

# Selection of differentiating cells by different levels of delta-like 1 among neural precursor cells in the developing mouse telencephalon

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During the neurogenic phase of mammalian brain development, only a subpopulation of neural precursor cells (NPCs) differentiates into neurons. The mechanisms underlying this selection remain unclear. Here we provide evidence that the Notch-Delta pathway plays an important role in this selection in the developing mouse telencephalon. We found that the expression patterns of the Notch ligand delta-like 1 (Dll1) and of the active form of Notch1 were mutually exclusive and segregated into distinct NPC subpopulations in the ventricular zone of the telencephalon. When Dll1 was overexpressed in a small, but not a large, proportion of NPCs, these cells underwent neuronal differentiation *in vitro* and *in vivo*. This Dll1-induced neuronal differentiation did not occur when cells were plated at lower densities in an *in vitro* culture. Importantly, conditional deletion of the *Dll1* gene in a small proportion of NPCs reduced neurogenesis *in vivo*, whereas deletion in a large proportion promoted premature neurogenesis. These results support the notion that different levels of Dll1 expression determine the fate of NPCs through cell-cell interactions, most likely through the Notch-Delta lateral inhibitory signaling pathway, thus contributing to the selection of differentiating cells.

**KEY WORDS:** Dll1, Notch, Lateral inhibition, Cell-cell interaction, Neural precursor cell, Telencephalon

## INTRODUCTION

Neural precursor cells (NPCs) produce neurons at a period called the neurogenic phase during mammalian brain development (Temple, 2001; Hirabayashi and Gotoh, 2005). Neocortical NPCs successively produce different types of neurons depending on the stage at which they differentiate, and this contributes to cortical layer formation (Molyneaux et al., 2007). A subset of NPCs is selected to differentiate into neurons at a given time point during the neurogenic phase, whereas other NPCs maintain their undifferentiated state. The ratio between undifferentiated and differentiating NPCs should thus be a key factor in determining the type and correct number of neurons produced at each stage. However, it remains largely unclear by what mechanisms NPCs are selected to undergo neuronal differentiation in the developing mammalian brain. The prevailing view is that the ratio of asymmetric to symmetric cell division contributes to the proportion of NPCs differentiating into neurons. This model assumes that asymmetric (vertical/oblique) cell division produces one differentiating and one undifferentiated daughter cell due to an asymmetric distribution of cell fate determinants, whereas symmetric (planar) cell division at the apical surface of the ventricular zone mainly produces two undifferentiated daughter cells (Chenn and McConnell, 1995; Götz and Huttner, 2005). However, perturbation of the ratio of vertical/oblique to planar division does not necessarily alter the rate of neuronal production (Konno et al., 2008), indicating that other mechanisms are also likely to contribute to the selection of differentiating cells. It is not known whether cell-cell interactions are important for the selection of differentiating cells in the mammalian brain.

The Notch signaling pathway plays a central role in the maintenance of the undifferentiated state of NPCs in the mammalian central nervous system (CNS) (Gaiano and Fishell, 2002; Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006), and has been implicated in the selection of differentiating cells in a number of systems (Artavanis-Tsakonas et al., 1995; Beatus and Lendahl, 1998; Greenwald, 1998). Notch is a transmembrane receptor that is activated by the binding of ligands (delta-like 1, 3, 4 and jagged 1, 2 in mammals) presented by neighboring cells, and thus mediates signaling generated by cell-cell interactions. This triggers the cleavage of the intracellular domain of Notch, which then translocates to the nucleus and binds to Rbpj, converting it from a transcriptional suppressor to an activator. During neurogenesis, Notch activation of Rbpj induces the expression of basic helix-loop-helix (bHLH) Hes proteins, which suppress proneural bHLH transcriptional regulators, such as Neurogenins and Mash1, and, thereby, suppress neuronal differentiation (Kageyama et al., 2005). Notch signaling has been proposed to contribute to binary cell fate specification from an equipotent/homogeneous population through a mechanism called lateral inhibition (Heitzler and Simpson, 1991; Muskavitch, 1994; Wilkinson et al., 1994; Artavanis-Tsakonas et al., 1995; Chitnis, 1995; Heitzler et al., 1996; Lewis, 1996; Beatus and Lendahl, 1998; Greenwald, 1998). This mechanism is based on feedback whereby Notch activation suppresses the expression of its ligand, Delta. If the expression levels of Delta are slightly different among cells, this difference is amplified because Delta-expressing cells receive fewer Notch signals and express more Delta, while the surrounding cells receive more Notch signals and express less Delta. This amplification ultimately segregates the equipotent/homogeneous cell population into two distinct cell populations: Delta-positive Notch-inactivated (differentiating) cells and Delta-negative Notch-activated (undifferentiated) cells. This binary cell fate specification by the Notch-Delta lateral inhibitory signaling pathway was first demonstrated in *Drosophila* neuroectoderm (Heitzler and Simpson, 1991; Heitzler et al., 1996) and *C. elegans* gonad (Wilkinson et al., 1994), and then in other systems such as chick and *Xenopus* retina

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(Dorsky et al., 1997; Henrique et al., 1997). It might also function in the mammalian CNS given that the proneural bHLH genes (*Mash1* and neurogenin 1/2) and anti-neural bHLH Hes genes positively and negatively regulate, respectively, the expression of delta-like 1 (*Dll1*) (Casarosa et al., 1999; Castro et al., 2006; Hatakeyama and Kageyama, 2006), and that *Dll1* exhibits non-homogeneous (so-called salt-and-pepper) expression patterns in the developing mouse CNS (Lindsell et al., 1996). However, the causal relationship between the expression levels of *Dll1* and cell fate in these systems has not been demonstrated. Thus, it is unclear whether the Notch-Delta lateral inhibitory system operates in uncommitted NPCs to determine which NPCs become neurons during the neurogenic phase in the mammalian CNS. Although numerous studies have reported the requirement of Notch ligands for the maintenance of undifferentiated NPCs, it is not clear whether Notch ligands are required for neuronal differentiation (via cell-cell interactions) in the CNS.

In this study, we found that expression of *Dll1* and activation of Notch1 occur in different cells in a mutually exclusive manner in the ventricular zone of the embryonic mouse telencephalon. Importantly, by the overexpression and conditional deletion of *Dll1*, we found that different levels of *Dll1* expression can determine the proportion of differentiating cells among NPCs through cell-cell interactions. In particular, *Dll1* deletion in a small proportion of NPCs revealed a prerequisite role of *Dll1* in neuronal differentiation. These results strongly support the notion that lateral inhibition of Notch signaling indeed operates in the developing mammalian brain and contributes to the selection of differentiating cells among uncommitted NPCs.

## MATERIALS AND METHODS

### Mice

Floxed *Dll1* mice (kind gift of Dr J. Lewis) (Hozumi et al., 2004) were crossed with mice expressing Cre under the control of the nestin promoter and enhancer (*nestin-Cre* mice) (kind gift of Dr R. Kageyama) (Isaka et al., 1999). Jcl:ICR strain mice (ICR mice) were purchased from CLEA Japan. All mice were maintained according to the protocol approved by the Animal Care and Use Committee of the University of Tokyo.

