Notch1 and Notch2 collaboratively maintain radial glial cells in mouse neurogenesis

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During mammalian corticogenesis, Notch signaling is essential to maintain neural stem cells called radial glial cells (RGCs) and the cortical architecture. Because the conventional knockout of either Notch1 or Notch2 causes a neuroepithelial loss prior to neurogenesis, their functional relationship in RGCs remain elusive. Here, we investigated the impacts of single knockout of Notch1 and Notch2 genes, and their conditional double knockout (DKO) on mouse corticogenesis. We demonstrated that Notch1 single knockout affected RGC maintenance in early to mid-neurogenesis whereas Notch2 knockout caused no apparent defect. In contrast, Notch2 plays a role in the RGC maintenance as Notch1 does at the late stage. Notch1 and Notch2 DKO resulted in the complete loss of RGCs, suggesting their cooperative function. We found that Notch activity in RGCs depends on the Notch gene dosage irrespective of Notch1 or Notch2 at late neurogenic stage, and that Notch1 and Notch2 have a similar activity, most likely due to a drastic increase in Notch2 transcription. Our results revealed that Notch1 has an essential role in establishing the RGC pool during the early stage, whereas Notch1 and Notch2 subsequently exhibit a comparable function for RGC maintenance and neurogenesis in the late neurogenic period in the mouse telencephalon.

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1. Introduction

The evolutionarily conserved Notch signaling pathway is one of the most essential signaling pathways for regulating the proliferation of stem cells in various developmental processes (Andersson et al., 2011; Siebel and Lendahl, 2017). Mammals have four Notch homologues, Notch1, 2, 3, and 4. Experiments on mice have shown that mutant mice show systemic developmental defects and embryonic lethality at earlier embryonic stages; this shows that in mammalian development, Notch1 and Notch2 largely contribute to embryogenesis (Conlon et al., 1995; Hamada et al., 1999; Krebs et al., 2003b, 2000; McCright et al., 2006, 2001; Radtke et al., 1999; Swiatek et al., 1994). Notch1 and Notch2 are thought to be redundant (Krebs et al., 2003a), and these two genes are expressed in radial glial cells (RGCs) of the dorsal cortex (Higuchi et al., 1995; Irvin et al., 2001). While previous studies have identified that Notch1 largely contributes to the maintenance of neural stem cells (NSCs) (Yang et al., 2004; Yoon et al., 2004), the early lethality of Notch1 and Notch2 knockout mice has limited the phenotypic analysis of telencephalon development from the mid-neurogenic stage (around E13.5) onward. The functional differences between Notch1 and Notch2 for the maintenance of NSCs throughout telencephalon development remains elusive. In the present study, we address how Notch signaling regulates NSC maintenance in the dorsal cortex throughout neurogenesis by combinations of the conditional knockout for Notch1 and Notch2 genes.

2. Materials and methods

2.1. Animals

All animal experiments were performed in compliance with the guidelines for animal experiments at the RIKEN Center for Biosys-
tems Dynamics Research. In order to find the time of pregnancy of mice, embryonic stages were calculated by observing the vaginal plug. The noon of the day when the vaginal plug was observed was defined as embryonic day 0.5 (±0.5). The sex of the embryos used was not examined in this study.

2.2. Crosses for making mutant mice

We examined the conditional mutant for Notch1 and Notch2 to investigate Notch1 and Notch2 single and double knockout (DKO) mice, based on the Cre-loxP system (Robinson and Sauer, 1998). An Emx1-CreR1 driver line (Guo et al., 2000) and Notch1flox/flox or Notch1flox/flox mouse lines (McCright et al., 2006; Yang et al., 2004) were crossed to produce a conditional deletion of the Notch1 or Notch2 gene in the dorsal cortex. A floxed gene is known to be deleted by Emx1 driver as early as E9.5, at which many NSCs have been formed (Guo et al., 2000). Notch1flox/flox; Emx1-CreR1+/- mice (hereafter referred to as Notch1flox/flox; Emx1-CreR1+/− mice) that were crossed to generate a conditional deletion of Notch1 were used. Notch2flox/flox mice with Notch1flox/flox; Emx1-CreR1+/- mice were acquired by crossing Notch1flox/flox mice with Notch1flox/flox; Emx1-CreR1+/- mice and Notch2flox/flox; Emx1-CreR1+/- mice (hereafter referred to as Notch2flox/flox; Emx1-CreR1+/− mice) that were crossed to generate a conditional deletion of Notch2 were used. Notch1flox/flox; Notch2flox/flox; Emx1-CreR1+/- mice were acquired by crossing Notch1flox/flox; Notch2flox/flox mice with Notch1flox/flox; Emx1-CreR1+/- mice and Notch2flox/flox; Emx1-CreR1+/- mice (hereafter referred to as Notch1flox/flox; Notch2flox/flox; Emx1-CreR1+/− mice) that were crossed to generate a conditional deletion of Notch1 and Notch2 were used. Notch1flox/flox; Notch2flox/flox; Emx1-CreR1+/- mice were used for obtaining heterozygotes, and the offspring were crossed to obtain each genotype for the experiments shown in Fig. 4 (see Table 1). C57BL/6 J mice were used for all the experiments in Figs. 1–4.

