STEM CELLS®
TISSUE-SPECIFIC STEM CELLS

Gα Subunit Coordinates with Ephrin-B to Balance Self-Renewal and Differentiation in Neural Progenitor Cells

KIYOHITO MURAI,[a] RUNXIANG QIU,[a] HEYING ZHANG,[a] JUN WANG,[a,b] CHEN WU,[a,b] RICHARD R. NEUBIG,[c] QIANG LU[a]

[a]Department of Neurosciences, [b]City of Hope Graduate School of Biological Sciences, Beckman Research Institute of the City of Hope, Duarte, California, USA; [c]Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan, USA

Key Words. Gα • subunit • Ephrin-B/RGS signaling • Neural progenitor cells • Cortical neurogenesis • Self-renewal and differentiation

ABSTRACT

Proper development of the mammalian brain requires that neural progenitor cells balance self-renewal and differentiation under precise temporal and spatial regulation, but the underlying mechanisms are not well understood. In this study, we identify Gα subunit as a positive regulator of mammalian neurogenesis, working with the regulator of G protein signaling (RGS)-mediated ephrin-B signaling pathway as two opposing forces to maintain a balance between self-renewal and differentiation in the developing mouse cerebral cortex. Multiple $G\alpha_i$ subunits are expressed by cortical neural progenitor cells during the course of cortical neurogenesis. Activation of $G\alpha_i$ signaling, through in utero electroporation-mediated expression of wild-type and constitutively active $G\alpha_i$ subunits, counteracts the function of ephrin-B in cortical neural progenitors to induce differentiation. Genetic knock-in of an RGS-insensitive G184S$G\alpha_{i2}$ causes early cell cycle exit and a reduction of cortical neural progenitor cells and leads to a defect in the production of late born cortical neurons, similar to what is observed in mutant mice with deficiency in ephrin-B reverse signaling pathway. This study reveals a role of Gα subunit in mammalian neurogenesis and uncovers a developmental mechanism, coordinated by the Gα and ephrin-B signaling pathways, for control of the balance between self-renewal and differentiation in neural progenitor cells. STEM CELLS *2010;28:1581–1589*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

In the developing mammalian cerebral cortex, neural progenitor cells located in the germinal ventricular zone (VZ) give rise to the majority of projection neurons. During cortical neurogenesis, neural progenitor cells progress from proliferation to differentiation with temporal and spatial precision and control of the balance between progenitor self-renewal and differentiation is essential for ensuring that correct numbers of neural cells are generated to make a functional brain [1–5]. A number of factors that either promote progenitor cell fate or promote differentiation have been documented, however, the mechanisms underlying the coordinated action by these opposing forces in balancing self-renewal and differentiation are not well understood [6, 7].

Genetic studies in invertebrates have implicated a central role of Gα subunit of the heterotrimeric G protein complex in controlling the mode of cell division during neurogenesis. In the *Drosophila* and *C. elegans*., Gα subunit has been found to be essential for specifying neural progenitor asymmetric versus symmetric cell divisions, mediated by a G protein-coupled receptor-independent pathway, which involves regulator proteins such as Pins/activator of G protein signaling 3 (AGS3)/LGN, GoLoco/G protein-coupled receptor (GPR), Ric-8, and regulator of G protein signaling (RGS) protein [8–12]. This suggests that Gα subunit can be a critical regulator for controlling the balance between neural progenitor self-renewal and differentiation. In the mammalian nervous system, however, whether Gα subunit is involved in the regulation of self-renewal versus differentiation during neurogenesis is unclear.

RGS proteins are a large family of modulators of the heterotrimeric G protein complex [13, 14]. The RGS domain acts as GTPase-activating protein (GAP) for Gα subunits, accelerating the Gα subunit catalytic cycle and thereby facilitating rapid turn off of Gα activation by extracellular signals [13, 14]. It was suggested that the catalytic cycle of Gα subunit regulated by RGS and other G protein modulators is also critically important for the noncanonical G protein function in cell division [8–12]. We previously found that an RGS-mediated ephrin-B signaling pathway is critical for promoting neural progenitor cell fate in the developing cerebral cortex [15]. Our observation of the involvement of an RGS protein in the maintenance of cortical neural progenitor cells suggests that Gα subunits might function in coordination with ephrin-B in the control of cortical neural progenitor homeostasis by promoting neural progenitor differentiation.

Author contributions: K.M. and R.X.Q.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; H.Y.Z., J.W., and C.W.: collection and assembly of data; R.R.N.: provision of study material, manuscript writing; Q.L.: conception and design, financial support, data analysis and interpretation, manuscript writing. K.M. and R.Q. contributed equally to this article.

Correspondence: Qiang Lu, Ph.D., Department of Neurosicences, Beckman Research Institute of City of Hope, 1,500 East Duarte Road, Duarte, CA 91,010. Telephone: 626-301-8357; Fax: 626-301-8310; e-mail: qlu@coh.org Received April 13, 2010; accepted for publication June 26, 2010; first published online in STEM CELLS *EXPRESS* July 13, 2010. © AlphaMed Press 1066-5099/2009/$30.00/0 doi: 10.1002/stem.474

In this study, we have examined the function of $G\alpha_i$ subunit in cortical neural progenitor cells using combined embryological and genetic approaches. We present evidence for a role of the $G\alpha_{i2}$ subunit in promoting cortical neurogenesis, and we further show that Gα and ephrin-B signaling pathways work as two counteracting forces to coordinately regulate neural progenitor homeostasis in the developing mammalian brain.

Materials and Methods

Plasmids and Antibodies

PDZ-RGS3N871D, $G\alpha_{i2}$QL, and $G\alpha_{i2}$ were expressed under the control of elongation factor two promoter. The parental plasmid also contains an ubiquitin promoter-enhanced green fluorescent protein (EGFP) expression cassette for easy identification and tracing of the transfected cells.

Primary antibodies used in this study included a rabbit polyclonal anti-ephrin-B antibody C18 (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com), goat-anti-ephrin-B1 monoclonal antibody (1:200, Sigma), anti-$G\alpha_{i2}$ (1:100, Santa Cruz Biotech), monoclonal anti-nestin Rat-401 (1:100, Developmental Studies Hybridoma Bank, Iowa, IA, http://dshb.biology.uiowa.edu/), anti-doublecortin (DCX) (1:50, Santa Cruz), anti-BrdU (1:500, Sigma, St. Louis, MO, http://www.sigmaaldrich.com), Sheep anti-Digoxigenin-AP (1:2,000, Roche, Basel, Switzerland, http://www.roche-applied-science.com), anti-green fluorescent protein (GFP) (1:200, Molecular Probe Inc., Eugene, OR, http://probes.invitrogen.com), Rabbit anti-Ki67 (1:100, Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K., http://www.novocastra.co.uk), and Rabbit anti-phospho-Histone H3 (1:200, Millipore, Billerica, MA, http://www.millipore.com/). All secondary antibodies were from Jackson ImmnunoResearch (West Grove, PA, http://www.jacksonimmuno.com/) (Rhod Red-X-AffiniPure and Cy2 AffiniPure conjugated) and were used with a dilution of 1:200.