### Expression constructs and antibodies

The plasmids pMX-enhanced green fluorescent protein (EGFP) (pMX-GFP), pMX-IRES-EGFP (pMX-IG) and pMX-SV40-puro were kindly provided by Dr T. Kitamura (University of Tokyo, Tokyo, Japan). The Cre recombinase construct was kindly provided by Dr S. Kato (University of Tokyo, Tokyo, Japan). HA-tagged rat *Dll1* was inserted into the *EcoRI/SnaBI* sites of pMX-IG (pMX-*Dll1*-IG). The plasmids pMX-SV40-GFP and pMX-Cre-SV40-GFP have been described previously (Yoshimatsu et al., 2006). HA-tagged rat *Dll1* intracellular domain (DICD) (Cys560 to Val714) was amplified by PCR from the HA-tagged rat *Dll1* construct, verified by DNA sequencing, and then inserted into the *BamHI/EcoRI* sites of pMX-IG (pMX-DICD-IG). Recombinant retroviruses were produced using the pMX vectors as described previously (Hirabayashi et al., 2004).

Antibodies used in immunocytochemistry and immunohistochemistry were: mouse monoclonal antibodies to  $\beta$ III-tubulin (TuJ1) (Babco) at 1:1000, nestin (BD Pharmingen) at 1:200, Gfap (Chemicon) at 1:500, phosphorylated histone H3 (pH3) (Cell Signaling Technology) at 1:1000, PcnA (Ab-1, Oncogene) at 1:500 and BrdU (BD Biosciences) at 1:50; and rabbit polyclonal antibodies to *Dll1* (H-265, Santa Cruz Biotechnology) at 1:100, cleaved Notch1 (Val 1744, Cell Signaling Technology) at 1:100, Sox2 (Chemicon) at 1:1000, Pax6 (Chemicon) at 1:1000, Tbr2 (Chemicon) at 1:1000 and GFP (MBL) at 1:1000. Alexa Fluor-labeled secondary antibodies, TO-PRO-3 and Hoechst 33342 (for nuclear staining) were from Molecular Probes.

### Primary NPC culture and immunostaining

Primary NPCs were prepared from the dorsal cerebral cortex of ICR mouse embryos at E12.5 (E1 was defined as 12 hours after detection of the vaginal plug) as described previously (Hirabayashi et al., 2004). The cells were cultured in medium comprising a 1:1 (v/v) mixture of Dulbecco's modified

Eagle's medium and F12 medium (Gibco) supplemented with B27 (Invitrogen) and with or without human FGF2 (R&D Systems). To obtain NPC-enriched populations, we plated the dissociated neuroepithelium directly on non-coated 100-mm dishes in culture medium containing FGF2 (20 ng/ml) or both FGF2 (20 ng/ml) and Egf (20 ng/ml, Upstate), and cultured the cells for 3 days (neurosphere culture). The resulting neurospheres were then dissociated and plated on poly-D-lysine-coated dishes in culture medium containing FGF2 (20 ng/ml) to yield an NPC culture. For retroviral infection, cells were mixed with recombinant viruses for 18 hours, washed with phosphate-buffered saline (PBS) and then incubated in the absence or presence of a low dose of FGF2 (2 ng/ml). Clonal analysis was performed as described previously (Hirabayashi et al., 2004). For immunostaining, cells were fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 for 10 minutes, incubated with primary antibodies overnight and then with secondary antibodies for 30 minutes, and mounted in Mowiol (Calbiochem).

### Immunohistochemistry

Immunohistochemistry was performed as described previously (Hirabayashi et al., 2004; Yoshimatsu et al., 2006). For staining with anti-*Dll1* and anti-cleaved Notch1, antigen retrieval was performed by autoclave treatment of sections for 5-10 minutes at 105°C in 0.01 M sodium citrate buffer (pH 6.0) and Target Retrieval Solution (TRS) (Dako), respectively. The TSA Kit (Molecular Probes) was used for signal amplification of *Dll1* or cleaved Notch1 staining. Staining with anti-*Dll1* and anti-cleaved Notch1 was performed using the ABC Kit (Vector Laboratories) and TSA Kit. After antigen retrieval in TRS, the samples were incubated sequentially with anti-cleaved Notch1, with biotinylated secondary antibody, with ABC reagent (ABC Kit) and then with tyramide-biotin (TSA Kit). The samples were then subjected to antigen retrieval by autoclaving for 10 minutes at 105°C in 0.01 M sodium citrate buffer (pH 6.0) and incubated with anti-*Dll1*, HRP-conjugated secondary antibody, then with streptavidin-Alexa Fluor 488 (for detection of cleaved Notch1) and tyramide-Alexa Fluor 555 (TSA Kit) (for detection of *Dll1*). The fluorescence images were obtained with a confocal laser microscope (LSM510, Zeiss).

### In situ hybridization

In situ hybridization on frozen brain sections was performed essentially as described previously (Nomura and Osumi, 2004). The digoxigenin-labeled antisense riboprobe for detecting mouse *Dll1* corresponds to a ~1.6 kb region spanning exons 8 and 11.

### Retrovirus infection in utero

The protocol for retrovirus infection in utero was a modification of a method for in utero electroporation (Tabata and Nakajima, 2001). At E12.5, mice were anaesthetized and the uterine horns were exposed. Recombinant retrovirus suspension (0.5-1.0  $\mu$ l) with Fast Green (0.01%) was injected into the cerebral ventricles of each littermate. The uterine horns were returned to the abdominal cavity to allow the embryos to continue normal development. Two or three days after the operation, the embryos were harvested and the brains examined by immunohistochemical analysis.

### BrdU labeling

For in vivo labeling of BrdU, a single injection of BrdU (50 mg/kg, intraperitoneally) was performed 30 minutes prior to sacrifice. BrdU-positive cells were detected by immunohistochemistry as described above.