Hes1-d2-ECFP5x+/- used for single cell analysis was contributed by R. Kageyama (Ohtsuka et al., 2006). Tails were used for genotyping.

2.3. Immunostaining

Brains were fixed in 1% paraformaldehyde in phosphate buffer, pH 7.4, at 4°C overnight, followed by quenching of paraformaldehyde with 0.1 M Glycine in phosphate buffer and cryoprotection in 25% sucrose overnight at 4°C, and then frozen in Tissue-Tek O.C.T. Compound (45833, Sakura Finetek Japan). Cryostat sections (12 µm thick) were treated with HistoVT One (06380-05, Nacalai tesque) following the manufacturer’s protocol, and subsequently with a blocking buffer (10% BSA, 0.1% Tween 20, and 0.2% Triton-X100 in PBS, pH 7.4) for 1 h at room temperature, and incubated with primary antibodies against Tbr2 (1:1000; AlexaFlour 660 labeled rat monoclonal antibody, clone Dan11mg, 50-4875-82, Thermo Fisher Scientific) and Sox2 (1:1000; AlexaFlour 488 labeled rat monoclonal antibody, clone Btge, 53-9811-82, Thermo Fisher Scientific) overnight at 4°C. These primary antibodies were diluted in the same buffer before use. We also used anti-Ki67 antibody (1:1000; rabbit monoclonal antibody, clone SP6, MA5-14520, Thermo Fisher Scientific), anti-GFP antibody (1:1000; goat polyclonal antibody, ab5450, Abcam), and DAPI (D9542, MERCK) for DNA staining. For EdU detection, Click-IT® EdU Imaging Kits (Alex Fluor 555 dye, C10338, Thermo Fisher Scientific) were used.

2.4. Microscopic observations

We observed antibody-stained cryostat sections in a confocal microscope (LSM880, inverted laser scanning confocal microscope, Carl Zeiss). Whole brains shown in Fig. S1 were photographed by a stereo fluorescence microscope (Macro Zoom Fluorescence Microscope System, MVX10 with DP71 digital microscope camera, Olympus).

2.5. Measurement of cell cycle exit rate

The cell-cycle exit rate is defined as the rate of cells that exit cell cycle during approximately one cell cycle length among mitotic cells (Chenn and Walsh, 2002). To label proliferating cells, EdU (1 mg/25 g body weight, A10044, Thermo Fisher Scientific) was administrated intraperitoneally into pregnant mice at E11. We fixed the embryos 12 h after the EDU administration to detect EDU incorporation in a single cell cycle, given that the cell cycle length at E11.5 was about 8.1 h (Takahashi et al., 1995). The brains were collected, fixed, followed by double staining for EdU and Ki67, which is a marker expressed in mitotic cells. No less than three sections from three different brains were counted for the fractions of EdU+/Ki67+ cells and all EdU incorporating cells. We calculated the cell cycle exit rate as the fraction of (EdU+ Ki67-) cells relative to all EdU incorporating cells (EdU+ Ki67+ plus EdU+ Ki67- cells) in the unit area of each dorsal cortex at E11.5. No less than three sections from three different brains were counted for the fractions of EdU+/Ki67+ cells and all EdU incorporating cells.

2.6. Single cell isolation, library construction and sequencing

Details for the single cell dataset analysis from E14 mouse have been described in the method section of a separately submitted manuscript (Wu et al., 2020). Single cell isolation and library construction from E11 and E16 mice have been described there.

2.7. Bioinformatics analysis of single cell data

Sequenced data was mapped to 10 mm using cellranger 2.0.2 (10X GENOMICS). All data were further analyzed by a bioinformatics pipeline Seurat (2.3.4) (Butler et al., 2018).

