RBPJκ-Dependent Signaling Is Essential for Long-Term Maintenance of Neural Stem Cells in the Adult Hippocampus

Oliver Ehm,1 Christian Göritz,1 Marcela Covic,1 Iris Schäffner,1 Tobias J. Schwarz,1 Esra Karaca,1 Bettina Kempkes,2 Elisabeth Kremmer,1 Frank W. Pfrieger,1 Luis Espinosa,6 Anna Bigas,6 Claudio Giachino,6 Verdon Taylor,7 Jonas Frisén,4 and D. Chichung Lie1

1Research Group Adult Neurogenesis and Neural Stem Cells, Institute of Developmental Genetics, 2Department of Gene Vectors, and 3Institute of Molecular Immunology, Helmholtz Zentrum München, German Research Center for Environmental Health, 85764 Munich-Neuherberg, Germany, 4Department of Cell and Molecular Biology, Medical Nobel Institute, Karolinska Institute, SE-171 77 Stockholm, Sweden, 5Centre National de la Recherche Scientifique Unité Propre de Recherche-3212, Institute of Cellular and Integrative Neurosciences, University of Strasbourg, 67084 Strasbourg, France, 6Institut Municipal d’Investigació Médica-Hospital del Mar, Parc de Recerca Biomèdica de Barcelona, 08003 Barcelona, Spain, and 7Department of Molecular Embryology, Max Planck Institute of Immunobiology, 79108 Freiburg, Germany

The generation of new neurons from neural stem cells in the adult hippocampal dentate gyrus contributes to learning and mood regulation. To sustain hippocampal neurogenesis throughout life, maintenance of the neural stem cell pool has to be tightly controlled. We found that the Notch/RBPJκ-signaling pathway is highly active in neural stem cells of the adult mouse hippocampus. Conditional inactivation of RBPJκ in neural stem cells in vivo resulted in increased neuronal differentiation of neural stem cells in the adult hippocampus at an early time point and depletion of the Sox2-positive neural stem cell pool and suppression of hippocampal neurogenesis at a later time point. Moreover, RBPJκ-deficient neural stem cells displayed impaired self-renewal in vitro and loss of expression of the transcription factor Sox2. Interestingly, we found that Notch signaling increases Sox2 promoter activity and Sox2 expression in adult neural stem cells. In addition, activated Notch and RBPJκ were highly enriched on the Sox2 promoter in adult hippocampal neural stem cells, thus identifying Sox2 as a direct target of Notch/RBPJκ signaling. Finally, we found that overexpression of Sox2 can rescue the self-renewal defect in RBPJκ-deficient neural stem cells. These results identify RBPJκ-dependent pathways as essential regulators of adult neural stem cell maintenance and suggest that the actions of RBPJκ are, at least in part, mediated by control of Sox2 expression.

Introduction

In the adult mammalian brain, neural stem cells (NSCs) in the subgranular zone (SGZ) of the hippocampal dentate gyrus continuously give rise to new functional granule neurons. There is growing evidence that adult hippocampal neurogenesis is important for hippocampus-dependent learning (Kee et al., 2007; Imayoshi et al., 2008; Clelland et al., 2009; Deng et al., 2009; Jessberger et al., 2009) and that impaired neurogenesis may contribute to hippocampal dysfunction observed in neuropsychiatric diseases such as cognitive decline during aging (Kuhn et al., 1996; Drapeau et al., 2003), anxiety and depression (Bergami et al., 2008; Revest et al., 2009), and epilepsy (Jessberger et al., 2005; Jakubs et al., 2006; Parent et al., 2006). The ability of NSCs to generate new neurons throughout life depends on the tight balance of stem cell maintenance and differentiation. Incomplete maintenance and premature differentiation will result in depletion of the NSC pool and, consequently, will lead to decreased levels of neurogenesis over time. Increased stem cell maintenance at the expense of neuronal differentiation will impair the ability of NSCs to generate neurons at a rate necessary for proper hippocampal function. Candidate pathways to control stem cell maintenance in the adult hippocampus include Notch-dependent pathways, which are essential for NSC maintenance, proliferation, and survival during development (Ohmatsu et al., 1999; Hitoshi et al., 2002; Androutsellis-Theotokis et al., 2006; Basak and Taylor, 2007; Mizutani et al., 2007) and control stem cell maintenance in several stem cell niches of the adult organism (Yamamoto et al., 2003; Duncan et al., 2005; Blanpain et al., 2006; Song et al., 2007). Ablation of Notch1 in hippocampal NSCs during the early postnatal period and during adulthood promotes cell cycle exit and neuronal fate determination of NSCs, whereas forced Notch1 signaling increases proliferation of the NSC pool (Breunig et al., 2007). Whether Notch signaling is necessary to maintain the NSC pool and hippocampal neurogenesis throughout adulthood has not been determined. It is also unknown whether Notch signaling controls adult NSCs through the
Materials and Methods

Animals. For all experiments, 8- to 12-week-old mice were used. Mice were housed in standard cages under a 12 h light/dark cycle and had ad libitum access to food and water. C57BL/6J mice and Tg(Cp-EGFP)25Gaia were obtained from Charles River. Hes5-GFP reporter mice were described previously (Basak and Taylor, 2007). Four male Tg(Cp-EGFP)25Gaia and 3 Hes5-GFP reporter animals were analyzed.

GLAST:CreERT2 mice (Slezak et al., 2007) allow for expression of tamoxifen (TAM)-inducible Cre-recombinase controlled by promoter elements of the astrocyte-specific glutamate aspartate transporter (GLAST). GLAST:CreERT2 mice were crossed with RBPJfl/fl mice, in which exons 6 and 7, which code for DNA- and Notch-binding domains, are flanked by loxP sites (Han et al., 2002) and with R26:YFP reporter mice (Srinivas et al., 2001) TAM was injected daily (2 mg) for 5 consecutive days. For loss of RBPJfl function experiments, four to six animals per group were analyzed. Male and female transgenic mice were included in the analysis. Experimental groups were matched for age and sex.

Tissue processing. Animals were killed using CO2. Mice were perfused transcardially with PBS, pH 7.4, for 5 min followed by 4% paraformaldehyde (PFA) for 5 min. Brains were postfixed in 4% PFA overnight at 4°C and were subsequently transferred to a 30% sucrose solution. Forty-micrometer-thick coronal brain sections were made using a sliding microtome (Leica).

Cell culture. Murine adult hippocampal stem/progenitor cells were kept under proliferating conditions in DMEM/F-12 (Invitrogen) supplemented with N2 supplement (Invitrogen), glutamine, and 1% penicillin/streptomycin/fungizone (Invitrogen) in the presence of 20 ng/ml FGF2, 20 ng/ml epidermal growth factor (EGF; PeproTech), and 0.5 μg/ml heparin (Sigma-Aldrich). Medium and growth factors were renewed every second day (Ray and Gage, 2006). Neurospheres were prepared as described previously (Haslinger et al., 2009).

Neurosphere assay. For expression of CRE-recombinase and Sox2, cDNAs for a CRE-recombinase carrying an additional nuclear localization signal (nls) (Kaspar et al., 2002) and murine Sox2 were cloned into the pCAG vector or the pCAG IRES-GFP (Tashiro et al., 2009) to generate pCAG-GFPnlsCRE (Tashiro et al., 2006) and pCAG-Sox2-Dsred.

For single-cell assay, neurospheres were dissociated to single cells and transduced with the retroviruses CAG-GFP, CAG-GFPnlsCRE, or CAG-Sox2-DSRED together with CAG-GFPnlsCRE retrovirus (multiplicity of infections, ∼1). Two days after the transduction, 25 μl of a suspension of 80 cells/ml in culture medium supplemented with 20 ng/ml FGF2 and EGF were plated on two 60-well microtiter plates. Three hours after plating, wells containing single transduced cells were selected. Five days after plating, the percentage of single cells that had generated neurospheres was determined. Cells were supplied with 20 ng/ml FGF2 and EGF every second day. Three independent experiments were conducted.

For assessment of primary and secondary neurosphere formation ability under low cell density conditions, RBPJfl fl neurons were dissociated to single cells and transduced with the retroviruses CAG-GFP, CAG-GFPnlsCRE, or CAG-Sox2-DSRED together with CAG-GFPnlsCRE retrovirus (multiplicity of infections, ∼1). Visual inspection of cells at 24 h after transduction revealed that virtually all cells were transduced with the respective retroviruses. One hundred twenty-five cells in 200 μl of medium were seeded in a 96-well plate and cultured for 7 d. The remaining cells were cultured at a density of 10 per microliter for 7 d. Cells in 96-well plates were analyzed with a fluorescent microscope, and the number of primary neurospheres was determined. Bulk seeded neurospheres were passaged and seeded in a 96-well plate as described above. After 7 d, the numbers of secondary neurospheres were determined. Three independent experiments were performed.

