© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

RESEARCH ARTICLE

STEM CELLS AND REGENERATION

Cadherin-based adhesions in the apical endfoot are required for active Notch signaling to control neurogenesis in vertebrates

Jun Hatakeyama[1,*], Yoshio Wakamatsu[2], Akira Nagafuchi[3], Ryoichiro Kageyama[4,5], Ryuichi Shigemoto[6,7] and Kenji Shimamura[1,*]

ABSTRACT

The development of the vertebrate brain requires an exquisite balance between proliferation and differentiation of neural progenitors. Notch signaling plays a pivotal role in regulating this balance, yet the interaction between signaling and receiving cells remains poorly understood. We have found that numerous nascent neurons and/or intermediate neurogenic progenitors expressing the ligand of Notch retain apical endfeet transiently at the ventricular lumen that form adherens junctions (AJs) with the endfeet of progenitors. Forced detachment of the apical endfeet of those differentiating cells by disrupting AJs resulted in precocious neurogenesis that was preceded by the downregulation of Notch signaling. Both Notch1 and its ligand Dll1 are distributed around AJs in the apical endfeet, and these proteins physically interact with ZO-1, a constituent of the AJ. Furthermore, live imaging of a fluorescently tagged Notch1 demonstrated its trafficking from the apical endfoot to the nucleus upon cleavage. Our results identified the apical endfoot as the central site of active Notch signaling to securely prohibit inappropriate differentiation of neural progenitors.

KEY WORDS: Notch signaling, Adherens junction, Neurogenesis, Neural stem/progenitor cell, Apical endfoot, Cadherin, Mouse, Chick

INTRODUCTION

During vertebrate development, specific progenitors in each organ primordium repeatedly undergo cell division with profound proliferative and differentiation potentials to produce an adequate number of cellular constituents for a given organ. Because the differentiated cells responsible for the physiological functions of the organ typically do not proliferate, excess temporal differentiation of progenitors often results in hypoplastic organ formation (e.g. Hatakeyama et al., 2004; Self et al., 2006; Zhu et al., 2006). Thus, proper preservation and differentiation of the progenitor cells is crucial for the growth and formation of organs, and how it is achieved cellularly and molecularly has been a major issue in developmental biology, as well as in regenerative medicine.

[1]Department of Brain Morphogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan. [2]Division of Developmental Neuroscience, Tohoku University, Graduate School of Medicine, 2-1 Seiryo-Machi, Aoba-ku, Sendai 980-8575, Japan. [3]Department of Biology, Nara Medical University, 840 Shijo, Kashiwara, Nara 634-8521, Japan. [4]Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan. [5]World Premier International Research Initiative–Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Kyoto 606-8501, Japan. [6]Division of Cerebral Structure, National Institute for Physiological Sciences, 5-1 Higashiyama, Myodaiji, Okazaki 444-8787, Japan. [7]Institute of Science and Technology Austria, Am Campus 1, Klosterneuburg A-3400, Austria.

*Authors for correspondence (jhatakey@kumamoto-u.ac.jp; simamura@kumamoto-u.ac.jp)

Received 23 August 2013; Accepted 11 February 2014

Differentiating cells participate in the regulation of progenitor differentiation by a feedback control through Notch signaling. Notch signaling is a juxtamembrane cell interaction system used in many contexts of metazoan development, which play a central role in lateral inhibition, a mechanism that coordinates the balance of differentiated and undifferentiated states within a progenitor pool (Louvi and Artavanis-Tsakonas, 2006). Although much is known about how Notch signaling is activated, transmitted and modulated (Fortini, 2009; Kopan and Ilagan, 2009), little is known about the regulation of Notch-ligand interactions between juxtaposed cells during massive cell rearrangements that dynamically occur in morphogenesis. In particular, how the signaling is turned off is poorly understood beyond the transcriptional control of the ligands and the post-transcriptional modulations by the auxiliary proteins of the pathway (Fortini, 2009). The termination of Notch signaling is crucial in order that progenitors are allowed to differentiate in the next round of cell division.

Cell-cell junctions act as physical connections between cells, and also play a role in juxtacrine communication between epithelial cells for controlling cell growth and differentiation (Fuchs et al., 2004). In *Drosophila*, Notch and its ligands are predominantly localized to the apical junction of epithelial cells (Fehon et al., 1991; Kooh et al., 1993). Notch forms a complex with E-cadherin, a transmembrane component of the adherens junction (AJ), depletion of which downregulates Notch signaling, indicating a functional link between Notch signaling and cadherin-mediated AJ in *Drosophila* (Sasaki et al., 2007). However, the details of this relationship remain elusive for the vertebrate central nervous system (CNS).

Neuroepithelial cells and radial glia residing in the ventricular zone (VZ) of the neural tube function as the stem/progenitor cells of the embryonic CNS. These cells are highly polarized epithelial cells connected through apically localized junctional complexes, including AJs and tight junctions (TJs). These pseudostratified epithelial cells span the ventricular wall with thin processes extending both apically and basally from the soma, and undergo interkinetic nuclear migration as the cell cycle progresses (Fujita, 2003; Rakic, 2007). In addition, these cells also serve as architectural scaffolds that physically support tissue integrity and morphogenesis (Hatakeyama et al., 2004). Neural progenitor cells (NPC) undergo extensive cell division to support the proliferation, self-renewal and production of neurons. These cells persist long enough to produce an adequate number of neurons with divergent properties. Thus, a quantitative balance between the proliferation and differentiation of NPCs must be exquisitely coordinated both spatially and temporally for the proper development of the CNS.

In the present study, we investigated the regulation of neurogenesis through interactions between differentiating cells and undifferentiated progenitors in the early phase of neurogenesis in the vertebrate CNS, when the preservation of NPC pools is crucial

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

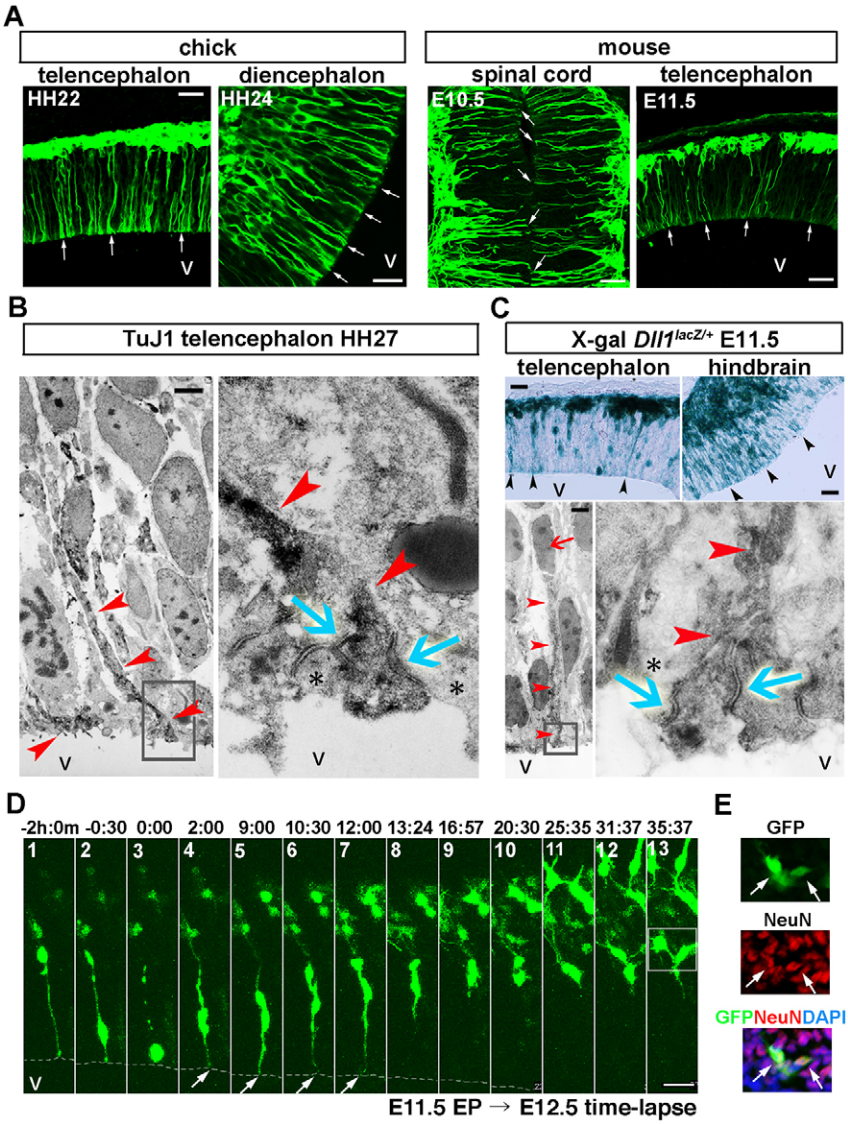

Fig. 1. Nascent neurons retain apical endfeet that form AJs with progenitors. (A) Sections of various CNS regions of chick and mouse embryos immunostained with TuJ1. Many TuJ1-positive cells retained apically elongated processes that reached the ventricular lumen (arrows). (B) Immuno-TEM images of a specimen from HH27 chick telencephalon stained with TuJ1. The darkly stained process reached the ventricular lumen (red arrowheads), and its endfoot retained AJs (blue arrows) with the adjacent progenitors (asterisk). The boxed area on the left is magnified on the right. (C) Light microscopy and TEM images of X-gal staining of the cortex and hindbrain of E11.5 *Dll1*$^{lacZ/+}$ mice. X-gal-stained processes from the mantle zone reached the ventricular lumen (black arrowheads). A darkly stained process (red arrowheads) extending from a Dll1-positive cell (red arrow) retained AJs (blue arrows) with the neighboring progenitors (asterisk). The boxed areas on the left are magnified on the right. (D) A representative case of the time-lapse images of differentiating neurons in E12.5 mouse cortical slice. The cells were electroporated with GFP at E11.5. Panel 3 (*t*=0) shows the cells immediately after mitosis. The apical contact to the ventricular lumen (arrows) was retained for up to 12 h (panel 7) after cell division. The endfoot detached from the apical lumen indicated by broken lines (panel 8). (E) The boxed area in panel 13 stained for GFP and NeuN, and with DAPI after the time-lapse observation. Scale bars: 20 μm in A,C,D; 2 μm in B,C (TEM). V, ventricle.

for subsequent robust neuron production. We observed that numerous nascent neurons retain apical contacts that form AJs with neighboring progenitors for a limited period of time. Our *in vitro* and *in vivo* experiments strongly suggest that AJ facilitates Notch signaling to maintain the undifferentiated state of NPCs. Based on these results, we hypothesize that the apical endfeet of differentiating cells with AJs could serve as a plug to shut down Notch signaling in NPCs and control the timing and pace of neuron production, thereby regulating the histogenesis of brain tissues.