2.8. Statistical analysis

No statistical methods were used to predetermine the sample sizes. Statistical analyses were performed using Prism 8 (GraphPad Software). The data were tested for normal distribution using the Shapiro-Wilk test (alpha = 0.05). For non-normal distribution data, following non-parametric test, the Kruskal–Wallis test with a Dunn’s test was performed to compare more than two data groups. For normal distribution data, parametric one-way ANOVA with a Tukey’s multiple comparison test was done for comparisons between more than two data groups. The significance of the comparisons is represented on graphs by asterisks; *P < 0.05; **P < 0.01; ***P < 0.001 and ****P < 0.0001.
Fig. 1. Defects in radial glial cell maintenance in cerebral cortices of the Notch1$^{1/2\Delta}$ and Notch2$^{1/2\Delta}$ single KO and Notch1$^{1/2\Delta}$; Notch2$^{1/2\Delta}$ DKO at early-to-mid-neurogenic stages. (A) Staining for Sox2 (green), Tbr2 (magenta) and DAPI (blue) of E11.5 dorsal cortices with the genotype of the double heterozygous Notch1$^{1/2\Delta}$; Notch2$^{1/2\Delta}$ as a control, a single KO of Notch1$^{1/2\Delta}$, Notch2$^{1/2\Delta}$, and Notch1$^{1/2\Delta}$; Notch2$^{1/2\Delta}$ DKO from top to bottom. The bottom of cryostat sections faces the ventricle in this and all following panels throughout figures unless mentioned. (B) The same staining as A of E13.5 dorsal cortices with the same genotypes. Scale bar, 10 μm. In Notch1$^{1/2\Delta}$; Notch2$^{1/2\Delta}$ DKO, Tbr2 (+) cells invade more apically into the middle region that lacks Sox2 (+) cells in the VZ, and the ventricular surface (white dot lines) is curled and rough, suggesting that the epithelial surface is compromised. White arrowhead indicates NSCs in medial-ventral cortex where Ems1-Cre does not expresses. (C) Histograms of Sox2 (+) cells (left) and Tbr2 (+) cells (right) in E11.5 dorsal cortices with the genotype of double heterozygous Notch1$^{1/2\Delta}$; Notch2$^{1/2\Delta}$ as a control, a single KO of Notch1$^{1/2\Delta}$ and Notch2$^{1/2\Delta}$, and DKO of Notch1$^{1/2\Delta}$; Notch2$^{1/2\Delta}$, respectively. Notch1$^{1/2\Delta}$; Notch2$^{1/2\Delta}$: Sox2 = 161.2 ± 12.6 cells, Tbr2 = 32.5 ± 5.2 cells (n = 6 embryos); Notch1$^{1/2\Delta}$; Sox2 = 133 ± 13.4 cells, Tbr2 = 50.5 ± 19.7 cells (n = 10 embryos); Notch2$^{1/2\Delta}$; Sox2 = 174.1 ± 20.3 cells, Tbr2 = 34.7 ± 2.3 cells (n = 5 embryos); Notch1$^{1/2\Delta}$, Notch2$^{1/2\Delta}$; Sox2 = 123.0 ± 11.1 cells,
3. Results

3.1. The loss of Notch1 but not Notch2 enhances neurogenesis during early neurogenesis

We first investigated the effects of Notch1Δ/Δ and Notch2Δ/Δ on the differentiation of RGCs during the early to mid-neurogenic period, by crossing Emx1-CreKI driver line (Guo et al., 2000) with Notch1floXfloX or Notch2floXfloX mouse lines (Table 1). The numerical ratio of RGCs and differentiating cells was measured at embryonic day E11.5 (a late proliferative stage) and E13.5 (the mid-neurogenic stage when layer 4 neurons are generated), by counting cells expressing Sox2, a marker expressed in NSCs and those expressing Tbr2, a marker for committed neural progenitors in the dorsal cortex. We found that at both E11.5 and E13.5, the elimination of Notch1 in the dorsal cortex greatly reduced cell number per unit width (100 μm) of cryosections (hereafter referred to as cell number/unit area) of RGCs, whereas Notch2Δ/Δ showed no significant change in RGC cell number/unit area (Fig. 1A–C).

3.2. The simultaneous loss of Notch1/2 functions causes synergistic defects

We also examined Notch1Δ/Δ; Notch2Δ/Δ DKO phenotype in corticogenesis. We observed that Notch1Δ/Δ; Notch2Δ/Δ brain showed more drastic defects than those of Notch1 single knockout. There was a remarkable increase in Tbr2 (+) differentiating cells even at E11.5 (Fig. 1A and C). At E13.5, only a small number of proliferating RGCs remained, and epithelial surface was compromised in the dorsal cortex of DKO brains (Fig. 1B and D). These DKO phenotypes suggest that the simultaneous loss of Notch1 and Notch2 synergistically affects the maintenance of RGCs and neurogenesis (see Discussion).