Retrovirus preparation. Retroviruses were generated as described previously. Virus-containing supernatant was harvested four times every 48 h after transfection and concentrated by two rounds of ultracentrifugation (Tashiro et al., 2006). Viral titers were about 1 × 106 colony-forming units ml−1.

Cell lines. DG75 Burkitt’s lymphoma cell line (Ben-Bassat et al., 1977) and SM224.9 (Maier et al., 2005) were used in electrophoretic mobility shift assays (EMSA). HEK293 cells were used in luciferase assays (Lengler et al., 2005).

Electroporation and luciferase assay. To generate the Sox2 luciferase reporter construct, 5.5 kb upstream of the Sox2 transcription start site were cloned into the pGL3basal luciferase vector (Promega). Two million cells were used per electroporation. Cells were electroporated using a Nucleofector II electroporation device (Lonza Cologne). Medium including all supplements was changed 24 h after electroporation. Cells were analyzed 48 h after the electroporation using the dual luciferase kit (Promega) and a Centro LB 960 luminometer (Berthold Technologies). For manipulation of the Notch/RBPJfl pathway, we cloned the murine cDNA for the Notch intracellular domain (NICD) or for the dominant-negative RBPJfl mutant protein (Kato et al., 1997) into the pCAG IRES-GFP expression vector (Jagasia et al., 2009) to generate pCAG-NICD-IRES-GFP or pCAG-RBPJKx2R218H-IRES-GFP. Cells were electroporated with equal molar amounts (500 fmol) of these vectors together with Sox2-Luciferase (3 μg) and Renilla-Luciferase (10 ng). HEK293 cells were transfected using CaCl2 and analyzed at multiple points in time between 6 and 48 h after transfection. Luciferase experiments were performed from three independent electroporations or transfections. For the time line, each point in time represents the mean of three independent experiments.

Histology and counting procedures. Sections were blocked in Tris-buffered saline (TBS) supplemented with 3% normal donkey serum and 0.25% Triton X-100 for 60 min. Brain sections were then incubated in blocking solution containing the primary antibodies at the appropriate dilutions at 4°C for 48 h. Primary antibodies against the following antigens were used: β-galactosidase (rabbit, 1:2000; Cappel/MP Biomedicals), doublecortin (DCX; goat, 1:250; Santa Cruz Biotechnology), DCX (guinea pig, 1:1000; Millipore), GFAP (guinea pig, 1:1000; Zytomed Systems), GFAP (mouse, 1:500; Sigma-Aldrich), GFAP (rabbit, 1:500; Dako), green fluorescent protein (GFP; chicken, 1:1000; Aves Labs), NeuroD1 (goat, 1:200; Santa Cruz Biotechnology), NeuN clone 66 (mouse, 1:100; Millipore), proliferating cell nuclear antigen (PCNA; mouse, 1:500; Santa Cruz Biotechnology), Sox2 (goat, 1:500; Santa Cruz Biotechnology), Sox2 (rabbit, 1:1000; Millipore), and 6-diamidino-2-phenylindole (DAPI; 1:10,000; Sigma-Aldrich). After three washes in TBS, samples were incubated in blocking solution containing the secondary antibody coupled to Cy5, Cy5, FITC, or Alexa 488 (The Jackson Laboratory) at a dilution of 1:250 for 2 h at room temperature. Samples were washed three times with TBS, incubated in 10 ng/ml DAPI (Sigma-Aldrich) for 10 min, and mounted in Aqua Polymount (Polysciences). Confocal single plane images and Z-stacks were taken on a Fluoview 1000 (Olympus) or on a SP5 confocal microscope (Leica). The number of Sox2-, Sox2/GFAP-, DCX-, PCNA-, and yellow fluorescent protein (YFP)-expressing cells in the dentate gyrus was determined in every sixth 40 μm section of the dorsal hippocampus. DAPI staining was used to trace the granule cell layer. For normalization, cell numbers were related to the analyzed granule cell layer volume. For phenotyping, all YFP+ cells were analyzed for coexpression with lineage-specific markers.

RNA isolation, cDNA production, and reverse transcription–PCR. Total RNA was isolated using the RNeasy kit (QiAGEN) or the Trizol reagent (Invitrogen). Isolated RNA was treated with DNase (Promega) according to the manufacturer’s protocol. cDNA was synthesized using the SuperScript First Strand Synthesis System for reverse transcription–PCR (RT-PCR; Invitrogen). Quantitative real-time PCRs were performed on a StepOne device (Applied Biosystems Deutschland). Primers for RT-PCR were as follows: Notch1: forward, GCTGACCTGCGCATGTCTGCCATG; reverse, CTTCTACTGCTCAGGATGCTGCTCCATG.
reverse, CATTTGTCCTGGATGTGGCCACAT; Notch2: forward, CACCCTTGACATCTGCTTCT; reverse, GACCTGGAGAAGAGACTTCC; Notch4: forward, GGAGGCGACACATCTGGTGG; reverse, CAACCGGCGACATCTGAGT; Dll3: forward, CAGTCGCGGCTCACTCCAGAG; reverse, TGGTAGACCAAGTCTGTAGG; Delta3: forward, CDAGGGTCGCTTCATGCACTC; reverse, CATGTTGTCCTGGATGTTGGCATCTG; Sox2: forward, GAAGGAGCTGATCTTATCGAGA; reverse, GAAGCGTGTACTTATCGAGA; Hes5: forward, AGATGCTCAGTGGCACTCGTTCATGCACTC; reverse, TGTTGTCGCTTCATGCACTC; Notch2: forward, ATGCTGAGAAATTCC; reverse, CTTCTATGGCAACAGCGATG; RBPJ: forward, TTCTATGGCAACAGCGATG; reverse, CGGGAAGCGTG- 

Western blotting. For nuclear extracts, cells were allowed to swell on ice in buffer A (in mM: 10 HEPES, pH 8, 10 KCl, 0.1 EDTA, 0.1 EGTA, 2 DTT) for 5 min. Thirty microliters of IGEPA (Sigma-Aldrich) and the cells were vortexed for 10 s. Cells were then centrifuged at 14,000 rpm at 4°C for 1 min. The supernatant constitutes the cytosolic cell fraction and was transferred into a new reaction tube. The pellet was resuspended in 3 vol of buffer A (in mM: 10 HEPES, pH 7.9, 10 KCl, 1.5 MgCl2) and incubated for 60 min on ice. Following homogenization, 10,000 rpm at 4°C for 1 min. The supernatant constitutes the nuclear cell fraction to get an isotonic suspension (—150 mM NaCl). Proteins were blotted on a 0.45 μm BioTrace polynylvinil difluoride-(polyvinylidenfluorid) membrane (Pall Corporation) and were blocked in 5% milk solution [slim milk powder in TBS with 0.1% NaN3] for 1 h at room temperature. Primary antibodies were used in TBST with 3% BSA. Primary antibody incubation was performed under constant shaking/rolling overnight at 4°C. Blots were washed three times with TBST. HRP-conjugated secondary antibodies were used in TBST with 3% BSA. Primary antibody incubation was performed for 1 h at room temperature. Blots were washed three times in TBST and one time in TBS. Protein bands were visualized using ECL solution (GE Healthcare) on ECL hyperfilms (GE Healthcare). The following primary antibodies were used: α-tubulin (mouse, 1:10000; Sigma), Notch1 (rabbit, 1:1000; Santa Cruz Biotechnology), PARP (mouse, 1:2000; Santa Cruz Biotechnology), and Vimentin (mouse, 1:5000; Sigma). Three independent experiments were performed. Preparation of nuclear protein cell extracts for EMSA. Adherent cells were washed with PBS, trypsinized, and spun down at 300 × g for 15 min at room temperature. Suspension cultures were spun down, and the pellet was resuspended in PBS and centrifuged again. The pellet was resuspended in 3 vol of buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2) and incubated for 60 min on ice. Following homogenization, cells were centrifuged for 10 s at 14,000 rpm at 4°C. Three hundred microliters of buffer A were added to the pellet and briefly vortexed. The resulting suspension was centrifuged for 10 s at 14,000 rpm at 4°C. The pellet was resuspended in 3 vol of buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 4.2 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, pH 8.5, 0.5 mM protease inhibitors), incubated for 30 min on ice, and centrifuged at 140,00 rpm for 20 min at 4°C. Aliquots were stored at —80°C. EMSA. The MatInspector and Eldorado algorithms in Genomatix software (Genomatix Software) were used for the prediction of potential binding sites for RBPJκ in the promoter sequence of the Sox2 gene. Predicted binding sites, with respect to the transcription start site, were as follows: #1: from —4475 to —4461, + strand; ATGCGTGGAAATTTCC; #2: from —3746 to —3732, + strand; CTAATAGAAGATAG; #3: from —2538 to —2524, + strand; AGCCCTGGGAAATGG; #4: from —1983 to —1969, — strand; GCTTGGGAGAATTG; #5: from —1472 to —1458, — strand; AGCGTGGGAAATGG.

