staining on dissociated cells derived from electroporated brains showed that more cells affected by shRNAB1.3 were negative of nestin but positive for DCX (Fig. 3 E), further indicating that RNAi of ephrin-B1 could promote differentiation of the affected progenitor cells.

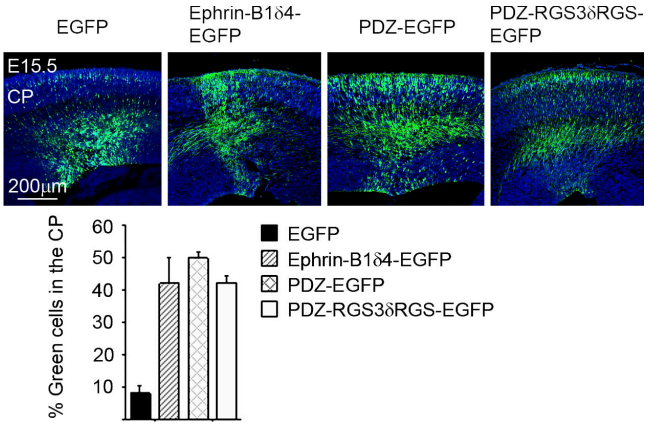

Figure 5. **PDZ-RGS3–dependent signaling mediates ephrin-B1 function in the cerebral cortex.** Dominant-negative inhibition of ephrin-B reverse signaling leads to a similar outcome to RNAi of ephrin-B1. Results are averaged from three to five brains per construct. EGFP, 8% (276/3,456); ephrin-$B1^{\delta4}$-EGFP, 42% (1,498/3,567); PDZ-EGFP, 50% (1,938/3,876); PDZ-$RGS3^{\delta RGS}$-EGFP, 42% (1,564/3,724). Error bars show SD.

To more directly assess the effect of RNAi of ephrin-B1 on cell fate, we looked at the electroporated cells at E14.5, when the earliest migrated cells seen in the CP at E15.5 were presumably still in the VZ area. RNAi of ephrin-B1 led to an outward shift of the affected cells away from the apical side of the VZ, as was also seen at E15.5 (Fig. 4 A). Analyses on the BrdU incorporation and the number of phospho–Histone $H3^+$ ($p\text{-}H3^+$) cells showed that RNAi of ephrin-B1 caused a decrease on both the BrdU labeling index (percentage of short pulse-labeled $BrdU^+$ S-phase cells in the total number of green cells; Chenn and Walsh, 2002) and the mitotic index (percentage of $p\text{-}H3^+$ mitotic cells in the total number of green cells; Li et al., 2003; Fig. 4 A). Cell cycle exit analysis further showed that RNAi of ephrin-B1 caused more cells to exit the cell cycle (Fig. 4 B).

To address further whether RNAi of ephrin-B1 could activate the differentiation, we transfected shRNAB1.3 or shRNAB1.3mt in vitro into isolated neural progenitor cells derived from early embryonic cortices (Johe et al., 1996). As shown in Fig. 4 C, compared with the mutant control shRNA, shRNAB1.3 reduced the population of nestin-positive and mitotic (BrdU-positive) cells, with a concomitant increase in the number of cells positive for DCX.

Collectively, the results from the knockdown of ephrin-B1 in vivo and in vitro indicate that ephrin-B1 is critical for maintaining the neural progenitor identity in the developing cerebral cortex.

PDZ-RGS3–dependent signaling mediates the function of ephrin-B

To further study the effect of blocking ephrin-B function on neural progenitor cells, we sought to alternatively take a dominant-negative approach by expressing mutant ephrin-B or PDZ-RGS3 proteins (Lu et al. 2001). These mutant proteins could provide an independent method for assessing the observed effect of RNAi of ephrin-B and could also offer insights on the contribution of reverse versus forward signaling. Ephrin-$B1^{\delta4}$-EGFP was a deletion mutant of ephrin-B1 with the last four amino acids of the cytoplasmic domain (which constitute the PDZ-binding