3.3. Notch2 is dispensable in the presence of Notch1 from the early to mid-neurogenic period

To evaluate the rate of neurogenesis, we measured cell-cycle exit rate for Notch1Δ/Δ and Notch2Δ/Δ single KO and Notch1Δ/Δ; Notch2Δ/Δ single KO. Notch1Δ/Δ; Notch2Δ/Δ DKO showed severe increases in the cell cycle exit compared to double heterozygotes (Fig. 2A and B); however, Notch2Δ/Δ single KO brains did not show such results.

Next, we investigated whether proliferating cells, including both apically dividing RGCs and basally dividing intermediate progenitors (IP), changed their cell number/unit area in those mutant brains. We measured the cell numbers of RGCs and IPs undergoing mitosis in the unit area of the developing lateral cortex by staining for phospho-histone H3. In Notch1Δ/Δ; Notch2Δ/Δ DKO brains, non-apical dividing cells, which were supposed to be IPs, were increased (Fig. 3A and C), suggesting precocious neurogenesis at E11.5. At E13.5, Notch1Δ/Δ brains also showed a significant increase in the IP cells, suggesting an enhanced differentiation of RGCs into IPs by Notch1 KO alone. Conversely, in Notch1Δ/Δ; Notch2Δ/Δ DKO brains at E13.5, RGCs and total dividing cells were largely decreased in cell number/unit area, suggesting that a large part of proliferating cells (RGCs and IPs) had been differentiated (Fig. 3B and D). We noted cell death in the ventricular zone (VZ) of Notch1Δ/Δ brain (Fig. 3B) as previously described (Mason et al., 2006). Interestingly, Notch2 knockout did not significantly alter the cell cycle exit rate nor affected the proliferation of progenitor cells, both at E11.5 and E13.5. Taken together, these data suggest that Notch2 is dispensable as far as the Notch1 locus is normal in the developing telencephalon during the early (E11.5)-mid (E13.5) neurogenic period. However, in the absence of Notch1 activity, Notch2 can partly take over Notch1 for RGCs self-renewal (see Discussion).

3.4. Notch1 and Notch2 might have a comparable level of signaling activity at late neurogenic stage

Next, we examined phenotypes of various Notch mutant brains at a much later stage such as E18.5, where neurogenesis had been nearly completed. To visualize the developing dorsal cortex where a various combination in the dosage of Notch1 and Notch2 genes was deleted, we crossed Notch1Δ/Δ and/or Notch2Δ/Δ mice with CAG-CAT-GFP mice (Kawamoto et al., 2000), where the Emx1-Cre active cells were visualized with GFP (Fig. 4A). Since we found an apparent variety in size of the brains depending on genotypes (Fig. S1), we counted the total number of Sox2+ cells in the VZ of the telencephalon in the genotypes shown in Fig. 4B. Interestingly, Notch1Δ/+; Notch2Δ/Δ brains showed a similar reduction of Sox2 (+) cell number to that in Notch1Δ/Δ; Notch2Δ/+ brains, suggesting an increase in Notch signaling activity by Notch2 to a comparable level with that by Notch1, in contrast to the early stages. We also found that RGCs were completely depleted in the Notch1Δ/Δ; Notch2Δ/Δ DKO mice. Furthermore, brains bearing a single Notch gene dosage such as Notch1Δ/+; Notch2Δ/Δ and Notch1Δ/+; Notch2Δ/Δ also exhibited a severe depletion of RGCs, compared to brains of double heterozygous Notch1Δ/+; Notch2Δ/+ or that of a single KO of Notch1Δ/+ or Notch2Δ/Δ, while it was less severe than that of the Notch1Δ/Δ; Notch2Δ/Δ DKO brains (Fig. 4B); when the dosage effects of Notch genes are examined, the wild type with the geno-type of Notch1Δ/+; Notch2Δ/Δ would be the appropriate control. We obtained only two wild type (Notch1Δ/+; Notch2Δ/Δ; Emx1Cre+) animals from the crosses for these experiments, which gave 1482, and 1386 for Sox2 (+) cell number. Whereas these values are larger than the average value for (Notch1Δ/+; Notch2Δ/Δ), these values cannot be statistically compared with numerical values of the other genotypes, because of too few sample numbers for the wild type. However, it is highly likely that phenotypic severity depends on the Notch gene dosage, being increased according to a decrease in the dosage of the four genes of Notch1 at Notch2 at the late embryonic stage, as far as we compare the Sox2 (+) cell numbers among the above genotypes available for statistical comparisons. Those mice that had only one dose of the Notch1 or Notch2 gene (Notch1Δ/+; Notch2Δ/Δ and Notch1Δ/Δ; Notch2Δ/+), showed a severe phenotype in Sox2 (+) cell number to a similar degree. This again implies that Notch1 and Notch2 have comparative levels of signaling activity (or expression) for RGC maintenance at the late embryonic stage, in contrast to their large difference at the earlier stage (Fig. 1)

