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G protein coupled receptor 50 promotes self-renewal and neuronal differentiation of embryonic neural progenitor cells through regulation of notch and wnt/β-catenin signalings

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ABSTRACT

G protein-coupled receptor 50 (GPR50), a risk factor for major depressive disorder and bipolar affective disorder, is expressed in both the developmental and adult brain. However, the function of GPR50 in the brain remains unknown. We here show GPR50 is expressed by neural progenitor cells (NPCs) in the ventricular zone of embryonic brain. Knockdown of GPR50 with a small interference RNA (siRNA) decreased self-renewal and neuronal differentiation, but not glial differentiation of NPCs. Moreover, overexpression of either full-length GPR50 or the intracellular domain of GPR50, rather than the truncated GPR50 in which the intracellular domain is deleted in, increased neuronal differentiation, indicating that GPR50 promotes neuronal differentiation of NPCs in an intracellular domain-dependent manner. We further described that the transcriptional activity of the intracellular domain of notch on *Hes1* gene was repressed by overexpression of GPR50. In addition, decreased levels of transcription factor 7-like 2 (TCF7L2) mRNA was observed in GPR50 siRNA-transfected NPCs, suggesting that knockdown of GPR50 impairs wnt/β-catenin signaling. Moreover, the mRNA levels of *neurogenin (Ngn) 1*, *Ngn2* and *cyclin D1*, the target genes of notch and wnt/β-catenin signalings, in NPCs were reduced by knockdown of GPR50. Therefore, GPR50 promotes self-renewal and neuronal differentiation of NPCs possibly through regulation of notch and wnt/β-catenin signalings.

1. Introduction

Neural progenitor cells (NPCs) are self-renewing and multipotent cells that generate neurons, astrocytes and oligodendrocytes in the central nervous system [1]. NPCs proliferate, differentiate, migrate and eventually integrate into the neural network. The abnormalities in any of these processes will cause dysfunctions of the brain and leads to neurological diseases such as schizophrenia [2], autism [3,4], Alzheimer's disease [5,6]. Recent studies indicate patients with major depressive disorder (MDD) or bipolar affective disorder (BPAD) also exhibit an abnormal proliferation and differentiation of NPCs [7–9], indicating a function of NPCs in the pathogenesis of MDD and BPAD.

G protein-coupled receptor 50 (GPR50), which is also known as H9 or melatonin-related receptor, is an X-linked orphan G protein-coupled receptor [10]. A recent study indicates that GPR50 is a genetic risk factor for MDD and BPAD which are associated with abnormality of cortical development in females [11–13]. GPR50 is expressed in the pituitary, hypothalamus and hippocampus of adult mammalian brain [14,15]. Moreover, expression of GPR50 in the developing mouse brain starts at embryonic day 13 (E13), peaks at E18 [16]. These lines of evidence suggest a potential role of GPR50 in brain development. However, the function of GPR50 in the brain remains unclear. In this study, we describe that GPR50 is expressed in NPCs in the ventricular zone (VZ) of embryonic (E) 14 day mouse brain, a region enriched with NPCs. We show that knockdown of GPR50 with small inference RNA (siRNA) reduces self-renewal, neuronal

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differentiation, but not of glial differentiation of NPCs. Transfection of plasmids expressing full-length GPR50, the intracellular domain of GPR50 increases neuronal differentiation of NPCs. In contrast, overexpression of the truncated GPR50 in which the intracellular domain is deleted, shows similar levels of neuronal differentiation to control NPCs. We further provide evidence that knockdown of GPR50 enhances notch signaling, whereas attenuating wnt/β-catenin signaling, two pathways play essential roles in NPCs in developmental brain [17–21]. Therefore, our study shows that GPR50 modulates self-renewal and neuronal differentiation of NPCs, possibly through regulating notch and wnt/β-catenin signalings.

2. Materials and methods

2.1. Antibodies

Rabbit anti-GPR50 (US-Biological), mouse anti-GPR50 (Santa Cruz), anti-Sox2 (Santa Cruz), anti-BrdU (Covance), anti-βIII tubulin (Sigma–Aldrich), anti-GFAP antibody (Millipore).

