The G protein-coupled receptor GPRC5B contributes to neurogenesis in the developing mouse neocortex

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SUMMARY

Neural progenitor cells in the developing brain give rise to neurons and glia. Multiple extrinsic signalling molecules and their cognate membrane receptors have been identified to control neural progenitor fate. However, a role for G protein-coupled receptors in cell fate decisions in the brain remains largely putative. Here we show that GPRC5B, which encodes an orphan G protein-coupled receptor, is present in the ventricular surface of cortical progenitors in the mouse developing neocortex and is required for their neuronal differentiation. GPRC5B-depleted progenitors fail to adopt a neuronal fate and ultimately become astrocytes. Furthermore, GPRC5B-mediated signalling is associated with the proper regulation of β-catenin signalling, a pathway crucial for progenitor fate decision. Our study uncovers G protein-coupled receptor signalling in the neuronal fate determination of cortical progenitors.

KEY WORDS: G protein-coupled receptor, Developing neocortex, Neurogenesis

INTRODUCTION

During development of the mammalian neocortex, cortical progenitor cells located in the ventricular zone generate both neurons and glia. Prior to the neurogenic phase, most progenitor cells self-renew to expand the progenitor pool. As corticogenesis proceeds, progenitor cells predominantly divide asymmetrically to self-renew and to produce either a neuron or an intermediate progenitor cell that undergoes additional symmetric divisions to generate neurons (Chenn and McConnell, 1995; Miyata et al., 2001; Noctor et al., 2001; Noctor et al., 2004; Miyata et al., 2004). The neurogenic phase is followed by the gliogenic phase during which cortical progenitors give rise to glial progeny (Schmechel and Rakic, 1979; Voigt, 1989; Malatesta et al., 2000; Marshall and Goldman, 2002; Noctor et al., 2004). The precise control of progenitor cell fate is key in yielding the correct number of neurons and glia during corticogenesis.

Fate decision instructed in cortical progenitor cells involves the action of both environmental and intrinsic cues (Edlund and Jessell, 1999; Qian et al., 2000). Recently, many environmental signalling molecules (e.g. Notch ligands, Wnts, fibroblast growth factor, platelet-derived growth factor and cytokines) and their cognate receptors have been identified to affect cell fate during corticogenesis (reviewed by Guillemot, 2007). Importantly, these signalling pathways often cross-talk and their integration determines proper cell fate. Thus, uncovering novel signalling pathways that affect progenitor cell fate constitutes an important step in our understanding of the complex mechanisms underlying neurogenesis.

G protein-coupled receptors (GPCRs) constitute the largest family of membrane receptors. Most GPCRs transduce extracellular signals into cells via activation of cognate heterotrimeric G proteins. GPCR/G-protein-mediated signalling cascades in neural development are now being uncovered. For instance, perturbations of G proteins and non-receptor type regulators for G proteins have been shown to affect the biology of neural progenitors in the developing neocortex (Shinohara et al., 2004; Sanada and Tsai, 2005; Konno et al., 2008; Postiglione et al., 2011). Moreover, mutations in GPR56, which encodes an orphan GPCR, are associated with a human brain malformation called bilateral frontoparietal polymicrogyria (Piao et al., 2004; Li et al., 2008). Also, exogenous lysophosphatidic acid (LPA) exposure in an ex vivo cerebral cortical explant results in decreased apoptosis, an increased number of mal-positioned M-phase cells, an increased number of neurons and eventually enhanced growth of the explant through LPA₁ and LPA₂ GPCRs (Kingsbury et al., 2003; Kingsbury et al., 2004). These observations implicate GPCR signalling in multiple aspects of neural progenitor biology. Although many major membrane receptor-mediated signalling pathways have been implicated in progenitor cell fate decision, the participation of GPCRs in this process remains unclear.

In the present study, we found that GPRC5B, an orphan GPCR, is predominantly expressed in neural progenitors in the developing mouse brain and that GPRC5B-depleted progenitors fail to adopt a neuronal fate. Further, GPRC5B-mediated signalling is coupled to the heterotrimeric G proteins G12/13 and affects β-catenin signalling, which is important for the neuronal differentiation of progenitors during the neurogenic phase. Together, the present study identified a GPCR that is required for neurogenic potential in neural progenitors and provided evidence that GPCR-mediated signalling, through cross-talk with β-catenin signalling pathways, contributes to the neuronal differentiation of neural progenitors.

MATERIALS AND METHODS

Plasmids and in situ hybridisation

The plasmid expressing GFP under the control of the CAG promoter (pCAGiG) was a kind gift from Takahiko Matsuda (Kyoto University, Kyoto, Japan). Plasmids encoding pertussis toxin (PTX), Gα12QL, Gα-CTs and TOP6GFP-CAGmCherry were generous gifts from Randall T. Moon (University of Washington, WA, USA), Hiroshi Itoh (Nara Institute of Science and Technology, Nara, Japan), Yoshihiko Take (Kanazawa University, Ishikawa, Japan) and Anjen Chenn (University of Illinois, IL, USA), respectively. pBS/U6 plasmid and mouse Gprc5b subcloned into the
pCDNA3 plasmid were generously provided by Yang Shi (Harvard Medical School, MA, USA) and Yuko Harada (Kobe University, Kobe, Japan), respectively.