RESULTS

Nascent neurons transiently retain the apical endfoot, which forms AJs with progenitor cells

Differentiating neurons typically migrate from the VZ soon after the final division of the mother cell (Rakic, 2007). Unexpectedly, the careful examination of neural tubes immunostained with the neuronal marker TuJ1 revealed numerous processes extending apically from the somata (Fig. 1A). We observed this phenomenon regardless of the CNS subdivision or vertebrate species (chick or mouse) examined, although there were clear differences in the densities of the processes depending on the developmental stage and region of the CNS (supplementary material Fig. S1A,B).

Many of these TuJ1-positive apical processes reached the ventricular lumen (Fig. 1A, arrows), suggesting that these cells retained apical cell junctions with progenitor cells. Indeed, transmission electron microscopy (TEM) revealed that the darkly stained TuJ1-positive processes form electron-dense junctional structures with the apical processes of the neighboring progenitors that are negative for TuJ1 (Fig. 1B). A similar observation was made using the tissues derived from Delta-like 1 (*Dll1*)$^{lacZ/+}$ mice (Hrabe de Angelis et al., 1997). *Dll1* is transiently expressed in early differentiating neurons (Henrique et al., 1995), and many X-gal-stained *Dll1*-expressing cells extended apical processes into the VZ (Fig. 1C). TEM also demonstrated that the X-gal-stained processes formed the junctional structures with the apical endfeet of the unstained progenitors (Fig. 1C). The neuronal identity of these cells and processes was further confirmed by immunostaining for MAP2 (supplementary material Fig. S1C).

To reconcile these findings with the generally accepted notion that neurons lose apical contacts upon differentiation, we analyzed the dynamics of apical endfeet in cultured slices of the embryonic day 12.5 (E12.5) mouse cerebral cortex using time-lapse microscopy (Fig. 1D). Whereas many cells retained apical contacts and exhibited typical interkinetic nuclear migration (Fujita, 2003; Rakic, 2007),

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

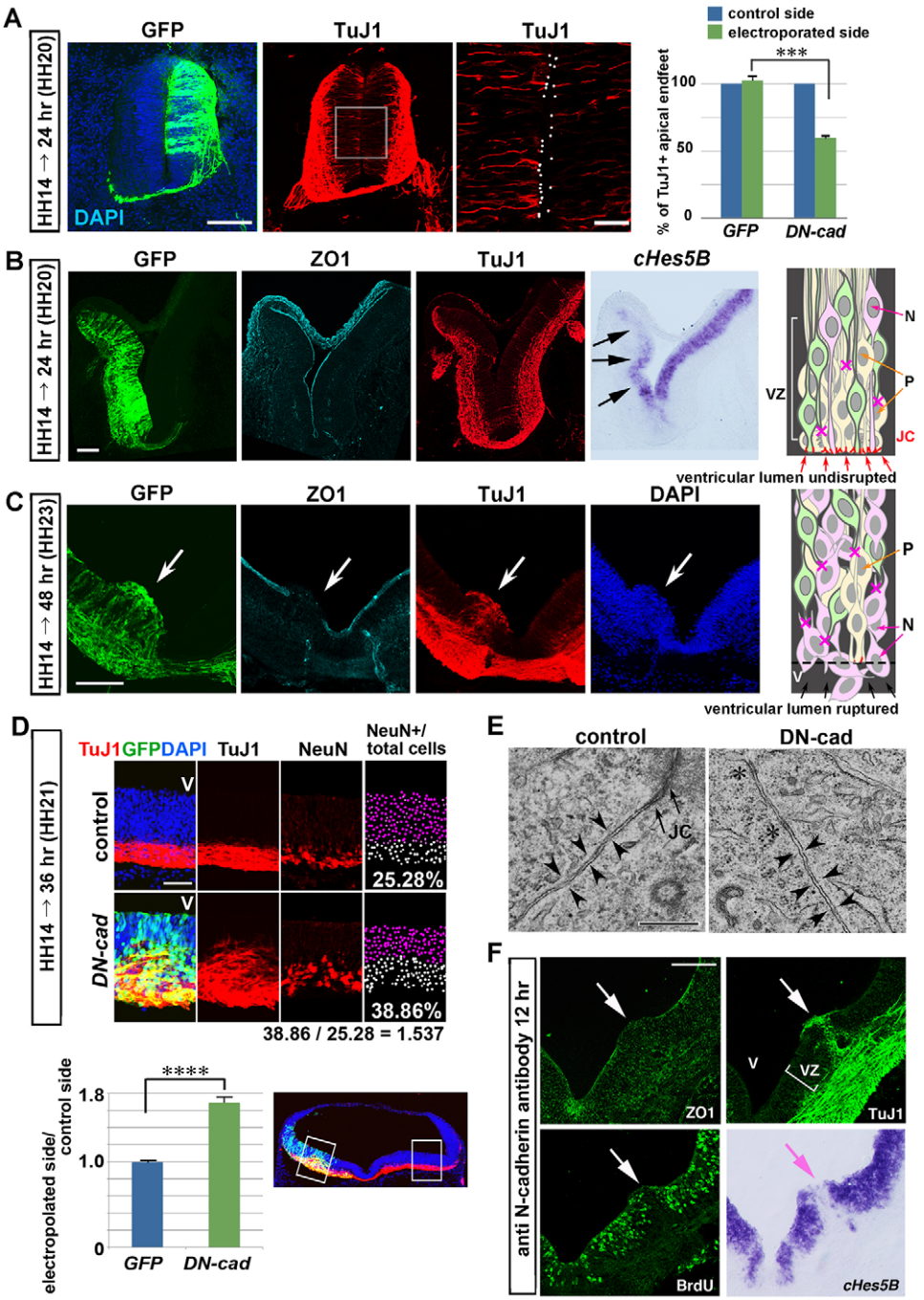

Fig. 2. Impact of junction disruption on neurogenesis in the chick CNS. (A) HH20 chick spinal cord electroporated with *DN-cad+GFP* at HH14 was stained with anti-GFP, TuJ1 and DAPI. The boxed area is magnified and the TuJ1-positive apical endfeet that reached the ventricular lumen (white dots) were quantified. The percentages of the electroporated/control (unoperated) are represented: *GFP*, 101.97±4.30%, *n*=4; *GFP+DN-cad*, 59.67±2.83%, *n*=7; ***P<0.001. The total numbers of processes analyzed were 195 (GFP)/193 (control) and 132 (DN-cad)/225 (control). Error bars indicate s.e.m. (B,C) HH20 (B) and HH23 (C) chick posterior hindbrain electroporated with *DN-cad+GFP* at HH14 were stained for GFP, βIII-tubulin, ZO-1 and chick *Hes5B*. Many ectopic neurons were detectable in the VZ at 48 h after electroporation (white arrows in C). The downregulation of chick *Hes5B* expression preceded ectopic TuJ1 immunoreactivity and the robust abolishment of ZO-1 localization along the ventricular lining (black arrows in B). These phenotypes are schematically depicted in the right columns, in which the green cells represent *DN-cad*-electroporated cells, showing the detachment of the endfeet (B) and the rupture of ZO-1-localized ventricular lining (C). (D) HH21 hindbrain unilaterally electroporated with *GFP* and *DN-cad* were stained for β-tubulin, NeuN, GFP and nuclei. Relative percentages of NeuN-positive cells over total cells were calculated between the equivalent areas of the electroporated and contralateral (unoperated) side of the neural tube (graph): *GFP*, 0.993±0.021; *DN-cad*, 1.689±0.069; *n*=8; ****P<0.0001. An example is shown on the right of the graph. (E) TEM images of the HH23 caudal hindbrain electroporated with *DN-cad* at HH14. The electron-dense junctional complexes (JC, arrows) are absent from cells expressing DN-cad, but some punctate adhesion structures (asterisk) remained. The distance between the apposed plasma membrane is similar with or without DN-cad (arrowheads). (F) HH22 chick posterior hindbrain to which the function-blocking anti-N-cad monoclonal antibody was injected in the ventricle at HH21 stained for ZO-1, β-tubulin, BrdU and chick *Hes5B*. BrdU was injected 3 h before fixation to label proliferating cells. Scale bars: 100 μm in A,B; 50 μm in C,D,F; 500 nm in E. JC, junctional complex; N, neuron; P, progenitor; V, ventricle; VZ, ventricular zone.

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

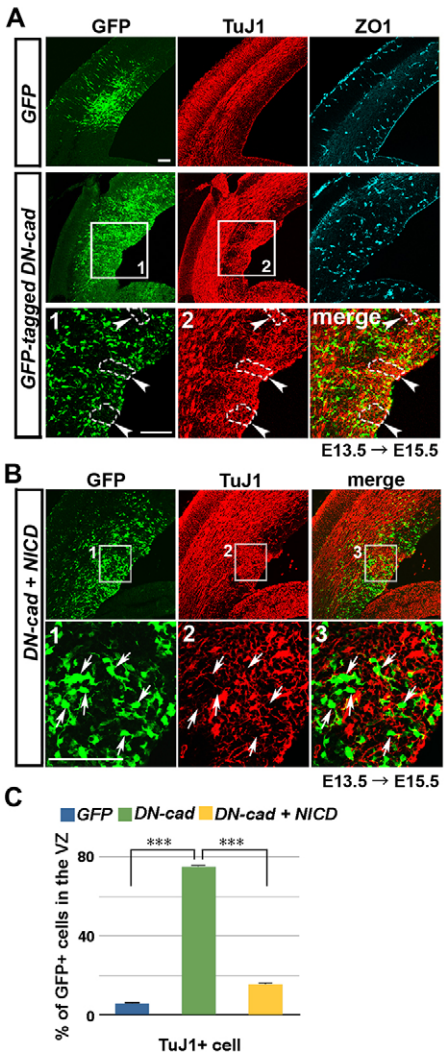

Fig. 3. Aberrant neurogenesis upon junction disruption and the restoration by Notch activation in the developing mouse cortex. (A,B) E15.5 mouse cerebral cortex electroporated at E13.5 with *GFP*, *GFP*-tagged *DN-cad* and *DN-cad+NICD+GFP* were co-immunostained with anti-GFP and TuJ1. The numbered boxed areas are enlarged in the lower panels. GFP-negative cells stained with TuJ1 were ectopically present in the VZ (arrowheads in A, encircled with dotted lines). The cells electroporated with *DN-cad+NICD*, shown in green, are no longer TuJ1 positive (B, arrows). (C) Quantification of the TuJ1+ cells in the VZ: *GFP*, 5.84±0.58%, 593 cells, *n*=10; *DN-cad-GFP*, 75.09±1.61%, 1216 cells, *n*=10; *DN-cad+NICD+GFP*, 15.53±1.70%, 1075 cells, *n*=10; ***P<0.001. Scale bars: 50 μm.

some cells did not show this behavior and migrated further into the subventricular zone (SVZ); the neuronal identity of these cells was confirmed by expression of NeuN (Fig. 1E). These neurons maintained apical contacts after mitosis, whereas their somata were translocating basally (average retention time at E12.5: 8.60±0.89 h; *n*=10). Eventually, the apical endfeet detached from the ventricular lumen and the processes were withdrawn (Fig. 1D, panel 8).