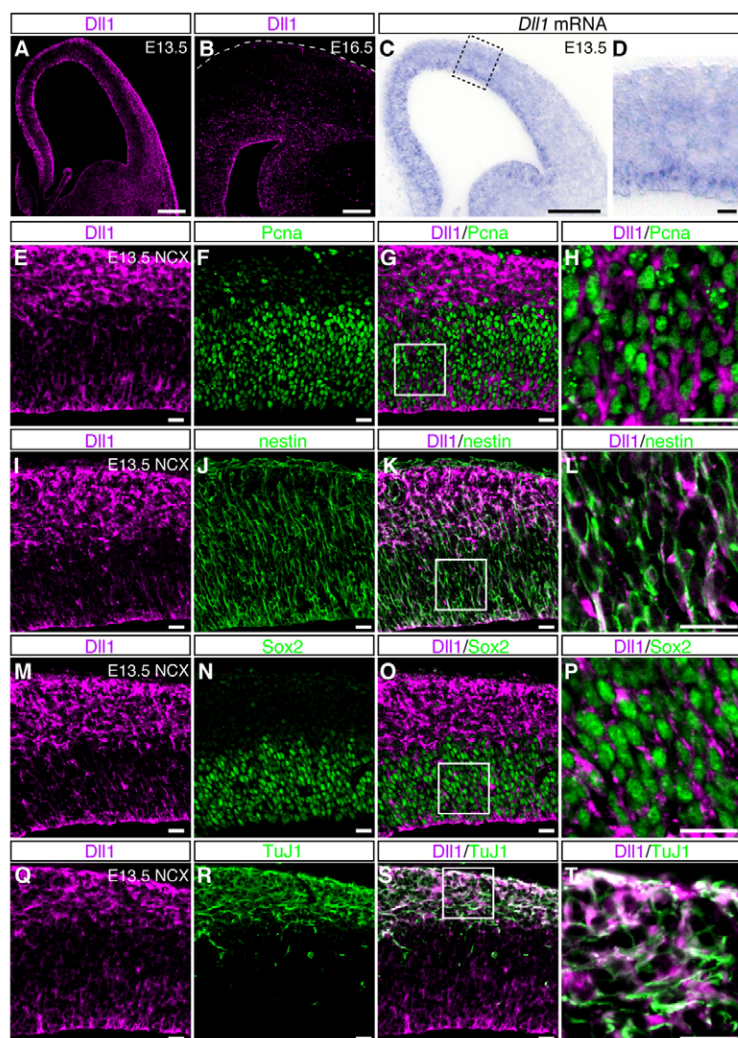
### Statistical analysis

Quantitative data are presented as the mean  $\pm$  s.e.m. from representative experiments. The experiments were repeated at least three times with similar results. Values were compared using the unpaired Student's *t*-test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### *Dll1* is expressed in a subpopulation of undifferentiated NPCs in the mouse embryonic telencephalon

Whereas previous reports have shown that *Dll1* mRNA is expressed only in postmitotic cells in the developing mouse telencephalon (Campos et al., 2001), a recent report has demonstrated the



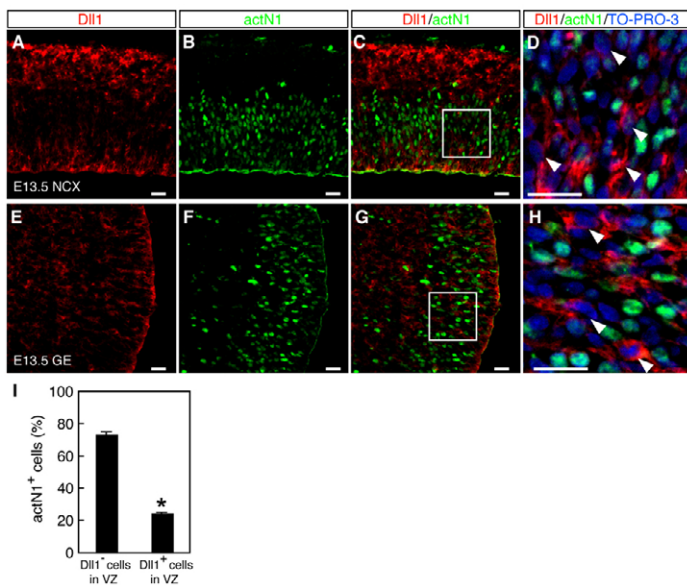
**Fig. 1. Expression of Dll1 in the developing mouse telencephalon.** (A,B,E-T) Brain sections from ICR mouse embryos at E13.5 (A,E-T) or E16.5 (B) were immunostained with anti-Dll1 alone (A,B) or together with anti-Pcna (E-H), anti-*nestin* (I-L), anti-*Sox2* (M-P) or anti- $\beta$  III-tubulin (TuJ1) (Q-T). (C,D) *Dll1* mRNA in sections of E13.5 ICR mouse brain detected by in situ hybridization. Higher magnifications of the boxed region in C,G,K,O and S are shown in D,H,L,P and T, respectively. NCX, neocortex. Scale bars: 200  $\mu$ m in A-C; 20  $\mu$ m in D-T.

oscillatory expression of *Dll1* mRNA at low levels in proliferating NPCs (Shimojo et al., 2008). In this study, we investigated the expression of Dll1 protein in the developing mouse telencephalon by immunohistochemical analyses. Dll1-immunopositive cells were detected in a subset of cells in the ventricular zone (VZ), the intermediate zone (IZ) and the cortical plate (CP) of neocortex and in the ganglionic eminences at E13.5 (Fig. 1A). At later stages, such as at E16.5, Dll1-expressing cells were found in the VZ and IZ, but at very low levels in the CP of the neocortex (Fig. 1B). The expression pattern of Dll1 protein was similar to that of *Dll1* mRNA (Fig. 1C,D). Importantly, most of the Dll1-expressing cells in the VZ were co-immunostained with antibodies to the proliferation marker *Pcna* and to the undifferentiated NPC markers *nestin* and *Sox2* (Fig. 1E-P). Consistent with this, Dll1-expressing cells in the VZ were mostly devoid of the neuronal marker  $\beta$  III-tubulin (TuJ1; Tubb3), whereas most of the Dll1-expressing cells in the IZ (and those in the CP at E13.5) were TuJ1-positive (Fig. 1Q-T). These results suggest that Dll1 is expressed in a subset of undifferentiated NPCs in the VZ, as well as in immature neurons localized at the IZ and CP.

### Mutually exclusive expression patterns of Dll1 and active Notch1

It has been reported that NPCs localized at the VZ are heterogeneous in terms of their Notch activity, as revealed by immunostaining with anti-cleaved Notch1 (active Notch1)

antibody, which recognizes the presenilin/ $\gamma$ -secretase-catalyzed cleavage site of Notch1, and by the use of a reporter gene that monitors the activity of Notch signaling (Tokunaga et al., 2004; Del Monte et al., 2007; Mizutani et al., 2007). We confirmed that only a subpopulation of NPCs in the VZ was immunostained for active Notch1 (Fig. 2B,F). However, a negative correlation between the Notch activity and Dll1 expression in the developing mouse brain has not been demonstrated, although this would provide key evidence to support the role of the Notch-Delta lateral inhibitory system in this system of differentiation. We thus carried out immunohistochemistry of active Notch1 and Dll1 together, and found that their expression patterns in the VZ of the telencephalon were segregated into distinct cells in a mutually exclusive manner (Fig. 2A-H). In the VZ of the neocortex at E13.5, 73.8% of Dll1-negative cells were positive for active Notch1 within the nucleus, whereas only 24.7% of Dll1-positive cells were positive for active Notch1 within the nucleus (Fig. 2I). Dll1 expression and Notch1 activation might have a rough correlation with the cell cycle, as active Notch1- and Dll1-expressing cells tended to localize basally and apically in the VZ, respectively (Fig. 2A-H). However, the percentage of active Notch1-positive cells was significantly lower among Dll1-positive cells than among Dll1-negative cells in both the basal and apical halves of the VZ (see Fig. S1 in the supplementary material). These results suggest that the mutually exclusive patterns of active Notch1 and Dll1 expression might be