The full-length open reading frame of mouse Gprc5b was subcloned into a pCAGEN plasmid (a kind gift from Takahiko Matsuoka) that directs subcellon gene expression from the CAG promoter. A plasmid encoding a silent mutant of GPRC5B was generated by the QuikChange mutagenesis technique using primers 5'-GGATGGCGTTTGATTTTCTCAGCATTCC-3' (forward) and 5’-GGAGTTGCACTGGAAATCACAAG-ACCACATCC-3' (reverse). Gprc5b subcloned into the pHBluescript II KS(-) vector was used for generating RNA probes, and in situ hybridisation was carried out as described (Asada et al., 2007). Plasmids encoding GPRC5B shRNA were generated by inserting the annealed oligonucleotides into a pBS/U6 plasmid (Su et al., 2002). Oligonucleotides were as follows: GPRC5B shRNA, 5’-GGCTTTTGCATCTTCCATGAAGCTTCATGGAAGACATCAGGACCCTTTTTG-3’ and 5’-AAATCTAAAGGGTCTTGTGATCCTTCCATGAGCTTGCAAAAGACC-3’; GPRC5B shRNA2, 5’-GGCTTTTCCATGGAAGCTTCATGGAAGACATCAGGACCCTTTTTG-3’ and 5’-AATCTAAAGGGTCTTGTGATCCTTCCATGAGCTTGCAAAAGACC-3’. GPRC5B shRNA#2, 5’-GGATGGCGTTTGATTTTCTCAGCATTCC-3’.

In utero electroporation

In utero electroporation was performed as described previously (Sanada and Tsai, 2005). Concentrations of plasmids were: GFP-expressing plasmid (pCAGIG: 5 μg/μl), GPRC5B shRNA plasmid (5 μg/μl), plasmid expressing silent mutant GPRC5B (1.5 μg/μl), control shRNA plasmid (pBS/U6: 5 μg/μl) and TOPdGFP-CAGmCherry (5 μg/μl). Animal experiments were conducted in accordance with guidelines set by The University of Tokyo.

GPRC5B antibody production and immunostaining

Antibody against GPRC5B was generated by Sigma Genosis as described below. Synthetic peptides corresponding to amino acids Gly395-Trp410 of GPRC5B were conjugated to keyhole limpet haemocyanin and used to immunize rabbits. GPRC5B antibody was affinity-purified from the serum by a column coupled with synthetic peptides Gly395-Trp410 (for western blotting and immunostaining of cultured cells) or Pro401-Trp410 (for immunohistochemistry of brain sections).

Brains were fixed with 4% paraformaldehyde in PBS for 30 minutes (for E14-18 brains) or 2 hours (for postnatal brains) at room temperature. Thick cryosections (20 μm for E15-18 brains; 30 μm for postnatal brains) were prepared. For immunostaining of postnatal day (P) 14 brain sections with anti-GPRC5B, brain sections (40 μm) were prepared with a vibratome. For NeuN (RbFOX3 – Mouse Genome Informatics) staining, brain sections were pretreated with a Tris/EDTA solution (10 mM Tris, 1 mM EDTA, pH 7.4) for 15 minutes, and blocked with 3% BSA/0.1% Triton X-100 in PBS, followed by immunostaining.

Cell culture, transfection and cell-based analyses

HEK293T cells on sterile coverslips in the wells of 24-well plates were transiently transfected using Lipofectamine 2000 (Invitrogen). For analysis of the subcellular distribution of β-catenin in Vero cells, cells on sterile coverslips in the wells of 24-well plates were transiently transfected with plasmids encoding GFP (pEFGP-C1, Clontech; 0.1 μg/well), GPRC5B/pCDNA3 (0.3 μg/well) and GnaI2QL (0.025 μg/well) using FuGENE 6 (Roche). Cultures were fixed 48 hours later and subjected to immunostaining. For analysis of the subcellular distribution of β-catenin in cortical progenitor cells, E14 embryos were electroporated with plasmids encoding GFP (pCAGIG; 5 μg/μl), GPRC5B (5 μg/μl), and GnaI2CT (10 μg/μl). Following electroporation, neocortical cells were prepared (Sanada and Tsai, 2005), plated at a density of ~2.0×10³ cells on sterile coverslips precoated with poly-ornithine and fibronectin in wells of 24-well plates and maintained in DMEM/F12 supplemented with N2, B21 and bFGF (FGF2) (20 ng/ml). Neocortical cells were fixed at 2 days in vitro (DIV2) with 2% paraformaldehyde in PBS for 20 minutes at 37°C after 4 hours of treatment with MG132 (25 μM), permeabilised with 0.5% Triton X-100 in PBS for 15 minutes, and blocked with 3% BSA/0.1% Triton X-100 in PBS, followed by immunostaining.

For analysis of the identity of GPRC5B-depleted cells in vitro (Fig. 4), neocortical cells were prepared by incubating neocortices with pankin in Hank’s balanced salt solution, plated at a density of 0.8×10⁶ cells on sterile coverslips precoated with poly-lysine in wells of 24-well plates, and maintained in DMEM/F12 supplemented with N2, B27 and 10% FBS. At DIV6, cultures were fixed and subjected to immunostaining.

RT-PCR

Total RNA (1 μg) was reverse-transcribed with SuperScript II (Invitrogen) using oligo(dT)₁₆ primer. cDNAs were then subjected to PCR analysis (35 cycles) using the primers listed in supplemental material Table S1.

β-catenin activity assay

E14 embryos were electroporated with a dual reporter plasmid, TOPdGFP-CAGmCherry, together with various additional plasmids. Embryos were harvested 24 hours later. Brain cryosections (20 μm) were immunostained with antibodies against PAX6 and GFP. Images of GFP and mCherry in individual sections were obtained under identical parameters with a 40× objective (EC Plan-Neofluor, Zeiss) on a Zeiss LSM5 confocal microscope. Mean fluorescence intensity of mCherry and GFP in the soma of individual cells was measured using Zeiss Zen2010 software. In addition, the average background fluorescence intensity was measured in individual sections using areas with no electroporated-cells present and was subtracted from the mCherry and GFP intensity measurements. Cells with saturated signal were not used for the analysis. The ratio of fluorescence intensity of GFP to that of mCherry in individual cells was defined as the β-catenin activity index.

Statistical analysis

All data are shown as mean ± s.e.m. Statistical analyses were carried out with Excel (Microsoft). Comparison of two groups was analysed using a two-tailed t-test. *P<0.05 was considered statistically significant.