Tbr2 = 105.6 ± 15.5 cells (n = 9 embryos). 10 pregnant females were used. (D) Histograms of Sox2 (+) cells (left) and Tbr2 (+) cells (right) in E13.5 dorsal cortices for the same genotype series as in C. Notch1Δ/+; Notch2Δ/+; Sox2 = 236.8 ± 29.3 cells; Tbr2 = 126.7 ± 18.7 cells (n = 7 embryos); Notch1Δ/Δ; Sox2 = 181.5 ± 17.1 cells, Tbr2 = 134.6 ± 8.6 cells (n = 3 embryos); Notch2Δ/Δ; Sox2 = 245.7 ± 28.9 cells, Tbr2 = 107.7 ± 16.8 cells (n = 5 embryos); Notch1Δ/Δ; Notch2Δ/Δ; Sox2 = 115.8 ± 18.2 cells, Tbr2 = 154.6 ± 9.5 cells (n = 3 embryos). Five pregnant females were used. Values are the mean ± S.D. Statistical significance calculated using one-way ANOVA with Tukey’s correction for multiple comparisons; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Fig. 2. Cell cycle exit rate of progenitor cells in the dorsal cortex at the transition stage from the proliferative to neurogenic state. (A) E11.5 dorsal cortices double staining for EdU (green) and Ki67 (magenta) 12 hours after EdU administration. Dorsal cortices of double heterozygous Notch1^{+/Δ}; Notch2^{+/Δ} as a control, a single KO of Notch1^{+/Δ} and Notch2^{+/Δ}, and DKO of Notch1^{+/Δ}; Notch2^{+/Δ} as marked in each panel. Scale bar, 10 μm. (B) Histograms of cell cycle exit rate of progenitor cells in E11.5 dorsal cortices with the genotype of the double heterozygous Notch1^{+/Δ}; Notch2^{+/Δ} as a control, a single KO of Notch1^{Δ/Δ} and Notch2^{Δ/Δ}, and DKO of Notch1^{Δ/Δ}; Notch2^{Δ/Δ}. Notch1^{+/Δ}; Notch2^{+/Δ}: 6.6 ± 2.6 % (n = 3 embryos); Notch1^{Δ/Δ}; Notch2^{Δ/Δ}: 26.6 ± 2.6 % (n = 4 embryos); Notch2^{Δ/Δ}: 5.2 ± 2.1 % (n = 8 embryos); Notch1^{Δ/Δ}; Notch2^{Δ/Δ}: 28.0 ± 3.2 % (n = 6 embryos). Nine pregnant females were used. Values are the mean ± S.D. Statistical significance calculated using one-way ANOVA with Tukey’s correction for multiple comparisons; ****P < 0.0001 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
Fig. 3. Distribution of mitotic cells in the germinat zone of the dorsal cortices of the single KO of Notch1$^{+/\Delta}$ and Notch2$^{+/\Delta}$, and DKO of Notch1$^{+/\Delta}$; Notch2$^{+/\Delta}$ at early- to mid-neurogenic stages. (A) Staining for phosphorylated histone 3 (PH3, green) and DAPI (blue) of E11.5 dorsal cortices with the genotype of the double heterozygous Notch1$^{+/\Delta}$; Notch2$^{+/\Delta}$ as a control, a single KO of Notch1$^{+/\Delta}$ and Notch2$^{+/\Delta}$, and DKO of Notch1$^{+/\Delta}$; Notch2$^{+/\Delta}$, from left to right. (B) The same staining as A of E13.5 dorsal cortices with the same genotypes. Scale bar, 10 μm. In both A and B, PH3(+) cells that are apically dividing cells are mostly RGCs whereas PH3(+) cells dividing at the subventricular zone are intermediate progenitor cells that have committed to the neuronal fate. Note that cell death frequently occurs in Notch1 knockout cortices indicated by white arrowheads consistent with the findings of Mason et al., 2006. (C) Histograms indicating PH3(+) cell number of the apical divisions and basal divisions, and their sum per 100 μm width.
3.5. Notch2 expression in RGCs increases from E14.5