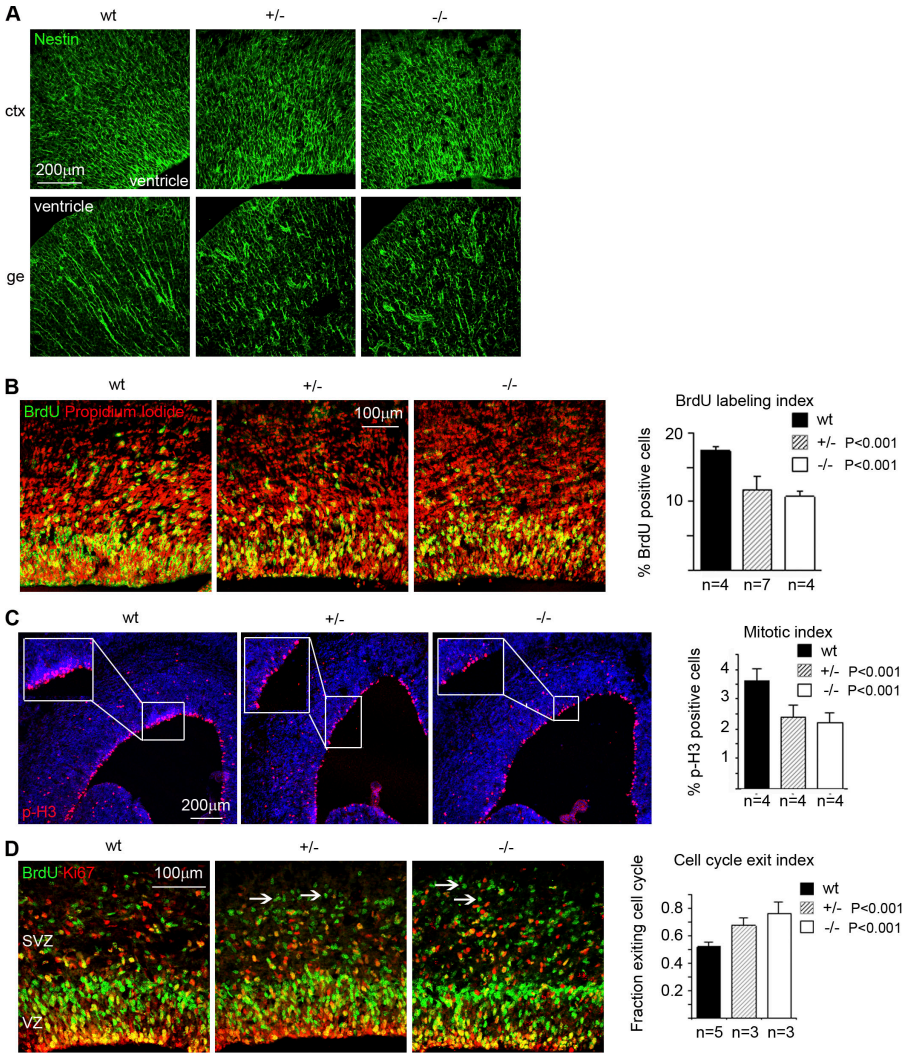

Figure 6. **Genetic loss of function of ephrin-B1 leads to a loss of cortical neural progenitor cells.** (A) Defects in progenitor cell radial organization in ephrin-B1 mutant brains. ctx, cortex; ge, ganglion eminence. (B) Percentage of $BrdU^+$ cells after a short pulse labeling. Wild type (wt), 17% (1,056/6,058); +/−, 12% (1,226/10,453); −/−, 11% (656/6,114). (C) Percentage of mitotic cells ($p\text{-}H3^+$). Wild type, 3.6% (285/7,895); +/−, 2.4% (206/8,642); −/−, 2.2% (156/7,015). Boxed areas are shown in enlarged images (insets). (D) Fraction of progenitor cells exiting cell cycle ($BrdU^+Ki67^-$ cells, some are indicated by arrows) in the population of $BrdU^+$ cells. Wild-type, 0.52 (1,672/3,216); +/−, 0.67 (1,589/2,358); −/−, 0.76 (1,461/1,913). Error bars show SD.