RNA In Situ and Immunohistochemistry

RNA probes for $G\alpha_i$ subunits were transcribed from cDNAs of each individual molecule. RNA in situ and immunohistochemistry were done essentially as described previously [15]. RNA In situ images were captured on Olympus IX81 inverted microscope. Immunofluorescence images were taken using a confocal microscope (Zeiss LSM 510 Upright two photon).

In Utero Electroporation Functional Analysis

In utero electroporation was performed essentially as described previously [15]. Embryos of Swiss Webster mice or the G184S$G\alpha_{i2}$ knock-in mice were electroporated at E13.5 and brains were removed for analyses at E14.5, or later stages. Survivals of electroporated embryos in the G184S$G\alpha_{i2}$ knock-in mice were poor. This technical limitation was due to the factor that electroporation of the entire litter of embryos (so to cover different genotypes of embryos in a het × het mating) often induced abortion before brains could be collected for analysis, compounded by the factor that homozygous ($G\alpha_{i2}$_G184S/G184S) embryos did not survive well after electroporation.

Pictures were taken using a confocal microscope (Zeiss LSM 510 Upright two photon). The VZ/SVZ, intermediate zone (IZ), and cortical plate (CP) areas of the cortex were defined by the Hoechst nuclear stain revealed cytoarchitectural demarcations. Center coronal sections along the anterior-posterior axis of the injected region in individual brains were used for quantification. Quantification was done using Image–Pro Plus 5.1 program (Media Cybernetics, Inc., Crofton, MD, http://www.mediacy.com) and was shown as mean ± SD. Multiple brain samples (ranging from 6 to 11 brains) were used for analyses in each individual electroporation experiment.

Acutely Dissociated Cell Culture and Immunocytochemistry

E15.5 cortices isolated from the electroporated embryos or from wild-type and mutant G184S$G\alpha_{i2}$ knock-in mice were dissociated in Hank's balanced salt solution (HBSS) and washed twice with HBSS. Cell pellets were resuspended in D-MEM/F12 medium supplemented with B27 (1:50 v/v), penicillin (100 units/ml) and streptomycin (100 μg/ml), counted, plated (5×10^5 cells/well) onto poly-D-lysine-coated coverslips placed in a 24-well plate, and cultured at 37°C. Two hours after incubation, cells were fixed with 4% paraformaldehyde and processed for immunocytochemistry of cellular markers.

G184S$G\alpha_{i2}$ Knock-in Mice Analysis

G184S$G\alpha_{i2}$ knock-in mice were reported previously [16]. Progenitor cell cycle exit and BrdU labeling were analyzed essentially as described previously [15]. In brief, to obtain BrdU labeling and progenitor cell cycle exit index, pregnant female mice were labeled with BrdU (50 mg/kg) for 30 minutes and 24 hours, respectively. For BrdU birth dating, E12.5 and E15.5 pregnant female mice were labeled with BrdU (100 mg/kg) and the labeled mice were sacrificed at postnatal day 0 (P0) for analysis. Cryosections of the brains (12–14 μm) were processed for BrdU, Ki67 Tbr2, Sox5, or Cux1 staining, and images were taken using a confocal microscope (Zeiss LSM 510 Upright two photon). For BrdU labeling, Pax6, and Tbr2 quantification, $BrdU^+$, $Pax6^+$, or $Tbr2^+$ cells were counted against the total cells stained by propidium iodide in ×40 optical view. For progenitor cell cycle exit index, $BrdU^+Ki67^-$ cells (cells exiting cell cycle) were counted against the total $BrdU^+$ cells in ×40 optical view. For late born neuron analyses at P0, the numbers of BrdU positive cells within the upper layer in ×20 optical view were quantified. Measurement of the thickness of Cux1 positive cell band was quantified using comparable sections (coronal sections at the similar locations along the anterior-posterior axis) of G184S/G184S homozygous brains or wild-type littermate brains. Quantifications were shown as mean ± SD.

Results

Gα Activation Counteracts the RGS-Mediated Function of Ephrin-B in Neural Progenitors

We started by testing the requirement for GAP activity in the RGS-dependent ephrin-B function [15]. Mutation of a conserved asparagine in the RGS domain is known to result in an RGS protein that lacks GAP activity. We made a mutant PDZ-regulator of G-protein signaling 3 (RGS3) (PDZ-RGS3N871D) which carries the corresponding asparagine to aspartate mutation. We previously found that coexpression of ephrin-B1 and PDZ-RGS3 in the cortex could prevent neural progenitor cell differentiation resulting in the retention of many affected progenitors in the area around the subventricular zone (SVZ) (Fig. 1B) [15]. When ephrin-B1 is coexpressed with PDZ-RGS3N871D, neural progenitor cells appeared to be not affected and were mostly translocated into the CP via outward migration (Fig. 1C), similar to control cells expressing EGFP alone (Fig. 1A). This result indicates

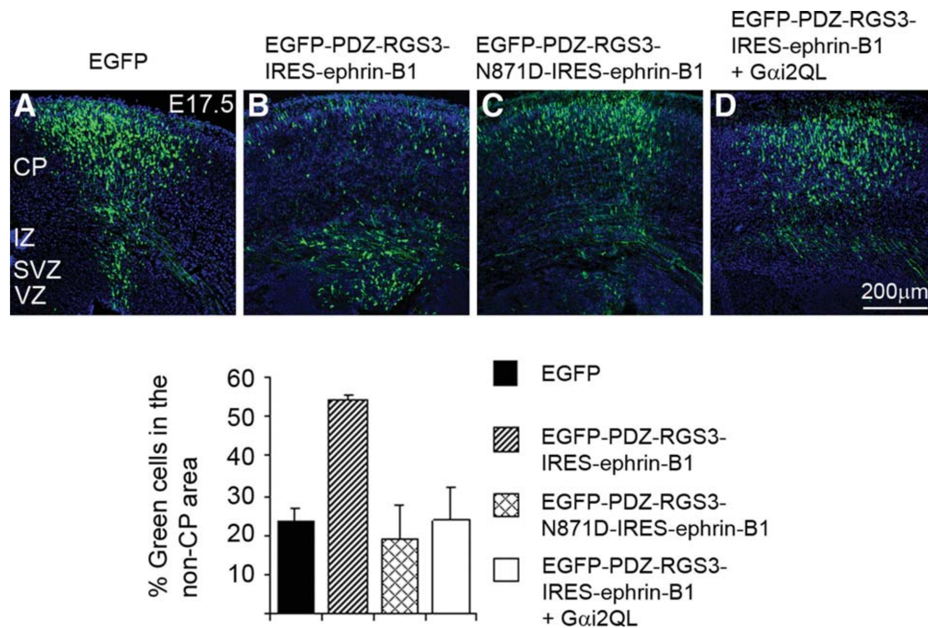

Figure 1. GTPase-activating protein activity of the regulator of G protein signaling domain is required for the function of ephrin-B in cortical neural progenitor cells. Combinations of different DNA constructs of ephrin-B, PDZ-RGS3, and Gα were electroporated into the cortex at E13.5. Brains were analyzed at E17.5 to visualize the affected cells. The graph shows the percentage of green cells in the non-CP area (VZ, SVZ, and IZ) among the total green cells through the entire thickness of the cortex. The VZ/SVZ, IZ, and CP were outlined based on the Hoechst stain revealed structural marks (examples shown in Fig. 3C). Error bars show SD. Abbreviations: CP, cortical plate; EGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