Impact of junction disruption on neurogenesis

As communication between differentiating neurons and progenitors is important for neural development, the above findings pose the question of whether the apical endfeet of the nascent neurons play any role in the neuron-progenitor communication. We assessed how neurogenesis was affected when the apical endfeet were forced to detach from the ventricular lumen through the disruption of AJs. A dominant-negative form of cadherin, a major extracellular constituent of AJs that lacks the extracellular domain (DN-cad), was shown to disrupt AJs from within the cell (Fujimori and Takeichi, 1993). We equally tested the dominant-negative version of both E-cadherin (E-cad) and N-cad, and equivalent results were obtained (data not shown), consistent with the highly conserved cytoplasmic domain of these molecules. Thus, we use the term DN-cad hereafter for simplicity. *DN-cad* was electroporated into the neural tube of Hamburger-Hamilton stage 14 (HH14) chick embryos and the disruption of apical junctions was examined after staining for ZO-1, a protein typically localized to AJs and TJs (Sugrue and Zieske, 1997; supplementary material Fig. S5C). As expected, the number of the TuJ1-positive apical processes touching the ventricular lumen was reduced upon the expression of DN-cad at 24 h post-electroporation (Fig. 2A). At this time point, the gross localization of ZO-1 along the ventricular lining did not appear to be markedly disrupted, but the expression of chick *Hes5B*, a marker for undifferentiated neural progenitors, was severely downregulated on the electroporated side (Fig. 2B; *n*=15). At 48 h post-electroporation, several TuJ1-positive cells were abnormally present in the VZ at sites where the ventricular localization of ZO-1 was abolished (Fig. 2C; *n*=11). Acceleration of neurogenesis was evident at 36 h post-electroporation such that the number of NeuN-positive cells was significantly increased in the *DN-cad*-electroporated areas (Fig. 2D; *n*=8). To verify the structural impact of DN-cad expression, we analyzed these same specimens using TEM and observed that DN-cad expression abolished AJs without changing the distance between the apposed plasma membranes, despite the distribution of cadherin along the lateral side of the cell (Fig. 2E). Consistently, injection of a function-blocking monoclonal antibody against N-cad into the ventricle of HH21 embryo resulted in disruption of AJs and aberrant neuronal differentiation at HH22 (Fig. 2F). Aberrant neurogenesis was also detected in the mouse after electroporation of *DN-cad* into the telencephalon at E13.5. A number of TuJ1-positive cells were abnormally present in the VZ at 48 h post-electroporation at sites where the apical junctions were disrupted (Fig. 3A; *GFP*, *n*=12; *GFP+DN-cad*, *n*=10; *GFP-tagged DN-cad*, *n*=7). Together, these results suggest that the cadherin-based cell junction is required for the maintenance of the undifferentiated state of NPCs in both chicks and mice.

Given that the downregulation of chick *Hes5B* expression, which is a read out of Notch signaling activity (Ohtsuka et al., 1999), precedes the precocious differentiation of neurons, we hypothesized that the impact of junction disruption on neurogenesis could be attributed to the downregulation of Notch signaling. Accordingly, we examined whether this phenotype is restored by simply activating Notch signaling. The intracellular domain of Notch1 (NICD), which constitutively activates the pathway (Fortini, 2009; Kopan and Ilagan, 2009), was co-introduced with DN-cad. As a result, the large majority of the GFP-positive co-transfectants no longer expressed the TuJ1 antigen (class III β-tubulin) (Fig. 3B,C), consistent with the idea that the neurogenic influence of the AJs is mediated by Notch signaling. However, DN-cad expression did not affect the canonical Wnt/β-catenin signaling, which was downregulated upon the depletion of N-cad in NPCs (Zhang et al., 2010), prior to the downregulation of Notch signaling (supplementary material Fig. S2).

We noted that DN-cad influenced neurogenesis in both a cell-autonomous and non-cell-autonomous manner, as GFP-negative/TuJ1-positive cells were present in abnormal locations (Fig. 3A, arrowheads). A GFP-tagged form of DN-cad was used for the strict assessment of cell-autonomy. DN-cad probably acts on the AJs

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

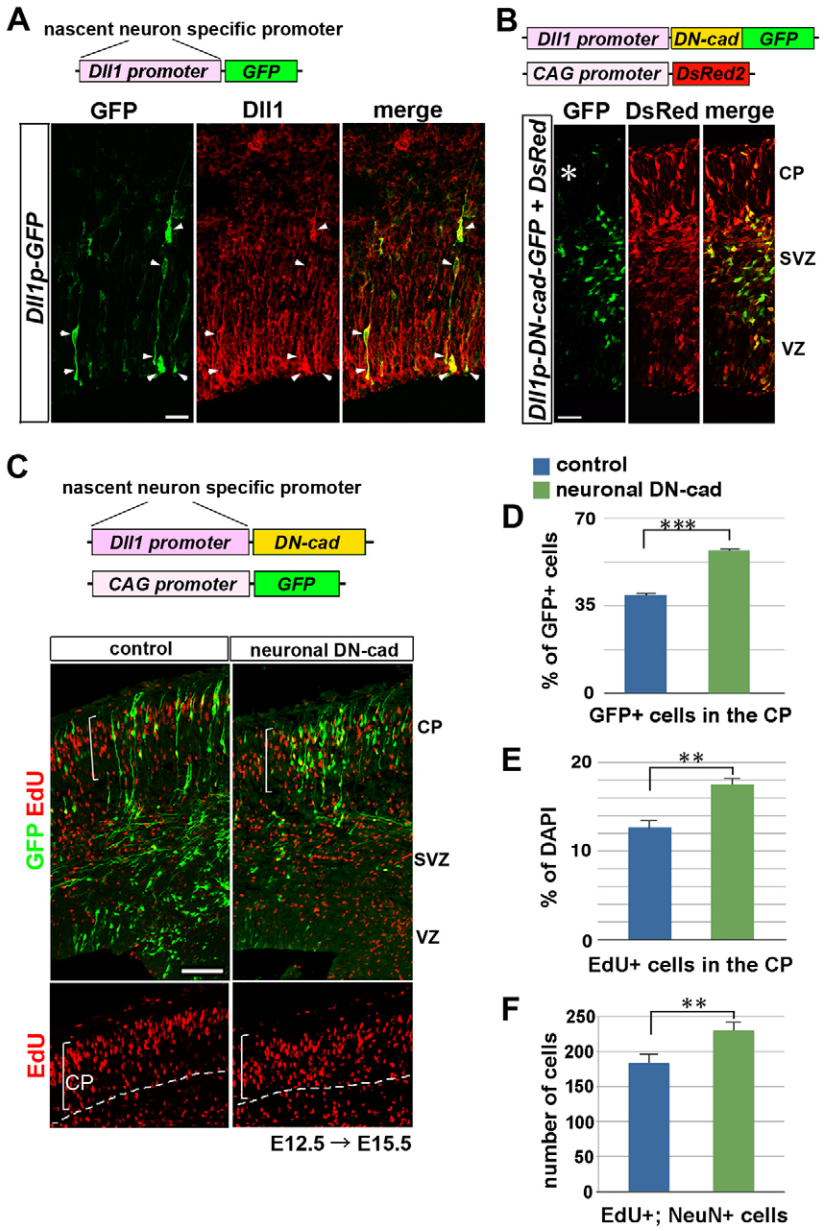

Fig. 4. Neuronal DN-cad expression is sufficient for precocious differentiation of NPCs. (A) E13.5 cortex carrying the *Dll1 promoter-GFP* (*Dll1p-GFP*) plasmid after electroporation at E12.5 was immunostained for GFP and Dll1. Dll1 protein is expressed by the GFP-positive cells (arrowheads). (B) E14.5 cortex electroporated with *Dll1p-DN-cad-GFP* and *pCAG-DsRed2* at E12.5. GFP expression was diminished as the cells migrated into the CP (asterisk). (C) E15.5 cortices electroporated at E12.5 with *Dll1p-GFP+pCAG-GFP* (control) or *Dll1p-DN-cad +pCAG-GFP* (neuronal DN-cad) were stained for GFP and EdU. EdU was administered at E12.75 for the cell cycle exit analysis. (D-F) Quantification of the results for GFP+ (D) and EdU+ cells (E) in the CP, and total EdU+; NeuN+ cells (F): (D) control, 39.32±2.78%, *n*=12; neuronal DN-cad, 57.18±1.77%, *n*=21; ***P<0.001; (E) control, 12.66±1.029%, *n*=8; neuronal DN-cad, 17.52±0.771, *n*=10; **P<0.01; (F) control, 183.5±8.26, *n*=8; neuronal DN-cad, 230.0±12.27, *n*=9; **P<0.01. EdU- and NeuN-double positive cells in a 250 μm wide column of the whole cerebral wall were counted (F). Scale bars: 50 μm. CP, cortical plate; SVZ, subventricular zone; VZ, ventricular zone.

primarily cell-autonomously, but eventually acts non-cell-autonomously, as the normal cadherin on the opposed plasma membrane fails to interact with the non-functional cadherin to maintain the AJ assemblies (Fujimori and Takeichi, 1993). Because NICD cell-autonomously functions to prevent neurogenesis, the non-cell-autonomous action of DN-cad was obvious in a rescue experiment in that many GFP-negative/TuJ1-positive cells remained in the VZ (Fig. 3B).

It was not clear, however, whether the above neurogenic phenotypes are caused by the disruption of AJs between the differentiating cells and NPCs or the NPCs themselves. To address this issue, DN-cad was expressed preferentially in the differentiating cells to perturb AJs between the differentiating cells and NPCs selectively. A *Dll1* promoter with activity in early differentiating cells was used (Castro et al., 2006; supplementary material Fig. S3A); the activity of this promoter recapitulated the endogenous expression of Dll1 (Fig. 4A) and was downregulated in the cells that have entered the cortical plate (CP) (Fig. 4B, supplementary material Fig. S3A). The promoter activity was also found in Tbr2-positive presumptive intermediate neurogenic progenitors (INPs) (supplementary material Fig. S3B). When DN-cad was expressed under control of this promoter (*Dll1p-DN-cad*), the significant acceleration of neurogenesis was again observed; the number of cells labeled with GFP and exiting the cell cycle in the CP (EdU+) was increased (Fig. 4C-E). Importantly, the total number of EdU/NeuN double-positive cells was increased, arguing against possibilities other than acceleration of neurogenesis being responsible, such as abnormal neuronal migration (Fig. 4F). However, the gross integrity of the VZ was preserved without the ectopically located neurons, unlike in the case of the ubiquitous promoter. These results further support the idea that AJs are necessary for the endfeet of differentiating cells to maintain progenitors in the undifferentiated state.

