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RBP-J promotes the maturation of neuronal progenitors

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ABSTRACT

During brain development, neurons and glias are generated from neural stem cells and more limited intermediate neural progenitors (INPs). Numerous studies have revealed the mechanisms of development of neural stem cells. However, the signaling pathways that govern the development of INPs are largely unknown. The cerebellum is suitable for examining this issue because cerebellar cortical inhibitory neurons such as basket and stellate cells are derived from small $Pax2^+$ interneuronal progenitors. Here, we show that $Sox2^-$/$Pax2^+$ and $Sox2^+$/$Pax2^-$ progenitors, 2 types of interneuronal progenitors of basket and stellate cells, exist in the cerebellar white matter (WM) and that the former arise from the latter during the first postnatal week. Moreover, RBP-J promotes the neurogenesis of stellate and basket cells by converting $Sox2^+$/$Pax2^-$ interneuronal progenitors to more mature $Sox2^-$/$Pax2^+$ interneuronal progenitors. This study shows a novel RBP-J function that promotes INP differentiation.

Introduction

During brain development, neurons and glias are generated from neural stem cells and more limited intermediate neural progenitors (INPs). In recent years, numerous studies have revealed the mechanisms of cell fate specification and differentiation of neural stem cells (Copray et al., 2006; Corbin et al., 2008; Fujimoto et al., 2009; Fukuda et al., 2007; Taylor et al., 2007). However, the signaling pathways that govern the differentiation of INPs are largely unknown. The cerebellum is the most suitable region of the brain for examining this issue because the majority of GABAergic interneurons in the cerebellum, including inhibitory deep nuclear neurons and Golgi, basket, and stellate cells, are derived from paired box gene 2-positive ($Pax2^+$) interneuronal progenitors. A discrete cluster of $Pax2^+$ interneuronal progenitors is detected in the medial aspect of the cerebellar anlage of embryonic day E12 mouse. Thereafter, these cells continue to divide and spread throughout the prospective white matter (WM), eventually settling in the deep nuclei and cortical layer. $Pax2^+$ interneuronal progenitors give rise to different types of GABAergic interneurons in an "inside–out" sequence in mice (Wang and Zoghbi, 2001): deep cerebellar nuclei (DCN) neurons at E10–E17, Golgi cells in the granular layer (GL) at E12–E15, and stellate/basket cells in the molecular layer (ML) postnatally (Miale and Sidman, 1961; Pierce, 1975). Although there has been no systemic analysis of the size of $Pax2^+$ interneuronal progenitors, it has been suggested that Golgi cells and GABAergic neurons in the DCN are generated from large $Pax2^+$ interneuronal progenitors, whereas basket and stellate cells arise from small $Pax2^+$ interneuronal progenitors (Maricich and Herrup, 1999). Recently, a study on the ontogenesis of $Pax2^+$ interneuronal progenitors has revealed that, at least at P0 and P3, the numeric increase in these cells is primarily due to the proliferation of the $Pax2^-$ interneuronal progenitor population (Weisheit et al., 2006). This study also suggests that 2 types of INPs exist in the cerebellar WM. However, the molecular markers that distinguish these 2 INP types remain poorly understood.

The Notch/RBP-J signaling pathway helps in maintaining neural stem cells in an undifferentiated state and promotes the maturation of glial cells (Furukawa et al., 2000; Gaiano and Fishell, 2002; Gaiano et al., 2000; Shimojo et al., 2008; Tanigaki et al., 2001; Taylor et al., 2007). When the Notch ligand binds to the Notch receptor, the latter is cleaved by the gamma-secretase complex, and the intracellular domain of Notch (NICD) is released, which then translocates to the nucleus and associates with the DNA-binding protein RBP-J and coactivators such as Mastermind-like (Maml) (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Fryer et al., 2002; Hansson et al., 2009; Lubman et al., 2007; Tamura et al., 1995; Wu et al., 2000; Wu et al., 2002). Previously, we have shown that Notch/RBP-J signaling plays an important role in Bergmann glial layering and morphology (Komine et al., 2007). We had generated conditional RBP-J knockout mice by using GFAP-Cre transgenic mice

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to induce Cre-mediated recombination in astrocytes and Bergmann glial cells. However, the mutant mouse showed not only disorganization of Bergmann glia and astrocytes but also a marked reduction in basket and stellate cells in the cerebellum because Cre-mediated recombination occurred in both the glial and basket and stellate cells (Komine et al., 2007). Recently, it was reported that RBP-J signaling plays an important role in determining the identity of GABAergic neurons over glutamatergic neurons in the spinal cord established by Ptf1a, a bHLH transcription factor (Hori et al., 2008). However, the mechanism by which the RBP-J signaling pathway contributes to the generation of GABAergic neurons has not yet been elucidated.

In this study, we show that 2 types of interneuronal progenitors exist in the cerebellar WM, and these can be identified on the basis of the expression of specific molecular markers, including Sox2, S100β, GLAST, and Pax2. Furthermore, we reveal that RBP-J promotes the neurogenesis of stellate and basket cells by converting $Sox2^+/Pax2^-$ interneuronal progenitors to more mature small $Pax2^+$ interneuronal progenitors.

Materials and methods

Mice

GFAP-Cre transgenic mice and Ptf1a-Cre mice have been described previously (Bajenaru et al., 2002; Kawaguchi et al., 2002), and these were used to drive Cre recombinase expression in the CNS of mice. GFAP-Cre mice and Ptf1a-Cre mice were bred with ROSA26R mice (Soriano, 1999) to map Cre activity in vivo. GFAP-Cre mice were bred with RBP-$J^{loxP/loxP}$ or ROSA26R-DN-MAML-GFP mice (Tanigaki et al., 2002; Tu et al., 2005) to generate RBP-J conditional knockout mice and DN-MAML-GFP-expressing mice. RBP-J conditional knockout mice were also bred with Sox2-GFP transgenic mice (Suh et al., 2007). The animal procedures were approved by the Animal Experiment Committee of Tokyo Medical and Dental University.