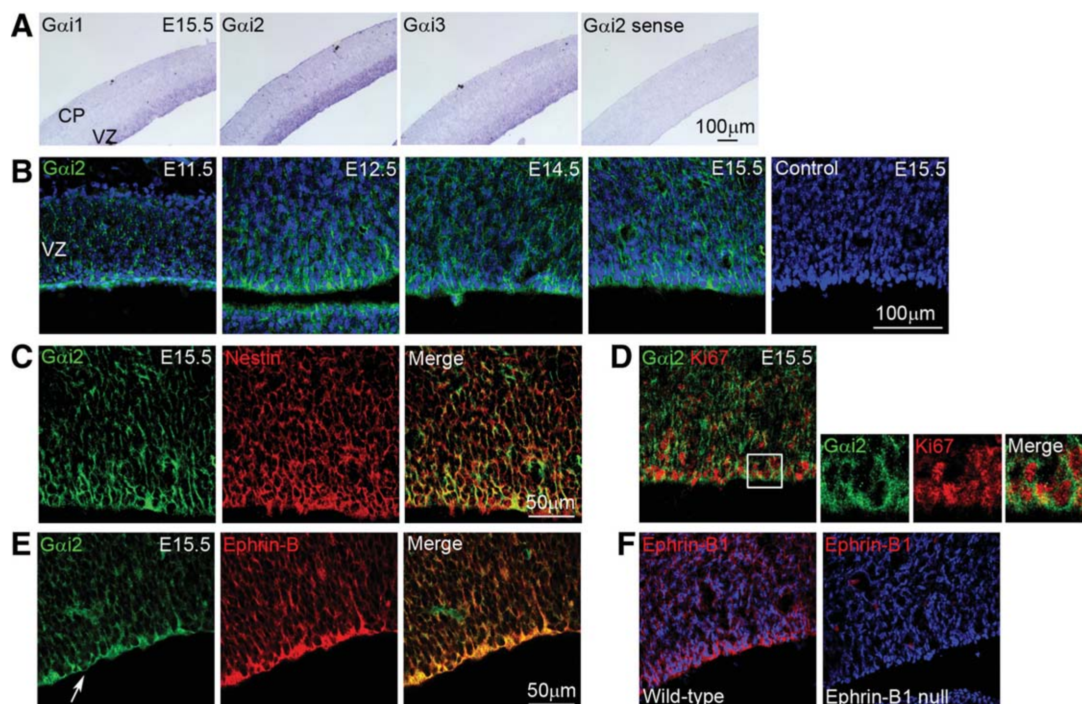

Figure 2. Multiple $G\alpha_i$ subunits are expressed in the cortical ventricular zone. **(A):** RNA in situ hybridization in the mouse developing cortex. **(B):** Immunohistochemistry of $G\alpha_{i2}$ at various stages of corticogenesis. The control shows staining without primary antibody. (C, D, **E**): Coexpression of $G\alpha_{i2}$ with nestin **(C)**, Ki67 **(D)**, and ephrin-B1 **(E)**. The arrow indicates the apical surface of the VZ. **(F):** Antiephrin-B1 staining on wild-type and ephrin-B1 null mutant cortices. Abbreviations: CP, cortical plate; VZ, ventricular zone.

that the GAP activity of PDZ-RGS3 is essential for the RGS-dependent ephrin-B function in the cortex, suggesting that a G protein is a likely signaling molecule working in cooperation with ephrin-B. To test this possibility further, we next coexpressed ephrin-B1 and PDZ-RGS3 with $G\alpha_{i2}$QL, a constitutively active form of the Gα subunit of the heterotrimeric G proteins. Expression of $G\alpha_{i2}$QL countered the action of ephrin-B1 and PDZ-RGS3, most cells were present in the CP (Fig. 1D), indicating that active Gα can antagonize ephrin-B function in cortical neural progenitor cells.

Multiple Gα Subunits Are Expressed by Cortical Neural Progenitors

To determine which Gα subunits are expressed during the period of cortical neurogenesis, we examined expression patterns of the $G\alpha_i$ subfamily of Gα subunits. The $G\alpha_i$ subfamily has been implicated by genetic studies in *C. elegans* and *Drosophila* to play an essential role in asymmetric cell division [8, 9], and they are known to interact with the RGS domain of RGS3 [13, 14]. RNA in situ analyses showed that all three

known $G\alpha_i$ subunits ($G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$) were preferentially expressed in the cortical VZ (Fig. 2A), with $G\alpha_{i2}$ expression most prominently detected. We further looked at protein expression of $G\alpha_{i2}$ subunit in relation to cortical neural progenitor cells, which are abundant in the VZ. $G\alpha_{i2}$ protein expression was detected at multiple stages during corticogenesis and was more strongly present in the VZ area of the cortex (Fig. 2B). $G\alpha_{i2}$ was found to coexpress with neural progenitor markers, including nestin (Fig. 2C), Ki67 (Fig. 2D) and ephrin-B (Fig. 2E), suggesting that $G\alpha_{i2}$ is expressed in cortical neural progenitor cells. Interestingly, $G\alpha_{i2}$ and ephrin-B were both seen often enriched at the apical surface of the VZ (Fig. 2E), which harbors proteins implicated in regulating apical-basal polarity or asymmetric cell division including Par3, atypical protein kinase C (aPKC), or cdc42 [17, 18]. This apical staining for ephrin-B1 was specific as it was absent in the ephrin-B1 null mutant cortex (Fig. 2F). These expression patterns are consistent with a possible role of $G\alpha_i$ subunits in neural progenitor cells during cortical neurogenesis.