Equimolar amounts of both single-stranded DNA oligonucleotides containing potential RBPJκ-binding sites were mixed in annealing buffer. Oligonucleotides comprising RBPJκ-binding sites were designed as follows: #1 forward, CTAATTAGAATGGTGGAGAAT; reverse, CCTGTGTAACTGGTTAATTTCAAG; #2 forward, TGAGAAATAGGTTTTGGTACCT; reverse, AATCTTGGGAGGATCCATTTGTTA; #3 forward, TTGAGAAATAGGTTTTGGTACCT; reverse, AATCTTGGGAGGATCCATTTGTTA; #4 forward, GGCTGTGGGAGAATGG; reverse, ATGCTGAGAAATTCC; #5 forward, AGCGTGGGAAATGG; reverse, ATGCTGAGAAATTCC. Equimolar amounts of both single-stranded DNA oligonucleotides containing potential RBPJκ-binding sites were mixed in annealing buffer. Oligonucleotides comprising RBPJκ-binding sites were designed as follows: #1 forward, CTAATTAGAATGGTGGAGAAT; reverse, CCTGTGTAACTGGTTAATTTCAAG; #2 forward, TGAGAAATAGGTTTTGGTACCT; reverse, AATCTTGGGAGGATCCATTTGTTA; #3 forward, TTGAGAAATAGGTTTTGGTACCT; reverse, AATCTTGGGAGGATCCATTTGTTA; #4 forward, GGCTGTGGGAGAATGG; reverse, ATGCTGAGAAATTCC; #5 forward, AGCGTGGGAAATGG; reverse, ATGCTGAGAAATTCC.
For quantitative PCRs, the following primers were used to amplify regions within the Sox2 promoter, which contain a potential RBPJκ-binding site (numbers correspond to the predicted RBPJκ-binding site; see above): #1 forward, GCAGTGAGGGGTTGCCAAGACT; reverse, GCAGTGAGGGGTTGCCAAGACT; #3 forward, GCGTGGAGGAGCGTGGACT; reverse, GCTCCGCTACTTGCTTAC; #4 forward, CATTGGGAGATCGGCTAAAA; reverse, AGCGGCAGGGTTGATGTCGAC; #5 forward, CCTGGTGTAGGGTGATGGG; reverse, CTCGTCCTCGGTCACAC. Primers for the HeS1 were as follows: forward, ACAGCCGAACACCAAGAGAC; reverse, GTCACCTCGTTGATGTCGAC. Primers for the amplification of an RBPJκ-unrelated region within the Sox2 promoter were as follows: forward, CCCAGGTAAGCAGGGATTCTT; reverse, GCATTGCTTTGGGAGAAGAGAC.

For detection, Brilliant II Fast SYBR Green qPCR Master Mix (Agilent) was used according to the manufacturer’s protocol. Three independent experiments were performed.

Statistical analysis. Unpaired Student’s t test was used for analysis of most experiments. Before the t test, an F test was performed. In those cases, in which the F test resulted in a difference in the variances, a Mann–Whitney–Wilcoxon rank sum test was used. Differences were considered statistically significant at p < 0.05. All data are presented as mean ± SEM.

Results

Notch signaling is differentially active in stem cells and neurally committed cells

In canonical Notch signaling, binding of ligands to the Notch receptor results in the cleavage of the NICD. NICD then translocates to the nucleus to interact with the transcriptional regulator RBPJκ to induce the expression of target genes (Baron, 2003). To determine the activity of Notch/ RBPJκ signaling in the adult hippocampal neurogenic niche, we analyzed two distinct Notch/RBPJκ signaling reporter mouse lines. Tg(Cp-EGFP)125Gaia mice (Duncan et al., 2005; Mizutani et al., 2007) and HeS5-GFP transgenic mice (Basak and Taylor, 2007) express enhanced GFP (EGFP) under the control of multimerized RBPJκ-binding sites and the HeS5 promoter, respectively. Both transgenic mouse lines have been successfully used to determine canonical Notch signaling activity in vivo (Duncan et al., 2005; Basak and Taylor, 2007; Mizutani et al., 2007). To determine the activity of canonical Notch signaling in NSCs and neurally committed cells, we stained hippocampal tissue from these reporter mice for the transcription factors SOX2 and NeuroD, which are sequentially expressed in the hippocampal neurogenic lineage where they control NSC maintenance (Favaro et al., 2009; Kuwabara et al., 2009) and neuronal fate commitment (Gao et al., 2009; Kuwabara et al., 2009), respectively. In both reporter mouse lines, EGFP was expressed in Sox2-positive NSCs in the SGZ (Fig. 1). Quantification of Sox2 EGFP coexpression in HeS5-GFP transgenic mice revealed reporter activity in the vast majority of Sox2-positive cells in the SGZ (94.8 ± 1.9%) (Fig. 1b,c). No EGFP expression was detected in NeuroD-expressing cells in the SGZ in either of the two reporter lines (Fig. 1). Thus, EGFP reporter activity in both transgenic mouse lines consistently indicate that...
Notch signaling through RBPJκ is active in Sox2-positive stem cells and that Notch signaling is inactivated in parallel with the loss of Sox2 expression, the initiation of NeuroD expression, and the neuronal fate commitment of NSCs. The additional reporter activity, which was observed in scattered cells in the granule cell layer of Tg(Cp-EGFP)25Gaia mice (Fig. 1a) raises the possibility that canonical Notch signaling may also be active in a subset of mature granule neurons.
Impaired stem cell maintenance and transient enhancement of neurogenesis after inactivation of RBPJκ

To test the role of Notch/RBPJκ signaling in adult hippocampal stem cell maintenance in vivo, we inactivated RBPJκ in NSCs of the adult hippocampus. We took advantage of the BAC transgenic mouse line that expresses TAM-dependent Cre recombinase (CreER<sup>T2</sup>) under the control of the GLAST promoter (GLAST::CreERT2). In these mice, the CreER<sup>T2</sup> transgene is strongly expressed during adulthood in Sox2-expressing radial glia-like stem cells of the adult hippocampus (type 1 cells) (Slezak et al., 2007). To generate mice, in which Notch signaling through RBPJκ can be conditionally ablated in type 1 cells, GLAST::CreER<sup>T2</sup> mice were crossed with mice carrying conditional alleles for RBPJκ (RBPJκ<sup>loxP/loxP</sup>) (Han et al., 2002) and R26::EYFP reporter mice (Srinivas et al., 2001) to generate GLAST::CreERT2; RBPJκ<sup>loxP/loxP</sup>; R26::EYFP (RBPJκ-cKO) (Fig. 2a). GLAST::CreERT2; RBPJκ<sup>loxP/loxP</sup>; R26::EYFP (control; i.e., mice in which only one RBPJκ allele can be deleted after induction of Cre recombinase activity) served as controls.

Twelve-week-old animals were treated for 5 consecutive days with TAM to induce recombination in the radial glia-like NSCs. Animals were killed 3 weeks after the final TAM injection. Intriguingly, the fraction of Sox2-expressing cells among the recombined cells, which were identified on the basis of YFP expression, was greatly reduced in RBPJκ-cKO (RBPJκ-cKO, 30.32 ± 4.87% vs control, 69.60 ± 3.67%; p < 0.001) (Fig. 2h). The Sox2-expressing stem cell population in the SGZ consists of quiescent or slowly dividing multipotent stem cells with a radial glia-like morphology (type 1 cells) and of nonradial stem/precursor cells (type 2 cells), which show higher levels of proliferation (Kempermann et al., 2004; Suh et al., 2007). We identified these populations using the following criteria: radial glia-like morphology (type 1 cells) and of nonradial stem/precursor cells (type 2 cells).
47,097 ± 1,231/mm³; p < 0.05) were significantly reduced in the SGZ of RBPJκ-cKO mice (Fig. 2b,d). Hence, loss of RBPJκ in NSCs decreases the NSC pool in the hippocampal neurogenic niche.