Subcellular distribution and dynamics of Notch1 protein in NPCs

To gain an insight into how AJ is involved in Notch signaling, we first analyzed the distribution of Notch and its ligands in the developing

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

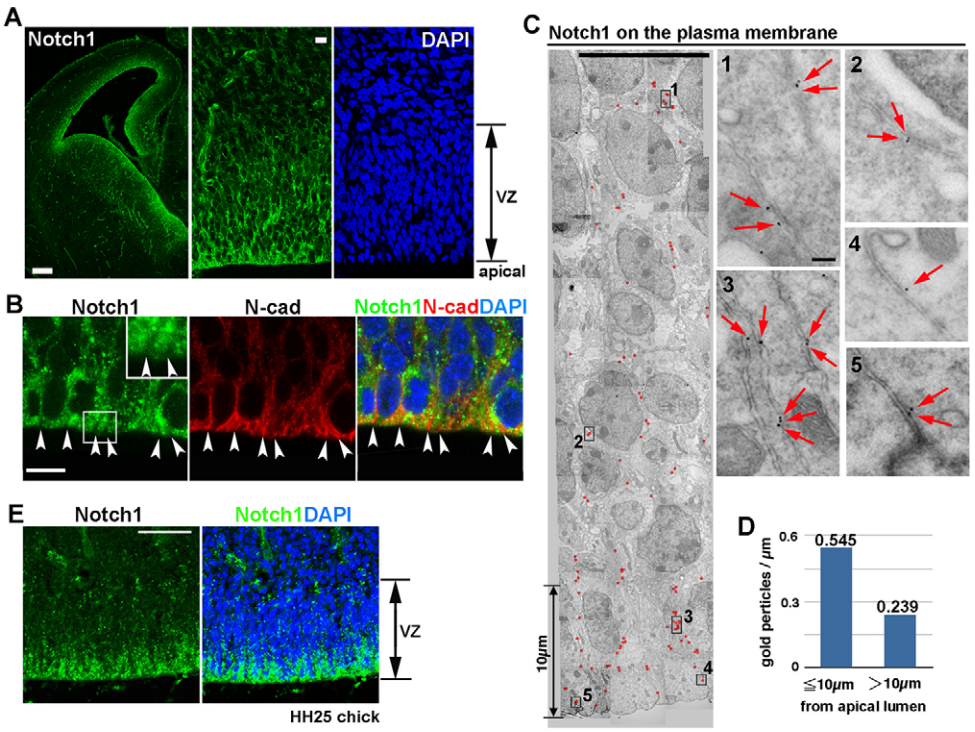

Fig. 5. Distribution of Notch1 protein in the developing mouse cortex. (A,B) E13.5 mouse cerebral cortex immunostained for Notch1 and N-cad. The cell nuclei were stained with DAPI. Notch1 was predominantly distributed in the apical region of the VZ. Notch1 exhibited distributions similar to N-cad (arrows), although Notch1 was decreased at the AJs, where N-cad was highly accumulated (arrowheads). The boxed area is magnified in the inset. (C) Immuno-TEM images of the E13.5 mouse cortex, showing the distribution of Notch1 labeled with gold particles on the plasma membrane (red dots and arrows). The numbered boxed areas are magnified on the right. (D) Quantification of the distribution in which the gold particles on the cell surface were counted with respect to the distance from the ventricular lumen along the plasma membrane (within 10 μm from the lumen, 0.545 particles/μm of membrane; beyond this range, 0.239 particles/μm of membrane). Quantification was carried out through five different fields. (E) HH25 chick telencephalon stained for Notch1. An apically enriched distribution of Notch1 within the VZ was observed. Scale bars: 50 μm in A, E; 10 μm in B,C; 100 nm in C1-5.

brain. Notch1 protein was distributed in an apically biased manner in the VZ both in the mouse and chick (Fig. 5A,E). The distribution of these proteins included AJs marked by accumulation of N-cad, although Notch1 appeared somewhat decreased at AJ itself (Fig. 5B, arrowheads). The apically biased distribution of Notch1 on the plasma membrane was further revealed by immuno-TEM analyses (Fig. 5C,D). Distribution of Dll1 and Jag1, which are ligands of Notch, and of presenilin 1, which cleaves Notch, were analyzed by immunostaining (Fig. 6). Those proteins also exhibited an apically biased distribution in the VZ (Fig. 6A,D,F), and immuno-TEM analyses for Dll1 and Jag1 revealed those on the plasma membrane were indeed present around AJs of the apical endfeet (Fig. 6C,E). The presenilin 1 localization in the VZ is consistent with the notion that presenilin 1 forms complexes with the cadherin/catenin cell-adhesion system (Georgakopoulos et al., 1999). The specificities of the antibodies against Notch1 and Dll1 were confirmed using *Notch1* conditional KO (Yang et al., 2004) and *Dll1* KO mice, respectively (supplementary material Fig. S4).

A more-detailed analysis was conducted using an *in vitro* culture in which NPCs often form clusters in a rosette-like arrangement (Fig. 7A, supplementary material Fig. S5A,B); ZO-1 and β-catenin were localized in a ring at the center of the rosettes (Fig. 7B, supplementary material Fig. S5C), indicating that the rosette center corresponds to the ventricular lumen of the neural tube. TuJ1-positive neurons resided on top of the NPC sheet and the processes of these neurons occasionally reached the center of the rosette (supplementary material Fig. S5A, arrows). Notch1 protein was enriched predominantly on the processes extending from the soma to the centers of the rosettes (Fig. 7B, supplementary material Fig. S5B) and, to a lesser extent, at the AJ itself (Fig. 7B, encircled area), as observed *in vivo* (Fig. 5B). To detect Dll1 exposed on the cell surface, live cells were stained using an antibody raised against the extracellular domain of Dll1 (Wakamatsu et al., 2000). Cell-surface Dll1 was frequently detected at the center of the rosettes overlapping with ZO-1 but rarely at other domains (Fig. 7D), indicating that a ligand capable of interacting with Notch extracellularly is present at or near the junction. Based on the apicolateral distribution of Notch1 and Dll1, we examined whether these proteins physically interact with the AJ complex. Immunoprecipitation experiments for E11.5 mouse brain lysate using anti-ZO-1 antibody revealed that both Notch1 and Dll1 form complexes with ZO-1 (Fig. 7E,F). N-cad and β-catenin were also detected in the precipitates (Fig. 7E,F). Although it is not clear whether those proteins form a single molecular complex or they independently interact with ZO-1, this result provide a molecular explanation for the presence of Notch and Dll1 at AJs.

To identify the site of active Notch signaling, the Notch1 protein dynamics in NPCs was analyzed. First, when Notch cleavage was suppressed by the γ-secretase inhibitor DAPT, Notch1 protein accumulated at the junction such that staining of Notch1 overlapped with that of ZO-1 (Fig. 7B,C, zone 1). The intensity of the anti-Notch1 immunoreactivity in other subcellular domains remained comparable with that of the control (Fig. 7C, zone 2). Although γ-secretase is involved in N-cad processing (Marambaud et al., 2003), we observed no significant alteration in the distribution of N-cad, ZO-1 or β-catenin through this short (4 h) treatment with DAPT (Fig. 7B, supplementary material Fig. S5D). The accumulation of Notch1 protein at the apical junction under DAPT treatment suggests that Notch1 processing takes place at or near the junctions under normal conditions. Similar results were obtained from experiments using cortical slice cultures (supplementary material Fig. S6). Next, we examined whether Notch processed at the apical endfeet is indeed transported to the nucleus for signaling. A photoconvertible monomeric fluorescent protein, Dendra2 (Chudakov et al., 2007), was fused to the Notch1 intracellular domain (Fig. 8A). A previous study in *Drosophila* has shown that such tagged molecules are fully functional and are successfully used to monitor the intracellular trafficking of Notch protein (Couturier et al., 2012). Indeed, this fusion construct rescued the neurogenic phenotypes of *Notch1* deficiency (supplementary material Fig. S7). Dendra2-tagged Notch1 was expressed in NPCs and photoconverted in small subcellular amounts by irradiating a 405 nm laser. The behavior of the photoconverted protein (red fluorescent) was tracked in culture