2.2. Plasmids and siRNA

The coding sequences of full-length mouse GPR50 (amino acids 1-592; FL-GPR50), the intracellular domain of GPR50 (amino acids 304-592; c-GPR50), the truncated GPR50 fragments deleted in its intracellular domain (amino acids 1-303; t-GPR50) and were amplified and ligated to PCDF-EGFP plasmid. PCDF-EGFP plasmid was purchased from System Biosciences, which expresses an enhanced green fluorescent protein (EGFP). Small interference RNA (siRNA) oligonucleotide duplexes were synthesized by Genepharma Biotech (Genepharma co., Ltd., Shanghai, China). Sequences of GPR50 siRNA duplexes: CCGCCUCUGUUCAUUUCAATT (sense) and UUGAAAUGAACAGAGGCGGTT (antisense); Sequences of scrambled control siRNA were: *UUCUCCGAACGUGUCAGGUTT* (sense) and *AGGUGACACGUUCGGAGAATT* (antisense).

2.3. Mice

The mice used in this study were C57/BL6J mice. All mice were handled and treated according to the animal care and handling protocols approved by the Institutional Animal Care and Use Committee of Soochow University. In all experiments, the pregnant mice were anaesthetized with 3.6% chloral hydrate.

2.4. Culturing neural progenitor cells

NPCs were isolated from the lateral ventricle walls of E14 C57/BL6J mice and cultured as described [6]. For differentiation, dissociated cells from primary neurospheres were seeded into 24-well dishes and were cultured in DMEM-F12 culture medium containing N2 and 0.5% fetal bovine serum (FBS, Gibco) for 3–5 days. For neurospheres formation, NPCs were cultured at a density of 500 cells/well in 96-well plates for 8 days. The numbers of neurospheres per well were counted. For quantification of neurosphere size, NPCs were cultured at a density of 2×10^4 cells/well in 24-well plates for 8 days.

2.5. Transfection

Transfection in N2a or CHO cells was performed with lipofectamin 2000 according to the protocols provided by the manufacturer. Due to low transfection efficiency with lipofectamin 2000, cultured NPCs were transfected with 5 μg DNA plasmid or 200 nM siRNA per cuvette using nucleofector system (Lonza) according to the protocol provided in the Amaxa Nucleofector kit.

2.6. Real-time Quantitative polymerase chain reaction (qPCR)

RNA was extracted using Trizol Reagent (Invitrogen life technologies) and RT reactions were performed with the RevertAid First Strand cDNA Synthesis Kit (Promega). The Primers used were shown as following: *GPR50*: *CAACATTACTGCCATTGCC* (forward), *TTTGGAAGCAGCCCTGTAATG* (reverse). *GAPDH*: *CAAGGTCATCCATGACAACTTTG* (forward), *GTCCACCACCCTGTTGCTGTAG* (reverse). mouse Hes1: *AGCCAACTGAAAACACCTGATT* (forward), *GGAGTTTATGATTAGCAGTGG* (reverse). *Ngn1*: *ATCACCACTCTCTGACCC* (forward), *GAGGAAGAAAGTATTGATGTTGCCTTA* (reverse). *Ngn2*: *ATAGAGAACGTATGTCGAGGTAGG* (forward), *GTTGGAGAAGGTGGAACCAA* (reverse). *Cyclin D1*: *TGTTACTTGTAGCGGCCTGTTG* (forward), *CCGGAGACTCAGAGCAAATCC* (reverse).

2.7. Luciferase reporter assay

The Hes1-luciferase system has been described [22]. CHO cells were seeded into 24-well plate and co-transfected with PGVB-Hes1-luciferase, PcDNA3.1-NICD, together with PCDF-GPR50 or PCDF-c-GPR50 or PCDF-EGFP (as control) plasmids in an amount of 0.2 μg/plasmid/well. 0.02 μg PCMV-Lacz plasmid per well was cotransfected as the internal control. Cells were harvested by Glo Lysis Buffer (Promega) at 24–36 h after transfection and analyzed using the Steady-Glo Luciferase Assay Kit (Promega). Luciferase activity was detected with a luminometer. β-galactosidase activity was detected through a filter with the absorbance at 420 nm wavelength using a Microplate Reader (TECAN infinit M200 Pro).

2.8. Immunocytochemistry, immunochemistry and quantification

Immunostaining of cultured cells and brain sections was performed as described [6]. In brief, images of fields of cultured cells were captured by digital photomicrograph under a 20× objective systematically from top-to-bottom and left-to-right across the entirety of each coverslip. All labeled cells were then counted in each photomicrograph. The percentage of neurons, astrocytes and proliferative cells was quantified as the numbers of βIII-tubulin$^+$, GFAP$^+$ and BrdU$^+$ cells divided by the total numbers of DAPI$^+$ cells in the same field, respectively. For quantification of the amount of neurospheres, the number of neurospheres present in each well of 96-well plates was counted manually. For quantification of the size of neurospheres, the neurospheres in 24 well-plates were selected randomly. The diameters of neurospheres were measured in photoshop with a calibration of the scale bar. The volume of each neurosphere was quantified. All images were relabeled and subjected to a blind analysis that the analyzers were unknown the information about the images.