RESULTS

Expression of GPRC5B in cortical progenitors of the developing neocortex

GPRC5 receptors, such as GPRC5A, GPRC5B and GPRC5C, are orphan GPCRs. GPRC5A was initially identified by differential display analysis of a small lung carcinoma cell line and is expressed almost exclusively in the human lung (Cheng and Lotan, 1998). GPRC5B and GPRC5C are mainly expressed in the central nervous system and peripheral tissues, respectively (Robbins et al., 2000). To examine the expression of these receptors in neural progenitor cells in the developing mouse brain, PCR analysis of cDNAs derived from cultured progenitor cells, E16 cortices, adult brains and lungs was performed. In agreement with previous reports (Cheng and...
Lotan, 1998), Gprc5a was detected in the lung, but not in the brain (Fig. 1A). By contrast, Gprc5b and Gprc5c were detected in the adult brain. Importantly, Gprc5b, but not Gprc5a and Gprc5c, was expressed in E16 cortex and by cultured cortical progenitors. GPRC5B possesses a brain-specific C-terminal splice variant (GPRC5B_v2) that differs in C-terminal 14 amino acids (Cool et al., 2010). PCR analysis showed that Gprc5b_v2 was hardly detectable in the neocortex during embryonic and perinatal periods but was expressed in the adult neocortex. These results are consistent with previous reports showing that the mRNA and protein levels of GPRC5B_v2 in the brain are detected at ~P14-15 and peak at adulthood (Cool et al., 2010; Sano et al., 2011). Thus, GPRC5B, but not GPRC5B_v2, is the predominant form expressed in the developing neocortex.

In situ hybridisation analysis using an antisense probe revealed strong expression of Gprc5b mRNA at proliferative regions surrounding the ventricles of E10 and E14 mouse forebrain (Fig. 1B-D). To localise GPRC5B protein in the developing neocortex, an affinity-purified rabbit anti-GPRC5B antibody was generated using its C-terminal region as the immunogen (see Materials and methods). The antibody specifically detected GPRC5B transiently expressed in HEK293T and COS7 cells, as assessed by western blotting and immunocytochemistry, respectively (supplementary material Fig. S1; see also Fig. 6A). When E14 neocortical sections were immunostained, GPRC5B immunofluorescent signals were mainly detected at the luminal surface of the ventricular zone (VZ) (Fig. 1E). Almost no GPRC5B immunoreactivity was observed in sections immunostained with antibody preincubated with its immunogen (supplementary material Fig. S2). Detailed analyses of GFP-labelled progenitors revealed that GPRC5B immunofluorescent signals were punctate and present near the apical surface of the descending processes (Fig. 1F). In addition, these signals were detected in close proximity to cadherin, which is highly concentrated at adherens junctions formed by descending processes of neighbouring progenitors (Chenn et al., 1998). The specificity of the antibody was also confirmed in neocortical sections that were electroporated with the GPRC5B shRNA-expressing plasmid (see below). In GPRC5B shRNA cells, cadherin was normally detected near the luminal surface of the VZ, whereas GPRC5B immunofluorescent signals were strikingly diminished (Fig. 1F). Together, these results indicate that GPRC5B is preferentially expressed in cortical progenitors of the developing brain, particularly at the luminal surface of progenitors.

We also examined the localisation of GPRC5B at P14. Intense GPRC5B immunoreactivity was detected at the apical surface of cells lining the lateral ventricle, which were positive for S100β, an
antigen that is highly expressed in ependymal cells (supplementary material Fig. S3). GPRC5B was not detected in SOX2-positive cells in the subependymal zone and in cells positive for GFAP, a marker for astrocytes and subventricular zone (SVZ) progenitors (Mirzadeh et al., 2008). These data indicate the expression of GPRC5B in ependymal cells in the postnatal brain.

**GPRC5B knockdown inhibits the generation of neurons**

To investigate a putative role for GPRC5B in cortical progenitors in the developing neocortex, we examined the effects of GPRC5B knockdown on cell fate. For this purpose, DNA-based RNAi plasmids that express shRNA against *Gprc5b* were generated. GPRC5B transiently expressed in HEK293T cells was efficiently silenced by the GPRC5B shRNA construct. By contrast, the expression of mutant GPRC5B, with two silent mutations within the target sequence of the GPRC5B shRNA, was not affected by the shRNA (supplementary material Fig. S1). When GPRC5B shRNA was electroporated into the VZ of E13 embryos, levels of GPRC5B were very low or barely detectable near the luminal surface of the shRNA-transfected progenitors (Fig. 1F), thereby confirming the efficacy of the shRNA in vivo. In addition, the radial polarity of progenitor cells appears to be preserved following GPRC5B depletion, as the normal apical localisation of cadherin was retained (Fig. 1F).

In another set of experiments, we introduced GPRC5B shRNA or control shRNA construct, together with the GFP-expressing plasmid, into the VZ of E13 mouse brains by *in utero* electroporation. At E15 neocortical sections were prepared and immunostained with an antibody against PAX6, a marker for neocortical progenitors. In GPRC5B shRNA-electroporated neocortices, a smaller fraction of GFP-labelled cells remained as undifferentiated PAX6-positive progenitors when compared with the fraction found in control shRNA-expressing neocortices (14.2±1.0% versus 23.1±0.7%, respectively; *n*=4 embryos, *P*<0.001) (Fig. 2A). These results suggest that GPRC5B knockdown slightly promotes the differentiation of cortical progenitors. Of note, following GPRC5B shRNA electroporation we did not observe an increase in the number of cleaved caspase 3-positive cells (data not shown). This result indicates that apoptosis did not account for the reduction in progenitors caused by GPRC5B depletion.