Despite the reduction in the NSC pool, higher numbers of YFP-positive cells were observed in RBPJκ-cKO compared with control mice (RBPJκ-cKO, 66,839 ± 9,785/mm³ vs 46,196 ± control, 5,662/mm³; p < 0.05) (Fig. 3b). Moreover, RBPJκ-cKO showed an overall increase in the number of cells expressing the proliferation marker PCNA (RBPJκ-cKO, 31,526 ± 5,816/mm³ vs control, 2,468 ± 797/mm³; p < 0.01) and an increased fraction of proliferating cells among the YFP-positive cells (RBPJκ-cKO, 30.0 ± 1.3% vs control, 2.8 ± 1.3%; p < 0.001), indicating that inactivation of RBPJκ in NSCs increased cell genesis (Fig. 3a,c,e). In RBPJκ-cKO, a much higher proportion of Sox2 cells expressed PCNA (RBPJκ-cKO, 29.2 ± 12.3 vs control, 3.3 ± 1.7; p < 0.05), demonstrating that the remaining NSCs were recruited into proliferation. RBPJκ-cKO mice showed a fourfold to fivefold increase in the fraction of DCX-expressing immature neurons among the recombined cells (RBPJκ-cKO, 81.87 ± 5.88% vs control, 25.47 ± 5.43%; p < 0.001) and in the total number of DCX-positive (RBPJκ-cKO, 121,345 ± 6,432/mm³ vs control, 16,871 ± 1,908/mm³; p < 0.001) and NeuroD-positive (RBPJκ-cKO, 22,585 ± 28,819/mm³ vs control, 24,026 ± 6,906/mm³; p < 0.001) immature neurons (Fig. 3d,f–h). A great proportion of the neurons that were generated in excess in response to loss of RBPJκ showed long-term survival, as evidenced by the fact that the density of YFP-labeled mature neurons was significantly increased in RBPJκ-cKO 2 months after recombination (RBPJκ-cKO, 17,383 ± 3,870 cells/mm³ vs 4,405 ± control, 2,042; p < 0.01).

Surprisingly, we also observed alterations in the behavior of YFP-negative cells in the dentate gyrus of RBPJκ-cKO. In fact, RBPJκ-cKO showed greatly increased numbers of YFP-negative, DCX-expressing immature neurons (RBPJκ-cKO, 39,924 ± 9,476 cells/mm³ vs control, 7,595 ± 327 cells/mm³; p < 0.05) and YFP-negative proliferating cells (RBPJκ-cKO, 11,619 ± 3,791 cells/mm³ vs control, 1,040 ± 248 cells/mm³; p < 0.05) (Fig. 4). Similar to the YFP-positive cell compartment, a high percentage of YFP-negative NSCs was found to be proliferating in RBPJκ-cKO (~30%). Moreover, the estimated density of YFP-negative type 1 and type 2 cells was decreased in RBPJκ-cKO compared with control (supplemental Table 1, available at www.jneurosci.org as supplemental material). We cannot fully exclude that the lower density of YFP-negative type 1 and type 2 cells is a function of different recombination efficiencies in RBPJκ-cKO and control mice. The fact that the density of YFP-negative proliferating cells and of YFP-negative, DCX-positive cells in RBPJκ-cKO exceeds the density of proliferating cells and DCX-positive newborn neurons in control animals, however, strongly indicates that inactivation of RBPJκ-cKO affects proliferation and neurogenesis also from cells with intact RBPJκ-mediated signaling.

Together, our results demonstrate that conditional ablation of RBPJκ results in a reduction in the hippocampal NSC pool and an increase in proliferation and the generation of new neurons 3 weeks after recombination. These findings indicate that RBPJκ-dependent pathways regulate the balance between stem cell maintenance and differentiation within the adult hippocampal neurogenic niche.

**RBPJκ is essential for long-term NSC maintenance in the adult hippocampus**

Next, we sought to determine the long-term consequences of loss of RBPJκ signaling in adult NSCs on hippocampal neurogenesis. To this end, 12-week-old RBPJκ-cKO and control mice were treated for 5 consecutive days with TAM. Mice were examined 2 months after the induction of recombination. Immunostaining with GFAP and Sox2 revealed an even more pronounced decrease at this time point in the density of Sox2-expressing cells (RBPJκ-cKO, 23,928 ± 4,788/mm³ vs 59,805 ± 4,852/mm³; p < 0.01) and radial glia-like stem cells in RBPJκ-cKO (RBPJκ-cKO, 5,503 ± 1,005/mm³ vs control, 16,386 ± 2,147/mm³; p < 0.01) (Fig. 5a,b). In striking contrast to the 3 week time point, neurogenesis was almost absent in RBPJκ-cKO as evidenced by the almost complete lack of PCNA-positive proliferating cells (Fig. 5f) and strong reduction in NeuroD- and DCX-positive cells (RBPJκ-cKO, 1,408 ± 466/mm³ vs control, 13,687 ± 4,296/mm³; p < 0.05) (Fig. 5d,e) in the dentate gyrus of RBPJκ-cKO mice. Consistent with these findings, the fraction of Sox2-negative cells in the dentate gyrus of RBPJκ-cKO mice was significantly reduced compared with control (Fig. 5c). These findings strongly indicate that inactivation of RBPJκ strongly decreases the NSC pool in the hippocampal neurogenic niche.
YFP-positive cells were NeuN-expressing neurons (RBPJ recombination. Cells were phenotyped according to the following criteria: type 1, Sox2, DCX, indicating that recombined cells do not contribute to the generation of new neurons 2 months after induction of recombination. The majority recombined radial glial like stem cells have left the stem cell compartment. Almost no YFP-positive cells express Sox2-expressing stem cells 2 months after recombination.

Loss of RBPJ in radial glia-like stem cells decreases hippocampal neurogenesis and leads to persistent loss of Sox2-expressing stem cells 2 months after recombination. a, Representative confocal images of RBPJκ-cKO (right) and control mice (left). In RBPJκ-cKO mice, YFP-positive recombined cells (green) do not express Sox2 (red) or GFAP (blue). Staining for GFAP demonstrates a strong reduction in the number of radial glia-like stem cells in the dentate gyrus. The vast majority of YFP-positive cells in RBPJκ-cKO is located in the granule cell layer. DAPI is shown in gray. Scale bar, 20 μm. b, Sox2-expressing cells, radial glia-like stem cells (type 1 cells), and nonradial stem cells (type 2 cells) are depleted from the SGZ of the dentate gyrus in RBPJκ-cKO mice (**p < 0.01). c, Phenotyping of YFP-positive cells demonstrates that the vast majority recombined radial glial like stem cells have left the stem cell compartment. Almost no YFP-positive cells express DCX, indicating that recombined cells do not contribute to the generation of new neurons 2 months after induction of recombination. Cells were phenotyped according to the following criteria: type 1, Sox2 + GFAP + and radial morphology; type 2, Sox2 + GFAP −; immature neurons, DCX +; mature neurons, NeuN +. d, Representative confocal images of RBPJκ-cKO and control mice. In RBPJκ-cKO mice, DCX-expressing immature neurons (red) are virtually absent. YFP is shown in green, and DAPI is shown in blue. Scale bar, 20 μm. e, The density of DCX-expressing immature neurons in the dentate gyrus is severely reduced (*p < 0.05). f, Representative confocal images of RBPJκ-cKO and control mice. In RBPJκ-cKO mice, proliferating cells identified by the expression of PCNA (red) are virtually absent. DAPI is shown in blue. Scale bar, 20 μm.