motif) replaced with EGFP. The deletion mutants of PDZ-RGS3 were PDZ-EGFP (the entire sequence C terminal to the PDZ domain was replaced by EGFP) and PDZ-RGS3$^{\delta RGS}$-EGFP (the RGS domain was replaced by EGFP). Similar to the effect of RNAi of ephrin-B1, electroporation-mediated cortical expression of these mutant proteins resulted in an outward shift of the affected cells away from the apical side of the VZ and led to more green cells translocating into the CP (Fig. 5). RNAi of PDZ-RGS3 also led to a similar phenotype (unpublished data). These results further support a role of ephrin-B in the maintenance of neural progenitor cells. The data from dominant-negative inhibition also provide evidence that the function of ephrin-B1 in progenitor cells is dependent on reverse signaling and that PDZ-RGS3 is an important downstream mediator.

Genetic loss of function of ephrin-B1 leads to a loss of neural progenitor cells

The electroporation studies suggest that genetic loss of function of ephrin-B reverse signaling might cause early differentiation and hence a reduction in the number of cortical neural progenitor cells. We therefore assessed neural progenitor cells in ephrin-B1 knockout mice (Davy et al., 2004) during the period of cortical neurogenesis. Ephrin-B1 is X linked, and previous studies (Compagni et al., 2003; Davy et al., 2004, 2006) showed that ephrin-B1 heterozygotes exhibited phenotypes not observed in homozygotes because of sorting out of ephrin-B1–positive and –negative cells generated by random X inactivation. We thus analyzed cortical neural progenitor cells both in heterozygous and homozygous ephrin-B1 mutant mice.

We first performed anti-nestin immunohistochemistry to look at the morphological appearance of neural progenitor cells (Fig. 6 A). In wild-type brains, nestin-positive cells typically extend radially oriented long processes in an orderly manner. In mutant mice (null or het), nestin-positive cells in the brain appeared to have a disrupted radial organization (e.g., an uneven nestin staining pattern in the cerebral cortex) or to have lost the radial organization (e.g., in the ganglion eminence, the processes of progenitor cells were shorter and projected in various directions). This abnormality showed a 90% penetrance in nulls and 65% penetrance in heterozygotes. These results indicate that deleting the ephrin-B1 gene causes defects in neural progenitor cell organization in the developing brain.

We next studied the cell cycle dynamics of the mutant neural progenitor cells. We first analyzed the BrdU labeling index

(percentage of short pulse-labeled $BrdU^+$ S-phase cells per 40× optical view; Chenn and Walsh, 2002) and the mitotic index (percentage of $p\text{-}H3^+$ mitotic cells per 40× optical view; Li et al., 2003) to look at the effects of ephrin-B1 knockout in neural progenitor cell population. We found that mutant (null or het) brains showed a lower BrdU labeling index (Fig. 6 B) and a decrease in the mitotic index (Fig. 6 C) compared with the wild-type brains, indicating that there were fewer neural progenitor cells in the ephrin-B1 mutant brains. Tunnel staining showed that there was no obvious difference in cell apoptosis between mutant and wild-type brains (unpublished data). To further address the possible mechanisms of neural progenitor cell depletion in the ephrin-B1 mutant mice, we performed a 24-h labeling of BrdU to examine cell cycle exit and reentry by measuring the fraction of $BrdU^+Ki67^-$ cells (cells that have exited cell cycle) in $BrdU^+$ cells per 40× optical view (Chenn and Walsh, 2002). As shown in Fig. 6 D, the mutant brains revealed a marked increase in the fraction of progenitor cells exiting the cell cycle (more $BrdU^+Ki67^-$ cells), thus reducing the pool of self-renewing progenitor cells. These results indicate that genetic loss of function of ephrin-B1 causes progenitor cells to leave the cell cycle and leads to a loss of cortical neural progenitor cells in development.

Discussion

Using cellular, embryological, and genetic approaches, we have observed that gain of function of ephrin-B/PDZ-RGS3 signaling prevents differentiation of cortical neural progenitor cells, whereas loss of function induces differentiation. Our findings thus identify ephrin-B1 as a functional surface marker for neural progenitor cells in the developing cerebral cortex and indicate that ephrin-B/PDZ-RGS3 signaling plays a critical role in the maintenance of the neural progenitor cell state during brain development.