2.9. Statistical analysis

All statistical analyses were performed using SPSS19.0. Data are presented as Mean ± SEM. Data between multiple groups were analyzed by one-way analysis of variance (ANOVA). Comparisons between two groups were made by independent samples t-test. $p < 0.05$ was considered as significance level for all analyses. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results

3.1. GPR50 is expressed in neural progenitor cells in the ventricular zone

We first examined whether GPR50 is expressed by NPCs in the VZ. Double-immunostaining for GPR50 and SRY (sex determining

region Y) -box 2 (Sox2), which is expressed in self-renewing NPCs and thus is a marker of NPCs [23], in E14 mouse dorsal telencephalon showed that GPR50 was expressed in Sox2$^+$ cells in the apical VZ (Fig. 1A). We further examined GPR50 expression in cultured NPCs. GPR50 (Figs. 1B and C) and Sox2 (Fig. 1B) or Paired box protein Pax-6 (Pax6) (Fig. 1C), a marker of NPC as well, were immunostained in cultured NPCs derived from E14 mouse telencephalic ventricular walls. GPR50$^+$ fluorescence signals were detected in Sox2$^+$ or Pax6$^+$ NPCs. Most of GPR50$^+$ fluorescence signals distributed in the cytoplasm and membrane of NPCs (Figs. 1B and C), which is consistent with that GPR50 is a transmembrane protein. The specificity of GPR50 antibody was validated by detection of immunoreactivity of GPR50 in GPR50-, but not in EGFP-transfected CHO cells (Supplementary Fig. 1). Moreover, GPR50 mRNA was detected in lysates of cultured NPCs as well as of E14 mouse cerebral cortex by RT-PCR analysis (Fig. 1D). Thus, these results indicate that GPR50 is expressed in NPCs in the VZ.

3.2. Knockdown of GPR50 decreases self-renewal of NPCs

We then asked whether GPR50 would regulate the proliferation and differentiation of NPCs. A small interfering RNA (siRNA) targeting GPR50 (siGPR50), which could downregulate GPR50 mRNA levels efficiently in cultured NPCs (Fig. 2A), were transfected to cultured NPCs. NPCs transfected with a scrambled siRNA (NC) were used as control. The transfected NPCs were cultured in medium containing Bromodeoxyuridine (BrdU), which is incorporated into DNA during S-phase of cell cycle (Fig. 2B). The proportion of BrdU$^+$ cells showed a decrease in siGPR50-transfected NPCs compared to that in NC-transfected cells (Fig. 2C), indicating that GPR50 promotes NPC proliferation. Self-renewal is the capability of NPCs to go through numerous cycles of cell division and thus expands their numbers. To investigate the role of GPR50 in self-renewal of NPCs, NPCs transfected with either siGPR50 or NC were cultured in low density in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) for 8 days. siGPR50-transfected