Immunohistochemistry

Animals were deeply anesthetized and perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). The brains were removed, postfixed with 4% PFA, and cryoprotected in 30% sucrose. These were then embedded in OCT compound (Sakura, Tokyo, Japan), and 12-μm-thick sagittal cryostat sections were prepared. The following antibodies were used for immunostaining: mouse monoclonal anti-β-galactosidase antibody (Promega), rabbit anti-β-galactosidase antibody (Cappel), mouse monoclonal anti-calbindin antibody (Sigma), rabbit anti-GABA antibody (Sigma), rabbit anti-GLAST antibody (Matsugami et al., 2006), rabbit anti-GFAP antibody (DAKO), mouse monoclonal anti-parvalbumin antibody (Sigma), rabbit anti-Pax2 antibody (Zymed), rabbit anti-Sox2 antibody (Chemicon), goat anti-Sox2 antibody (Santa Cruz Biotechnology), mouse anti-Hu antibody (Chemicon), rabbit anti-GFP antibody (Molecular Probes), and mouse anti-S100β antibody (Sigma). The sections were rinsed and incubated in the appropriate secondary antibodies—goat antibodies against either mouse or rabbit Alexa 488 and 568, goat antibodies against rat Alexa633 (Molecular Probes), or donkey antibodies against either rabbit or goat FITC and Cy3 (Jackson ImmunoResearch). Images were recorded with an LSM-510 META confocal laser microscope (Carl Zeiss).

LacZ staining

For LacZ staining, the mice were transcardially perfused with 2% PFA and 0.2% glutaraldehyde in PBS. The brains were postfixed in the same fixative for 4 h. Then 50-μm vibratome sections were prepared and stained with X-Gal.

BrdU staining

Postnatal mice were injected intraperitoneally with a BrdU solution (50 μg/g body weight; Sigma) in PBS. Three hours after injection, the mice were anesthetized and perfused with 4% PFA. Their cerebella were then dissected and sagittally cryosectioned into sections that were 12 μm thick. Sections were treated with 2 N HCl at 37 °C for 30 minutes and washed in 0.1 M sodium borate for 10 minutes. The sections were then immunostained with the anti-BrdU antibody (Oxford Biotechnology) and with the anti-Sox2 and anti-S100β antibodies.

TUNEL staining

TUNEL staining was performed with cryosections (12 μm thick) according to the manufacturer's instructions (Promega). Fluorescence detection was carried out using Alexa-fluor 568-conjugated streptavidin (Molecular Probes) instead of HRP-conjugated streptavidin. Immunohistochemical analysis was performed with the anti-Sox2 antibody using an Alexa-fluor 488-conjugated secondary antibody (Molecular Probes) for detection.

In situ hybridization

In situ hybridization was performed on cryosections (12 μm thick) using digoxigenin-labeled GLAST, CD133, Hes1, Hes5, and Hey1-specific cRNA probe as described previously (Yamada et al., 2000).

Cell counts

To determine the number of basket cells (inner one-third of the ML) and stellate cells (outer two-thirds of the ML), parvalbumin-positive cells were counted per 10^3 μm^2 from 3 sections of each mouse ($n=4$ per genotype). The number of Purkinje cells per millimeter of Purkinje cell layer was counted by HE staining from 3 sections of each mouse ($n=3$ per genotype). The number of $Pax2^+$ interneuronal progenitors in the WM and IGL was counted by Pax2 staining from 3 sections of each mouse ($n=3$ per genotype). To quantify $Pax2^+/\beta\text{-gal}^+$ cells in the WM, $Pax2^+/\beta\text{-gal}^+$ cells were counted per unit area from 3 sections of each mouse ($n=3$ per genotype). To determine the number and proliferation of $Sox2^+/S100\beta^-$ cells, the numbers of $Sox2^+/S100\beta^-$ and $Sox2^+/S100\beta^-/BrdU^+$ cells in the WM were calculated from 3 sections of each mouse ($n=3$ per genotype). The data are represented as mean ± SD. The P values were calculated using Student's t test.

Single-cell RT-PCR

To clarify the deletion of RBP-J expression from $Sox2^+$ interneuronal progenitor, we mated GFAP-Cre/RBP-$J^{loxP/loxP}$ mice with Sox2-GFP transgenic mice in that Sox2-expressing cells express GFP (Suh et al., 2007). Cerebellums were dissociated by using the Papain Dissociation System (Worthington Biochemical Corporation). Single-GFP-expressing cell was picked up by mouth pipetting into pulled glass microcapillary. Collected cell was transferred to the PCR tube and then single-cell RT-PCR was performed using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). The following primers were used for cDNA detection:

GLAST FWD: 5′ AGA ATT CTG ACC TGA ACT TTG GCA GAT TA 3′; REV: 5′ TGG ATC CTC TTG AAA GTT GAT TTT AAA ACT 3′

RBP-J FWD: 5′ GAG AAT TGT GTG CAT TGC TTC AGG AAC GAA 3′; REV: 5′ GAT CTA GAC AAT CTT GGG AGT GCC ATG CCA 3′

GFP FWD: 5′ AGC AAG GGC GAG GAG CTG TT 3′; REV: 5′ GTA GGT CAG GGT GGT CAC GA 3′

GAPDH FWD: 5′ ACT TTG TCA AGC TCA TTT CC 3′; REV: 5′ TGC AGC GAA CTT TAT TGA TG 3′