Ephrin-B reverse signaling in the cerebral cortex

Our study has uncovered a previously unknown function of ephrin-B in development: maintaining the pool of cortical neural progenitor cells. This role of ephrin-B was first suggested by the specific expression pattern of ephrin-B1 and its downstream signaling mediator PDZ-RGS3 in cortical neural progenitor cells. Cellular and embryological studies next showed that ephrin-B/PDZ-RGS3 signaling can sufficiently block differentiation and is important for maintaining neural progenitor cell identity. Consistent with these observations, knockout of ephrin-B1 causes a reduction in the number of neural progenitor cells in the embryonic cortices.

The ability of ephrin-B to initiate signaling in both directions (to activate a forward signaling via Eph and initiate a reverse signaling through its own cytoplasmic domain; Flanagan and Vanderhaeghen, 1998; Drescher, 2002; Poliakov et al., 2004; Davy and Soriano, 2005; Egea and Klein, 2007; Arvanitis and Davy, 2008; Pasquale, 2008) implies that an ephrin-B mutant phenotype could be caused by a cell-autonomous or -nonautonomous function. For example, the functions of ephrin-B in hindbrain segmentation (Xu et al., 1999), in the formation of the venous and arterial vasculature (Wang et al., 1998; Adams et al., 1999), and in the skeletal defects (polydactyly and cranial foramen) observed in the heterozygote females of the ephrin-B1 mutant mice (Compagni et al., 2003; Davy et al., 2004, 2006) were based on EphB–ephrin-B–mediated cell sorting. In contrast, ephrin-B1 could play a cell autonomous role in neural crest cells to control their migration (Davy et al., 2004). Our data of ephrin-B in regulating the self-renewal of cortical neural progenitor cells appear to be also consistent with a cell-autonomous ephrin-B function.

In the postnatal (postnatal day 23) ephrin-B1 knockout mice, the overall brain morphology, including cortex and hippocampus, appeared to be largely comparable among the null and wild-type brains, although the surviving ephrin-B1 nulls were, on average, 40% lighter in body weight compared with wild-type mice, which suggests growth retardation (unpublished data). As cortical VZ/SVZ expression of ephrin-B2 persisted in the ephrin-B1–null brains (unpublished data), double knockout of ephrin-B1 and -B2 or knockout of PDZ-RGS3 will need to be further analyzed to alleviate possible functional redundancy in the cortex. It would also be interesting to investigate whether the postnatal cortex might have a similar self-repair plasticity in fixing early defects as revealed in the adult SVZ (Kuo et al., 2006).

Signaling of B and A subfamily of ephrins in neural progenitor/stem cells

Our current results lend further support to the recently reported roles of ephrins in neural progenitor/stem cells. In addition, our data reveal functional differences between the two subfamilies of ephrins in neural progenitor cells. Although our study indicates that ephrin-B reverse signaling is critical for the maintenance of cortical neural progenitor cells, transgenic expression of ephrin-A5 in the cortex causes a decrease in the number of neural progenitor cells (Depaepe et al., 2005), suggesting that ephrin-A/EphA signaling (activation of EphA or gain of ephrin-A reverse signaling) negatively regulates the progenitor numbers. In the adult SVZ, infusion of EphB-Fc/ephrin-B-Fc (Conover et al., 2000) or EphA-Fc/ephrin-A-Fc (Holmberg et al., 2005) leads to a similar outcome: more proliferation of neural stem cells, which suggests that either activation of ephrin-B/EphB and ephrin-A/EphA signaling or the disruption of it activates neural stem cell proliferation. Because the knockout of ephrin-A2 or EphA7 causes increased numbers of adult neural stem cells, it was suggested (Holmberg et al., 2005) that ephrin-A reverse signaling normally negatively regulates adult neural stem cell proliferation. It is not yet clear whether ephrin-B reverse signaling functions to promote or block the maintenance of adult neural stem cells. Future analyses on single or double knockout mice of ephrin-B1 and -B2 (ephrin-B1 and -B2 are both expressed in the SVZ neural stem cells; Conover et al., 2000; Ricard et al., 2006) will help to better understand this.