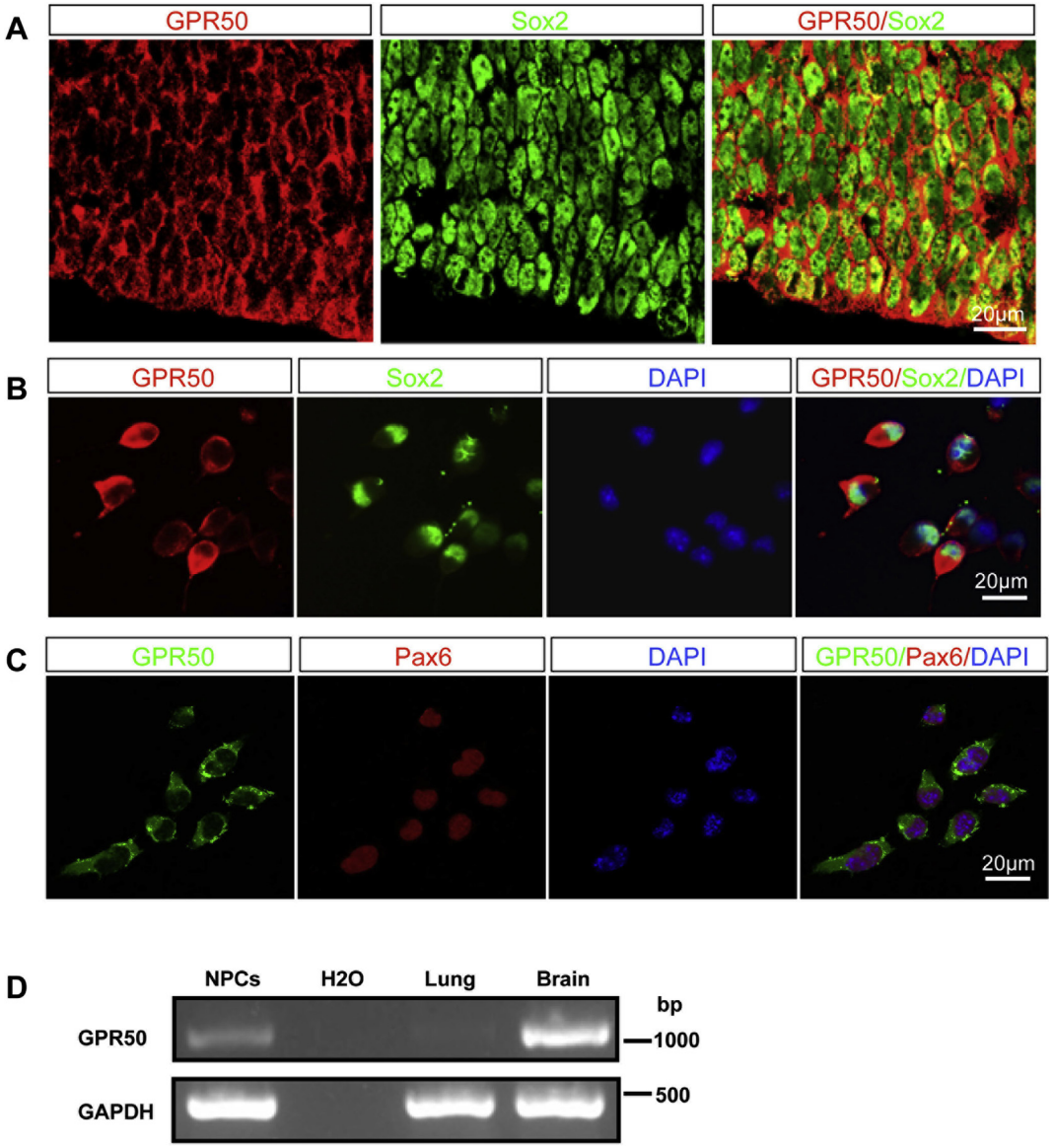

Fig. 1. Expression of GPR50 in the VZ and neural progenitor cells (NPCs). A, Coronal sections of the VZ of E14 mouse were immunostained for GPR50 and Sox2. B and C, NPCs isolated from the lateral ventricles of E14 mouse brain and maintained in culture for 4 days were immunostained for GPR50, Sox2 (B) or Pax6 (C) and DAPI. D, RT-PCR analysis of GPR50 mRNA in NPCs maintained in culture for 4–5 days. mRNA extracts from E14 mouse brain (brain) and adult mouse lung (lung) serve as controls. Scale bars: 20 μm.

NPCs form less amounts (Fig. 2D and E) and smaller size (Fig. 2F and G) of neurospheres, compared to NC-transfected NPCs. Thus, these results indicate that knockdown of GPR50 impairs self-renewal of NPCs.

3.3. Knockdown of GPR50 inhibits neuronal differentiation, but not astroglial differentiation of NPCs

We further examined whether knockdown of GPR50 would affect the differentiation of NPCs. NPCs transfected with either siGPR50 or NC were cultured in differentiation condition for 5 days, and were then stained for βIII-tubulin (Fig. 3A), a marker of neuron, or glial fibrillary acidic protein (GFAP) (Supplementary Fig. 2A), a maker of astrocyte, and DAPI. The proportion of βIII-tubulin$^+$ cells (Fig. 3B) decreased in siGPR50-transfected NPCs compared to that in NC-transfected NPCs. In contrast, the proportion of GFAP$^+$ cells (Supplementary Fig. 2B) showed identical in between NC- and siGPR50-transfected NPCs. These results indicate that knockdown of GPR50 inhibits neuronal, but not astroglial differentiation of NPCs.