Figure 5. Loss of RBPJκ in radial glia-like stem cells decreases hippocampal neurogenesis and leads to persistent loss of Sox2-expressing stem cells 2 months after recombination. a, Representative confocal images of RBPJκ-cKO (right) and control mice (left). In RBPJκ-cKO mice, YFP-positive recombined cells (green) do not express Sox2 (red) or GFAP (blue). Staining for GFAP demonstrates a strong reduction in the number of radial glia-like stem cells in the dentate gyrus. The vast majority of YFP-positive cells in RBPJκ-cKO is located in the granule cell layer. DAPI is shown in gray. Scale bar, 20 μm. b, Sox2-expressing cells, radial glia-like stem cells (type 1 cells), and nonradial stem cells (type 2 cells) are depleted from the SGZ of the dentate gyrus in RBPJκ-cKO mice (**p < 0.01). c, Phenotyping of YFP-positive cells demonstrates that the vast majority recombined radial glial like stem cells have left the stem cell compartment. Almost no YFP-positive cells express DCX, indicating that recombined cells do not contribute to the generation of new neurons 2 months after induction of recombination. Cells were phenotyped according to the following criteria: type 1, Sox2 + GFAP + and radial morphology; type 2, Sox2 + GFAP −; immature neurons, DCX +; mature neurons, NeuN +. d, Representative confocal images of RBPJκ-cKO and control mice. In RBPJκ-cKO mice, DCX-expressing immature neurons (red) are virtually absent. YFP is shown in green, and DAPI is shown in blue. Scale bar, 20 μm. e, The density of DCX-expressing immature neurons in the dentate gyrus is severely reduced (*p < 0.05). f, Representative confocal images of RBPJκ-cKO and control mice. In RBPJκ-cKO mice, proliferating cells identified by the expression of PCNA (red) are virtually absent. DAPI is shown in blue. Scale bar, 20 μm.

positive radial glia-like stem cells (RBPJκ-cKO), 10.7 ± 3.3% vs control, 26.7 ± 8.2%; p < 0.05) (Fig. 5c) and nonradial stem/precursor cells (RBPJκ-cKO, 9.6 ± 2.7% vs control, 32.8 ± 3.1%; p < 0.01) (Fig. 5c). d) was greatly decreased, and DCX-expressing immature neurons were almost absent (RBPJκ-cKO, 0.2 ± 0.4% vs control, 16.3 ± 5.5%; p < 0.01) (Fig. 5c) among the recombined cells in the dentate gyrus of RBPJκ-cKO. The majority of YFP-positive cells were NeuN-expressing neurons (RBPJκ-cKO, 76.2 ± 18.4% vs control, 15.5 ± 6.2%; p < 0.01) (Fig. 5c). Because NeuN is expressed in the hippocampal neurogenic lineage predominantly by mature neurons, it is likely that these YFP-positive neurons were generated early after induction of recombination. The long-term depletion of NSCs and the persistent loss of neurogenesis after inactivation of RBPJκ in adult NSCs strongly support the notion that RBPJκ-dependent pathways are essential for stem cell maintenance in the adult hippocampal neurogenic niche.

RBPJκ-dependent signaling controls NSC maintenance directly

The perturbation of maintenance, proliferation, and neurogenesis in recombined and nonrecombined NSCs suggests that the inactivation RBPJκ has a pronounced effect on the hippocampal microenvironment. To investigate, whether RBPJκ also contributes to stem cell maintenance independently of the niche (i.e., through cell-autonomous mechanisms), we sought to determine the effects of RBPJκ inactivation on stem cell maintenance in a “niche-free” system. To this end, we established neurosphere cultures from adult RBPJκloxp/loxp mice and performed single-cell neurosphere-forming assays as a measurement of stem cell maintenance and self-renewal. Recombination of the RBPJκ locus was induced via transduction with retrovirus encoding for CRE-GFP fusion protein (Tashiro et al., 2006); retrovirus encoding for GFP served as a control. Previous comparison of CRE-GFP and GFP transduced neurospheres derived from wild-type mice had shown that transduction with CRE-GFP does not impair survival and neurosphere-forming capacity (I. Schaeffner and D. C. Lie, unpublished results). Cells were left for 48 h to allow for transgene expression and recombination of the RBPJκ locus. Quantitative PCR analysis showed loss of RBPJκ mRNA expression, indicating that expression of CRE-GFP resulted in efficient recombination of the RBPJκ locus (Fig. 6b).

Although we transduced equal numbers of cells with CRE-GFP virus and control virus, cell numbers in CRE-GFP transduced cultures were repeatedly reduced to ~20% of controls (data not shown). Single cells were seeded into miniwells 48 h after transduction. Cultures were visually inspected and marked for the presence of a single-cell and transgene expression 3 h after seeding (Fig. 6f). Five days after seeding, a large fraction of control single cells had generated neurospheres (38.6 ± 1.8%). In contrast, CRE-GFP transduced cells showed an ~50% reduced ability to generate neurospheres in single-cell neurosphere assays (20.5 ± 6.4%; p < 0.01) (Fig. 6c). Moreover, the remaining neurospheres were significantly smaller in diame-
Recent work by Favaro et al. (2009) has demonstrated that the transcription factor Sox2 is essential for maintenance of hippocampal NSCs. Intriguingly, we found that a large proportion of CRE-GFP transduced cells were Sox2 negative, whereas almost all control transduced cells expressed Sox2 (CRE-GFP transduced cells, 65.9 ± 15.3% vs control, 95.6 ± 0.8%; *p < 0.05) (Fig. 6a). This and our previous observation that RBPJκ-signaling reporters are active in Sox2-expressing hippocampal NSCs in vivo raised the question whether Sox2 expression may be regulated by RBPJκ-dependent signaling. A 5.5 kb region upstream of the Sox2 transcription start site in the mouse Sox2 gene has previously been shown to control Sox2 expression in telencephalic NSCs during development (Zappone et al., 2000) and to be sufficient to mimic endogenous Sox2 expression in the adult neurogenic zones (Suh et al., 2007). Interestingly, in silico analysis of the 5.5 kb Sox2 promoter fragment using the MatInspector and Eldorado algorithms of the Genomatix software predicted five RBPJκ-binding sites (Fig. 7a). To determine whether Notch signaling can enhance the activity of the Sox2 promoter, a reporter construct (5.5 kb Sox2-luciferase) was generated in which the expression of the firefly luciferase is controlled by this 5.5 kb Sox2 promoter. To investigate whether the Sox2 promoter is activated by Notch signaling, we determined Sox2–luciferase activity in HEK293 cells after cotransfection with an expression construct for activated Notch receptor 4 (i.e., NICD) at multiple time points after transfection (6–48 h). Compared with control cells, which were transfected with an expression construct for GFP, NICD-transfected cells showed reproducible significant induction of Sox2 promoter activity starting from 18 h after transfection (Fig. 7b). We also sought to determine whether activation of the Notch pathway can stimulate the Sox2 promoter and Sox2 expression in an NSC context. To this end, we used neural stem/progenitor cell cultures isolated from the hippocampus of adult mice (Ray and Gage, 2006), as expression plasmids and reporter plasmids can be introduced into these cells with high efficiency via electroporation. RT-PCR, Western blot, and immunocytochemical analysis revealed that Sox2 was highly expressed in these cultures (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). In addition, RT-PCR analysis revealed expression of essential components of the canonical Notch-signaling cascade including Notch receptors 1–4 and RBPJκ and expression of the canonical Notch–signaling targets Hes1 and Hes5 (Ohtsuka et al., 1999) (supplemental Fig. 1, available at

![Figure 6](image_url). a, Immunocytochemical analysis of neurospheres derived from adult RBPJκloxp/loxp mice 48 h after transduction with the CAG-GFP (control) or the CAG-GFPnlsCRE (CRE-GFP) retrovirus (green). Cells were dissociated and fixed onto slides. Note that a number of CRE-GFP transduced cells are negative for Sox2 (red, arrowheads). b, Quantitative PCR reveals significantly decreased (p < 0.001) expression of RBPJκ mRNA in CAG-GFPnlsCRE (CRE) transduced RBPJκloxp/loxp neurospheres. c–f, Neurosphere assays of RBPJκloxp/loxp NSCs after transduction with CAG-GFP (control), CAG-GFPnlsCRE (CRE), or CAG-GFPnlsCRE and CAG-SOX2 IRES dsRED (CRE + SOX2). c, Analysis of neurosphere-forming efficiency of RBPJκloxp/loxp NSCs in single-cell assays (*p < 0.05; **p < 0.01). d, Analysis of neurosphere diameter of RBPJκloxp/loxp NSCs 7 d after transduction (*p < 0.05). e, Analysis of primary and secondary neurosphere-forming efficiency of RBPJκloxp/loxp NSCs in low-density cell assays (*p < 0.05; **p < 0.01). f, Left, Representative image of a CAG-GFP transduced single cell 3 h after seeding into a minicell. Right, Representative images of neurospheres formed by RBPJκloxp/loxp NSCs 5 d after plating. Top, Bright field; bottom, fluorescent analysis. Scale bar, 100 μm.
using a 5.5 kb Sox2-luciferase demonstrate that the Sox2 promoter is activated by Notch signaling. This activation was inhibited by significant activation 18 h after transfection with NICD (*). Reporter assays in HEK293 cells using the Sox2 promoter revealed the presence of a 120 kDa NICD fragment in nuclear extracts, which suggested that Notch signaling is active in adult hippocampal NSCs (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Finally, Western blot analysis of adult hippocampal NSCs after overexpression of NICD or GFP as control indicated that Notch signaling increased Sox2 expression, adult hippocampal stem cells and that RBPJκ is present in the nucleus of adult hippocampal NSCs.