**Fig. 2. GPRC5B knockdown promotes progenitor differentiation.** Plasmid expressing either GPRC5B shRNA (right) or control shRNA (left) was electroporated, together with the GFP-expressing plasmid, into E13 embryos. E15 brain sections were immunostained with antibodies against (A) PAX6, (B) TBR2 or (C) CUX1. Images of the entire cerebral wall are shown, with the boxed regions magnified to the right. Images are projections of four serial z-sections (1 μm intervals) for PAX6/TBR2 and two z-sections (1 μm interval) for CUX1. The graphs on the right show the fraction of cells positive for PAX6 (A), TBR2 (B) or CUX1 (C) among GFP-positive cells. Mean ± s.e.m. (*n*=3-5). *P*<0.05, ***P*<0.001. Con, control shRNA; RNAi, GPRC5B shRNA. Scale bars: 20 μm for entire cerebral wall; 10 μm in magnified views. CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone.
To determine whether the neuronal differentiation of progenitors is promoted upon GPRC5B knockdown, we assessed the production of intermediate/basal progenitors by immunohistochemistry using an antibody to TBR2 (EOMES – Mouse Genome Informatics), a specific marker for intermediate progenitors that are destined to become neurons (Englund et al., 2005). Remarkably, TBR2-positive populations of GFP-labelled cells in GPRC5B shRNA brains were decreased as compared with control shRNA brains (Fig. 2B). In addition, whereas the majority of GFP-labelled cells in control shRNA brains were positive for CUX1 (Fig. 2C), which is a marker for both progenitors and neurons destined for layers 2-4 (Nieto et al., 2004; Shen et al., 2006), a significantly smaller fraction of GPRC5B shRNA cells showed detectable levels of CUX1 (Fig. 2C). Thus, introduction of GPRC5B shRNA into progenitors impairs neurogenesis in the developing neocortex.

To assess the long-term effects of GPRC5B knockdown on neurogenesis in vivo, E18 cortices electroporated at E14 were immunostained with antibodies against neuronal markers. Almost all GPRC5B shRNA cells were abnormally located in the intermediate zone (IZ), instead of being correctly positioned in the cortical plate (CP) (Fig. 3). Whereas the vast majority of control shRNA cells were immunostained with MAP2 (>98%) and CUX1 (76.2±2.1%; n=3 embryos), only a small fraction of GPRC5B shRNA cells were positive for MAP2 (<5%) and CUX1 (26.8±2.0%; n=3 embryos, P<0.001) (Fig. 3A,B). In addition, GFP-labelled cells in the IZ were negative for PAX6 (Fig. 3C). These phenotypes (i.e. decreased CUX1-positive cells, mislocalisation of GFP-labelled cells in the IZ) were recapitulated with a second shRNA construct (GPRC5B shRNA#2) (supplementary material Figs S1, S4), confirming that the observed phenotypes are specific to GPRC5B. Taken together, these results indicate that GPRC5B-depleted progenitors, rather than remaining undifferentiated, differentiate into non-neuronal cells.

To confirm that impaired neurogenesis results specifically from GPRC5B knockdown, we performed a rescue experiment in which GPRC5B shRNA was co-expressed with a mutant GPRC5B that has two silent mutations within the GPRC5B shRNA target sequence (supplementary material Fig. S1). Co-expression of shRNA-insensitive GPRC5B almost completely reversed the decrease in CUX1-positive cells caused by GPRC5B shRNA (supplementary material Fig. S4C,D). Thus, impaired neurogenesis in GPRC5B shRNA progenitors is caused by loss of GPRC5B function.

To further support the notion that GPRC5B is crucial for neuronal differentiation, cells electroporated with GPRC5B shRNA/control shRNA at E14 were isolated from E16 neocortices, cultured until DIV6 and immunostained with an antibody against the neuronal

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Fig. 3. GPRC5B knockdown impairs neurogenesis. Plasmid expressing either GPRC5B shRNA or control shRNA was electroporated, together with the GFP-expressing plasmid, into E14 embryos. E18 brain sections were immunostained with antibodies against (A) MAP2, (B) CUX1 or (C) PAX6. Images of the entire cerebral wall are shown. Magnified views of the CP or IZ regions (boxed areas) are shown to the right. Con, control shRNA; RNAi, GPRC5B shRNA. Scale bars: 20 μm.
marker TUJ1. More than 90% of control cells were TUJ1 positive, whereas a smaller population of GPRC5B shRNA cells were TUJ1 positive (Fig. 4). Surprisingly, a significantly larger population of GPRC5B-depleted cells was positive for GFAP (GPRC5B shRNA, 51±0.7%; GPRC5B shRNA#2, 32±4%; control shRNA, 5.9±1.4%; n=3 embryos). In addition, almost all of the cells were negative for PDGFRα, an oligodendrocyte lineage marker (data not shown).

There was no significant difference in the percentage of apoptotic cells between control and GPRC5B knockdown GFP-labelled cells (<2.6%). Importantly, the increase in the GFAP-positive population among GFP-labelled cells upon GPRC5B depletion was almost completely reversed by co-expression of the GPRC5B mutant insensitive to GPRC5B shRNA (Fig. 4). These results indicate that GPRC5B is required for the proper neuronal differentiation of progenitors and that GPRC5B depletion generates cells with the potential to differentiate into astrocytes in vitro.

**GPRC5B knockdown cells ultimately become astrocytes**

We then examined the fate of GPRC5B shRNA-electroporated cells in the postnatal period. The control or GPRC5B shRNA construct was electroporated into E14 embryos and the pups were harvested at P14 or P27. In P14 control brains, almost all GFP-labelled cells were located in layers 2-4 and were positive for the mature neuronal marker NeuN (Fig. 5A,C). By contrast, at P14 ~50% of GPRC5B knockdown cells were located within or near the SVZ, with the remaining cells found in the lower part of the CP (Fig. 5A, arrowheads). Importantly, 66.0±3.3% (n=3 embryos) of the cells in the CP displayed the bushy morphology that is reminiscent of mature astrocytes (Fig. 5B,C). Immunohistochemical analysis confirmed that almost all bushy cells were positive for the mature astrocyte markers GFAP and S100 (Fig. 5D,E), but negative for NeuN (Fig. 5C) and PDGFRα (supplementary material Fig. S5). Of
NOTE, the vast majority of GFP-labelled cells without bushy morphology in the SVZ and CP were negative for GFAP, NeuN and PDGFRα (Fig. 5C; supplementary material Fig. S5; data not shown). Collectively, among the GPRC5B-depleted cell populations, we found a very small fraction positive for NeuN (6.2±3.1%, n=3 embryos) and a much larger fraction positive for GFAP (29.4±12.8%, n=3 embryos) and S100 (29.2±12.9%, n=3 embryos). Moreover, in P27 brains, ~50% of GPRC5B-depleted cells were positive for GFAP (49.9±0.3%, n=3 embryos), 22% were positive for NeuN (22.5±4.0%, n=4 embryos) and none were positive for PDGFRα (supplementary material Fig. S6). Finally, the increase in the GFAP-positive cell population upon GPRC5B depletion observed at P27 was consistently reversed by co-expression of the GPRC5B mutant insensitive to the GPRC5B shRNA (GFAP, 7.5±2.4%; NeuN, 85±6.1%; n=6 embryos; supplementary material Fig. S7). Together with the fact that almost all GFP-labelled cells in control shRNA cortices were positive for NeuN (>96%) and negative for GFAP (>99%), we conclude that a significantly large population of cells derived from GPRC5B-depleted progenitors fail to adopt a neuronal fate and instead ultimately become astrocytes.