Activation of Gα Signaling in Cortical Neural Progenitors Causes Differentiation

To examine the function of Gα subunits, we tested the effect of expression of Gα in the embryonic cortex. We electroporated $G\alpha_{i2}$QL or $G\alpha_{i2}$ into the cortex at E13.5 and analyzed the transfected neural progenitor cells at E14.5 and E15.5, two peak stages of cortical neurogenesis. Expression of $G\alpha_{i2}$QL or $G\alpha_{i2}$ in cortical neural progenitor cells caused an effect similar to the inhibition of ephrin-B signaling [15]. When compared with control cells expressing EGFP, expression of $G\alpha_{i2}$QL or $G\alpha_{i2}$ induced an outward shift of the affected cells away from the apical surface of the VZ (Fig. 3A and 3C), an indication of neural progenitor differentiation. At E14.5, assessment of BrdU incorporation and expression of phospho-Histone H3 (p-H3) in the electroporated progenitors showed that expression of $G\alpha_{i2}$QL caused a reduction in the number of progenitors, reflected in both the BrdU labeling index (percentage of $BrdU^+$ S-phase cells, labeled with a short pulse of BrdU, out of the total number of green cells; Fig. 3A) and the mitotic index (percentage of $p\text{-}H3^+$ apically localized mitotic cells out of the total number of green cells; Fig. 3B), suggesting that $G\alpha_i$ signaling negatively regulates the number of neural progenitors. At E15.5, more cells expressing $G\alpha_{i2}$QL or $G\alpha_{i2}$ were found leaving the VZ and entering the IZ and the CP, compared with control cells expressing EGFP (Fig. 3C). $G\alpha_{i2}$QL expressing cells that had reached the CP were positive for doublecortin and βIII-tubulin (Fig. 3D), suggesting that the earlier appearance of these cells in the CP was a result of differentiation and subsequent outward migration rather than a premature migration of the affected neural progenitors. Consistent with the observed cell distribution, quantification of acutely dissociated cells derived from electroporated cortices showed an increase of doublecortin positive cells in $G\alpha_{i2}$QL-expressing cells (Fig. 3D). Together, these results suggest that activation of $G\alpha_i$ signaling in the cortical neural progenitor cells promotes cell cycle exit and neuronal differentiation, counteracting the function of ephrin-B. The fact that both $G\alpha_{i2}$ and $G\alpha_{i2}$QL have the same effect implies, respectively, that there is ongoing G protein activation by a receptor or a nonreceptor guanine nucleotide exchange factor and that the primary mediator is the Gα subunit and not $G\beta\gamma$.

Knock-in of an RGS Insensitive $G\alpha_i2$ Subunit Causes Early Cell Cycle Exit and Neuronal Differentiation

We next looked at a strain of RGS-insensitive G184S$G\alpha_{i2}$ knock-in mice [16] to examine the effect of a genetic mutation of Gα signaling in mice. The G184S mutation prevents RGS binding to the Gα subunit and GAP activity. The mutation does not affect other functions of the Gα subunit, such as the intrinsic GTPase activity of the G protein or its coupling to $G\beta\gamma$ subunits, receptors, or effectors (adenylyl cyclase) [19]. We reasoned that in the knock-in mice, the RGS-insensitive G184S$G\alpha_{i2}$ mutant would elicit a gain-of-function (GOF) effect of $G\alpha_{i2}$ and would prevent the ephrin-B/RGS-mediated maintenance of the neural progenitor state resulting in progenitor cell differentiation. Analysis of the cytoarchitecture in E15.5 brains of the G184S$G\alpha_{i2}$ knock-in mice revealed thinning of the cortical radial dimension in the $G\alpha_{i2}$_G184S/G184S homozygous brains, compared with their wild-type or heterozygous littermates (data not shown), suggesting a defect in neurogenesis.

To look at neural progenitor cells more directly, we examined BrdU incorporation in the cortex of the G184S$G\alpha_i$2 knock-in mice. We first examined a short pulse of BrdU incorporation (30-minutes BrdU labeling). The $G\alpha_{i2}$_G184S/G184S homozygous brains showed a decrease in the number of $BrdU^+$ cells when labeled with BrdU for a short period (Fig. 4A). Immunohistochemistry on Pax6 and Tbr2, markers of radial glia and basal progenitor [20], also showed a reduction in the number of $Pax6^+$ and $Tbr2^+$ progenitor cells (Fig. 4A). The loss of basal progenitor cells might be due to the loss of radial glial cells, as Tbr2 was regulated by Pax6 [21]. Anticleaved caspase three staining did not show obvious difference between brains of $G\alpha_{i2}$_G184S/G184S homozygote and wild-type littermate (data not shown), suggesting that the loss of neural progenitors was not likely a result of cell death.

We further performed an analysis of cell cycle exit and reentry [22], as this would be expected to more readily reveal any possible defect in the balance between self-renewal and differentiation in development, that is, an increase or a decrease in the cell cycle exit index suggests induced or reduced differentiation, respectively. Pregnant mice were labeled with BrdU for 24 hours. Cell cycle exit and re-entry were analyzed by measuring the fraction of $BrdU^+Ki67^-$ cells (cells that have exited cell cycle) of total $BrdU^+$ cells in ×40 optical view. We found that in the homozygous ($G\alpha_{i2}$_G184S/G184S) brains, more $BrdU^+Ki67^-$ cells (green cells) were present (e.g., in the area of the SVZ and the IZ where newly differentiated cells are migrating out of the VZ) compared with their wild-type littermates (Fig. 4B), indicating there were more cells exiting the cell cycle in the homozygous mutant cortices. The $BrdU^+$ cells in the region of IZ were positive for doublecortin (Fig. 4C), suggesting neuronal differentiation in the cells having exited cell cycle. This was also supported by quantification of acutely dissociated cells derived from the wild-type and homozygous brains, which showed that more $BrdU^+$ cells in the homozygous brain were positive for doublecortin (Fig. 4C). We next wanted to look at the effect of genetic GOF of $G\alpha_{i2}$ on the ephrin-B/RGS pathway. This was tried by coexpression of ephrin-B1 and PDZ-RGS3 in the G184S$G\alpha_{i2}$ knock-in mice via in utero electroporation at E13.5 and analyses of injected brains at E17.5. The recovery of electroporated embryos were poor in this test (discussed in "Materials and Methods"), however, in one surviving litter that we were able to obtain for analysis, the result showed that genetic GOF of $G\alpha_{i2}$ could also counteract the function of ephrin-B. As shown in Figure 4D, coexpression of ephrin-B1 and PDZ-RGS3 resulted in more affected cells to stay outside the CP in the wild-type embryos, but this effect of ephrin-B was inhibited in the homozygous embryo, consistent with the electroporation result shown in Figure 1. These genetic data thus indicate that GOF of $G\alpha_{i2}$ signaling causes early cell cycle exit thereby reducing the pool of self-renewing progenitor cells, a phenotype similar to what was observed in ephrin-B1 knockout mice [15] and PDZ-RGS3 knockout mice (Qiu et al., unpublished data).