**Fig. 2. Dll1 expression and Notch1 activation are segregated into distinct cells in the VZ of the developing mouse telencephalon.**

(A-H) Brain sections from ICR mouse embryos at E13.5 were immunostained with anti-active Notch1 (actN1) and anti-Dll1 and TO-PRO-3 (nuclear staining). Higher magnifications of the boxed regions in C and G are shown in D and H, respectively. Arrowheads in D,H indicate Dll1-positive actN1-negative cells in the VZ. (I) The proportions of active Notch1-positive cells among Dll1-negative or Dll1-positive cells in the VZ of the neocortex were determined by immunohistochemical analysis. Data are the mean  $\pm$  s.e.m. of values from three sections of each brain, and similar results were obtained from six independent brains. \* $P < 0.0005$ . NCX, neocortex; GE, ganglionic eminence. Scale bars: 20  $\mu$ m.

established at different phases of the cell cycle. This negative correlation between active Notch1 and Dll1 expression strongly supports the existence of the Notch-Delta lateral inhibitory system in the developing mouse telencephalon.

### Dll1 expression regulates neuronal fate specification in vitro

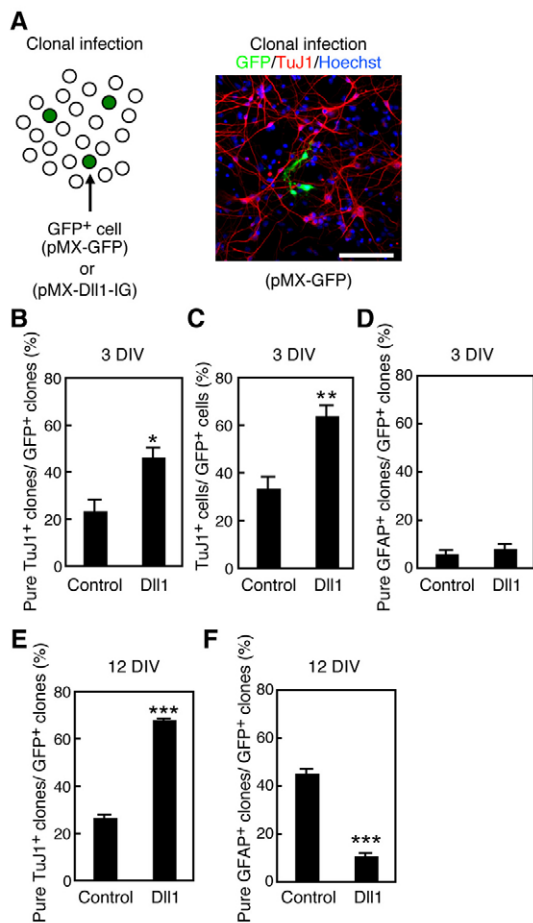
It has been proposed that *Dll1* expressed in immature neurons suppresses excess neuronal differentiation by activating Notch in the neighboring NPCs (Henrique et al., 1997; Campos et al., 2001). However, the expression of Dll1 in undifferentiated NPCs, as observed in Fig. 1, suggests an additional role of Dll1, i.e. in neuronal fate specification among NPCs through the lateral inhibitory system. If this is the case, ectopic expression of Dll1 in a subpopulation of NPCs should lead to neuronal differentiation of these cells, as ectopic Dll1 activates Notch signaling and reduces Dll1 expression in the surrounding cells, resulting in a reduction of Notch signaling in the Dll1-expressing cells (Heitzler and Simpson, 1991; Dorsky et al., 1997; Henrique et al., 1997). We examined this hypothesis by introducing Dll1 into an in vitro culture of NPCs. NPC cultures (see Materials and methods) prepared from E12.5 mouse neocortex were infected with retroviruses encoding either green fluorescent protein (GFP) alone, or GFP together with *Dll1*, at a low titer so that only a small number (less than 0.1%) of the cells were infected. Under this condition, the fate of each infected clone could be traced as a cluster of GFP-positive cells (Fig. 3A). The expression of Dll1 significantly increased the proportion of clones containing only TuJ1-positive neurons (pure TuJ1-positive clones) among GFP-positive clones (Fig. 3B). The expression of Dll1 also increased the percentage of TuJ1-positive cells among GFP-positive cells (Fig. 3C). The expression of Dll1 did not appear to alter the size of the clones (see Fig. S2 in the supplementary material). Since the stage of this culture roughly corresponds to the neurogenic phase, the percentage of cells expressing the astroglial marker Gfap among infected cells was small ( $\sim 7\%$ , regardless of Dll1 expression) (Fig. 3D), indicating that the Dll1-induced neuronal differentiation shown in Fig. 3B was not due to a suppression of an alternate (astroglial) fate of NPCs. The contribution of cell death was also negligible in these cultures (less than 2% among GFP-positive cells).

The fate switch of NPCs from neurogenic to astroglial can be recapitulated in an in vitro culture. When Dll1 was overexpressed in a subpopulation of NPCs prepared from a 12 days in vitro (DIV) culture of E12.5 neocortical neuroepithelial cells, which correspond to the astroglial phase, the proportion of clones containing only Gfap-positive astrocytes was reduced and the proportion of clones containing only TuJ1-positive neurons was increased (Fig. 3E,F). This suggests that high levels of Dll1 were sufficient to change the astroglial fate into the neurogenic fate when expressed at the astroglial phase. Interestingly, the levels of *Dll1* mRNA were reduced at around the onset of the astroglial phase (Campos et al., 2001; Irvin et al., 2004). This reduction might contribute to the suppression of neurogenesis during the astroglial phase.

### Dll1-induced neuronal differentiation requires cell-cell interactions among NPCs

If neuronal differentiation induced by Dll1 expression can be ascribed to the lateral inhibition model, it should be dependent on cell-cell interaction and the difference in Dll1 levels between the cells. To examine this, we introduced Dll1 into a large proportion of NPCs by retroviral infection at a high titer (more than 70% of NPCs were infected under this condition), so that there would be no major differences in Dll1 levels among the cells. In this case, the expression of Dll1 did not promote neuronal differentiation (Fig. 4A). We further examined whether cell-cell interactions are essential for Dll1-induced neuronal differentiation by reducing the cell density. We introduced Dll1 into a small proportion of NPCs by retroviral infection at a low titer, as in Fig. 3, but changed the seeding cell density. At a lower cell density ( $0.26 \times 10^5$  cells/cm<sup>2</sup> at seeding), the expression of Dll1 no longer increased the percentage of pure TuJ1-positive clones among GFP-positive clones (Fig. 4B). These results together support the idea that Dll1 induces neuronal differentiation of NPCs via cell-cell interaction, consistent with the lateral inhibition model.