Next, chromatin immunoprecipitation (ChIP) analysis was performed to directly investigate association of Notch-signaling components with the endogenous Sox2 promoter in adult NSCs. Chromatin was precipitated with antibodies specific for RBPJκ, whereas no shift was detected from RBPJκ knock-out cell line (SM224.9) (Fig. 7e). These experiments showed that RBPJκ is present in the nucleus of adult hippocampal stem cells and that RBPJκ can bind to at least some of the predicted binding sites in the Sox2 promoter.

To further analyze potential RBPJκ-binding sites in the 5.5 kb Sox2 promoter, we conducted EMSAs with nuclear extracts from adult hippocampal stem cells. Oligonucleotides corresponding to the predicted RBPJκ-binding site sequences from the 5.5 kb Sox2 promoter were used in these assays. Repeated failure to radioactively label oligonucleotide for the predicted RBPJκ-binding sites #2 (−3746 to −3732) and #3 (−2538 to −2524) precluded EMSA analysis of these sequences. Oligonucleotides encompassing the predicted binding sites #1 (−4475 to −4461), #4 (−1983 to −1699), and #5 (−1472 to −1458) were shifted on the gel after previous incubation with nuclear extracts from mouse stem/progenitor cells. Additional incubation with an antibody directed against RBPJκ resulted in a supershifted signal on the gel demonstrating that the observed shift was caused by binding of RBPJκ. The same results were obtained with recombinant RBPJκ protein and with nuclear extracts from RBPJκ overexpressing cells (DG75), whereas no shift was detected with nuclear extracts from an RBPJκ knock-out cell line (SM224.9) (Fig. 7f). These experiments showed that RBPJκ is present in the nucleus of adult hippocampal stem cells and that RBPJκ can bind to at least some of the predicted binding sites in the Sox2 promoter.
ers against an unrelated sequence were used as controls. Lack of suitable antibodies against Notch 2–4 precluded ChIP analysis of the Sox2 promoter for these proteins. As expected, enrichment of RBPJκ and Notch1 was observed on RBPJκ sites in the Hes1 promoter. Importantly, enrichment of RBPJκ and Notch1 was found on the predicted RBPJκ sites #1 and #5 within the endogenous 5.5 kb Sox2 promoter, demonstrating that Sox2 is a direct target of Notch/RBPJκ signaling in adult NSCs (Fig. 7g). Because only activated Notch translocates to the nucleus to interact with the transcriptional regulator RBPJκ to induce the target gene expression, the association of Notch1 and RBPJκ on the Sox2 promoter indicates that active Notch/RBPJκ signaling directly targets the Sox2 promoter in adult hippocampal NSCs.

Having identified the essential stem cell maintenance gene as a Notch/RBPJκ signaling target and given the observation that Sox2 expression is decreased after inactivation of RBPJκ, we asked the question whether Sox2 expression can compensate for the stem cell maintenance/self-renewal defect of RBPJκ-deficient NSCs. To this end, RBPJκloxP/loxP neurospheres were transduced with CRE-GFP in combination with a retrovirus encoding for Sox2 and red fluorescent protein and subjected to the single-cell neurosphere assay 48 h later. Independent experiments showed that Sox2 expression in nonrecombined NSCs does not enhance the efficiency of neurosphere formation (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Strikingly, Sox2 rescued the neurosphere-forming defect of RBPJκ-deficient NSCs in single-cell assays (Fig. 6c,d,f) and greatly enhanced the ability of RBPJκ-deficient NSCs to generate primary and secondary neurospheres in low cell density neurosphere assays (Fig. 6e). These results indicate that Sox2 can, at least in part, functionally compensate for loss of RBPJκ signaling with regard to stem cell self-renewal and strongly suggest that a Notch/RBPJκ/Sox2 pathway is contributing to adult NSC maintenance.

Discussion
The balance of NSC maintenance and neurogenesis is essential to ensure the generation of new hippocampal granule neurons throughout lifetime at a functionally relevant rate. Our study demonstrates that RBPJκ is an essential component of the regulatory network controlling this balance, as inactivation of RBPJκ in NSCs results in depletion of the stem cell compartment and a transient burst in proliferation and the production of new neurons. The long-term consequence of impaired stem cell maintenance after loss of RBPJκ is an almost complete loss of hippocampal neurogenesis.

RBPJκ is the central transcriptional downstream effector in canonical Notch signaling. We observed that Notch/RBPJκ 32 signaling is active in NSCs in vivo and in vitro, which indicates that the phenotype of RBPJκ-cKO is the consequence of loss of canonical Notch signaling. This argument is supported by the findings that loss of Notch1 receptor in adult NSCs also results in loss of the radial glia-like stem cell population (Ables et al., 2010) and increased neuronal differentiation of stem cells (Breunig et al., 2007). It is, however, important to note that inactivation of RBPJκ and of Notch1 does not produce completely similar phenotypes. Loss of Notch1 in stem cells of the early postnatal hippocampus does not lead to a transient increase in stem cell proliferation but, rather, promotes cell cycle exit (Breunig et al., 2007). Moreover, overexpression of activated Notch in stem cells of the early postnatal hippocampus strongly enhances proliferation (Breunig et al., 2007). It is possible that Notch/RBPJκ 32 signaling has distinct functions during the early postnatal period and during adulthood. It is, however, more likely that the phenotypic differences are caused by the fact that Notch signaling was perturbed at different levels. We and others have found that other Notch receptors are expressed in NSCs (supplemental Fig. 1, available at www.jneurosci.org as supplemental material) and the hippocampal neurogenic niche (Breunig et al., 2007), raising the possibility that signaling of other Notch receptors through RBPJκ in the Notch1 mutants may be responsible for the phenotypic differences. It has also been demonstrated that RBPJκ-independent Notch signaling promotes stem cell proliferation in the adult CNS (Androutsellis-Theotokis et al., 2006). Because RBPJκ-independent pathways are left intact in the RBPJκ-cKO, the phenotypic differences could also be the result of differences in the activity of noncanonical Notch signaling in NSCs.

Recent studies revealed considerable heterogeneity among NSCs from distinct neurogenic regions with regard to their differentiation patterns (Hack et al., 2005; Merkle et al., 2007; Brill et al., 2009) and their proliferative/self-renewal behavior (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). In addition, a number of signaling pathways appear to have region-specific functions on adult NSC proliferation and differentiation (Kuhn et al., 1997; Lie et al., 2005; Adachi et al., 2007). Imayoshi et al. (2010) recently reported that inactivation of RBPJκ in stem cells of the adult subventricular zone results in a neurogenesis phenotype, which is highly similar to the hippocampal neurogenesis phenotype observed in this study. Together, these studies provide strong evidence for the notion that the mechanisms of stem cell maintenance are shared between the principal adult neurogenic niches. In contrast to the study of Imayoshi et al. (2010), RBPJκ conditional knock-out mice in this study also carried a R26::EYFP reporter. Analysis of the YFP-positive and -negative populations surprisingly revealed that proliferation and neurogenesis from YFP-negative cells was substantially altered in RBPJκ-cKO mice and that YFP-negative stem cells were not able to sustain significant levels of neurogenesis. Several studies have indicated that stem cells themselves create a neurogenic microenvironment that provides signals for proliferation and differentiation (Lim and Alvarez-Buylla, 1999; Lim et al., 2000; Song et al., 2002; Lie et al., 2005; Favaro et al., 2009). Hence, we propose that the loss of RBPJκ and the reduction of stem cells alters signaling in the neurogenic microenvironment, which in turn resulted in global dysregulation of neurogenesis from the remaining nonrecombined stem cells. It will be interesting to determine in the future whether RBPJκ 32 signaling controls the neurogenic microenvironment through the transcriptional regulation of, for example, secreted and cell-surface-bound signaling molecules.