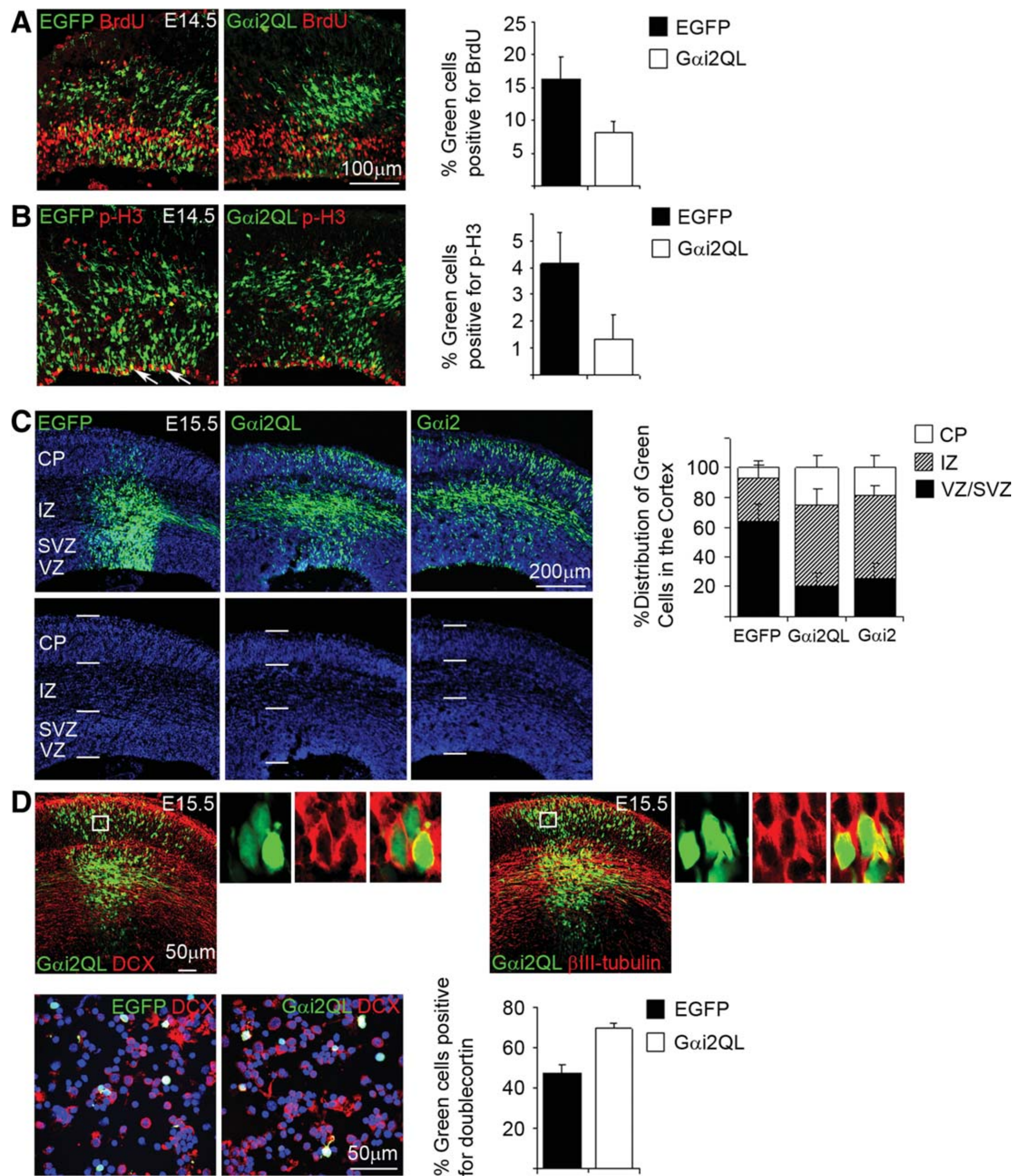

Figure 3. Activation of Gα signaling in the cortex leads to neural progenitor cell differentiation. **(A, B):** DNA construct of $G\alpha_{i2}$QL or EGFP was electroporated into the cortex at E13.5. Brains were analyzed at E14.5 and immunostained for BrdU (pulse-labeled for 2 hours) or phospho-Histone H3 (p-H3). The percentage of $BrdU^{+}$ or apical p-$H3^{+}$ cells (indicated by arrows) in the total number of green cells was determined. **(C):** DNA construct of $G\alpha_{i2}$QL or $G\alpha_{i2}$ was electroporated into the cortex at E13.5. Brains were analyzed at E15.5 to visualize the affected cells. The distributions of green cells in VZ/SVZ, IZ, and CP were determined and shown in the graph. The VZ/SVZ, IZ, and CP areas of the cortex were defined by the Hoechst revealed cytoarchitectural demarcations. **(D):** Immunohistochemistry of doublecortin and βIII-tubulin on electroporated brain sections (upper panels) or acutely dissociated cortical cells derived from electroporated cortices (bottom panels). Quantification from acutely dissociated cells shows more doublecortin positive cells in $G\alpha_{i2}$QL-expressing cells. Error bars show SD. Abbreviations: BrdU, bromodeoxyuridine; CP, cortical plate; EGFP, enhanced green fluorescent protein; EGFP-PDZ-RGS3, enhanced green fluorescent protein-PDZ-regulator of G-protein signaling 3; IRES, internal ribosome entry site; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

Knock-in of G184S$G\alpha_{i2}$ Subunit Causes a Defect in the Generation of Late Born Cortical Neurons

We next looked at cortical lamination and neuron generation in G184S$G\alpha_{i2}$ knock-in mice at birth, when embryonic cortical neurogenesis approached an end. To examine cortical lamination, we labeled littermates with BrdU at E12.5 or E15.5 and looked at the distribution of BrdU positive cells in the cortices at P0. As shown in Figure 5A, E12.5- and E15.5-labeled BrdU positive cells were concentrated in the deep layers VI and V, and the upper layers II–IV, respectively, suggesting that cortical lamination in mutant cortices were comparable to wild-type littermates. However, in the homozygous mutant cortices, there were fewer BrdU-labeled neurons in the upper layers, suggesting a defect in the generation of late born neurons. To confirm that this was not simply due to a variation in BrdU labeling efficiency among different littermates, we further examined the deep and upper layer neurons that were