Reduction of the cell density did not significantly increase the percentage of TuJ1-positive clones among GFP-positive clones in control cultures (Fig. 4B), suggesting the existence of a trans-acting signal that promotes neuronal differentiation in a non-cell-autonomous manner. Such a mechanism might involve Wnt signaling, as Wnt ligands, including Wnt7a, were expressed in

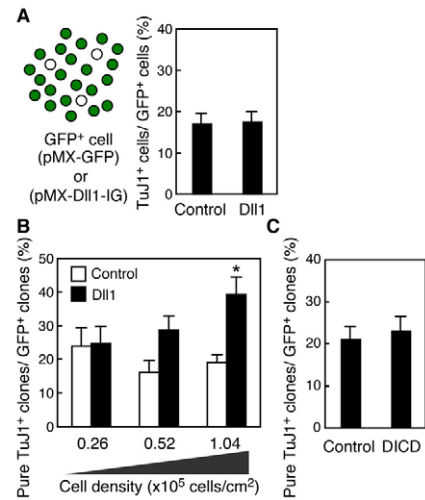


**Fig. 3. Dll1 overexpression in a small proportion of NPCs induces neuronal differentiation in vitro.** Primary NPCs were prepared from (A–D) 3-day sphere cultures [3 days in vitro (3 DIV)] or (E, F) 12-day sphere cultures (fourth passage, 12 DIV) of the neocortical cells of E12.5 ICR mice. The NPCs were infected with a retrovirus encoding GFP (pMX-GFP, control), or both GFP and *Dll1* (pMX-Dll1-IG), at a low titer and subjected to clonal analysis (see Materials and methods). (A) A representative GFP-positive clone stained with anti-GFP, TuJ1 and Hoechst. After incubation for 2 days in the presence of a low dose of human FGF2 (2 ng/ml) (B–D) or in the absence of FGF2 (E, F), cells were stained with anti-GFP and TuJ1 or anti-GFP and anti-Gfap. The percentage of clones containing only TuJ1-positive (B, E) or only Gfap-positive (D, F) cells among GFP-positive clones was then determined by immunocytochemical analysis. The percentage of TuJ1-positive cells among GFP-positive cells was also determined by immunocytochemistry (C). \* $P < 0.02$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ . Scale bar: 50  $\mu\text{m}$  in A.

this culture (data not shown), and the prevention of Wnt signaling suppresses the neuronal differentiation of NPCs cultured under similar conditions (Hirabayashi et al., 2004).

### The intracellular domain of Dll1 does not affect neuronal differentiation

Dll1 is cleaved at a transmembrane site upon Notch binding, and the intracellular domain of Dll1 has been shown to translocate into the nucleus and transmit some signals cell-autonomously (Bland et al., 2003; Ikeuchi and Sisodia, 2003; LaVoie and Selkoe, 2003; Six et al., 2003; Kolev et al., 2005). A recent study has shown that expression of the Dll1 intracellular domain (DICD) promotes neurogenesis cell-autonomously in P19 cells (Hiratochi et al., 2007). However, we

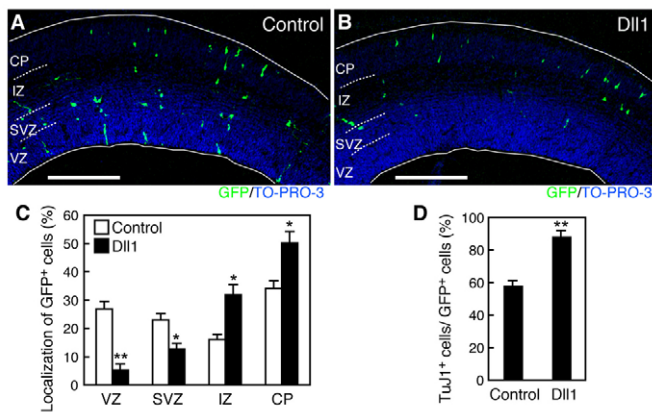


**Fig. 4. Dll1 overexpression induces neuronal differentiation in a non-cell-autonomous manner.** (A) Primary NPCs were prepared from 3-day sphere cultures (3 DIV) of the neocortical cells of E12.5 ICR mice, infected with a retrovirus encoding GFP (pMX-GFP, control), or both GFP and *Dll1* (pMX-Dll1-IG), at a high titer and analyzed as in Fig. 3C. (B) Primary NPCs were prepared from 3-day sphere cultures (3 DIV) of the neocortical cells of E12.5 ICR mice and plated at different cell densities (0.26 $\times$ , 0.52 $\times$  and 1.04 $\times$   $10^5$  cells/cm<sup>2</sup>). These cells were infected with a retrovirus encoding GFP (pMX-GFP, control), or both GFP and *Dll1* (pMX-Dll1-IG), at a low titer and analyzed as in Fig. 3B (clonal analysis). \* $P < 0.05$ . (C) Primary NPCs were prepared from 3-day sphere cultures (3 DIV) of the neocortical cells of E12.5 ICR mice and infected with a retrovirus encoding GFP (pMX-GFP, control), or both GFP and the delta-like 1 intracellular domain (pMX-DICD-IG), at a low titer and analyzed as in Fig. 3B (clonal analysis).

found that overexpression of DICD, even when expressed in a small number of NPCs, did not induce neuronal differentiation in primary telencephalic NPC culture (Fig. 4C) or in vivo (see Fig. S3 in the supplementary material). These results again suggest that neuronal differentiation induced by Dll1 expression is not due to a cell-autonomous mechanism.

### Different levels of Dll1 expression regulate neuronal fate specification in the developing mouse neocortex

In addition to the in vitro experiments, we wished to examine the effects of Dll1 expression on NPC fate in vivo. We therefore introduced Dll1 into a small number of NPCs in the telencephalon by injecting retroviruses encoding GFP alone, or both GFP and Dll1, into the telencephalic ventricle at E12.5. Two days after infection, the numbers of GFP-positive cells localized at each area [VZ, subventricular zone (SVZ), IZ or CP] were determined. When control retroviruses were introduced, 26.8 $\pm$ 2.7% of GFP-positive cells were found within the VZ 2 days after infection. When Dll1-expressing retroviruses were introduced, the percentage of GFP-positive cells within the VZ was markedly reduced (5.3 $\pm$ 2.1%) (Fig. 5A–C). Dll1 expression also decreased the percentage of GFP-positive cells located in the SVZ, and increased the percentages of GFP-positive cells in the IZ and CP. All of the GFP-positive cells in the IZ and CP were negative for the NPC marker Pax6, and more than 97% of these cells were positive for TuJ1 in both control and Dll1-expression samples. In fact, the expression of Dll1 significantly increased the proportion of TuJ1-positive cells among GFP-positive



### Fig. 5. *Dll1* overexpression in a small number of NPCs in the developing mouse neocortex induces neuronal differentiation.