To further support this conclusion, we performed a clonal analysis of GPRC5B-depleted progenitor cells (supplementary material Fig. S8). GFP-labelled progenitor cells electroporated with either control shRNA or GPRC5B shRNA at E14 were prepared at E15 and cultured at clonal density. The cellular composition of the GFP-labelled clones was analysed at DIV5. GFP-labelled control cells gave rise to 56±4.0% neuronal clones, 11±1.0% astroglial clones and 33±3.0% other clones. In contrast, GFP-labelled GPRC5B-shRNA-depleted cells gave rise to 2±1.0% neuronal clones, 48±3.0% astroglial clones and 50±3.0% other clones. These data suggest that GPRC5B depletion in progenitors results in a differentiation into a non-neuronal lineage.

Fig. 5. GPRC5B knockdown generates astrocytes. Plasmid expressing either GPRC5B shRNA or control shRNA was electroporated, together with the GFP-expressing plasmid, into E14 embryos, and embryos were harvested at P14. (A) Representative images of GFP-labelled cells throughout the entire cerebral wall in brains electroporated with control shRNA (left) and GPRC5B shRNA (middle and right). Arrowheads indicate GFP-labelled bushy cells. (B) Typical morphology of GFP-labelled cells in the CP and SVZ regions in GPRC5B shRNA neocortices. Shown are cells with bushy morphology in the CP (top), cells that do not have bushy morphology in the CP (middle), and cells in the SVZ region (bottom). (C) GFP-labelled cells in electroporated neocortices immunostained with anti-NeuN antibody. Shown are high-magnification images of GFP-labelled cells in control shRNA brain (row 1) and GPRC5B shRNA brains (row 2, cells with bushy morphology; row 3, cells without bushy morphology; row 4, cells in the SVZ region). (D,E) GFP-labelled cells in GPRC5B shRNA neocortices immunostained with antibodies against GFAP (D) and S100 (E). High-magnification images of GFP-labelled cells with bushy morphology are shown. WM, white matter; SVZ, subventricular zone. Scale bars: 100 μm in A; 20 μm in C-E.
and 22±6.0% mixed clones (containing neurons and astrocytes). Depletion of GPRC5B by shRNA dramatically reduced the percentage of neuronal clones (41±3.9%) and increased the percentage of astroglial clones (30.0±2.7%). Expression of the GPRC5B insensitive to GPRC5B shRNA almost completely abolished the increase in astroglial clones. Importantly, the average size of total clones and astroglial clones was not significantly different between GPRC5B shRNA and control cultures (GPRC5B shRNA: total 4.0±0.8 cells/clone, astroglial 3.3±0.2 cells/clone; control: total 4.1±0.2 cells/clone, astroglial 4.0±0.3 cells/clone). These results indicate that the increased number of astrocytes upon GPRC5B depletion in vivo (Fig. 5) is unlikely to be due to enhanced cell proliferation. Of note, the percentage of apoptotic cells was not significantly changed upon GPRC5B depletion (1.3±0.2% versus 0.8±0.4% in control and GPRC5B shRNA, respectively). Taken together, these results strengthen the idea that GPRC5B depletion generates cells with reduced neurogenic potential that are prone to differentiate into astrocytes.

β-catenin signalling is potentiated by GPRC5B signalling

Most GPCRs transmit signals through the activation of heterotrimeric G proteins. G protein α-subunits are generally divided into four families (Gi/o, Gs, Gq/11 and G12/13) based on their sequence similarity. Overexpression of many of the GPCRs in cells results in the activation of their cognate G proteins in a ligand-independent manner (Milligan, 2003). We found that overexpression of GPRC5B induces significant rounding of COS7 cells (~80%) (Fig. 6A,B), a phenotype reminiscent of cells expressing mutational activated Gα12-CT or Gα13-CT (Kranenburg et al., 1999; Meigs et al., 2005; Stemmle et al., 2006). Peptides corresponding to C-terminal regions of Gα (Gα-CTs) can serve as competitive blockers for GPCR-G protein signalling, as they selectively bind to the G protein-binding site on cognate GPCRs and suppress signal transduction through a given G protein (Gilchrist et al., 2002). Based on this mechanism, we examined which G proteins mediate GPRC5B signalling by using C-terminal peptides of α-subunits (Gαs-CT, Gαq-CT, Gα12-CT and Gα13-CT) (Sugimoto et al., 2003) as well as pertussis toxin, which inactivates Gα/o via the specific catalysis of ADP-ribosylation of the α-subunit. When either Gαs-CT or Gαq-CT was co-transfected with GPRC5B, no effect on GPRC5B-induced cell rounding was observed (Fig. 6A,B). Likewise, co-transfection of the plasmid encoding pertussis toxin did not affect cell morphology. By contrast, GPRC5B-induced cell rounding was largely prevented by co-expression of Gα12-CT or Gα13-CT (Fig. 6A). When plasmid encoding pertussis toxin or Gα-CTs was solely transfected into COS7 cells, the morphology of the transfected cells was normal (data not shown). These results indicate that GPRC5B couples with the G12/13 class of heterotrimeric G proteins.