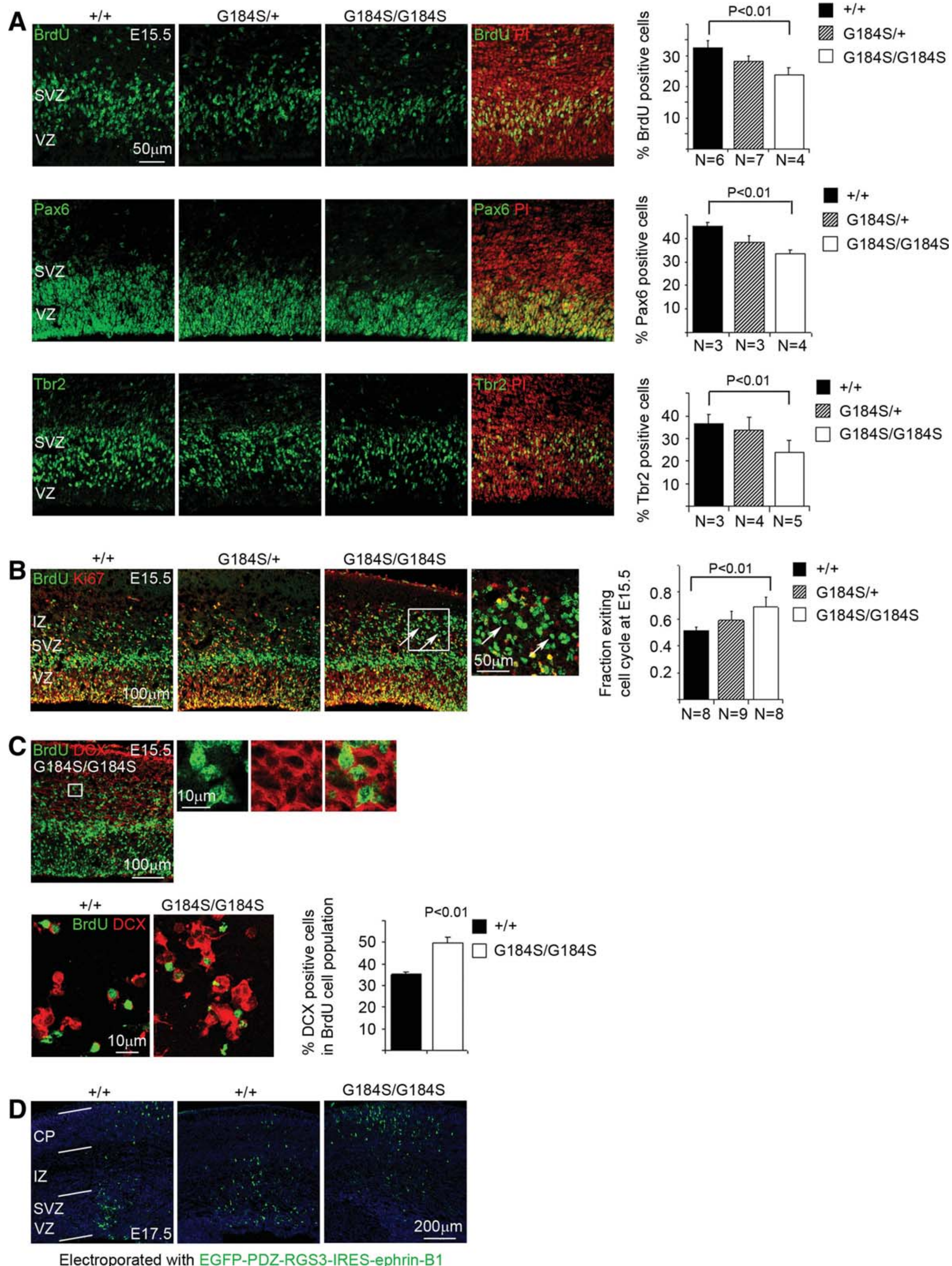

Figure 4. Genetic gain-of-function of Gα signaling leads to cell cycle exit and neuronal differentiation in cortical neural progenitors. Images show brain sections from littermates. **(A):** BrdU (30-minutes labeling), Pax6, and Tbr2 staining in the cortex. Quantification shows the percentage of $BrdU^+$, $Pax6^+$, or $Tbr2^+$ cells in the total number of cells/×40 optical view. **(B):** Cell cycle exit analysis—fraction of progenitor cells exiting cell cycle ($BrdU^+Ki67^-$ cells, indicated by arrows) in the population of $BrdU^+$ cells in brains labeled with BrdU for 24 hours. **(C):** BrdU and doublecortin costaining on E15.5 mutant cortex (labeled with BrdU for 24 hours; upper panels), and quantification of doublecortin positive BrdU-labeled cells in acutely dissociated cultures (bottom panels). **(D):** Coexpression of ephrin-B1 and PDZ-RGS3 in wild-type embryos results in more cells to stay outside the cortical plate, but this effect is inhibited in the G184S/G184S homozygous littermate embryo. Error bars show SD. Abbreviations: BrdU, bromodeoxyuridine; CP, cortical plate; DCX, doublecortin; EGFP-PDZ-RGS3, enhanced green fluorescent protein-PDZ-regulator of G-protein signaling 3; IRES, internal ribosome entry site; IZ, intermediate zone; Pax6, paired box 6; SVZ, subventricular zone; Tbr2, t box brain 2; VZ, ventricular zone.

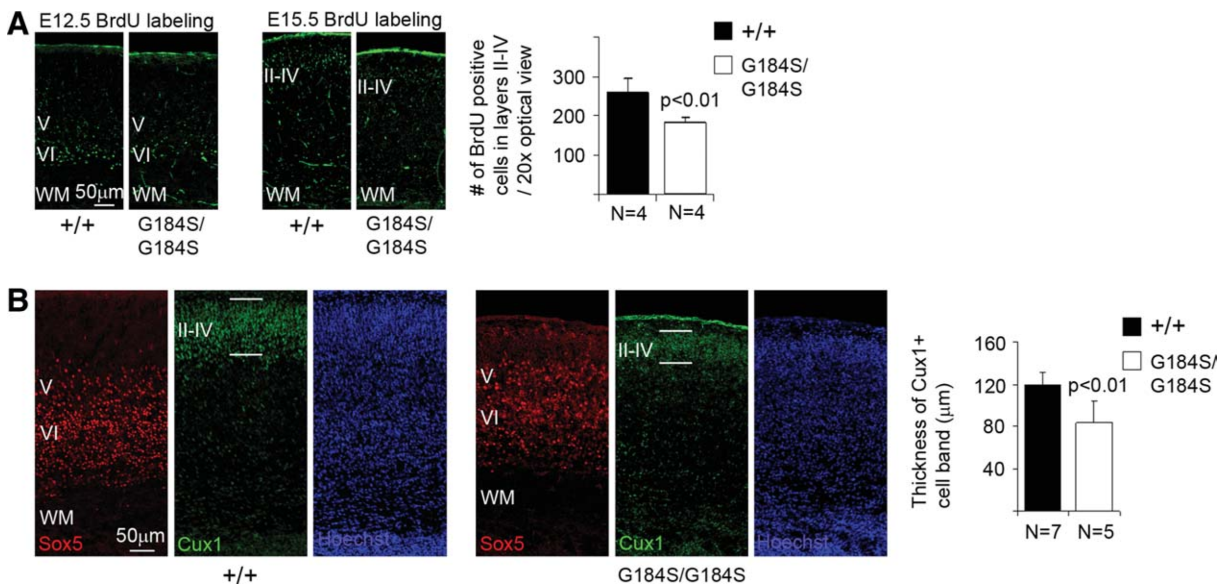

Figure 5. Defect in the production of late born neurons in G184S/G184S homozygous mutant cortices. **(A):** Cortical lamination in homozygous mutant mice appeared normal based on BrdU birth dating observed at P0, but production of late born neurons (revealed by BrdU labeling at E15.5) was reduced compared with the wild-type littermates. **(B):** Fewer late born neurons ($Cux1^{+}$) were present in the mutant cortices, as reflected in the reduction of the thickness of the upper cortical layers (II–IV). Abbreviations: BrdU, bromodeoxyuridine; WM, white matter; Sox5, SRY (sex determining region Y)-box 5; Cux1, cut-like homeobox 1.

marked by the expression of Sox5 and Cux1, respectively. We found that in homozygous mutant cortices, there was a consistent reduction in the thickness of the $Cux1^{+}$ cell band compared with the wild-type littermate brains (Fig. 5B), while the difference in the $Sox5^{+}$ cell band was more subtle. These results suggest that the production of late born cortical neurons were affected due to activation of $G\alpha_{i2}$ signaling.