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

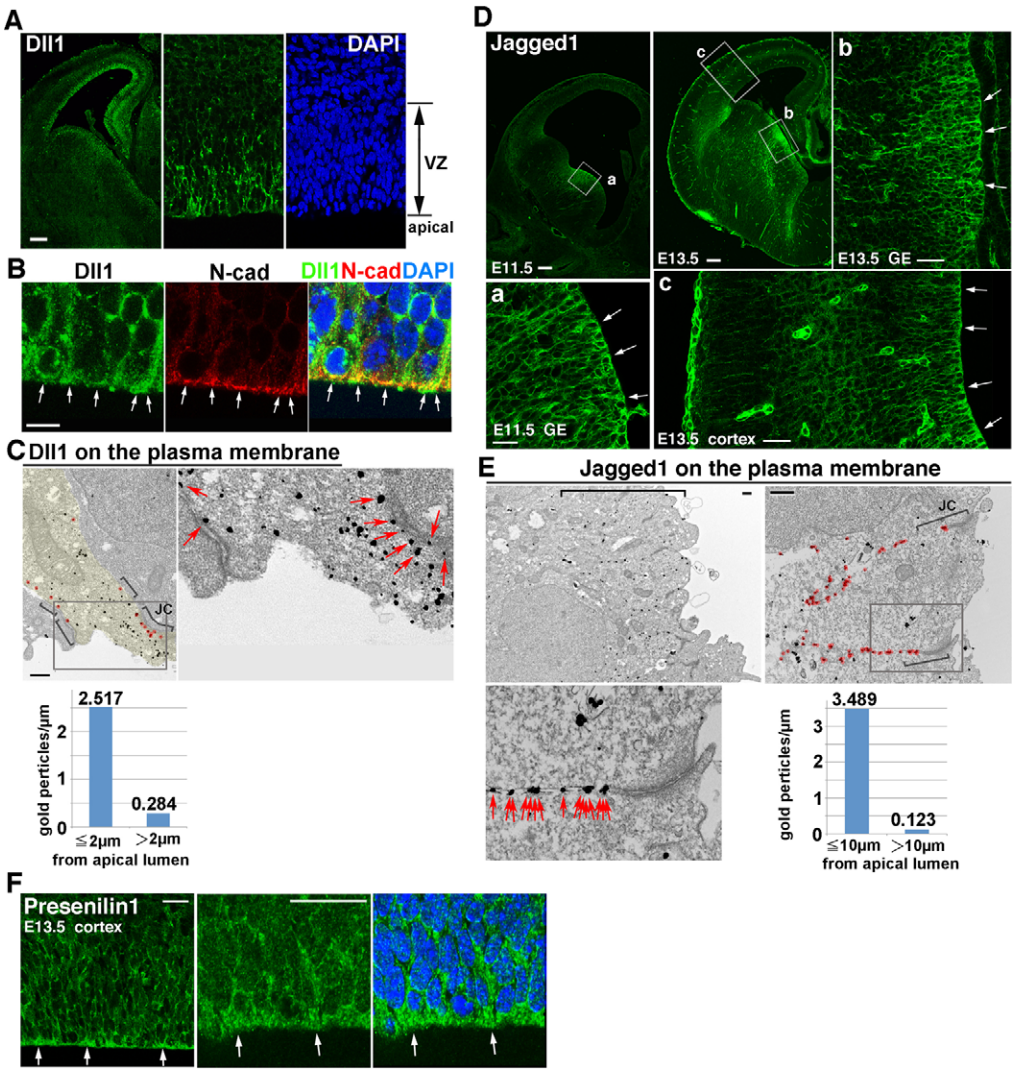

Fig. 6. Distribution of Notch signaling constituents in the embryonic telencephalon. (A,B,D,F) E13.5 mouse cerebral cortex immunostained for Dll1, Jag1, N-cad and presenilin 1. The cell nuclei were stained with DAPI. In D, boxed areas (a-c) are magnified. Dll1 was colocalized with N-cad at AJs in the apical endfeet (arrows in B). Jag1 and presenilin 1 were distributed at the apical side of the VZ (arrows in D,F). (C,E) Immuno-TEM images of the apical region of E13.5 cortices immunogold stained for Dll1 (C) and Jag1 (E). Dll1-expressing cells distinguished by the cytoplasmic signals are shaded in thin yellow (C). Signals on the plasma membrane were indicated as red dots. Boxed areas are enlarged. The numbers of gold particles on the cell surface (red arrows) per μm were counted with respect to the distance from the ventricular lumen along the plasma membrane. Quantification was carried out through seven and ten different fields for Dll1 and Jag1, respectively. Scale bars: 50 μm in A,D,F; 10 μm in B; 300 nm in C,E. GE, ganglionic eminence; JC, junctional complex.

(Fig. 8B). When Notch1-Dendra2 was photoconverted at the apical tip of the processes, the intensity of red fluorescence in the cell body rapidly increased (Fig. 8B, middle panel). As the nucleus of an NPC is large enough to occupy most of the soma mass, the signals detected in the soma at these confocal planes were likely in the nucleus. This rapid accumulation in the nucleus was not observed when Notch1-Dendra2 was photoconverted in other parts of the cell (Fig. 8B, left panel). Moreover, DAPT treatment markedly inhibited the nuclear accumulation of Notch1-Dendra2 photoconverted at the apical endfeet, but induced the accumulation of unconverted Notch1-Dendra2 at the apical endfeet (Fig. 8B, right panel), consistent with the results shown in Fig. 7.

Taken together, these results strongly support the idea that the apical endfeet provide a microenvironment in which Notch interacts with its ligands, and is cleaved and transported to the nucleus rapidly through the apical process of NPCs in order to prevent differentiation.

DISCUSSION

Here, we provide evidence that the numerous nascent neurons retain the apical endfeet transiently to communicate with NPCs for the regulation of neurogenesis through Notch signaling. For physical disengagement of the apical endfeet, we prefer expressing DN-cad to depleting cadherins or other junction constituents, because it is the only mean currently available to disrupt AJs with minimal effects, if any, directly on other signaling systems such as Wnt or Hedgehog signaling (see Fig. 2E, supplementary material Fig. S2; data not shown for Hh signaling; Lien et al., 2006; Zhang et al., 2010).

Because NPCs undergo extensive cell division and interkinetic nuclear migration, the distance between the apposed plasma membranes of neighboring cells changes dynamically, except at the AJs, which are maintained even through mitosis. Therefore, it is reasonable to use apical endfeet as stable contact sites for signaling between adjacent cells. Moreover, a molecular mechanism that restricts Notch signaling to AJs in zebrafish neuroepithelial cells was reported (Ohata et al., 2011). AJs may assure the efficiency of the Notch-ligand interaction by increasing the local concentrations of these transmembrane proteins possibly through the interaction with ZO-1. Alternatively, AJs may hold the apical endfeet of neurons long enough for the progenitors to receive Notch signals during the cell cycle to remain undifferentiated. The significance of the temporal and quantitative Notch signaling dose has been reported in zebrafish retinal development (Del Bene et al., 2008).

Possible implications of focused Notch signaling facilitated by AJs are depicted in Fig. 9. First, this system could temporally control Notch signaling; the detachment of the neuronal apical endfeet from the ventricular lumen would rapidly shut down Notch signaling, allowing the surrounding progenitors to differentiate (Fig. 9A). Second, the

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

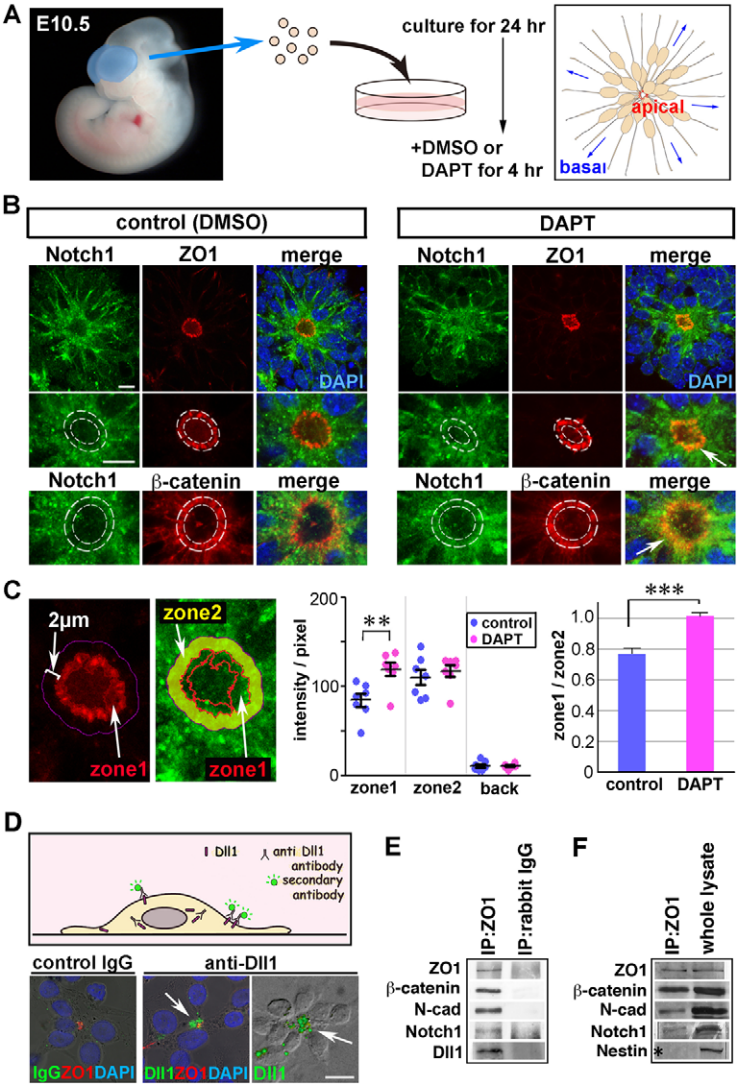

Fig. 7. Notch1 and Dll1 distribution in cultured NPCs and their interaction with the junctional proteins. (A) Schematic of the experiment and structure of an NPC rosette. (B) Rosettes of mouse NPCs cultured with DMSO (control) or DAPT, were stained for Notch1, ZO-1, β-catenin and with DAPI. The central area of the rosettes is enlarged in the lower panels; the right columns display the merged images (Notch1, green; ZO-1 or β-catenin, red; nucleus, blue). Notch1 was diminished at the apical junctions in the control and accumulated at the junctions in the presence of DAPT. The zone of the apical junction was encircled by dotted lines. (C) Quantification of the results. Zone 1 represents the region marked by ZO-1 localization, and zone 2 is the adjacent complementary domain located at 2 μm from zone 1. The average fluorescence intensities in zones 1 and 2 are represented: control, 84.26±7.31 (zone 1), 110.1±8.58 (zone 2), 0.767±0.045 (background), n=7; DAPT, 119.0±7.43 (zone 1), 117.0±6.42 (zone 2), 1.014±0.024 (background), n=7; $^{**}P$<0.01 (middle panel); the ratio of zone 1/zone 2 is shown in a histogram: control, 0.767±0.045; DAPT, 1.014±0.024; $^{***}P$<0.001 (right panel). Error bars indicate s.e.m. (D) Immunodetection of Dll1 exposed to the surface of NPCs derived from the HH25 chick telencephalon. The cell-surface Dll1 is concentrated (arrows) at the center of the rosette where the signal of ZO-1 overlaps. (E,F) Immunoprecipitation of E11.5 mouse telencephalon lysate using an anti-ZO-1 antibody. Immunoprecipitation with the normal rabbit IgG and immunoblotting for nestin (asterisk) are represented as negative controls. The band for Notch1 represents the full-length product. Scale bars: 10 μm.

restriction of the efficient Notch signaling to the apical-most thin plane of the VZ may be beneficial for the planar organization of the progenitor sheet (i.e. evenly spaced differentiation of neurons) through lateral inhibition (Fig. 9B). As neurons contact the processes of NPCs through cell-adhesion molecules during radial migration (Elias et al., 2007; Kawauchi et al., 2010), Notch signaling in NPCs could otherwise be activated continuously by multiple migrating neurons at different apicobasal positions until they all detach from the radial fibers. However, such cell contacts, which were unaffected upon DN-cad expression (see Fig. 2E), were not sufficient to prevent precocious neurogenesis.