3.4. GPR50 promotes neuronal differentiation through its intracellular domain

GPR50 has the longest carboxyl tail among known GPCR [24,25] and contains at least one putative proteolytic cleavage site [25]. The intracellular domain of GPR50 translocates into the nucleus after binding to TIP60, a transcriptional co-activator with histone acetyltransferase [26]. We thus investigated whether GPR50 would promote neuronal differentiation through its intracellular domain of GPR50. We constructed PCDF-EGFP plasmids expressing full-length GPR50 (FL-GPR50), the intracellular domain of GPR50 (c-GPR50) or the truncated fragment in which the intracellular domain of GPR50 is deleted (t-GPR50) (Fig. 3D) and transfected them into cultured NPCs. Upon differentiation, NPCs transfected with either FL-GPR50 or c-GPR50 differentiated into more βIII-tubulin$^+$ cells, compared to those cells transfected with EGFP alone (Fig. 3C and E). In contrast, the proportion of βIII-tubulin$^+$ cells differentiated from t-GPR50-transfected NPCs is similar to that from EGFP-transfected NPCs (Fig. 3C and E). These results indicate that overexpression of GPR50 promotes neuronal differentiation of NPCs in an intracellular domain-dependent manner.

3.5. GPR50 represses notch signaling, whereas enhancing wnt/β-catenin signaling

We further investigated the mechanisms whereby GPR50 modulates the proliferation and differentiation of NPCs. Notch signaling and wnt/β-catenin signaling play essential roles in NPCs during development. Notch signaling inhibits [17,27], while wnt/β-catenin signaling promotes neuronal differentiation [19]. Both signalings enhance self-renewal of NPCs [21,28]. We thus