Wnt/β-catenin signalling has been shown to promote the neuronal differentiation of progenitors at later stages of corticogenesis (Hirabayashi et al., 2004; Israsena et al., 2004). Interestingly, Gα12/13 signalling is linked to β-catenin signalling. Indeed, activated Gα12/13 is known to bind to the cytoplasmic tails of several members of the cadherin family and to induce the dissociation of cadherin-bound β-catenin, which in turn causes an increase in β-catenin-mediated transcriprional activation (Meigs et al., 2001). These observations raise the hypothesis that β-catenin signalling might be one of the downstream pathways elicited by GPRC5B-G12/13 in neural progenitors. In support of this, GPRC5B alters the subcellular distribution of β-catenin in Vero cells (Fig. 7A,B). In mock-transfected cells stained with β-catenin antibody, β-catenin signals were predominantly localised at the cell cortex. Expression of GPRC5B induced a marked shift in β-catenin immunoreactivity: β-catenin signals, which are normally found at the cell periphery, adopted a more diffuse pattern throughout the cell (Fig. 7A,B). Consistent with a previous report (Meigs et al., 2001), a similar redistribution of β-catenin was induced upon expression of mutationaly activated Gα12 [Gα12(Q229L)] (Fig. 7A,B).
It is known that the expression of activated Gα12 causes a substantial increase in β-catenin-mediated transcription in SW480 human colon carcinoma cells that lack functional APC, as well as a slight increase in Rat-1 fibroblast cells (Meigs et al., 2001). We then sought to examine whether the expression of GPRC5B in Vero cells causes an increase in β-catenin-mediated transcription. We utilised a dual reporter construct (TOPdGFP-CAGmCherry) that drives the expression of destabilised GFP (dGFP) under the control of a β-catenin-responsive promoter (TOP, a TCF/LEF consensus site upstream of a minimal c-Fos promoter) and directs mCherry expression through a constitutive CAG promoter (Mutch et al., 2009). In this system, mCherry serves as a transfection control, and the ratio of dGFP to mCherry fluorescence intensity was used to quantify the β-catenin signalling levels in individual cells (Mutch et al., 2009; Yokota et al., 2009; Woodhead et al., 2006). Consistent with observations made on Rat-1 fibroblast cells (Meigs et al., 2001), a slight but significant increase in β-catenin-mediated transcription was detected in Vero cells upon expression of GPRC5B as well as upon expression of activated Gα12 (supplementary material Fig. S9). We also examined the distribution pattern of β-catenin in cultured progenitor cells. In dividing progenitor cells, intense β-catenin immunoreactivity was detected at the cell cortex. Introduction of GPRC5B increased β-catenin immunoreactivity in the cytosol of neural progenitors (Fig. 7C,D). This phenotype was prevented by co-expression of Gα12-CT (Fig. 7C,D), thereby confirming a potential role for GPRC5B-G12/13 signalling in the regulation of β-catenin.

To evaluate the role for GPRC5B in β-catenin signalling in vivo, we electroporated the TOPdGFP-CAGmCherry reporter plasmid into E14 embryos and harvested the embryos 24 hours later. In
cortices electroporated with both TOPdGFP-CAGmCherry and control shRNA plasmids, PAX6-positive cortical progenitors in the VZ showed significantly higher dGFP expression than PAX6-negative differentiated cells (Fig. 8). This is consistent with a previous study showing that β-catenin signalling levels are downregulated in differentiated cells (Woodhead et al., 2006). Importantly, a marked suppression of dGFP expression was detected in GPRC5B-depleted PAX6-positive progenitor cells (Fig. 8). In addition, downregulation of β-catenin signalling in GPRC5B knockdown progenitors was significantly reversed by co-expression of the mutant GPRC5B insensitive to GPRC5B shRNA (Fig. 8). These results underscore a role for GPRC5B in regulating the β-catenin-mediated transcription that is important for the neuronal differentiation of progenitors. Thus, GPRC5B signalling may converge with β-catenin signalling to promote the neuronal differentiation of progenitors.

DISCUSSION

The present study demonstrates that GPRC5B is selectively expressed in neural progenitor cells of the developing neocortex. GPRC5B-depleted cortical progenitors give rise to cells that fail to adopt a neuronal fate. Instead, they are prone to differentiate into astrocytes as revealed by the analysis of cell type identity at E18, P14 and P27. In brief, our findings implicate GPCR-mediated signalling in the control of the neurogenic potential and cell fate of cortical progenitors.

In the developing neocortex, β-catenin signalling plays multiple roles in such as the neuronal differentiation and proliferation of
Role of GPRC5B in neurogenesis

Cortical progenitors. At early stages of cortical development, β-catenin signalling is essential for progenitor proliferation (Chenn and Walsh, 2002; Zechner et al., 2003; Mutch et al., 2009; Mutch et al., 2010). At later stages of cortical development, β-catenin signalling contributes to neuronal differentiation (Hirabayashi et al., 2004; Israsena et al., 2004). It is well known that β-catenin is an essential component of two distinct cellular systems: the Wnt signalling pathway and the cadherin-based adherens junctions. The cadherin-bound pool of β-catenin is thought to serve as a reservoir for signalling-competent β-catenin and to be functionally connected to the Wnt/β-catenin signalling pathway (Barth et al., 1997; Nelson and Nusse, 2004). It is also noteworthy that activated Gα12/13 can induce the dissociation of cadherin-bound β-catenin, which in turn causes an increase in β-catenin-mediated transcriptional activation (Meigs et al., 2001). Our study shows that GPRC5B is coupled with Gα12/13 and that its signalling induces a redistribution of β-catenin in cortical progenitors. Furthermore, GPRC5B depletion attenuates β-catenin-mediated transcriptional activation in the cortical progenitors of E14 neocortices in vivo. Thus, one of the downstream actions of GPRC5B in progenitors is to reinforce β-catenin signalling, probably by releasing cadherin-bound β-catenin. In support of this model, GPRC5B localises in close proximity to cadherin at adherens junctions of progenitors. Potential cross-talk between GPRC5B and Wnt/β-catenin signalling is also supported by the fact that GPRC5B physically associates with the Wnt receptor Frizzled in HeLa cells (Harada et al., 2007).