Our data indicates that Notch2 contributes to RGC maintenance at late stages to the same extent as Notch1 (Fig. 4), whereas Notch1 is important during the proliferative stage as well as at the early to mid-neurogenic stage (Fig. 1). Thus, we examined the temporal pattern of Notch1 and Notch2 transcription by single cell RNA sequencing of RGCs. To enrich the population of RGCs, we sorted 
Hes1 positive RGCs from 
Hes1-∆EGFP+/– reporter mice (Ohtsuka et al., 2006). We collected EGFP + cells at E11, E14, and E16 and analyzed the transcriptome of these cells. These cells were roughly clustered by the developmental time using t-SNE analysis (Fig. 5A). We also observed some 
Hes1 negative IP and neurons at E14 and E16 (Fig. 5B). These differentiated cells might have had a weak GFP signal because of the incomplete degradation of GFP. Therefore, we focused on 
Hes1+Sox2+Pax6+ cells as RGCs when we compared Notch1/2/3 expression (Fig. 5F–H). A large population of RGCs at E11 was detected as Notch1 mRNA positive (64.6 %), and that population relatively increased toward E14 (78.3 %), and E16 (81.5 %) (Fig. 5C). The averaged expression level of Notch1 in RGCs was also increased at later stages: from E11 to E14 (the ratio of E14 vs E11 = 1.43, p = 1.4E-48; see the legend of Fig. 5 for calculation of the averaged expression level and p-value), and from E11 to E16 (the ratio of E16 vs E11 = 1.78, p = 5.8E-113) (Figs. 5F and I). In contrast, a small population of RGCs expressing Notch2 mRNA at E11 (12.8 %) rapidly expanded at E14 (27.6 %) and then at E16 (44.9 %) (Fig. 5D). The averaged expression level of Notch2 in RGCs was gradually increased toward later stages: from E11 to E14 (the ratio of E14 vs E11 = 1.29, p = 3.1E-16), from E14 to E16 (the ratio of E16 vs E14 = 1.54, p = 2.3E-13), and E11 to E16 (the ratio of E16 vs E11 = 1.99, p = 9.1E-82) (Figs. 5G and J). Some of RGCs at E11 expressed Notch3, but the population of Notch3 positive RGCs dramatically decreased at E14 and E16 (Fig. 5H).

It might not be straightforward to compare the Notch1 and Notch2 activities from these data because a previous study showed a differential transcriptional activity between Notch1 and Notch2 (Shimizu et al., 2002). However, the simplest interpretation is that the difference between Notch1 and Notch2 in the slope of mRNA increases over time; that is, an almost zero level at E11.5 to a certain degree of Notch2 expression is a major reason why the relative activity between Notch1 and Notch2 changes temporally. Notch1 plays a major role at early stage, but the overall contribution of Notch1 and Notch2 is comparable to each other in RGC maintenance represented by Sox2 (+) cell abundance.

4. Discussion

4.1. Notch1 is the major Notch signaling receptor at early neural development

In this study, we have obtained multiple lines of evidence that Notch1 and Notch2 act collaboratively in RGC maintenance. Notch1 plays a more substantial role than Notch2 in the early developmental stage because we do not detect a significant defect in the progenitor pool size or neurogenesis in Notch2Δ/Δ brains during E11.5–E13.5. This is consistent with the observation of Notch1Δ/Δ brains by a previous study (Yang et al., 2004). We observed that combinatorial knockout of Notch1 and Notch2 exhibits a severe RGC depletion in the telencephalon than that of defects by Notch1 single knockout, indicating that Notch2 has some activity as a Notch signaling receptor. Alternatively, Notch1 knockout may possibly induce an upregulation of the Notch2 gene, and consequently compensate the decrease in Notch signaling activity by the loss of Notch1 to some degree, compared with the simple situation of the depletion of Notch1 activity plus the normal level of Notch2 activity. In such a case, Notch1Δ/Δ; Notch2Δ/Δ DKO brains show the phenotypic sum due to the loss of both wild type Notch1 activity and an overactivated Notch2. Whether the lack of one Notch gene affects the expression of the other Notch genes will be an interesting issue to be tested. Especially for Notch2 expression level in cortical progenitor cells of the Notch1 mutant at E13.5, needs to be examined. Taken together, Notch1 and Notch2 are major receptors of Notch signaling in the embryonic telencephalon development, while Notch1 accounts for the major signaling receptor activity for NSC self-renewal during the proliferative and early neurogenic stages.