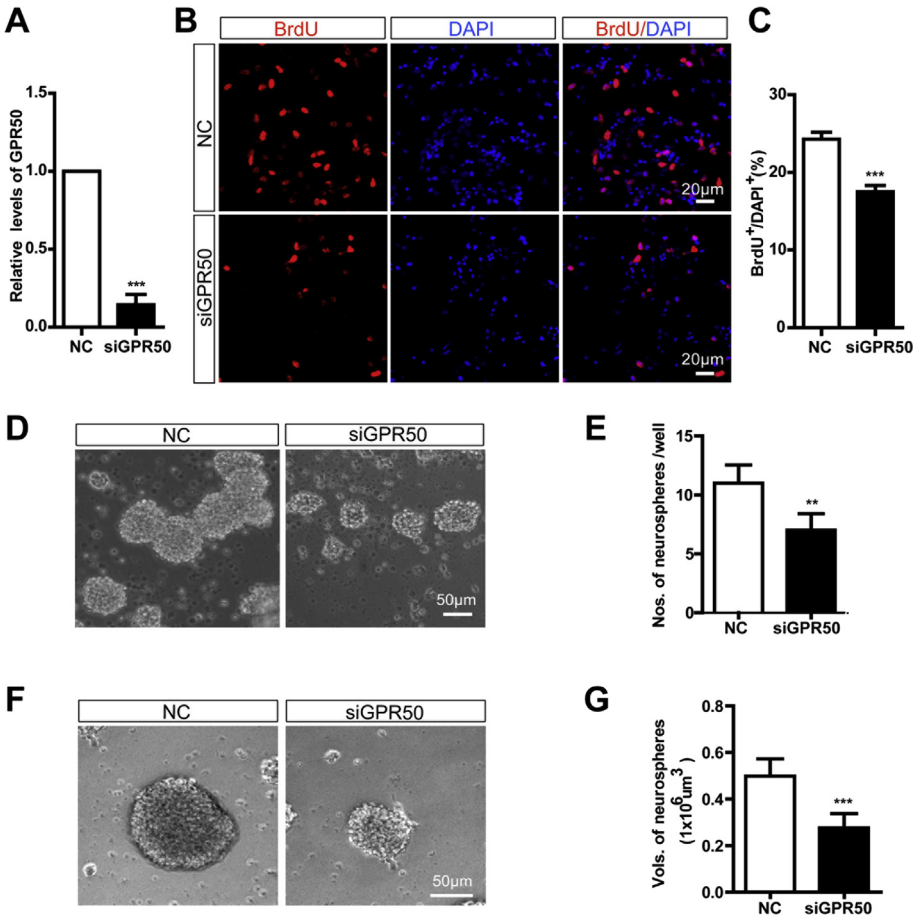

Fig. 2. Knockdown of GPR50 impairs proliferation and self-renewal of NPCs. A, Quantitative PCR analysis of the levels of GPR50 mRNA in cultured NPCs transfected with either GPR50 siRNA or its scrambled control siRNA (NC). B and C, NPCs transfected with GPR50 siRNA or NC were stained for BrdU and DAPI after culturing in medium containing BrdU for 4 h (B). The numbers of BrdU$^+$ cells were counted and expressed as the percentage of the numbers of DAPI$^+$ cells (C). Scale bars: 20 μm. D–G, siGPR50-or NC-transfected NPCs were cultured at low density for 8 days. The numbers (D, E) and size (F, G) of neurospheres were counted and expressed as the amount of neurospheres per well (E) and the volume per neurosphere (G), respectively. Scale bars: 100 μm. Values are presented as Mean ± SEM. n = 3. ***$p < 0.001$; Student's t-test.