Retroviruses encoding GFP alone (pMX-GFP, control), or both GFP and *Dll1* (pMX-*Dll1*-IG), were injected into the lateral ventricle of ICR mice at E12.5. Under these conditions, only a small proportion of cortical NPCs were infected. Brains were excised 2 days later and examined. (A, B) The location of the GFP-positive cells was determined by immunohistochemistry with anti-GFP and TO-PRO-3 (nuclear staining). Typical results are shown. (C) Quantification of the distribution of GFP-positive cells in the neocortex. Data are the mean  $\pm$  s.e.m. of values from eight sections of each brain compared between littermates. Similar results were obtained from seven independent littermates. (D) Brain sections were stained with anti-GFP and TuJ1, and the percentage of TuJ1-positive cells among GFP-positive cells in the whole neocortex was determined. Data are the mean  $\pm$  s.e.m. of values from eight sections of each brain compared between littermates. Similar results were obtained from three independent brains. \* $P < 0.01$ , \*\* $P < 0.0001$ . VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bars: 200  $\mu$ m.

cells in the whole neocortex (Fig. 5D). These results strongly suggest that *Dll1* expression in a small population of cells at the VZ promotes neuronal differentiation in the developing neocortex.

### Conditional deletion of *Dll1* results in aberrant neuronal differentiation in vivo

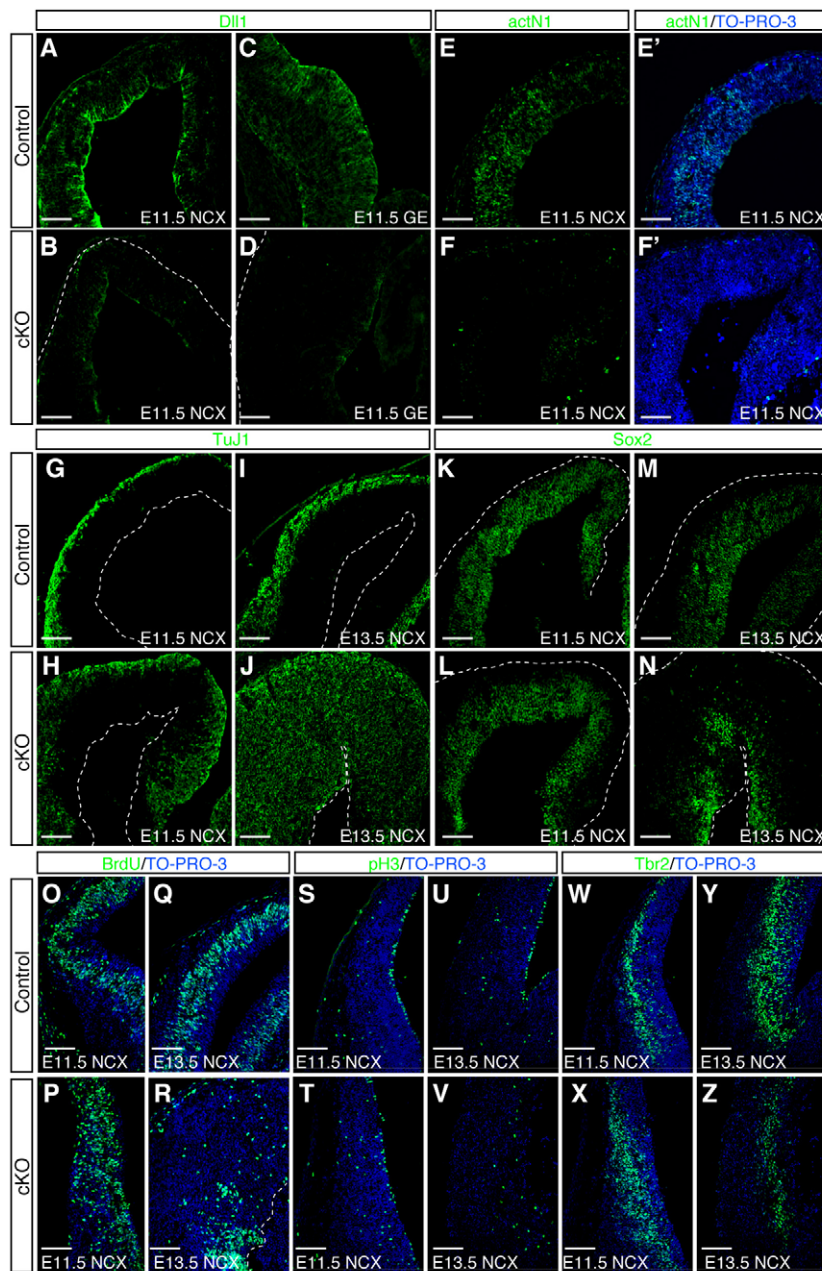
It has previously been reported that deletion of *Dll1* results in embryonic lethality at E11.5, accompanying hyperplasia of the CNS (Hrabe de Angelis et al., 1997) and increased early production of GABA-positive neurons at ganglionic eminences (Yun et al., 2002). However, it has remained unclear whether *Dll1* is a major Notch ligand in other parts of the telencephalon such as the neocortex, especially given that other Notch ligands such as jagged 2 and *Dll3* are also expressed in the developing neocortex (Luo et al., 1997; Campos et al., 2001; Irvin et al., 2004). Thus, to avoid the embryonic lethality at early developmental stages, we utilized a conditional deletion of the *Dll1* gene in the CNS. Conditional deletion was achieved by crossing mice carrying both *Dll1* alleles flanked by loxP sequences (*Dll1*<sup>fllox/fllox</sup>) (Hozumi et al., 2004) with mice harboring a transgene of *Cre* recombinase under the control of the nestin promoter and enhancer (Isaka et al., 1999). In these mice, *Dll1* immunoreactivity was already decreased at E11.5 in telencephalon (Fig. 6A-D). By E13.5, the *Dll1*-deficient neocortex became thicker than the control neocortex in the radial axis, but thinner in the tangential axis (Fig. 6I, J, M, N, and data not shown), with a slight increase in cell death (data not shown). The levels of active (cleaved) Notch1 in NPCs were significantly reduced in the *Dll1* conditional

KO mice at E11.5 (Fig. 6E, E', F, F'). These mice exhibited robust premature neurogenesis in the neocortex as well as in the subcortical regions by E13.5, as detected by an increase in TuJ1-positive cells and a reduction in Sox2-positive cells (Fig. 6G-N). We also observed a transient increase of Tbr2 (Eomes)-positive basal progenitors at E11.5 and a decrease at E13.5 in the lateral region of *Dll1*-deficient neocortex (Fig. 6W-Z, see also Fig. 6O-V showing the aberrant location of mitotic cells), probably owing to the premature neurogenesis and subsequent exhaustion of NPCs. At E16.5, fewer NPCs were found in the VZ of the *Dll1* conditional KO mice as compared with the control mice (data not shown), also suggesting the exhaustion of NPCs owing to the premature neurogenesis. Since these phenotypes are consistent with the premature neurogenesis observed in mice defective in the canonical Notch pathway (e.g. Notch1- and Rbpj-deficient mice) (de la Pompa et al., 1997), these results suggest that *Dll1* is a major ligand for the Notch receptor in the developing neocortex and that it contributes to the maintenance of the undifferentiated state of NPCs.

