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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

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