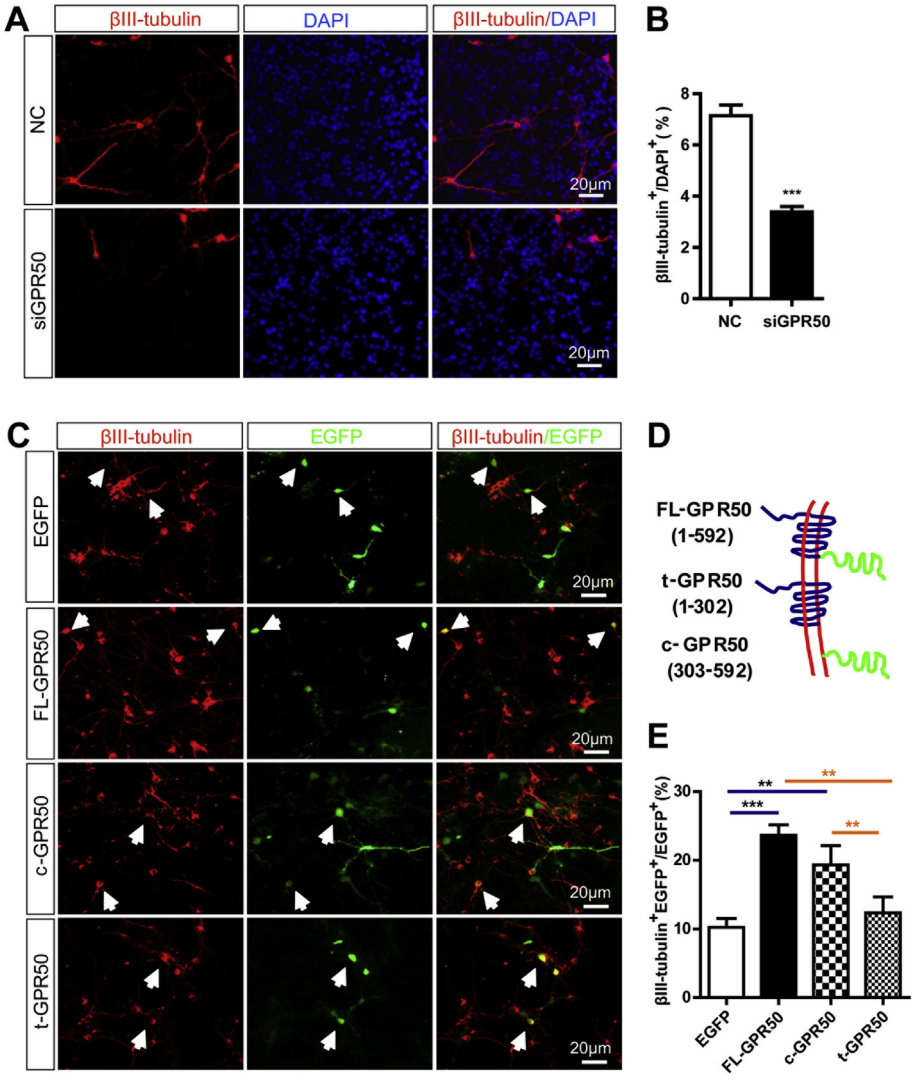

Fig. 3. GPR50 promotes neuronal differentiation through its intracellular domain. A, Cultured NPCs were transfected with GPR50 siRNA and its scrambled control siRNA (NC). After 3–4 days under differentiation condition *in vitro*, cells were stained for βIII-tubulin and DAPI. B, The numbers of βIII-tubulin$^+$ cells were counted and expressed as the percentage of the numbers of DAPI$^+$ cells. C, Cultured NPCs were transfected PCDF-EGFP plasmids expressing FL-GPR50, c-GPR50 and t-GPR50. The empty plasmid expressing EGFP alone was transfected as control (EGFP). The cells were stained for βIII-tubulin after 3–4 days under differentiation condition *in vitro* D, Schematic presentation of mouse full length GPR50 (FL-GPR50; a.a.1–592), of the intracellular domain of GPR50 (c-GPR50; a.a.303–592) and of the truncated GPR50 fragments deleted in its intracellular domain (t-GPR50; a.a.1–302). E, The numbers of βIII-tubulin$^+$ EGFP$^+$ cells were counted and expressed as the percentage of the numbers of EGFP$^+$ cells. Scale bars: 20 μm. Values are presented as Mean ± SEM. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; Student's t-test (B). One-way Anova (E).

investigated whether GPR50 modulates these two signaling pathways in NPCs. Knockdown of GPR50 with siRNA resulted in increased *hes1* mRNA levels (Fig. 4A), the effector gene of notch signaling, but decreased *transcription factor 7-like 2* (*TCF7L2*) mRNA, the effector gene of wnt/β-catenin signaling [19], in cultured NPCs (Fig. 4B). These results suggest GPR50 modulates notch and wnt/β-catenin signalings, possibly *via* regulating the transcriptional activity of *hes1* or *TCF7L2*. Considering that a nuclear localization of the intracellular domain of GPR50 has been observed [26], we examined whether GPR50 would affect the transcriptional activity of *hes1* using a *hes1* promoter driven-luciferase reporter [22]. Upon binding to its ligand, notch is cleaved by several secretases and releases its intracellular domain (NICD), which translocates into the nucleus to form a transcriptional complex that enhances transcription of *hes1* [17]. We co-transfected PCDF-EGFP plasmids encoding a hes1-luciferase reporter construct and NICD together with plasmids encoding either FL-GPR50, c-GPR50 or t-GPR50 into N2a cells. Consistent with previous reports [17], NICD enhances the transcriptional activity of *hes1*, as shown by the increased luciferase intensity detected in NICD-transfected compared to that in empty vector-transfected N2a cells (Fig. 4C). However, FL-GPR50 and c-GPR50 suppressed the activation of NICD on *hes1* transcriptional activity (Fig. 4C), indicating that GPR50 through its intracellular domain suppresses *hes1* transcriptional activity in coordination with NICD. Consistent with the fact that both notch and wnt/β-catenin signalings regulate expression of *Ngns* and *cyclin D1* [21,28] and that GPR50 promotes neuronal differentiation and self-renewal, the mRNA levels of *Ngn1*, *Ngn2* and *cyclin D1* decreased by knockdown of GPR50 in cultured NPCs (Fig. 4D). Thus, GPR50 promotes neuronal differentiation and self-renewal of NPCs, possibly through both notch and wnt/β-catenin signalings.

4. Discussion

As an orphan GPCR, the known function of GPR50 is limited in lipid metabolism, energy homeostasis [29,30], adaptive