Single-cell neurosphere assays demonstrated that RBPJκ 32 signaling controls stem cell maintenance, at least in part, through cell-autonomous mechanisms. Recent work by Favaro et al. (2009) has demonstrated that the transcription factor Sox2 is essential for adult hippocampal NSC maintenance. We found that activation of the Notch-signaling pathway, which leads to the transcriptional activation of RBPJκ-targets, enhances the activity of the Sox2 promoter and the expression of Sox2 in adult hippocampal NSCs. In addition, we show that the Sox2 promoter contains multiple RBPJκ-binding sites and that RBPJκ and activated Notch are enriched on the Sox2 promoter in adult hippocampal NSCs. Thus, we identify Sox2 as a direct target of Notch/RBPJκ 32 signaling in adult hippocampal NSCs. Importantly, Sox2 overexpression is sufficient to rescue the self-renewal defect of RBPJκ-deficient adult NSCs in culture, indicating that the Notch/RBPJκ/Sox2 pathway is functionally relevant for NSC...
Ehm et al. • RBPJκ in Adult Neural Stem Cell Maintenance

J. Neurosci., October 13, 2010 • 30(41):13794–13807 • 13805

We expect that the characterization of the transcriptional targets of RBPJκ signaling in NSCs will provide a deeper insight into the complex regulatory mechanisms underlying stem cell behavior in the adult hippocampus.

The strongly reduced generation of new dentate granule neurons in the aged hippocampus is thought to contribute to age-dependent hippocampal dysfunction. In vitro assays for NSC frequency (Walker et al., 2008), histological studies in primates (Aizawa et al., 2010), and magnetic resonance spectroscopy studies in humans (Manganas et al., 2007) indicated that the stem cell pool in the aged dentate gyrus is reduced and that complete stem cell maintenance may play a role in age-associated reduction of hippocampal neurogenesis. Interestingly, analysis of A53T α-synuclein transgenic mice, a model for age-dependent neurodegeneration, revealed a pronounced decrease in hippocampal neurogenesis that is paralleled by a significant decrease in Notch1 expression (Crews et al., 2008). Together with our finding that RBPJκ-dependent signaling is essential for NSC maintenance in the dentate gyrus during adulthood, these observations raise the intriguing possibility that perturbations of Notch/RBPJκ signaling contribute to stem cell loss, decreased neurogenesis, and impaired hippocampal function during aging and that pharmacological stimulation of the Notch/RBPJκ pathway may potentially prevent age-associated loss of NSCs and its consequences for hippocampal function.

References

Ables IL, Decarolis NA, Johnson MA, Rivera PD, Gao Z, Cooper DC, Radtke F, Hsieh J, Eisj AJ (2010) Notch1 is required for maintenance of the reservoir of adult hippocampal stem cells. J Neurosci 30:10484–10492.

Adachi K, Mirzadeh Z, Sakaguchi M, Yamashita T, Nikolcheva T, Gotoy T, Pelzt G, Gong L, Kawase T, Alvarez-Buylla A, Okano H, Sawamoto K (2007) Beta-catenin signaling promotes proliferation of progenitor cells in the adult mouse subventricular zone. Stem Cells 25:2827–2836.

Aizawa K, Ageyama N, Terao K, Hisatsune T (2010) Primate-specific alterations in neural stem/progenitor cells in the aged hippocampus. Neurobiol Aging, in press.

Andreu-Agullo C, Morant-Redolat JM, Delgado AC, Farinas I (2009) Vasculature niche factor PDEF modulates Notch-dependent stemness in the adult subependymal zone. Nat Neurosci 12:1514–1523.

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Ruesger MA, Bae SK, K Pittappa R, McKay RD (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442:823–826.

Baron M (2003) An overview of the Notch signalling pathway. Semin Cell Dev Biol 14:113–119.

Basak O, Taylor V (2007) Identification of self-replicating multipotent progenitors in the embryonic nervous system by high Notch activity and Hes5 expression. Eur J Neurosci 25:1006–1022.

Ben-Bassat H, Goldblum N, Mitrani S, Goldblum T, Yoffey JM, Cohen MM, Peltz G, Gong L, Kawase T, Alvarez-Buylla A, Okano H, Sawamoto K (2007) Beta-catenin signaling regulates stem cell numbers in vitro and in vivo. Nature 442:823–826.

Blancpain C, Lowry WE, Pasolli HA, Fuchs E (2006) Canonical notch signalling functions as a commitment switch in the epidermal lineage. Genes Dev 20:3022–3035.

Breunig JJ, Silbereis J, Vaccarino FM, Sestan N, Rakic P (2007) Notch regulates cell fate and dendrite morphology of newborn neurons in the postnatal dentate gyrus. Proc Natl Acad Sci U S A 104:20558–20563.

Brill MS, Ninkovic J, Winpenny E, Hodge RD, Ozen I, Yang R, Leiper A, Gascon S, Erdelyi F, Szabo G, Parras C, Guillomet F, Frotscher M, Berninger B, Heverner RF, Baineteau O, Gottz M (2009) Adult generation of glutamatergic olfactory bulb interneurons. Nat Neurosci 12:1524–1533.

maintenance. Together with the finding that Sox2 inhibits Wnt/β-catenin-induced fate determination (Lie et al. 2005) and expression of the neuronal fate determinant NeuroD (Kuwabora et al., 2009), our data suggest a model in which the activity of the Notch/RBPJκ/Sox2 pathway and the activity of the Wnt/β-catenin/NeuroD pathway are key regulators to control the balance between stem cell maintenance and neuronal fate determination in the adult hippocampal neurogenic niche.

Notch1 was identified as a direct transcriptional target of Sox2 in retinal progenitor cells (Taranova et al., 2006), raising the possibility of a positive Notch–Sox2–Notch feedback loop. Our ChIP analysis using chromatin from adult NSCs revealed no significant enrichment of Sox2 on the Notch1 promoter. Hence, we have no evidence for a Notch–Sox2–Notch feedback loop in adult NSCs, which is in line with the finding that the expression of Notch1, RBPJκ, and Notch targets such as Hes5 is unaltered in Sox2-deficient adult NSCs (Favaro et al., 2009).

Notch signaling interacts with a number of other pathways to control stem cell maintenance in the hematopoietic system (Duncan et al., 2005), the embryonic nervous system (Takizawa et al., 2003; Shimizu et al., 2008), and the adult subventricular zone (Andreu-Agullo et al., 2009). In addition, it has been found that Sox2 expression in the developing nervous system is influenced by sonic hedgehog (Takanaga et al., 2009) and fibroblast growth factor signaling (Takemoto et al., 2006). We therefore consider it likely that Notch/RBPJκ signaling controls stem cell maintenance in the adult hippocampus in cooperation with additional signals. In preliminary studies, we have found that modulation of the PI3kinase pathway and of the Wnt/β-catenin pathway potentiates Notch-induced Sox2 promoter activity in adult NSCs (O. Ehm, T. J. Schwarz, and A. Khan, unpublished results), which indeed suggests that multiple pathways converge onto Sox2 and regulate NSC maintenance in the hippocampus. Activity of such interacting pathways in the absence of Notch/RBPJκ signaling may be sufficient to sustain Sox2 expression for a short period of time, which could explain the observation that a fraction of the YFP reporter-positive cells in RBPJκ-cKO mice showed residual Sox2 protein expression.

RBPJκ-cKO and Sox2-conditional mouse mutants both show loss of hippocampal NSCs and loss of neurogenesis. Sox2 mutants, however, display proliferation and neurogenesis defects almost immediately after recombination, which is different from the transient increase in proliferation and neurogenesis in RBPJκ-cKO. It is likely that these phenotypic differences are attributable to the fact that RBPJκ-dependent signaling regulates NSC behavior not only through Sox2 but also through other downstream targets. This idea is supported by the observations that the Notch targets Hes1 and Hes5 are expressed in adult neurogenic niches (Stump et al., 2002; Crews et al., 2008) and that stem cells in the adult hippocampus (Fig. 1) and the adult subventricular zone (Imayoshi et al., 2010) show robust activity of the Hes5 promoter. In addition, we demonstrate that adult NSCs express Hes1 and Hes5 (supplemental Fig. 1, available at www.jneurosci.org as supplemental material), that overexpression of activated Notch increases Hes1 mRNA expression, and that activated Notch is enriched on the Hes1 promoter (Fig. 7). The fact that Hes1 is essential for reversible quiescence in primary cells and tumor cells in vitro (Sang et al., 2008) and the fact that inactivation of RBPJκ leads to loss of the quiescent radial gli-like stem cell population and a transient increase in proliferation (this study) raise the intriguing possibility that Notch/RBPJκ regulate quiescence of radial gli-like stem cells through the control of Hes gene expression.
Bull ND, Bartlett PF (2005) The adult mouse hippocampal progenitor is neurogenic but not a stem cell. J Neurosci 25:10815–10821.