4.2. Role of Notch3 in NSC maintenance and neurogenesis at the embryonic development

Notch1/Notch3 double mutant has no significant difference from Notch1 single knockout in the brain phenotype (Krebs et al., 2003b), suggesting that Notch3 does not appear to have a role in the embryonic brain development, whereas Notch3 is essential for the maintenance of quiescent neural stem cells (Basak et al., 2012; Kawai et al., 2017). We note the possibility that the embryonic Notch3 expression (Fig. 5H) may contribute to NSC proliferation, given that the depletion of Delta-like1 (Dll1), a major ligand of Notch signaling, shows a much severe depletion of NSC at an earlier stage, compared with the Notch1/Notch2 double mutant (data not shown).

4.3. Notch1 and Notch2 share Notch signaling activity at late-embryonic stage

Our results suggest that Notch2 appears to become a comparable player with Notch1 during late neural development (at E18.5). This temporal change in the relative role of Notch2 most likely comes from an increase in Notch2 activity at the late stage from its almost null level at E11.5. There are two pieces of supporting evidence for this hypothesis. First, our single cell transcriptome analysis revealed that Notch2 mRNA quantity increased from an almost null level at E11.5 to a certain level during the period of E14.5 and the late stage (E18.5, see Fig. 5). Second, Notch activity that was evaluated by the total Sox2 (+) cell number in the VZ was dose-dependent of Notch1 and Notch2 genes (at least at E18.5), and did not necessarily depend on the Notch isotype, Notch1 or Notch2, as shown in Fig. 4B. Interestingly, under the situation where Notch
Fig. 4. Dependence of RGC maintenance on the gene-dosage of Notch1 and Notch2 in cerebral cortices at late neurogenic stages, E18.5. (A) Lateral view of dorsal-medial cerebral cortices including the hippocampal region. Notch1 and/or Notch2 knockout area was specified with GFP(+) cells and/or deletion of Notch staining. Tissue sections of various genotypes shown in A, in which all cells express EGFP (green, see Material and Methods), were stained for Sox2 (magenta) and DNA by DAPI (blue). The total length of the ventricular zone along the entire cortex (dorsal to medial) appears to correlate with the total dosage of Notch1 and Notch2 genes. Top is dorsal and bottom is ventral. Scale bar, 100 μm. (B) Sox2(+) cell number in the whole dorsal cortex that expressed Emx1-Cre in various combination of dosages of Notch1 and Notch2 genes at the late embryonic stage. See Table 1 for each genotype. Notch1^{+/+}; Notch2^{+/+}: 1,180 ± 157 cells (n = 7 embryos); Notch1^{+/Δ}; Notch2^{+/Δ}: 1,094 ± 138 cells (n = 7 embryos); Notch2^{Δ/Δ}: 929 ± 129 cells.
Fig. 5. Single cell transcriptome analysis for Notch1, Notch2 and Notch3 in E11, E14 and E16 RGCs. (A) Plot of single-cell transcriptome of progenitor cells after reduction of dimension by t-distributed stochastic neighbor embedding (t-SNE), colored by stages. (B-E) Genes (Hes1, Notch1, Notch2 and Notch3) expression level showed in t-SNE plots. Blue and gray colors indicate a high and low expression level, respectively. Raw gene counts in a cell were normalized to total gene counts of that cell and multiplied by the scale factor (10000) and then log-transformed. Proportions of cells expressing Notch1 or Notch2 are 64.6 % (E11), 78.3 % (E14) and 81.5 % (E16) for Notch1; 12.8 % (E11), 27.6 % (E14) and 44.5 % (E16) for Notch2. (F–H) Quantitative expression level of genes (Notch1, Notch2, and Notch3) in each single cell. Data are presented as box-whiskers (left) and bee swarm plots (right). The box-whiskers plot represents maximum (except outliers), third quartile to first quartile, median and minimum (except outliers). Outliers are plotted as points outside of the box. Outliers are defined as 1.5 folds larger or smaller than interquartile range from third quartile or first quartile, respectively. Cells that were identified as (Hes1+ Pax6+ Sox2+) by t-SNE analysis were only used as RGCs (n = 1322 at E11, n = 729 at E14 and n = 1,165 at E16). The averaged expression level of Notch1 or Notch2 at each stage were compared between E11 versus E14, E11 versus E14 and E14 versus E16, respectively. To test whether Notch1 and Notch2 are differentially expressed in RGCs at different stages, statistical significance was calculated using non-parametric Wilcoxon signed-rank test and p-value was adjusted by Bonferroni correction; ****P < 0.0001, and ns means not-significant. (I, J) The ratio of averaged expression levels of Notch1 (I) and Notch2 (J) at E11.5, E14.5 and E16.5 relative to that of E11.5 were calculated from the data shown in F–H. 1.43 (E14 to E11), and 1.78 (E16 to E11) for Notch1; 1.29 (E14 to E11), and 1.99 (E16 to E11) for Notch2 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
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264 cells, (n = 6 embryos); Notch1Δ/Δ: Notch2Δ/Δ: 511 ± 114 cells (n = 3 embryos); Notch1Δ/Δ: Notch2Δ/Δ: 436 ± 63 cells (n = 6 embryos); Notch1Δ/Δ: Notch2Δ/Δ: 10.2 ± 9.3 cells (n = 3 embryos). Seven pregnant females were used. Values are the mean ± S.D. Statistical significance calculated using one-way ANOVA with Tukey’s correction for multiple comparisons; ****P < 0.0001 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
has only one gene dosage (Notch1Δ/Δ; Notch2+/-; or Notch1+/-; Notch2Δ/Δ), a single Notch1 and Notch2 gene activity gives rise to a similar level of the total Sox2 (+) cell number, which is a good measure for NSC self-renewal and maintenance. Since there is a limited NSC population that expresses Notch2, but not Notch1, in wild type E16.5 (Wu et al. 2020), the dose dependent NSC loss by Notch1Δ/Δ; Notch2Δ/Δ DKO in later stage can be attributed to the cell autonomous decrease of the net Notch activity. Whereas the quantity of Notch2 transcripts appears to be much lesser on average than Notch1 (Fig. 5), a previous study demonstrated that Notch2 has a higher efficiency to go to the cell membrane than Notch1 (Liu et al. 2013), and Notch2 has a higher affinity than Notch1 for DLL1, which is a major ligand expressed in the developing brain (Kakuda et al. 2020; Kawaguchi et al. 2008). Therefore, Notch2 might have a comparable activity to Notch1 in the total in the later stage, even if the quantity of Notch2 transcripts is much less than that of Notch1 transcripts. At late embryonic stage like E18.5, neurogenesis in the dorsal cortex is mostly terminated, and self-renewing RGCS have shifted to act as glial stem cells that mainly produce astrocytes. This continues after birth for a while (Levison et al., 1993).