Discussion

Taken together, our results from electroporation and genetic analyses reveal a critical role of Gα subunits in mammalian neurogenesis. On the basis of the data obtained here and our study of the ephrin-B signaling pathway, we propose a working model for how Gα and ephrin-B signaling pathways function in coordination in neural progenitor cells (Fig. 6): The balance between self-renewal and differentiation is expected to be determined by equilibriums of molecular interactions within neural progenitor cells. Our data suggest this can be achieved, at least in part, by the balance of two intricately linked and opposing signaling pathways [23]. The ephrin-B signaling pathway, mediated by PDZ-RGS3 [15] and zinc fingers and homeoboxes 2 (ZHX2) [28], promotes neural progenitor maintenance, the Gα signaling pathway encourages differentiation, and the two are linked by the RGS. As both ephrin-B/RGS and $G\alpha_i$ subunits are specifically enriched in the cortical VZ, the antagonistic effect of the two pathways on self-renewal and differentiation suggest that they likely act directly as a switch mechanism. This raises an interesting question as how $G\alpha_i$ subunits, after initiating differentiation, keep driving neural progenitors to move further along the differentiation path. We speculate that $G\alpha_i$ subunits may continue in some way to function in maintaining the differentiation state, either by acting directly in newborn neurons (RNA in situ or immunohistochemistry we performed here might not sensitive enough to detect

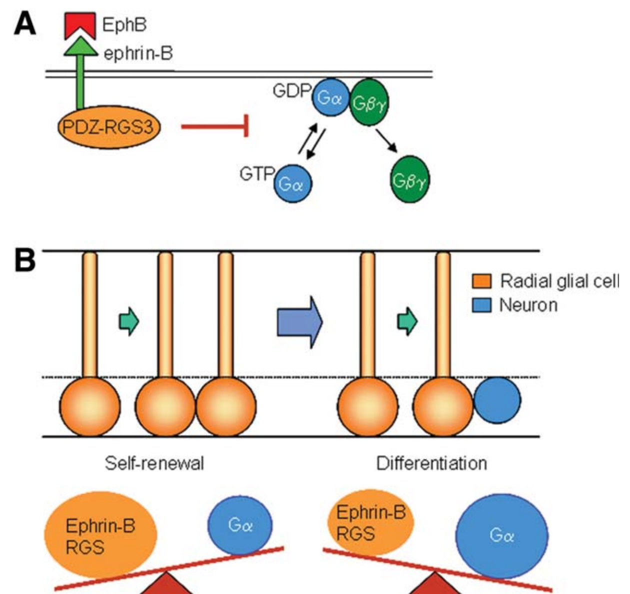

Figure 6. A model of ephrin-B and Gα pathways in balancing self-renewal and differentiation. **(A):** A molecular pathway of ephrin-B and Gα interaction: PDZ-RGS3 binds to the cytoplasmic domain of ephrin-B and links ephrin-B to the modulation of Gα subunit [23]. **(B):** Our results from ephrin-B and Gα suggest that the balance between these two signaling pathways helps maintain a balance between self-renewal and differentiation and regulates the developmental progression of neural progenitor cells from self-renewal to neurogenesis in the developing cerebral cortex. The cartoon of radial glia cell (RGC) symmetric and asymmetric cell divisions was modified from Fishell and Kriegstein [24]. Neurogenesis of RGCs takes both a direct (neuron generation) and an indirect (basal progenitor generation) route [25–27]. Whether ephrin-B and $G\alpha_i$ signaling pathways can act through both neurogenesis schemes requires further study. Abbreviations: EphB, ● ● ●; GDP, guanosine diphosphate; RGS, regulator of G protein signaling; PDZ-RGS3, PDZ-regulator of G-protein signaling 3.

continued expression in neurons), or by activating certain signaling programs or gene expressions that are responsible for the maintenance of differentiation. Another question is when the interaction between ephrin-B/RGS and $G\alpha_i$ pathways come into play in balancing self-renewal and differentiation, as ephrin-B/RGS and $G\alpha_i$ subunits were found to be expressed in the cortical VZ at the start of neurogenesis (E10-E11). In a separate study (Qiu et al., Stem Cells, 2010) [29], we found that knockout of PDZ-RGS3, which was expected to uniformly block RGS-mediated ephrin-B function, caused significant increase in the production of neurons at E11.5, suggesting that the action of ephrin-B/RGS and Gα pathways may coincide with the progression of cortical neurogenesis. In ephrin-B1 knockout mice and $G184SG\alpha_{i2}$ knock-in mice, precocious differentiation at E11.5 was much subtler than observed in PDZ-RGS3 knockout mice, likely due to a compensatory effect in ephrin-B1 knockout mice (multiple ephrin-Bs are expressed in neural progenitor cells) or due to the level of expression of $G184SG\alpha_{i2}$ (relative to other $G\alpha_i$ subunits) in $G184SG\alpha_{i2}$ knock-in mice. Although more studies are required to define the precise mechanisms of ephrin-B and Gα subunit in self-renewal versus differentiation, it is conceivable, based on the data from *Drosophila* and *C. elegans* [8–12], that the two pathways exert their functions via regulating neural progenitor symmetric and asymmetric cell division. In this regard, it is interesting to note that the mammalian $G\alpha_i$ protein can be subcellularly localized to structures critical for cell division, such as centrosomes and midbody [30].

The decision of cortical neural progenitor cells to either self-renew or differentiate is known to be influenced by multiple signaling pathways, such as growth factor pathways [epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF)], Notch and Wnt pathways, and their downstream transcription factors. Here, we identify the ephrin-B and Gα subunit pathways as an important axis in the control of neural progenitor cell homeostasis during cortical neurogenesis. The list of pathways is expected to keep going up with new factors important either for self-renewal or for differentiation being identified in future studies. An important question then is how these multiple pathways coordinately guide neural progenitor cells during development through the progression from progenitor expansion to neurogenesis and move further to switch to gliogenesis. Redundancy of multiple pathways in controlling self-renewal and differentiation may be critical for keeping neural progenitor/stem cells in check, as dysregulation of this process is expected to lead to abnormal brain development or perhaps even tumorigenesis. On the other hand, crosstalk among different pathways may be able to impose positive or negative feedback regulations to better respond to the environmental cues or provide a level of plasticity in regulation. In this regard, it is interesting to note the interaction between the Wnt and FGF pathways and the ephrin-B pathway in several progenitor/stem cell systems. In small intestine stem cells, the canonical Wnt signaling pathway-mediated transcriptional regulation controls the expression of ephrin-B1 [31]. In xenopus eye field, disheveled can mediate ephrinB1 signaling in the progenitors through the planar cell polarity pathway [32] while the interaction between FGF and ephrin-B signaling pathways are important for the proper formation of the eye field [33]. It will be interesting to further investigate how such crosstalk between multiple signaling pathways can impact on the homeostasis of neural progenitor cells in the context of cortical development.