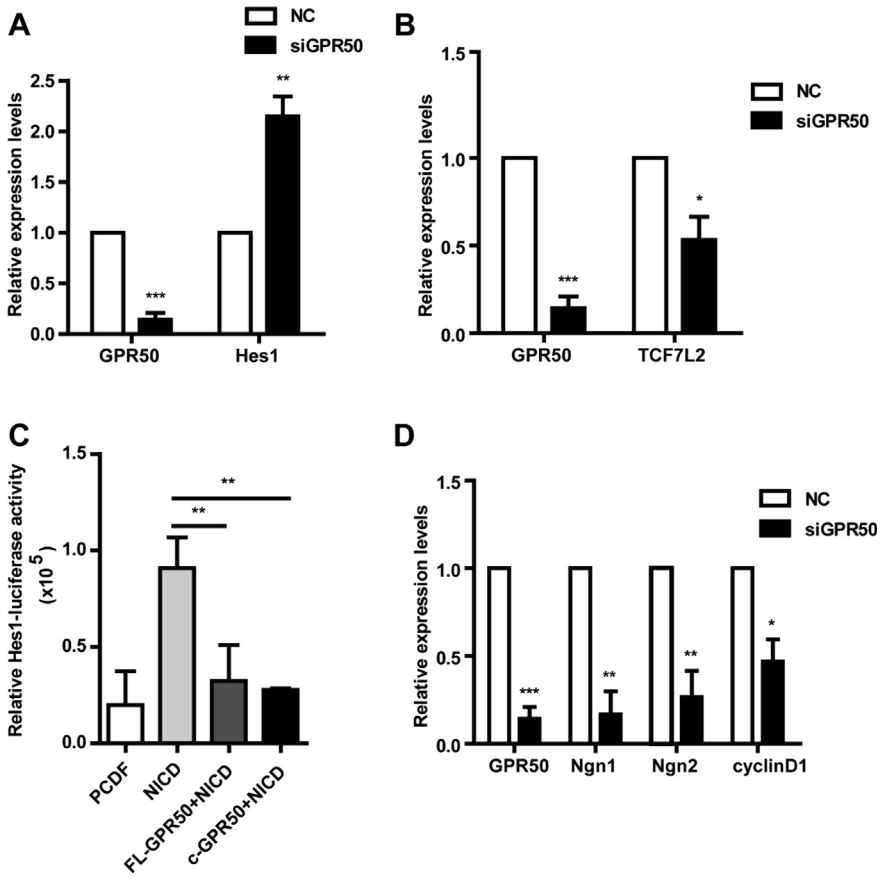

Fig. 4. GPR50 represses notch signaling, whereas enhancing wnt/β-catenin signaling. A, B, D, Quantitative PCR analysis of the mRNA levels of GPR50 (A, B, D), Hes1 (A), TCFL2 (B), Ngn1 (D), Ngn2 (D), cyclin D1 (D) in cultured NPCs transfected with siGPR50 or its scrambled control siRNA (NC) for 36 h. C, Luciferase assay in N2a cells transfected with PGVB-Hes1-luciferase reporter, notch intracellular domain (NICD), and full-length GPR50 (FL-GPR50), intracellular domain of GPR50 (c-GPR50). Values are presented as Mean ± SEM $^{**}p < 0.01$; $^{***}p < 0.001$; One-way Anova (A). Student's t-test (B–D).

thermogenesis and torpor [31]. Expression of GPR50 has been detected in both the adult and embryonic brain [14,16]. However, the function of GPR50 in the brain remains unknown. In this study, we show a novel function of GPR50 in the developmental brain by regulating the proliferation and differentiation of NPCs. Considering that GPR50 is a genetic factor for MDD and BPAD, modulation of NPCs by GPR50 may provide a potential mechanism underlying the function of GPR50 in the pathogenesis of MDD and BPAD.

GPR50 has a long intracellular domain, which translocates to the nucleus after binding to TIP60 [32,33]. These results suggest that the intracellular domain of GPR50 may participate with transcriptional signaling. Consistently, we show the intracellular domain of GPR50 represses the transcription of hes1 by NICD. Moreover, we observe an impairment of wnt/β-catenin signaling in GPR50-knockdown NPCs. However, it remains to be further investigated how GPR50 modulates both Notch signaling and wnt/β-catenin signaling, which are also crosstalked [34]. We further find a requirement of the intracellular domain of GPR50 in promoting neuronal differentiation of NPCs. A truncated GPR50 which lacks of the intracellular domain fails to induce neuronal differentiation. These results further emphasize the essential role of the intracellular domain of GPR50, since Δ502-505 polymorphism in the intracellular domain of GPR50 is associated with both MDD and BPAD [13].

Both notch and wnt/β-catenin signalings play essential roles in modulating NPCs in developmental cortex. Notch signaling through hes1 inhibits neuronal differentiation while enhancing self-renewal [17]. In contrast, wnt/β-catenin signaling promotes neuronal differentiation in the late embryonic stage [19] and self-renewal [20,21]. Knockdown of GPR50 results in suppressing neuronal differentiation and self-renewal, which is accompanied with increased hes1 and decreased TCF7L2 expression. Both pathways promote self-renewal through enhancing *cyclin D1* transcription [20,21,28]. It seems that decreased TCF7L2 overrides the effects of increased hes1 on self-renewal of NPCs. Consistent with this idea, mRNA levels of *cyclin D1* are decreased by knockdown of GPR50. Considering the crosstalk between notch and wnt/β-catenin signaling [34], it requires further investigation how these two pathways coordinate in modulation of self-renewal of NPCs. In contrast, hes1 inhibits, while β-catenin/TCF complex enhances transcription of *Ngns* [17,19,27]. These facts are consistent with our observation on decreased *Ngn1*, *Ngn2* mRNA levels, and suppressed neuronal differentiation in GPR50-knockdown NPCs. However, as a transcriptional factor, we could not exclude the possibility that GPR50 may modulate other proteins/signalings. It requires further investigation on how GPR50 modulates NPCs *in vivo* and thus the development of the cortex.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.040.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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