4.4. Interaction between Notch and various signaling pathway

At the downstream of Notch, combinatorial Hes1, Hes3 and Hes5 signaling play an important role in the maintenance of NSCs (Hatakeyama and Kageyama, 2006). These Hes genes play a pivotal role by interacting with not only Notch but also other signaling proteins assuming regional and context dependent expression such as BMP/SMAD, SHH and JAK/STAT to converge various signaling inputs into the NSC property (Kriegstein and Alvarez-Buylla, 2009; Mehler, 2002). Thus, it is intriguing issue to address the question of whether the differential temporal pattern of Notch1 and Notch2, in cooperation with spatially and temporally varying signaling pathways, contributes to the control of the NSC fate determination such as active cycling, gliogenesis, and quiescence.

In this study, we demonstrate that Notch1 is a major player during stem cell proliferation and neurogenesis while Notch2 shows up in the later stages of neurogenesis. In this sense, the situation might resemble the ventral side of the telencephalon, whose subventricular zone maintains adult NSCs, when they are quiescent. Notch2 relatively works for their maintenance, whereas Notch1 mainly functions for the activation of adult NSCs (Engler et al., 2018). It is intriguing to assume a higher dependency on Notch2 to maintain adult neural stem cells gradually appears from embryonic stage. whereas we need to examine the temporally differential expression pattern of Notch1 and Notch2 in the dorsal cortex is also preserved in the dentate gyrus primordium, as well as in the ventral side of the embryonic cerebral cortex.

Author contributions

S.M. and F.M. designed the project and wrote the manuscript. S.M. performed experiments for Figs. 1–4. A.S statistically analyzed quantitative data. Q.W. performed single cell transcriptome analysis for Fig. 5. T.G. provided Notch1Δ/+ and Notch1 Δ/Δ mouse line. M.M. provided Notch1+/- mice and genetic information of both mutants. All co-authors contributed to the discussion of the present study.

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Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.neures.2020.11.007.

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