A previous study [34] showed that overexpression of $G\alpha_{i3}$, but not $G\alpha_{i3}QL$, could cause changes in mitotic spindle orientations, indicating that activation of Gα signaling was not involved in the regulation of self-renewal versus differentiation. In our study, we consistently observed that both $G\alpha_{i2}$ and $G\alpha_{i2}QL$ could cause differentiation and subsequent outward migration of the affected neural progenitor cells. One possible explanation for this discrepancy might be that two different $G\alpha_i$ subtypes were used in the electroporation-mediated analyses. $G\alpha_{i3}$ (in the previous study) and $G\alpha_{i2}$ (in this study) could have distinct functions making them behave differently in the cortex. An alternative explanation for the contradictory results would be that changes of mitotic spindle orientation, which were scored in the previous study, may not serve as a reliable functional criterion for predicting the status of neural progenitor cell fate. Several recent studies [35–38] have found that the predominant mitotic spindle orientations in cortical neural progenitors are planar divisions throughout the entire period of cortical neurogenesis, suggesting that mitotic spindle orientation is not tightly linked to cell fate choice in the mammalian brain.

Conclusion

In conclusion, our data identify $G\alpha_i$ subunits and ephrin-B signaling pathways as an important axis in neural progenitor cells for balancing self-renewal and differentiation during brain development.

Acknowledgments

We thank Donna Isbell, Armando Amaya, Kelley Carpenter, Jeremy Ladou, Pauline Heath, and Raelene Charbeneau for assistance with animal breeding and care; Mariko Lee and Brian Armstrong for helping with confocal imaging. We also thank the Developmental Studies Hybridoma Bank. This work was supported by NIH Grant NS52388 (to Q.L.) and NIH Grant GM39561 (to R.R.N).

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

References

1 McConnell SK. Constructing the cerebral cortex: Neurogenesis and fate determination. Neuron 1995;15:761–768.
2 Temple S. The development of neural stem cells. Nature 2001;414: 112–117.
3 Molyneaux BJ, Arlotta P, Menezes JR et al. Neuronal subtype specification in the cerebral cortex. Nat Rev 2007;8:427–437.
4 Pontious A, Kowalczyk T, Englund C et al. Role of intermediate progenitor cells in cerebral cortex development. Dev Neurosci 2008;30: 24–32.
5 Rakic P. Evolution of the neocortex: A perspective from developmental biology. Nat Rev 2009;10:724–735.
6 Gotz M, Huttner WB. The cell biology of neurogenesis. Nat Rev Mol Cell Biol 2005;6:777–788.
7 Kriegstein A, Alvarez-Buylla A. The glial nature of embryonic and adult neural stem cells. Annu Rev Neurosci 2009;32:149–184.

8 Wilkie TM, Kinch L. New roles for Galpha and RGS proteins: Communication continues despite pulling sisters apart. Curr Biol 2005;15: R843–R854.
9 Yu F, Kuo CT, Jan YN. Drosophila neuroblast asymmetric cell division: Recent advances and implications for stem cell biology. Neuron 2006;51:13–20.
10 Doe CQ. Neural stem cells: Balancing self-renewal with differentiation. Development (Cambridge, England) 2008;135:1575–1587.
11 Knoblich JA. Mechanisms of asymmetric stem cell division. Cell 2008;132:583–597.
12 Zhong W, Chia W. Neurogenesis and asymmetric cell division. Curr Opin Neurobiol 2008;18:4–11.
13 Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: Regulators of G protein signaling (RGS) and RGS-like proteins. Annu Rev Biochem 2000;69:795–827.
14 Siderovski DP, Willard FS. The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits. Int J Biol Sci 2005;1:51–66.
15 Qiu R, Wang X, Davy A et al. Regulation of neural progenitor cell state by ephrin-B. J Cell Biol 2008;181:973–983.
16 Huang X, Fu Y, Charbeneau RA et al. Pleiotropic phenotype of a genomic knock-in of an RGS-insensitive G184S Gnai2 allele. Mol Cell Biol 2006;26:6870–6879.
17 Lin D, Edwards AS, Fawcett JP et al. A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. Nature Cell Biol 2000;2:540–547.
18 Cappello S, Attardo A, Wu X et al. The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. Nat Neurosci 2006;9: 1099–1107.
19 Fu Y, Zhong H, Nanamori M et al. RGS-insensitive G-protein mutations to study the role of endogenous RGS proteins. Methods Enzymol 2004;389:229–243.
20 Englund C, Fink A, Lau C et al. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 2005; 25:247–251.
21 Sansom SN, Griffiths DS, Faedo A et al. The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet 2009;5:e1000511.
22 Chenn A, Walsh CA. Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. Science (New York, NY) 2002; 297:365–369.
23 Lu Q, Sun EE, Klein RS et al. Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. Cell 2001;105:69–79.
24 Fishell G, Kriegstein AR. Neurons from radial glia: The consequences of asymmetric inheritance. Curr Opin Neurobiol 2003;13:34–41.
25 Haubensak W, Attardo A, Denk W et al. Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: A major site of neurogenesis. Proc Natl Acad Sci USA 2004;101:3196–3201.
26 Miyata T, Kawaguchi A, Saito K et al. Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development (Cambridge, England) 2004;131:3133–3145.
27 Noctor SC, Martinez-Cerdeno V, Ivic L et al. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci 2004;7:136–144.
28 Wu C, Qiu R, Wang J et al. ZHX2 Interacts with Ephrin-B and regulates neural progenitor maintenance in the developing cerebral cortex. J Neurosci 2009;29:7404–7412.
29 Qiu R, Wang J, Tsark W, Lu Q. Essential role of PDZ-RGS3 in the maintenance of neural progenitor cells. Stem Cells 2010;28: 1602–1610.
30 Cho H, Kehrl JH. Localization of Gi alpha proteins in the centrosomes and at the midbody: Implication for their role in cell division. J Cell Biol 2007;178:245–255.
31 Batlle E, Henderson JT, Beghtel H et al. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 2002;111:251–263.
32 Lee HS, Bong YS, Moore KB et al. Dishevelled mediates ephrinB1 signalling in the eye field through the planar cell polarity pathway. Nat Cell Biol 2006;8:55–63.
33 Moore KB, Mood K, Daar IO et al. Morphogenetic movements underlying eye field formation require interactions between the FGF and ephrinB1 signaling pathways. Dev Cell 2004;6:55–67.
34 Sanada K, Tsai LH. G protein betagamma subunits and AGS3 control spindle orientation and asymmetric cell fate of cerebral cortical progenitors. Cell 2005;122:119–131.
35 Morin X, Jaouen F, Durbec P. Control of planar divisions by the G-protein regulator LGN maintains progenitors in the chick neuroepithelium. Nat Neurosci 2007;10:1440–1448.
36 Noctor SC, Martinez-Cerdeno V, Kriegstein AR. Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. J Comp Neurol 2008;508:28–44.
37 Konno D, Shioi G, Shitamukai A et al. Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. Nat Cell Biol 2008;10: 93–101.
38 Stricker SH, Meiri K, Gotz M. P-GAP-43 is enriched in horizontal cell divisions throughout rat cortical development. Cereb Cortex 2006; 16(suppl 1):i121–i131.