Roles of the cell-cell junction in neurogenesis

The aberrant neurogenesis upon AJ disruption may also have resulted from several distinct mechanisms other than Notch signaling. First, apically localized cell junctions and the apicobasal polarity complexes have been implicated in the asymmetric cell division of NPCs (Farkas and Huttner, 2008). Mutations or experimental manipulations that affect the apical complex or the apical-basal polarity of NPCs often result in neurogenic abnormalities, such as precocious neuronal differentiation (Bultje et al., 2009; Cappello et al., 2006; Costa et al., 2008; Kim et al., 2010; Yokota et al., 2009). As the disruption of AJs may affect the apicobasal polarity of NPCs, the neurogenic phenotypes described here were potentially caused by this cell-autonomous mechanism. Second, the effects of DN-cad on neuronal migration may be attributed to the abnormal positioning of the TuJ1-positive cells in the VZ (see Figs 2 and 3). The substantial depletion of NPCs due to defective Notch signaling might result in the loss of guides for the migrating neurons. Similarly, the disorganization of the VZ upon AJ disruption would also contribute to the arrest of neurons in the VZ (Gänzler-Odenthal and Redies, 1998; Kadowaki et al., 2007). Finally, the global disruption of AJs in the VZ likely causes detachment of NPCs from the ventricular niche that maintains them proliferative and undifferentiated, leading to precocious neuronal differentiation (Rousso et al., 2012; Zhang et al., 2010). Thus, the phenotypes resulting from the indiscriminate disruption of AJs were perhaps produced through the composite and/or synergistic actions of these mechanisms. Nevertheless, DN-cad also induced neurogenesis in a non-cell-autonomous manner (Fig. 3A,B), and DN-cad expression only in differentiating cells was sufficient for the precocious neurogenesis of NPCs without displacement of neurons (Fig. 4C-F). These are best explained by a defect in cell-cell interaction, namely Notch signaling, rather than defects in cell-intrinsic processes, such as asymmetric cell division, disengagement from the stem-cell niche or neuronal migration.

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

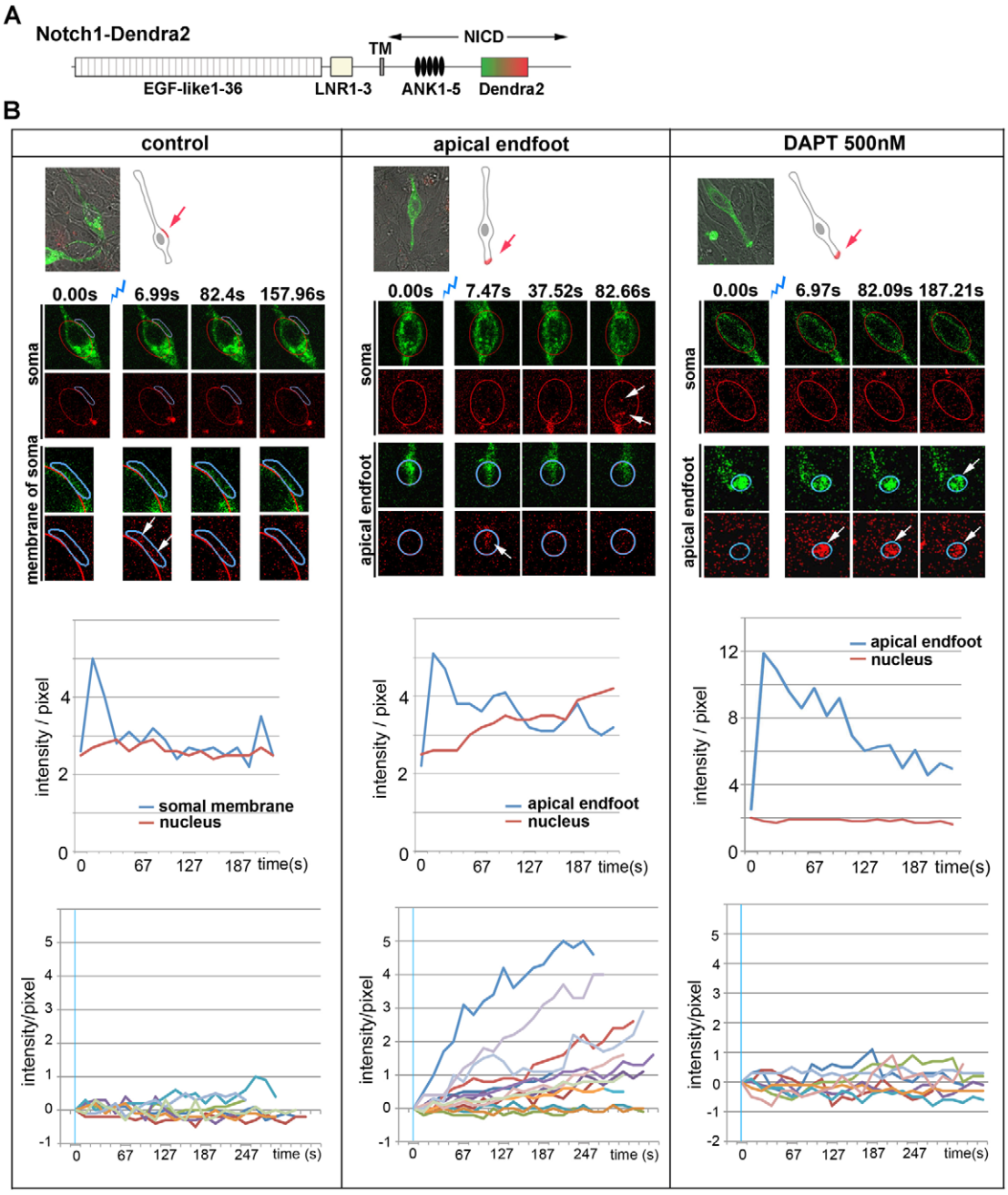

Fig. 8. Live imaging of Notch protein trafficking. (A) The structure of Notch1-Dendra2. LNR, Lin12-Notch repeat; ANK, ankyrin repeat. (B) Notch1-Dendra2 was photoconverted by irradiating a 405 nm laser at a small area (red arrow, encircled with blue line) of the cell body (control, left panel) or the apical endfoot (middle and right panels) of cultured telencephalic NPCs. 500 nM of DAPT was added in the culture to inhibit Notch processing (right panel). The intensities of the red fluorescence were measured over time at the photoconversion sites (blue) and the nuclei (red). Representative plots are shown above other examples in which only the nuclear intensities are presented (control, *n*=8; apical endfoot, *n*=14; DAPT, *n*=8).

Recent studies have shown that the apical junctional complex is inherited by the daughter cell that becomes a neuron upon mitosis of the mother cell (Alexandre et al., 2010; Konno et al., 2008; Shitamukai et al., 2011). The present findings are fully compatible with these observations and further provide its functional significance in neurogenesis. The apical contact of neurons has been described previously, albeit at slightly later stages with a much lower incidence (Minaki et al., 2005; Ochiai et al., 2007). It has been reported that INPs also transiently retain the apical endfeet (Borrell et al., 2013; Nelson et al., 2013; Noctor et al., 2008). Because INPs express Dll1 (Kawaguchi et al., 2008; Nelson et al., 2013; supplementary material Fig. S3B,C), these populations likely regulate the differentiation of NPCs (radial glia) to INPs themselves and/or to neurons in a manner similar to the nascent neurons.

Control of Notch signaling by cellular ‘skills’

In the fly mechanosensory organ development, Delta-expressing cells extend actin-based filopodia to activate Notch signaling in distant cells by ‘touch’ (de Joussineau et al., 2003). Together with the present findings, these cellular ‘skills’ that use cellular protrusions would make the actions of Notch signaling, which by nature only mediates a juxtamembrane communication, more

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

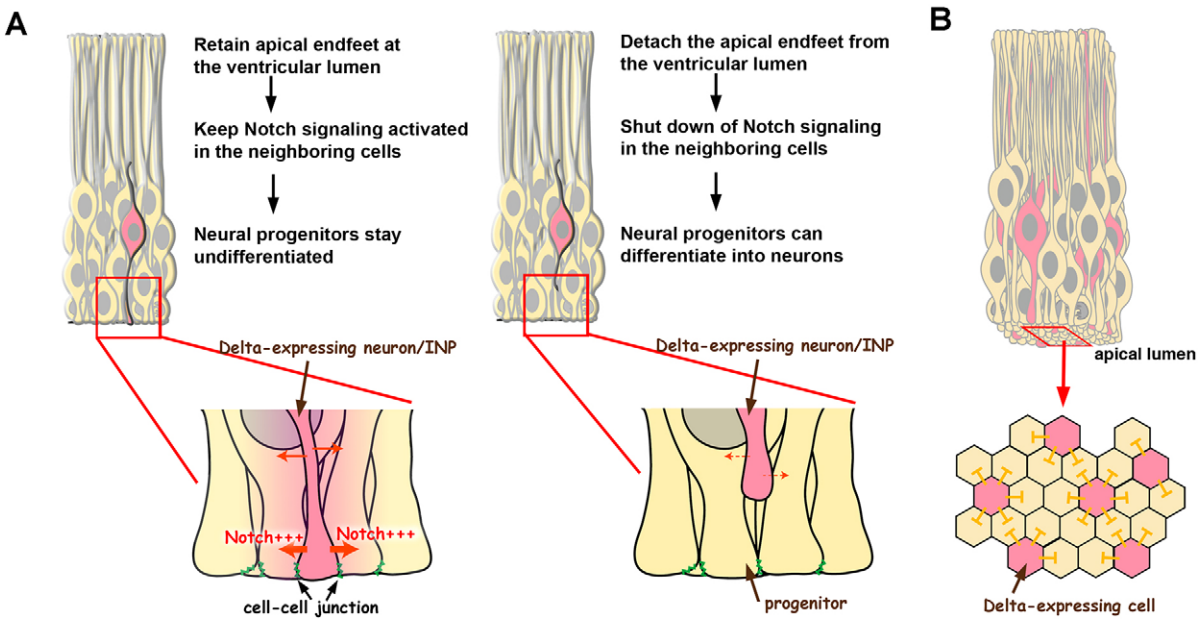

Fig. 9. Models for the roles of AJs in the regulation of neurogenesis. (A) The apical endfoot of neurons and/or INPs may serve as a plug to shut off Notch signaling. When the apical endfeet of the differentiating neurons/INPs maintain contact with the apical lumen, Notch signaling is kept activated in the neighboring cells (left). Once AJs are inactivated, the apical endfeet detach from the ventricular lumen and Notch signaling is rapidly shut down in the neighboring cells, allowing them to differentiate (right). (B) The restriction of Notch signaling at the apical plane of the ventricular wall, where the endfeet of both progenitors and neurons/INPs are tightly packed (lower diagram), would be beneficial for the planar arrangement of the pattern of differentiation.

versatile in diverse biological contexts. Although Notch signaling operates in the VZ tissues throughout the CNS, quantitative and temporal variations in active Notch signaling controlled by the apical endfeet of neurons and INPs might contribute to the generation of the regionally divergent histogenic organization in the CNS. In this regard, it is worth noting that the distribution and perhaps the duration of the neuronal apical endfeet appears regionally different (supplementary material Fig. S1B).