Mechanism of the ephrin-B/RGS function in cortical neural progenitor cells

During cortical neurogenesis, neural progenitor cells in the VZ go through consecutive rounds of cell cycles over an extended period of time. Detailed cell cycle measurements reveal that

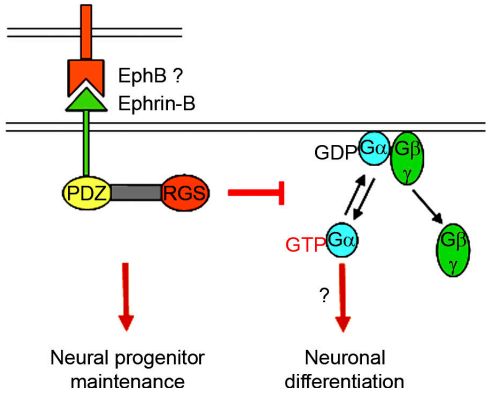

Figure 7. **A working model of the function of ephrin-B–RGS pathway.** Our results indicate that the RGS-mediated ephrin-B reverse signaling pathway is required for the maintenance of neural progenitor cells. As the RGS domain acts as an inhibitor of Gα subunit signaling, our data suggest that Gα subunit may function as an activator of progenitor differentiation. Future studies are required to investigate this potential role of Gα subunit. In addition, whether and how EphB functions in the activation of ephrin-B–RGS pathway in neural progenitor cells will also need to be further studied.

early in neurogenesis, neurons are generated relatively slowly and the VZ expands rapidly through accumulation of progenitor cells but the reverse happens at a later stage (Takahashi et al., 1996). A general model of cortical neurogenesis (McConnell, 1995; Takahashi et al., 1996; Monuki and Walsh, 2001; Temple, 2001; Gupta et al., 2002; Fishell and Kriegstein, 2003; Gotz and Huttner, 2005; Rakic, 2006; Molyneaux et al., 2007) suggests that in the early phase, neural progenitor cells predominantly go through symmetrical divisions to generate more progenitor cells. Then cell divisions progressively shift to asymmetrical generation of one progenitor and one neuron. In the final phase of neurogenesis, progenitor cells might also divide symmetrically to give rise to two daughter neurons to expand the neuronal population more quickly. Our results suggest that ephrin-B/PDZ-RGS3 signaling is likely to have a regulatory role at the stage when neural progenitor cells are shifting from progenitor self-renewal by symmetrical cell division to generation of neurons by asymmetrical cell division.

The dominant-negative inhibition by a mutant PDZ-RGS3 protein that lacks the RGS domain (Fig. 5) indicates that the function of ephrin-B/PDZ-RGS3 signaling in neural progenitor cells is likely mediated through inhibition of the heterotrimeric G protein signaling (De Vries et al., 2000; Ross and Wilkie, 2000; Siderovski and Willard, 2005). Our results thus suggest the following working model: the RGS-mediated ephrin-B signaling is required to promote neural progenitor maintenance via inhibition of G protein signaling (Fig. 7). This model proposes that the Gα subunit of the heterotrimeric G proteins functions as an activator of neuronal differentiation during cortical neurogenesis, perhaps through promoting asymmetrical cell division. It is interesting to note that ephrin-Eph signaling was recently shown to mediate the establishment of asymmetrical cell fates in the *Ciona intestinalis* notochord and neural tube (Picco et al., 2007; Shi and Levine, 2008).

The involvement of heterotrimeric G proteins in the mammalian neural progenitor cell fate determination is not clear. One study suggested that the Gβγ subunits control the mitotic spindle orientation in cortical neural progenitor cells thereby inhibiting differentiation (Sanada and Tsai, 2005). In *Caenorhabditis elegans* and *Drosophila melanogaster*, increasing genetic evidence has implicated a fundamental role for G proteins in controlling neural progenitor asymmetrical cell division (Wilkie and Kinch, 2005; Yu et al., 2006), which governs self-renewal and differentiation. In this function, Gα subunit, Pins/AGS3/LGN, GoLoco/GPR, Ric-8, and RGS proteins work together in a noncanonical G protein pathway to control asymmetrical division in progenitor differentiation (Wilkie and Kinch, 2005; Yu et al., 2006). In view of the G protein pathway from *D. melanogaster* and *C. elegans*, our results suggest that a similar mechanism of Gα subunits and RGS proteins may function in the mammalian neural progenitor cells, including likely candidate participants PDZ-RGS3 and Gαi2 subunit, which was shown to be selectively expressed in the VZ of the developing cerebral cortex (Asano et al., 2001; unpublished data). Our results would further suggest that the RGS function in the noncanonical G protein pathway may be modulated by an upstream receptor molecule, such as ephrin-B. This was reflected in our gain-of-function study that both ephrin-B and PDZ-RGS3 are required for maintaining the neural progenitor state.