Given that *Dll1* is a major Notch ligand in the developing telencephalon, we next examined whether different levels of endogenous *Dll1* among undifferentiated NPCs could determine the fate of NPCs in vivo, and whether the Notch-Delta lateral inhibitory system indeed operates in vivo. We deleted the *Dll1* gene in a small proportion of NPCs in vivo by injecting retroviruses harboring GFP alone, or GFP with Cre recombinase, into the telencephalic ventricle of *Dll1*<sup>fllox/fllox</sup> mice at E12.5. When examined 3 days after infection, expression of Cre recombinase increased the percentage of GFP-positive cells within the VZ (from 19.2% to 36.3%) and reduced that of GFP-positive cells within the IZ and CP (IZ, from 26.7% to 19.3%; CP, from 36.1% to 26.2%) (Fig. 7A-C). Since more than 96% of GFP-positive cells in the VZ were Pax6-positive and TuJ1-negative in both control and Cre-infected samples, this result indicates that the reduction of endogenous *Dll1* in a small proportion of NPCs suppressed the neuronal differentiation of these cells. In fact, the deletion of *Dll1* significantly increased the proportion of Pax6-positive cells among GFP-positive cells in the whole neocortex (Fig. 7D). Since the reduction of endogenous *Dll1* in all NPCs dramatically promoted neuronal differentiation (Fig. 6), these results strongly suggest that different levels of endogenous *Dll1* regulate the neuronal differentiation of NPCs via cell-cell interaction, most likely through the lateral inhibitory system.

### DISCUSSION

A classical view of the role of the Notch pathway in *Drosophila* neuroectoderm is that it selects differentiating cells based on a 'competition' between equipotent progenitor cells through amplifying differences in Notch ligand levels (Heitzler and Simpson, 1991; Heitzler et al., 1996). By contrast, it has been proposed that the Notch pathway in the developing vertebrate CNS mainly contributes to the negative-feedback signal emanating from nascent neurons to suppress excess neuronal differentiation of NPCs. This view was supported by the observation that *Dll1* mRNA is found predominantly in nascent neurons/intermediate progenitors (neuron-committed progenitors), but is scarce in NPCs in the developing retina, spinal cord and telencephalon (Henrique et al., 1997; Campos et al., 2001; Minaki et al., 2005; Kawaguchi et al., 2008). A recent study of mind bomb 1, an activator of *Dll1*, also supports this view because of its expression in nascent neurons/intermediate progenitors (Yoon et al., 2008). However, another study has recently revealed low-level expression of *Dll1* mRNA in NPCs (Shimojo et al., 2008), although its role in NPC differentiation remained unclear. In this study, we present a series of

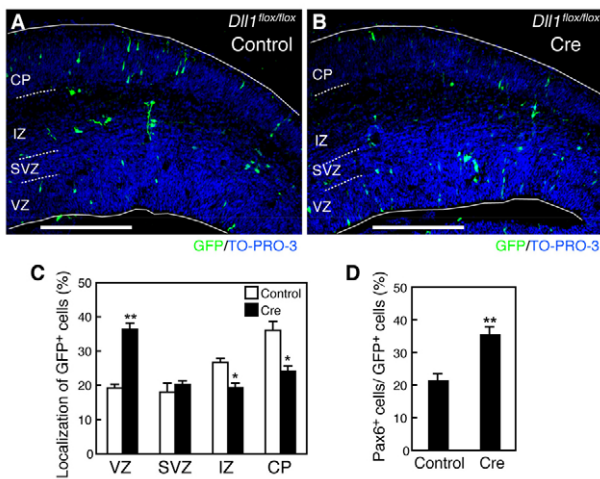


**Fig. 6. *Dll1* deletion in a large number of NPCs in the developing mouse telencephalon induces premature neurogenesis.** The *Dll1* gene was ablated in the majority of NPCs by crossing *Dll1* floxed with *nestin-Cre* mice. (**A-Z**) Immunoreactivity was compared between *Dll1*-intact mice (control, these mice were littermates of *Dll1* conditional KO mice of genotype *Dll1*<sup>flx/flx</sup> or *Dll1*<sup>flx/wt</sup> without Cre) (A, C, E, E', G, I, K, M, O, Q, S, U, W, Y) and *Dll1* conditional KO (cKO) mice (genotype was *Dll1*<sup>flx/flx</sup> with *nestin-Cre*) (B, D, F, F', H, J, L, N, P, R, T, V, X, Z). Brain sections from each mouse at E11.5 (A-H, E', F', K, L, O, P, S, T, W, X) or E13.5 (I, J, M, N, Q, R, U, V, Y, Z) were immunostained with anti-Dll1 (A-D), anti-active Notch1 (actN1) (E-F'), anti- $\beta$ III-tubulin (TuJ1) (G-J), anti-Sox2 (K-N), anti-BrdU (O-R), anti-phosphorylated histone H3 (pH3) (S-V) or anti-Tbr2 (W-Z). TO-PRO-3 was used for nuclear staining (E', F', O-Z). Dorsal side is up in all panels. NCX, neocortex; GE, ganglionic eminence. Scale bars: 100  $\mu$ m.

results supporting the notion that the Notch-Delta pathway contributes to the lateral signaling between NPCs that segregates equipotent mouse neocortical NPCs into two alternative fates: NPCs or neurons. Firstly, we found that Dll1 protein is expressed in a substantial population of NPCs that are positive for the undifferentiated cell markers nestin and Sox2 and for the proliferation marker PcnA in the developing mouse telencephalon. Secondly, we have shown that active Notch1 and Dll1 in the VZ of the developing telencephalon are expressed in different cells in a mutually exclusive manner, extending previous findings showing non-homogenous (salt-and-pepper) expression patterns of active Notch and Dll1 in the VZ of various nervous systems. This is consistent with the prediction of the lateral inhibition model: in the initially homogenous/equipotent cell population there might be small differences in the levels of ligands and receptor activity among cells, and these initial changes are amplified so that some cells

express high levels of ligands and low levels of receptor activity, whereas other cells express low levels of ligands and high levels of receptor activity. We do not yet know whether the levels of active Notch and Dll1 are initially homogenous in the NPCs in the earlier stages of development.