In E12 brains electroporated at E11, the fate of GPRC5B-depleted cells was not altered in terms of Pax6-positive neural progenitor populations (42.1±1.4% versus 38.9±4.4% in control versus GPRC5B shRNA, respectively; n=3-4 embryos, P=0.46), Tbr2-positive intermediate progenitor populations (34.5±2.5% versus 42.5±5.3% in control and GPRC5B shRNA, respectively; n=3-4 embryos, P=0.19) and Tbr1-positive early neuronal populations (10.1±0.8% versus 13.8±4.2% in control and GPRC5B shRNA, respectively; n=3-5 embryos, P=0.30). These results suggest that the GPRC5B signalling cascade might not be activated at early stages of corticogenesis, but rather underscore the integration of the GPRC5B and β-catenin signalling pathways at later stages of corticogenesis to regulate the neurogenic potential of cortical progenitors.

Noticeably, GPRC5B-depleted progenitors, instead of remaining undifferentiated, gave rise to cells that were prone to differentiate into GFAP-positive astrocytes in vitro and in vivo. This phenotype cannot be explained by attenuation of β-catenin signalling, as disruption of Wnt/β-catenin signalling in cortical progenitors at later stages of corticogenesis inhibits neuronal differentiation and causes progenitors to remain undifferentiated (Hirabayashi et al., 2004; Israsena et al., 2004). Thus, we suggest that GPRC5B signalling contributes to normal neuronal differentiation through multiple pathways. Of note, GPRC5B-depleted cells seem to require environmental cues to differentiate into GFAP-positive astrocytes, as the formation of GFAP-positive mature astrocytes upon GPRC5B depletion was not detected until the postnatal period at which extensive gliogenesis takes place.

Recently, GPRC5B-deficient mice have been generated. The mice do not show overt histological abnormalities in the brain, although they exhibit partial perinatal lethality, altered spontaneous activity pattern and a decreased response to new environment in the adult (Sano et al., 2011). In utero electroporation of shRNA allows the acute knockdown of genes in the developing neocortex, circumventing the molecular compensatory responses that may be activated in a genetic knockout (e.g. doublecortin (Bai et al., 2003)). Hence, acute reduction of GPRC5B has enabled us to dissect out its function in neural progenitor cells. It has been revealed that mice with a conditional knockout for Gα12 and Gα13 in the nervous system display neuronal ectopia in the developing neocortex (Moers et al., 2008). This phenotype is mainly caused by the overmigration of neurons and is accompanied by a disrupted pial basement membrane, the abnormal protrusion of radial processes of neural progenitors into the ectopia and the displacement of Cajal-Retzius cells in the subarachnoid space (Moers et al., 2008). As the effects of Gα12 and Gα13 knockout on progenitor fate were not studied by Moers and collaborators, our study reveals for the first time a potential contribution of Gα12 and Gα13 in the neuronal differentiation of progenitors.

In conclusion, our study sheds light on the roles of GPCR signalling in neurogenesis and fate determination. Our findings of a novel GPRC5B-G12/13-β-catenin signalling pathway in progenitor cell fate regulation could provide unique insights into the complex mechanisms underlying neurogenesis. The requirement of GPCR-mediated signalling, which is a main target for drugs, for the neurogenic potential of neural progenitors also has implications in the context of novel avenues to manipulate stem cells with a view to therapeutical value.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

N.K., M.D.N. and K.S. designed experiments and wrote the manuscript; N.K. and K.S. performed experiments.