Clelland GD, Choi M, Romberg C, Clemenson GD Jr, Fragniere A, Tyers P, Jessberger S, Sakaida LM, Barker RA, Gage FH, Bussey TJ (2009) A functional role for adult hippocampal neurogenesis in spatial pattern separation. Science 325:210–213.

Crews L, Mizuno H, Desplats P, Rockenstein E, Adame A, Patrick C, Winner Clelland CD, Choi M, Romberg C, Clemenson GD Jr, Fragniere A, Tyers P, Kato H, Taniguchi Y, Kurooka H, Minoguchi S, Sakai T, Nomura-Okazaki Jessberger S, Clark RE, Broadbent NJ, Clemenson GD Jr, Consiglio A, Lie DC, Jakubs K, Nanobashvili A, Bonde S, Ekdahl CT, Kokaia Z, Kokaia M, Lindvall Jagasia R, Steib K, Englberger E, Herold S, Faus-Kessler T, Saxe M, Gage FH, Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Han H, Tanigaki K, Yamamoto N, Kuroda K, Yoshimoto M, Nakahata K, Shimizu T, Kagawa T, Inoue T, Nonaka A, Takada S, Aburatani H, Taga T (2008) Stabilized beta-catenin functions through TCF/LEF proteins and contains restricted progenitors. J Neurosci 28:1047–1059.

Yoon K, Cook JM, Willert K, Gaiano N, Reya T (2005) Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance. Nat Immunol 6:314–322.

Favaro R, Valotta M, Ferri AL, Latorre E, Mariani J, Giachino C, Lancini C, Tosetti V, Ottolenghi S, Taylor V, Nicolas SK (2009) Hippocampal development and neural stem cell maintenance require Sox2-dependent regulation of Shh. Nat Neurosci 12:1248–1256.

Gao Z, Ure K, Ables JL, Lagace DC, Nave KA, Goebbels S, Eisch AJ, Hsieh J (2009) Neuro1 is essential for the survival and maturation of adult neurons. Nat Neurosci.

Hacker MA, Saghatelayan A, de Chevigny A, Pfeifer A, Ashery-Padan R, Lledo Duncan AW, Rattis FM, DiMascio LN, Congdon KL, Pazianos G, Zhao C, Squire LR, Gage FH (2009) Dentate gyrus-specific knockdown of adult neurogenic precursors in the adult hippocampus. J Neurosci 29:1203–2114.

Hitoshi S, Alexsson T, Tropea V, Donoviel D, Elia AJ, Nye JS, Conlon RA, Mak TW, Bernstein A, van der Kooy D (2002) Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev 16:846–858.

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Mak TW, Bernstein A, van der Kooy D (2002) Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev 16:846–858.

Kuwabara T, Hsieh J, Muotri A, Yeo G, Warashina M, Lie DC, Moore L, Nakashima K, Asashima M, Gage FH (2009) Wnt-mediated activation of Notch1 and retro-elements during adult neurogenesis. Nat Neurosci 12:1097–1105.

Lengler J, Bittner T, Munster D, Gawah Ael D, Graw J (2005) Agonistic and antagonistic action of AP2, Mxs2, Pax6, Proxi ANDSix3 in the regulation of Sox2 expression. Ophthalmic Res 37:301–309.

Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A, Lein E, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437:1370–1375.

Lim DA, Alvarez-Buylla A (1999) Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. Proc Natl Acad Sci U S A 96:7526–7531.

Lim DA, Troudnit AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla A (2000) Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron 28:713–726.

Maier S, Santak M, Mantik A, Grabusic K, Kremers E, Hammerschmidt W, Kempske B (2005) A somatic knockout of CBFI in a human B-cell line reveals that induction of CD21 and CCR7 by EBNA-2 is strictly CBFI-dependent and that downregulation of immunoglobulin M is partially CBFI independent. J Virol 79:8784–8792.

Mangoas LN, Zhang X, Li Y, Hazel RD, Smith MD, Waghul ME, Henn F, Benveniste H, Djuric PM, Enikolopov G, Maletic-Savatic M (2007) Magnetic resonance spectroscopy identifies neural progenitor cells in the live human brain. Science 318:980–985.

Merkle FK, Mirzadeh Z, Alvarez-Buylla A (2007) Mosaic organization of neural stem cells in the adult brain. Science 317:381–384.

Mizutani K, Yoon K, Kang L, Tokunaga A, Gaiano N (2007) Differential neurosignalling distinguishes neural stem cells from intermediate progenitors. Nature 449:351–355.

Ohtsuka T, Iwashishi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. EMBO J 18:2196–2207.

Parent JM, Elliott RC, Pleasure SJ, Barbaro NM, Lowenstein DH (2006) Aberrant seizure-induced neurogenesis in experimental temporal lobe epilepsy. Ann Neurol 58:891–901.

Ray J, Gage FH (2006) Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. Mol Cell Neurosci 31:560–573.

Revest JM, Dupret D, Koehl M, Funk-Reiter C, Grosjean N, Piazza PV, Abrous DN (2009) Adult hippocampal neurogenesis is involved in anxiety-related behaviors. Mol Psychiatry.

Sang L, Coller HA, Roberts JM (2008) Control of the reversibility of cellular quiescence by the transcriptional repressor HES1. Science 321:1095–1100.

Seaberg RM, van der Kooy D (2002) Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. J Neurosci 22:1784–1793.

Shimizu T, Kagawa T, Inoue T, Nonaka A, Takada S, Aburatani H, Taga T (2008) Stabilized beta-catenin functions through TCF/LEF proteins and the Notch/RBP-J kappa complex to promote proliferation and suppress differentiation of neural precursor cells. Mol Cell Biol 28:7427–7441.

Slezak M, Goritz C, Niemiec A, Frisen J, Chambon P, Metzger D, Pfrieger FW (2003) Spatial memory performances of aged rats in the water maze predict levels of hippocampal neurogenesis. Proc Natl Acad Sci U S A 100:14385–14390.

Kuhn HG, Dickinson-Anson H, Gage FH (1996) Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci 16:2027–2033.

Kuhn HG, Jessberger S, Thal LJ, Gage FH (1997) Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci 17:5820–5829.

Kuwabara T, Hsieh J, Mustri A, Teo G, Warashina M, Lie DC, Moore L, Nakashima K, Asashima M, Gage FH (2009) Wnt-mediated activation of Notch1 and retro-elements during adult neurogenesis. Nat Neurosci 12:1097–1105.
Costantini F (2001) Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 1:4.

Stump G, Durrer A, Klein AL, Lutolf S, Suter U, Taylor V (2002) Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain. Mech Dev 114:153–159.

Suh H, Consiglio A, Ray J, Sawai T, D’Amour KA, Gage FH (2007) In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2(+) neural stem cells in the adult hippocampus. Cell Stem Cell 1:515–528.

Takanaga H, Tsuchida-Straeten N, Nishide K, Watanabe A, Aburatani H, Kondo T (2009) Gli2 is a novel regulator of sox2 expression in telencephalic neuroepithelial cells. Stem Cells 27:165–174.

Takemoto T, Uchikawa M, Kamachi Y, Kondoh H (2006) Convergence of Wnt and FGF signals in the genesis of posterior neural plate through activation of the Sox2 enhancer N-1. Development 133:297–306.

Takizawa T, Ochiai W, Nakashima K, Taga T (2003) Enhanced gene activation by Notch and BMP signaling cross-talk. Nucleic Acids Res 31:5723–5731.

Taranova OV, Maness ST, Fagan BM, Wu Y, Surzenko N, Hutton SR, Pevny LH (2006) SOX2 is a dose-dependent regulator of retinal neural progenitor competence. Genes Dev 20:1187–1202.

Tashiro A, Zhao C, Gage FH (2006) Retrovirus-mediated single-cell gene knockout technique in adult newborn neurons in vivo. Nat Protoc 1:3049–3055.

Walker TL, White A, Black DM, Wallace RH, Sah P, Bartlett PF (2008) Latent stem and progenitor cells in the hippocampus are activated by neural excitation. J Neurosci 28:5240–5247.

Yamamoto N, Tanimaki K, Han H, Hiai H, Honjo T (2003) Notch/RBP-J signaling regulates epidermis/hair fate determination of hair follicular stem cells. Curr Biol 13:333–338.

Zappone MV, Galli R, Catena R, Meani N, De Biasi S, Mattei E, Tiveron C, Vescovi AL, Lovell-Badge R, Ottolenghi S, Nicolis SK (2000) Sox2 regulatory sequences direct expression of a (beta)-geo transgene to telencephalic neural stem cells and precursors of the mouse embryo, revealing regionalization of gene expression in CNS stem cells. Development 127:2367–2382.