Materials and methods

Plasmids and antibodies

Hairpin shRNAs were cloned into pNUTS (a gift from W. Lu and D. Baltimore, California Institute of Technology, Pasadena, CA). Dominant-negative mutants of ephrin-B1 and PDZ-RGS3, including ephrin-B1$^{\delta 4}$-EGFP, PDZ-RGS3$^{\delta RGS}$-EGFP, and PDZ-EGFP, were cloned into pEF-BOS (provided by T. Ohtsuka and R. Kageyama, Kyoto University, Kyoto, Japan). The bicistronic expression construct EGFP-PDZ-RGS3-IRES2-HA-ephrin-B1 was cloned into pEF-BOS or a vector that contains the DCX promoter/enhancer (Wang et al., 2007).

Primary antibodies used in this study included a rabbit polyclonal anti–ephrin-B antibody C18 (1:100; Santa Cruz Biotechnology, Inc.), anti–PDZ-RGS3 (described in Lu et al. [2001]), monoclonal anti-nestin rat-401 (1:100; Developmental Studies Hybridoma Bank), RC2 (1:100; Developmental Studies Hybridoma Bank), anti–βIII tubulin (1:400; Sigma-Aldrich), anti-DCX (1:50 Santa Cruz Biotechnology, Inc.), anti-BrdU (1:500; Sigma-Aldrich), rat anti-HA (1:100; Roche), sheep anti–digoxigenin-AP (1:2,000; Roche), anti-GFP (1:200; Invitrogen), rabbit Ki67 (1:100; Novocastra Laboratories Ltd), and rabbit anti–p-H3 (1:200; Millipore). All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (Rhod Red-X-AffiniPure and Cy2 AffiniPure conjugated) and were used with a dilution of 1:200.

Selection of shRNAs

Candidate shRNAs of ephrin-B1 were cotransfected into HEK cells with psi-CHECK (Promega)–ephrin-B1. Cell extracts were made 48 h after transfection and the potency of shRNAs was assessed using dual luciferase activities. Inhibition on ephrin-B1 expression was confirmed using Western blot on transfected cells (EGFP was coexpressed with shRNAs in pNUTs and was used as a loading control) or immunocytochemistry on cortical progenitor cells. The sequences of shRNAs used in the functional assays were the following (the loop of shRNA and the mutations introduced are underlined and bolded, respectively): shRNAB1.3, ACCAUCAAGUUCCAAGAGUUUCAAGAGAACUCUUGGAACUUGAUGGU; shRNAB1.3mt, ACCAUCAA**UCC**CCAAGAGUUUCAAGAGAACUCUUGG**GGA**UUGAUGGU; shRNAB1-3UTR, GACUGCUCGUCCACUAUCAUUCAAGAGAUGAUAGUGGACGAGCAGUC; and shRNAB1-3UTRmt, GA**UCAU**UCGUCCACUAUCAUUCAAGAGAUGAUAGUGGACGA**AUGA**UC.

RNA in situ and immunohistochemistry

RNA probes for ephrin-B1, PDZ-RGS3, and EphBs were described in Lu et al. (2001). RNA in situ and immunohistochemistry were done essentially as in

Lu et al. (2001). Fluorescent images were taken using a confocal microscope (LSM 510 Upright 2 photon: Carl Zeiss, Inc.).