MATERIALS AND METHODS

Animals

Dll1^{lacZ} and *Notch1*^{flox} mice were kindly provided by Dr Achim Gossler (Institute for Molecular Biology, Hannover, Germany) and Dr Raphael Kopan (Washington University, St Louis, MO, USA), respectively. The pregnant ICR mice were purchased from Japan SLC (Hamamatsu, Japan). The day of vaginal plug detection was regarded as embryonic day 0 (E0). EdU were intraperitoneally injected into the pregnant mothers at 50 µg/g of body weight. The fertilized chicken eggs were incubated at 39°C to obtain the desired embryonic stage, which was determined according to the normal table (Hamburger and Hamilton, 1951). To label cycling cells, 2 µl of a 10 mg/ml BrdU solution was injected into the vitelline vein *in ovo* 3 h before fixation. All animals were handled in accordance with the Kumamoto University Guide for the Care and Use of Laboratory Animals.

Plasmids

Expression plasmids for the following molecules were used: DN-E-cad [*pCAG-dE(HA)*], DN-cad-GFP [*pCAG-dE(HA)-GFP*] and DN-N-cad [*pCMV-cN390Δ-FLAG-pA*] from Dr Masatoshi Takeichi (Riken CDB, Japan); and NICD (*pCL-FNIC*), GFP (*pCAGGS-GFP*), DsRed (*pCAGGS-dsRed2*), Cre (*pCAGGS-Cre*), Cre reporter (*pAcLCL-AFP*) and Histone H2B-mRFP (*pCAG-H2B-mRFP*) from Dr Kazuo Yamagata (Riken CDB, Japan). *pCAG-dE(HA)* was constructed from *pCAG-EM2*, a mouse E-cad expression vector (Shimizu et al., 2008), by replacing the *Hin*cII-*Xho*I fragment with a PCR product including HA-tag sequence and several restriction enzyme sites. *pAcLCL-AFP* was constructed by replacing the *lacZ*-cassette of *pAcCATZ* (Araki et al., 1995) with GFP by blunt ligation. The Dll1 promoter (a 4.3 kb 5′ fragment of mouse *Dll1* gene) (Castro et al., 2006) was provided by Dr Francois Guillemot (National Institute for Medical Research, London, UK). *Dll1p-DN-cad* was constructed by inserting a *Eco*RV-*Cla*I fragment of SV40 late polyA by cohesive ligation, and a *Pvu*II-*Eco*RV fragment of the intron derived from the *pCI* (Promega) vector and a *Kpn*I-*Bgl*II cassette of *dE(HA)* or *dE(HA)-GFP* into the *Eco*RV site of the *Dll1* promoter by blunt ligation. *Notch1*-Dendra2 plasmid was constructed by inserting 690 bp of *Hin*dIII fragment of *Dendra2* (Takara Bio, Shiga, Japan) into the *Hin*dIII site of the full-length mouse *Notch1* with the 3′UTR, which was kindly provided by Dr Shigeru Chiba (University of Tsukuba, Japan). This fusion construct was subcloned into a *pCAGGS* vector.

Antibodies

The following antibodies used in this study were kindly provided or purchased: anti-Notch1 raised against the cytoplasmic domain (Novus Biologicals, NB110-57273; 1:100); anti-Dll1 (R&D Systems, AF3970; 1:100); anti-N-cad (BD Transduction Laboratories, #610920; 1:100); anti-N-cad monoclonal (Takara Bio, M110; 2 mg/ml); anti-Jag1 (R&D Systems, AF599; 1 µg/ml); anti-presenilin 1 (G1Nr2; Takasugi et al., 2002; 1:1000) from Dr Taisuke Tomita (The University of Tokyo, Japan); anti-β-catenin (BD Transduction Laboratories, #610153; 1:500); anti-nestin (BD Transduction Laboratories, #611659; 1:500); TuJ1 (Covance, MMS-435P; 1:500); anti-GFP (Invitrogen, A11122; 1:500); anti-BrdU (Sigma, B8434; 1:500); anti-NeuN (Chemicon, MAB377; 1:200); anti-MAP2 (Sigma, AB5622; 1:500); and anti-ZO-1 (Kiuchi-Saishin et al., 2002; 1:5).

Histochemical analysis

Immunohistochemistry of the cryosectioned specimens was performed according to a standard protocol without any antigen retrieval method. The stained specimens were analyzed using epifluorescent (BX52, Olympus) and laser scanning confocal microscopes (TCSSP2, Leica; LSM780, Zeiss).

In situ hybridization

In situ hybridization using digoxigenin-labeled cRNA probes was performed as described previously (Hatakeyama et al., 2001). The probes for chick

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

Axin2 and mouse *Dll1* were cloned using PCR and the probe for chick *Hes5B* was provided by Dr Harukazu Nakamura (Tohoku University, Japan).

Electroporation

In vivo electroporation was performed according to Nakamura (Nakamura, 2009). An electroporator (CUY21SC and NEPA21, NEPA GENE, Japan) was used to deliver five 50 ms pulses of 15 V and 25 V at 100 ms intervals for the chick neural tube and telencephalon, respectively; five 50 ms pulses of 25 V, 28 V and 30 V at 950 ms intervals for E11.5, E12.5 and E13.5 mouse telencephalon, respectively.

Statistical analysis

Quantitative and statistical analyses of the micrographic images were performed using MetaMorph (Molecular Devices) and Prism (GraphPad) software. The quantitative data are presented as the mean±s.e.m., and the values were compared using an unpaired Student's *t*-test. $P<0.05$ is considered statistically significant.

Neural progenitor cell culture

NPCs from the E10.5 mouse telencephalon or HH25 chick telencephalon were dissociated by trypsin digestion, followed by gentle pipetting. Approximately 2.0×10^6 cells were plated onto a 15 mm diameter cover slip placed in a 35 mm diameter dish. The cover slips were coated with laminin (Sigma) and poly-L-lysine (Sigma) prior to plating. The NPCs were cultured as described previously (Ohtsuka et al., 2001) and treated with 500 nM of DAPT for 4 h when necessary. For the study of Notch protein trafficking, NPCs were prepared from embryos electroporated with *Notch1*-Dendra2, cultured, photoconverted and live imaged under a laser confocal microscope (LSM780, Zeiss).

Detection of cell surface Dll1 in the chick NPCs

Live chick NPC culture was incubated with anti-cDll1 antibody (1:50) (Wakamatsu et al., 2000) for 1 h at 37°C, washed and briefly incubated with Alexa488-conjugated anti-rabbit IgG (Invitrogen) for 10 min on ice. After several washes, the NPCs were fixed with 4% paraformaldehyde for 10 min.

TEM analysis

EM analysis and pre-embedding immuno-EM analysis were performed as described previously (Hatakeyama et al., 2004; Parajuli et al., 2012). The specimens were immunostained with TuJ1, detected by HRP-DAB reaction or stained with X-gal for $Dll1^{lacZ}$-derived tissues. For the analysis of the protein distribution on the plasma membrane, the signals were detected by immunogold-conjugated secondary antibody (Aurion). The specimens were examined using an H-7000-type electron microscope (Hitachi) or a tecnai12-type electron microscope (FEI) operated at 75 kV or 100 kV, respectively.

Immunoprecipitation

E11.5 mouse telencephali were dissociated in the lysis buffer containing 2 mM EDTA, 50 mM NaCl and 1% Triton X-100 with protease inhibitors and centrifuged at 100,000 ***g*** for 30 min. The supernatant was incubated with protein G-Sepharose beads (GE Healthcare) pre-coupled with anti-ZO-1 antibodies, for 2 h on ice, and centrifuged at 20,000 ***g*** for 1 min, followed by washing five times with HEPES-buffered (pH 7.5) saline containing 0.5% NP-40 and 1 mM $CaCl_2$ with protease inhibitors. The anti-ZO-1 polyclonal (Zymed) and monoclonal (Kiuchi-Saishin et al., 2002) antibodies were used at 1:200 and 1:1 dilution, respectively.

Slice culture and live imaging

The slice culture of the embryonic cerebral cortex was performed as described previously (Miyata et al., 2002). For live imaging, the slices were observed under an epifluorescence inverted microscope (IX81, Olympus) or confocal microscopes (LSM780, Zeiss; CV1000, Yokogawa Electric, Japan). The slices were exposed to 5 µM DAPT for 4 h when necessary.

Acknowledgements

We thank T. Muroi-Higuchi, T. Magarikaji and J. Kusuura for excellent technical assistance, and L. Kumar Parajuli, K. Furuta and M. Fujioka for assistance with the EM studies. We also thank Drs K. Araki, S. Chiba, A. Gossler, F. Guillemot, R. Kopan, M. Morimoto, H. Nakamura, Y. Saga, H. Sasaki, M. Takeichi, T. Tomita and K. Yamagata for providing mice or reagents.

Competing interests

The authors declare no competing financial interests.

Author contributions

J.H., Y.W. and K.S. designed the study. J.H. and K.S. carried out the experiments and analyzed the data. J.H., Y.W., A.N., R.K., R.S. and K.S. contributed reagents, materials and analysis tools. J.H. and K.S. wrote the manuscript. All authors read and approved the final manuscript for publication.

Funding

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; from RIKEN CDB (to K.S.); from the Joint Usage/Research Center of Institute for Virus Research at Kyoto University (to J.H.); from the Japan Society for the Promotion of Science (to J.H.); and from the Kumamoto University GCOE program. J.H. received a postdoctoral fellowship from the Japan Society for the Promotion of Science (JSPS).

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.102988/-/DC1

References

Alexandre, P., Reugels, A. M., Barker, D., Blanc, E. and Clarke, J. D. W. (2010). Neurons derive from the more apical daughter in asymmetric divisions in the zebrafish neural tube. *Nat. Neurosci.* **13**, 673-679.

Araki, K., Araki, M., Miyazaki, J. and Vassalli, P. (1995). Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 160-164.

Borrell, V., Cárdenas, A., Ciceri, G., Galcerán, J., Flames, N., Pla, R., Nóbrega-Pereira, S., García-Frigola, C., Peregrín, S., Zhao, Z. et al. (2013). Slit/Robo signaling modulates the proliferation of central nervous system progenitors. *Neuron* **76**, 338-352.

Bultje, R. S., Castaneda-Castellanos, D. R., Jan, L. Y., Jan, Y.-N., Kriegstein, A. R. and Shi, S.-H. (2009). Mammalian Par3 regulates progenitor cell asymmetric division via notch signaling in the developing neocortex. *Neuron* **63**, 189-202.

Cappello, S., Attardo, A., Wu, X., Iwasato, T., Itohara, S., Wilsch-Bräuninger, M., Eilken, H. M., Rieger, M. A., Schroeder, T. T., Huttner, W. B. et al. (2006). The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. *Nat. Neurosci.* **9**, 1099-1107.