Thirdly, we found that manipulation of Dll1 expression levels in NPCs was sufficient for the alteration of cell fate. Ectopic overexpression of Dll1 in undifferentiated NPCs leads to their neuronal differentiation only when the NPC is surrounded by NPCs in which Dll1 is not misexpressed. This effect is observed in both in vitro neocortical cultures and in the developing neocortex. Conversely, conditional deletion of *Dll1* in undifferentiated NPCs results in the maintenance of the undifferentiated state only when this NPC is surrounded by NPCs with the intact *Dll1* gene. These results are striking considering the well-established anti-neurogenic effect of Dll1 and the Notch pathway, as revealed, for instance, by



**Fig. 7. *Dll1* deletion in a small number of NPCs in the developing mouse neocortex suppresses neuronal differentiation.** Retroviruses encoding GFP alone (pMX-SV40-GFP, control), or both GFP and Cre recombinase (pMX-Cre-SV40-GFP), were injected into the lateral ventricle of *Dll1*<sup>flox/flox</sup> mice at E12.5. Under these conditions, only a small proportion of cortical NPCs were infected. Brains were excised 3 days later and examined. (A,B) The location of the GFP-positive cells was determined by immunohistochemistry with anti-GFP and TO-PRO-3 (nuclear staining). Typical results are shown. (C) Quantification of the distribution of GFP-positive cells in the neocortex. Data are the mean $\pm$ s.e.m. of values from eight sections of each brain compared between littermates. Similar results were obtained from five independent littermates. (D) Brain sections were stained with anti-GFP and anti-Pax6, and the percentage of Pax6-positive cells among GFP-positive cells in the whole neocortex was determined. Data are the mean $\pm$ s.e.m. of values from six sections of each brain compared between littermates. Similar results were obtained from three independent brains. \* $P < 0.005$ , \*\* $P < 0.0005$ . VZ, ventricular zone; SVZ, subventricular zone; IZ, intermediate zone; CP, cortical plate. Scale bars: 200  $\mu$ m.

the loss-of-function phenotype (i.e. premature neurogenesis) observed in *Dll1*-deficient brain (Yun et al., 2002) (this study). These opposing effects of Dll1 on neurogenesis depending on the context can be explained by the lateral inhibitory signaling that takes place non-cell-autonomously, as the feedback signaling from surrounding cells ultimately reduces the Notch activity in the Dll1-expressing cells (Heitzler and Simpson, 1991; Dorsky et al., 1997; Henrique et al., 1997). The requirement of cell-cell interaction for the induction of neurogenesis by Dll1 is also supported by the observation that induction was not found in cells cultured at a low cell density. Together, these results strongly support the idea that the lateral inhibitory signals between NPCs contribute to the determination of cell fate in addition to the negative-feedback signals emanating from nascent neurons, and that different levels of Dll1 among NPCs play a crucial determining role.

Since the level of Dll1 is a key determinant of the NPC versus neuronal cell fate, the regulation of Dll1 expression should be critical in determining the place, timing and rate of neurogenesis. If so, what triggers the initial differences in Dll1 levels? Stochastic differences in Dll1 expression or intracellular distribution could result in the random choice of cell fate after rapid amplification as described above. In this case, oscillatory expression of *Dll1* in NPCs, which is likely to be due to the oscillatory expression of neurogenin 2 (which in turn is regulated by the oscillatory expression of *Hes1*), may play an important role (Shimojo et al., 2008). Alternatively,

some NPCs might be biased or predetermined to become neurons by additional mechanisms leading to *Dll1* induction. For instance, *Dll1* might be regulated by the Wnt-Lef1 pathway, as is the case in the developing somites (Galceran et al., 2004; Hofmann et al., 2004). Since the Wnt pathway promotes neurogenesis in the neocortex (Hirabayashi et al., 2004), it would be interesting to examine the possible involvement of Dll1 in this. We have previously reported that Stat3 induces *Dll1* and maintains NPCs in an undifferentiated state in a non-cell-autonomous manner (Yoshimatsu et al., 2006). Since Stat3 appears to be expressed homogeneously in most NPCs, it might not provide a 'biased' cue but rather confers 'competence' to NPCs to start lateral inhibition via expression of Dll1.

It is crucial to understand where Notch-Dll1 interaction takes place at a subcellular level because the amount of Dll1 present at the location of Notch interaction affects the process of lateral inhibition. We and others have observed that Dll1 is localized at the apical junctions (data not shown) (Mizuhara et al., 2005), but further studies will be needed to reveal the site(s) at which it activates the Notch receptor. If the Dll1-Notch interaction occurring between NPCs takes place at some particular site within the VZ, migration after neuronal commitment (or an increase in Dll1 levels) would remove Dll1-positive cells from that site, which would restart the selection process among NPCs. Therefore, the speed of migration of committed neurons might contribute to the rate of neurogenesis.

To our knowledge, this is the first report describing the conditional deletion of the Notch ligand *Dll1* in the mammalian CNS. Although several Notch ligands are expressed in the developing telencephalon, *Dll1* appears to play a major role among them, given that its conditional deletion reduces the immunoreactivity of active (cleaved) Notch1 and results in premature neurogenesis, recapitulating the phenotype of mice defective in Notch signaling (de la Pompa et al., 1997).

Although this is not a major focus of this paper, one of the prominent phenotypes of the CNS-specific *Dll1* gene deletion is the severe hemorrhage found throughout the entire brain as early as E11.5 (data not shown). Since an endothelium-specific deletion of *Notch1* using *Tie2-Cre* causes lethality at  $\sim$ E10.5, with vascular defects and hemorrhage (Limbourg et al., 2005), our result unexpectedly suggests that Dll1 expressed in nestin-positive cells (most likely cells in a neural lineage) might serve as a ligand for supporting this function of Notch in vascular development.

It has been proposed that in mammalian CNS development, the orientation of the mitotic spindle of NPCs (radial glial cells) regulates the fate of daughter cells. That is, horizontal or oblique cleavage is coupled to asymmetric division that produces one neuron and one NPC, whereas vertical cleavage is coupled to symmetric division that produces two NPCs (Chenn and McConnell, 1995; Götz and Huttnner, 2005). Indeed, depletion of *Ags3* (*Gpsm1*), which is responsible for maintaining correct spindle orientation, affects NPC fate (Sanada and Tsai, 2005). However, recent studies have shown that the vertical cleavage is predominant at all stages of the neurogenic phase (Stricker et al., 2006; Konno et al., 2008; Noctor et al., 2008), and that spindle orientation might be important for the position of the daughter cells but not for neuronal production rate, as revealed by manipulation of some of the components responsible for spindle orientation [LGN (*Gpsm2*), *Insc*] (Konno et al., 2008). This implies that mechanisms other than spindle orientation/asymmetric division also contribute to the determination of neuronal fate in NPCs. We propose that owing to cell-cell interactions that mediate Notch-Dll1 lateral inhibition, equipotent



NPCs can differentiate into neurons at a certain rate even without asymmetric division. This is based on the finding that differential Dll1 expression levels between NPCs are sufficient for determining the neuronal fate. It is also conceivable that asymmetric division (caused by an asymmetric inheritance of fate determinants, if any) can bias the outcome of lateral inhibition between adjacent cells. However, because Dll1-deficient NPCs surrounded by NPCs with an intact *Dll1* gene are less likely to undergo neurogenesis, the Notch-Dll1 lateral inhibitory mechanism might be dominant over fate determination by asymmetric division and could be prerequisite for neuronal differentiation.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/23/3849/DC1>

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