Image acquisition and processing

Immunohistochemistry was performed on frozen sections using Cy2 or Rhodmine Red-X–conjugated secondary antibodies. Slides were mounted in aqueous mounting media. Fluorescent images were all acquired using the confocal microscope (LSM 510 Upright 2 photon) with 0.5 NA FLUAR10×, 0.8 NA Plan-APOCHROMAT 20×, 0.75 NA Plan-NEOFLUAR 40×, 1.4 NA Plan-APOCHROMAT (digital image contrast) 63× oil immersion, and 1.4 NA Plan-APOCHROMAT 100× oil immersion objectives. Digital images were processed (brightness/contrast adjusted and color balanced) using Image-Pro Plus 5.1 software (Media Cybernetics, Inc.). RNA in situ images were captured on a dissection scope (S6D; Leica) connected to a camera (Spot Insight; GE Healthcare). Spot basic Version 3.5.5 was used for RNA In situ image processing.

In utero electroporation functional analysis

In utero electroporation was performed on E13.5 embryos as described previously (Ohtsuka et al., 2001; Saito and Nakatsuji, 2001). Electroporated brains were dissected out at E14.5 or at a later stage, cryoprotected, sectioned, and stained with HOECHST for microscopic analyses. For BrdU pulse labeling, 50 mg/kg BrdU was intraperitoneally injected into the female mouse 1.5 h before brain dissection. For cell cycle exit analysis, BrdU was administered at E14.5 and the electroporated brains were dissected out for analysis at E15.5. Electroporated cells were detected by direct visualization of EGFP expressed from the shRNA vector or by immunohistochemistry on EGFP that was expressed as a fusion protein to ephrin-B1 or PDZ-RGS3. Quantification of the distribution of green cells in the cortex was done using Image-Pro Plus 5.1.

Neural progenitor cell culture, transfection, and immunostaining

Cerebral cortices of E11.5 mouse embryos were dissected out in HBSS. Cells were dissociated by trituration using a pipette with a blue tip. After centrifugation at 1,000 rpm for 5 min, cells were washed twice with HBSS. Cell pellets were then resuspended in DME/F12 medium supplemented with 1:50 vol/vol B27, 100 units/ml penicillin, and 100 µg/ml streptomycin, counted, plated (2×10^5 cells/well) onto 15 µg/ml polyornithine in H_2O and 2 µg/ml fibronectin (in PBS)-coated coverslips placed in a 24-well plate, and cultured at 37°C. 1 d after plating, cells were transfected in triplicate with shRNAB1.3 or shRNAB1.3mt using Fugene-6 (Roche). 2 d after transfection, cells were fixed with 4% PFA for immunocytochemistry. For BrdU labeling, BrdU was added to the culture at a final concentration of 10 µM, 16 h (overnight) before fixation. The acutely dissociated cells were prepared as described in a previous study (Hartfuss et al., 2001).

Transwell analysis

EGFP or EGFP-PDZ-RGS3-IRES-HA-ephrin-B1 electroporated brains were removed at E15.5. The cortical areas that express the electroporated plasmid were dissected out under a fluorescent microscope and dissociated in HBSS. After being washed twice, cells were resuspended in DME/F12 medium supplemented with B27. Transwell migration assay was performed essentially as described previously (Lu et al., 2001).

Mutant ephrin-B1 mice analysis

Ephrin-B1 knockout mice were reported previously (Davy et al., 2004). BrdU labeling index, mitotic index, and progenitor cell cycle exit index were analyzed essentially as described previously (Chenn and Walsh, 2002; Li et al., 2003). In brief, to obtain BrdU labeling index and progenitor cell cycle exit index, pregnant female mice were labeled with 50 mg/kg BrdU for 2 and 24 h, respectively. Cryosections of the brains (12–14 µm) were processed for BrdU and Ki67 staining and images were taken using a confocal microscope (LSM 510 Upright 2 photon). For BrdU labeling index, $BrdU^+$ cells were counted against the total cells stained by propidium iodide in a 40× optical view of each section. For progenitor cell cycle exit index, $BrdU^+Ki67^-$ cells (cells exiting cell cycle) were counted against the total $BrdU^+$ cells/40× optical view. For mitotic index, $p\text{-}H3^+$ cells were counted against the total cells stained by HOECHST in 40× optical views.

Online supplemental material

Fig. S1 shows electroporation-mediated gain of function of ephrin-B1 and PDZ-RGS3 in the cortex using EF promoter–based vector. Fig. S2 shows specific inhibition of ephrin-B1 expression by two different shRNAs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200708091/DC1.

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