Supplementary material

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099754/-/DC1

References


Fig. S1. Evaluation of GPRC5B knockdown by shRNA constructs in HEK293T cells. HEK293T cells were transiently transfected with plasmids encoding GFP (pEGFP-C1), wild-type GPRC5B (WT), GPRC5B shRNA, GPRC5B shRNA#2, and mutant GPRC5B (Res; GPRC5B which contains two silent mutations within the GPRC5B shRNA-targeted sequence). The cell lysate was prepared 48 hrs later and subjected to immunoblotting with antibodies against GFP (1:2000; MBL), GPRC5B (1:5000), and dynein intermediate chain (DIC; 1:2000, Santa Cruz Biotechnology). Note that mutant GPRC5B is insensitive to GPRC5B shRNA (lane 6), but sensitive to shRNA#2 (lane 7).
Fig. S2. **GPRC5B immunoreactivity was detected at the ventricular surface at E15.** Images of E15 neocortical sections immunostained with anti-GPRC5B (green) and anti-Cadherin (red) antibodies. GPRC5B immunoreactivity was detected at the ventricular surface (left panels). The immunoreactivity is diminished by pre-incubation of the antibody with its immunogen (right panels). Scale bar = 50 μm. CP: cortical plate, IZ: intermediate zone, VZ: ventricular zone, LV: lateral ventricle.
Fig. S3. GPRC5B immunoreactivity was detected at cells lining the lateral ventricle at P14. (A) Images of P14 brain sections immunostained with anti-GPRC5B (green) and anti-S100β (red) antibodies. Nuclei were stained with TOPRO-3 iodide. GPRC5B immunoreactivity was detected at cells lining the lateral ventricle (left panels). The immunoreactivity was diminished by pre-incubation of the antibody with its immunogen (right panels). LV: lateral ventricle, NCX: neocortex, HIP: hippocampus. Scale bar = 100 μm. (B, C) High-magnification images of regions around the lateral ventricle immunostained with GPRC5B and S100β (B) and with GPRC5B, SOX2 and GFAP (C). LV: lateral ventricle, MW: medial wall, LW: lateral wall, NCX: neocortex, HIP: hippocampus.
Fig. S4. Effects of GPRC5B shRNA#2 on progenitor differentiation and rescue of GPRC5B knockdown-induced cell-fate alteration by shRNA-resistant GPRC5B. (A) Plasmids expressing GPRC5B shRNA#2 and GFP were electroporated in E14 embryos, and brains were fixed at E18. Images of the entire cerebral wall stained with antibodies against Pax6 (left two panels) and Cux1 (right two panels) are shown. Almost all of the GFP-labelled cells are negative for Pax6. Scale bar = 20 μm. (B) Graph showing quantification of fraction of GFP-labelled cells that were also positive to Cux1 (mean ± SEM, n=3-4 embryos). Similar quantification was performed in control shRNA-introduced brains (see Figure 3), and the data are also presented in the graph. **p < 0.01. (C, D) Rescue of GPRC5B knockdown-induced cell-fate alteration by shRNA-resistant GPRC5B. E14 embryos were electroporated with plasmids encoding GFP and GPRC5B shRNA, with or without a plasmid expressing GPRC5B<sup>res</sup> (GPRC5B which contains two silent mutations within the GPRC5B shRNA-targeted sequence), as indicated. Brain were fixed at E18. Brain sections were then immunostained with Cux1 antibody. Images of the entire cerebral wall immunostained are shown, and magnified views of the IZ or CP region are also shown to the right. The graph shows quantification of fraction of GFP-labelled cells that were also positive to Cux1 (mean ± SEM, n=3-4 embryos). Similar quantification was performed in control shRNA-introduced brains (see Fig. 3), and the data are also presented in the graph. ***p < 0.001 versus control shRNA. n.s., not significant. Scale bars = 20 μm.
Fig. S5. GPRC5B-depleted cells at P14 are not oligodendrocytes. Plasmid expressing GPRC5B shRNA was electroporated, together with the GFP-expressing plasmid, in E14 embryos. Brains were fixed at P14. Brain sections were then immunostained with an antibody against PDGFRα. High-magnification images of GFP-labelled cells with bushy morphology in the CP (1st and 2nd rows), cells without bushy morphology in the CP (3rd row), and cells in the SVZ/white matter region (4th row) are shown. Almost all of the GFP-labelled cells are negative for PDGFRα. Scale bar = 20 μm.
Fig. S6. GPRC5B-depleted cells ultimately differentiate into astrocytes. (A-D) Plasmid expressing GPRC5B shRNA was electroporated, together with the GFP-expressing plasmid, in E14 embryos, and brains were fixed at P27. Brain sections were then immunostained with antibodies against GFAP, NeuN and PDGFRα. (A) Representative images of GFP-labelled cells in the entire cerebral wall stained with GFAP antibody. (B) Magnified images of GFP-labelled cells in the area marked by a-d in (A). (C) High-magnification images of GFP-labelled cells in the CP region (1st-3rd rows) and SVZ region (4th row) stained with NeuN antibody. Some of the cells without bushy morphology in the CP are positive to NeuN. Almost all of the cells with bushy morphology are negative for NeuN. (D) Almost all of the GFP-labelled cells are negative for PDGFRα. Scale bars = 100 μm in (A) and 20 μm in (B-D).
Fig. S7. Rescue of cell-fate alteration of GPRC5B-depleted cells by shRNA-resistant GPRC5B. Plasmids expressing GPRC5B shRNA and GFP were electroporated in E14 embryos, with or without a plasmid expressing GPRC5B^{res}, and brains were fixed at P27. Brain sections were then immunostained with an antibody against NeuN. Representative images of GFP-labelled cells were shown. Scale bar = 100 μm.
Fig. S8. Progeny derived from GPRC5B-depleted progenitors have increased potential to differentiate into astrocytes in vitro.

(A) Plasmids expressing control shRNA, GPRC5B shRNA or GPRC5B shRNA/GPRC5Bres were electroporated, together with the GFP-expressing plasmid, in E14 embryos. Neocortical cells containing GFP-labelled cells were prepared at E15 and diluted with neocortical cells derived from non-electroporated littermates. Cells were plated at a density of 0.5 x 10^6 cells on sterile coverslips pre-coated with poly-D-lysine in wells of 24-well plates and were maintained in DMEM/F12 supplemented with N2 and 5% FBS. GFP expression was used to confirm that GFP-labelled cells were well isolated from each other so that clones could be unambiguously identified. GFP-labelled clones at DIV5 were stained with antibodies against Tuj1 and GFAP. Representative images are shown. Clones were divided into four groups: neuronal (containing neurons but not astrocytes), astroglial (containing astrocytes but not neurons), mixed (containing both neurons and astrocytes) and non-neuronal/astroglial (containing no neurons and astrocytes). Scale bar = 20 μm. (B) Percentage of each type of clone (neuronal, astroglial and mixed) is presented as mean ± SEM (n=3-8 experiments, 85-300 clones analyzed). *p < 0.05, **p < 0.01, n.s., not significant.
Fig. S9. Overexpression of GPRC5B activates β-catenin-dependent transcription in Vero cells. (A) Vero cells were transiently transfected with plasmids encoding GPRC5B (0.3 μg/well) and Gα12QL (0.025 μg/well) as indicated, along with the β-catenin activity reporter construct, TOPdGFP-CAGmCherry (0.1 μg/well), using Fugene 6 (Roche). Cultures were fixed 48 hours later. Images of dGFP and mCherry were obtained under identical parameters with a 20x objective (Plan-Apochromate; Zeiss) on a Zeiss LSM 5 confocal microscope. Scale bar = 50 μm. (B) Mean fluorescence intensity of mCherry and GFP in individual cells expressing mCherry was measured. In addition, the average background fluorescence was measured in areas with no mCherry-positive cells present and was subtracted from the mCherry and GFP intensity measurements. Cells with saturated signal were not used for the analysis. The ratio of fluorescence intensity of GFP to that of mCherry in individual cells was calculated and defined as β-catenin activity index. Data are presented as mean ± SEM (n=3). *p < 0.05, **p < 0.01.
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