Castro, D. S., Skowronska-Krawczyk, D., Armant, O., Donaldson, I. J., Parras, C., Hunt, C., Critchley, J. A., Nguyen, L., Gossler, A., Göttgens, B. et al. (2006). Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. *Dev. Cell* **11**, 831-844.

Chudakov, D. M., Lukyanov, S. and Lukyanov, K. A. (2007). Tracking intracellular protein movements using photoswitchable fluorescent proteins PS-CFP2 and Dendra2. *Nat. Protocols* **2**, 2024-2032.

Costa, M. R., Wen, G., Lepier, A., Schroeder, T. and Götz, M. (2008). Par-complex proteins promote proliferative progenitor divisions in the developing mouse cerebral cortex. *Development* **135**, 11-22.

Couturier, L., Vodovar, N. and Schweisguth, F. (2012). Endocytosis by Numb breaks Notch symmetry at cytokinesis. *Nat. Cell Biol.* **14**, 131-139.

Del Bene, F., Wehman, A. M., Link, B. A. and Baier, H. (2008). Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal Notch gradient. *Cell* **134**, 1055-1065.

Elias, L. A. B., Wang, D. D. and Kriegstein, A. R. (2007). Gap junction adhesion is necessary for radial migration in the neocortex. *Nature* **448**, 901-907.

Farkas, L. M. and Huttner, W. B. (2008). The cell biology of neural stem and progenitor cells and its significance for their proliferation versus differentiation during mammalian brain development. *Curr. Opin. Cell Biol.* **20**, 707-715.

Fehon, R. G., Johansen, K., Rebay, I. and Artavanis-Tsakonas, S. (1991). Complex cellular and subcellular regulation of notch expression during embryonic and imaginal development of Drosophila: implications for notch function. *J. Cell Biol.* **113**, 657-669.

Fortini, M. E. (2009). Notch signaling: the core pathway and its posttramslational regulation. *Dev. Cell* **17**, 811-822.

Fuchs, E., Tumbar, T. and Guasch, G. (2004). Socializing with the neighbors. *Cell* **116**, 769-778.

Fujimori, T. and Takeichi, M. (1993). Disruption of epithelial cell-cell adhesion by exogenous expression of a mutated nonfunctional N-cadherin. *Mol. Biol. Cell* **4**, 37-47.

Development (2014) 141, 1671-1682 doi:10.1242/dev.102988

Fujita, S. (2003). The discovery of the matrix cell, the identification of the multipotent neural stem cell and the development of the central nervous system. *Cell Struct. Funct.* **28**, 205-228.

Gänzler-Odenthal, S. I. and Redies, C. (1998). Blocking N-cadherin function disrupts the epithelial structure of differentiating neural tissue in the embryonic chick brain. *J. Neurosci.* **18**, 5415-5425.

Georgakopoulos, A., Marambaud, P., Efthimiopoulos, S., Shioi, J., Cui, W., Li, H.-C., Schütte, M., Gordon, R., Holstein, G. R., Martinelli, G. et al. (1999). Presenilin-1 forms complexes with the cadherin/catenin cell-cell adhesion system and is recruited to intercellular and synaptic contacts. *Mol. Cell* **4**, 893-902.

Hamburger, V. and Hamilton, H.L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol* **88**, 49-92.

Hatakeyama, J., Tomita, K., Inoue, T. and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. *Development* **128**, 1313-1322.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F. and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. *Development* **131**, 5539-5550.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-790.

Hrabě de Angelis, M., McIntyre, J. and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue Dll1. *Nature* **386**, 717-721.

de Joussineau, C., Soulé, J., Martin, M., Anguille, C., Montcourrier, P. and Alexandre, D. (2003). Delta-promoted filopodia mediate long-range lateral inhibition in Drosophila. *Nature* **426**, 555-559.

Kadowaki, M., Nakamura, S., Machon, O., Krauss, S., Radice, G. L. and Takeichi, M. (2007). N-cadherin mediates cortical organization in the mouse brain. *Dev. Biol.* **304**, 22-33.

Kawaguchi, A., Ikawa, T., Kasukawa, T., Ueda, H. R., Kurimoto, K., Saitou, M. and Matsuzaki, F. (2008). Single-cell gene profiling defines differential progenitor subclasses in mammalian neurogenesis. *Development* **135**, 3113-3124.

Kawauchi, T., Sekine, K., Shikanai, M., Chihama, K., Tomita, K., Kubo, K.-i., Nakajima, K., Nabeshima, Y.-i. and Hoshino, M. (2010). Rab GTPases-dependent endocytic pathways regulate neuronal migration and maturation through N-cadherin trafficking. *Neuron* **67**, 588-602.

Kim, S., Lehtinen, M. K., Sessa, A., Zappaterra, M. W., Cho, S.-H., Gonzalez, D., Boggan, B., Austin, C. A., Wijnholds, J., Gambello, M. J. et al. (2010). The apical complex couples cell fate and cell survival to cerebral cortical development. *Neuron* **66**, 69-84.

Kiuchi-Saishin, Y., Gotoh, S., Furuse, M., Takasuga, A., Tano, Y. and Tsukita, S. (2002). Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. *J. Am. Soc. Nephrol.* **13**, 875-886.

Konno, D., Shioi, G., Shitamukai, A., Mori, A., Kiyonari, H., Miyata, T. and Matsuzaki, F. (2008). Neuroepithelial progenitors undergo LGN-dependent planar divisions to maintain self-renewability during mammalian neurogenesis. *Nat. Cell Biol.* **10**, 93-101.

Kooh, P. J., Fehon, R. G. and Muskavitch, M. A. (1993). Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development. *Development* **117**, 493-507.

Kopan, R. and Ilagan, M. X. G. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216-233.

Lien, W.-H., Klezovitch, O., Fernandez, T. E., Delrow, J. and Vasioukhin, V. (2006). Alpha-catenin controls cerebral cortical size by regulating the Hedgehog signaling pathway. *Science* **311**, 1609-1612.

Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat. Rev. Neurosci.* **7**, 93-102.

Marambaud, P., Wen, P. H., Dutt, A., Shioi, J., Takashima, A., Siman, R. and Robakis, N. K. (2003). A CBP binding transcriptional repressor produced by the PS1/ε-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell* **114**, 635-645.

Minaki, Y., Mizuhara, E., Morimoto, K., Nakatani, T., Sakamoto, Y., Inoue, Y., Satoh, K., Imai, T. and Takai, Y. (2005). Migrating postmitotic neural precursor cells in the ventricular zone extend apical processes and form adherens junctions near the ventricle in the developing spinal cord. *Neurosci. Res.* **52**, 250-262.

Miyata, T., Kawaguchi, A., Saito, K., Kuramochi, H. and Ogawa, M. (2002). Visualization of cell cycling by an improvement in slice culture methods. *J. Neurosci. Res.* **69**, 861-868.

Nakamura, H. (2009). *Electroporation and Sonoporation in Developmental Biology*. Tokyo, Berlin, Heidelberg, New York: Springer.

Nelson, B. R., Hodge, R. D., Bedogni, F. and Hevner, R. H. (2013). Dynamic interactions between intermediate neurogenic progenitors and radial glia in embryonic mouse neocortex: potential role in Dll1-Notch signaling. *J. Neurosci.* **33**, 9122-9139.

Noctor, S. C., Martinez-Cerdeno, V. and Kriegstein, A. R. (2008). Distinct behaviors of neural stem and progenitor cells underlie cortical neurogenesis. *J. Comp. Neurol.* **508**, 28-44.

Ochiai, W., Minobe, S., Ogawa, M. and Miyata, T. (2007). Transformation of pin-like ventricular zone cells into cortical neurons. *Neurosci. Res.* **57**, 326-329.

Ohata, S., Aoki, R., Kinoshita, S., Yamaguchi, M., Tsuruoka-Kinoshita, S., Tanaka, H., Wada, H., Watabe, S., Tsuboi, T., Masai, I. et al. (2011). Dual roles of Notch in regulation of apically restricted mitosis and apicobasal polarity of neuroepithelial cells. *Neuron* **69**, 215-230.

Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F. and Kageyama, R. (1999). Hes1 and Hes5 as Notch effectors in mammalian neuronal differentiation. *EMBO J.* **18**, 2196-2207.

Ohtsuka, T., Sakamoto, M., Guillemot, F. and Kageyama, R. (2001). Roles of the basic helix-loop-helix genes Hes1 and Hes5 in expansion of neural stem cells of the developing brain. *J. Biol. Chem.* **276**, 30467-30474.

Parajuli, L. K., Nakajima, C., Kulik, A., Matsui, K., Schneider, T., Shigemoto, R. and Fukazawa, Y. (2012). Quantitative regional and ultrastructural localization of the Ca(v)2.3 subunit of R-type calcium channel in mouse brain. *J. Neurosci.* **32**, 13555-13567.

Rakic, P. (2007). The radial edifice of cortical architecture: from neuronal silhouettes to genetic engineering. *Brain Res. Rev.* **55**, 204-219.

Rousso, D. L., Pearson, C. A., Gaber, Z. B., Miquelajauregui, A., Li, S., Portera-Cailliau, C., Morrisey, E. E. and Novitch, B. G. (2012). Foxp-mediated suppression of N-cadherin regulates neuroepithelial character and progenitor maintenance in the CNS. *Neuron* **74**, 314-330.

Sakai, D., Tanaka, Y., Endo, Y., Osumi, N., Okamoto, H. and Wakamatsu, Y. (2005). Regulation of Slug transcription in embryonic ectoderm by beta-catenin-Lef/Tcf and BMP-Smad signaling. *Dev. Growth Differ.* **47**, 471-482.

Sasaki, N., Sasamura, T., Ishikawa, H. O., Kanai, M., Ueda, R., Saigo, K. and Matsuno, K. (2007). Polarized exocytosis and transcytosis of Notch during its apical localization in Drosophila epithelial cells. *Genes Cells* **12**, 89-103.

Self, M., Lagutin, O. V., Bowling, B., Hendrix, J., Cai, Y., Dressler, G. R. and Oliver, G. (2006). Six2 is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. *EMBO J.* **25**, 5214-5228.

Shimizu, M., Fukunaga, Y., Ikenouchi, J. and Nagafuchi, A. (2008). Defining the roles of beta-catenin and plakoglobin in LEF/T-cell factor-dependent transcription using beta-catenin/plakoglobin-null F9 cells. *Mol. Cell. Biol.* **28**, 825-835.

Shitamukai, A., Konno, D. and Matsuzaki, F. (2011). Oblique radial glial divisions in the developing mouse neocortex induce self-renewing progenitors outside the germinal zone that resemble primate outer subventricular zone progenitors. *J. Neurosci.* **31**, 3683-3695.

Sugrue, S. P. and Zieske, J. D. (1997). ZO1 in corneal epithelium: association to the zonula occludens and adherens junctions. *Exp. Eye Res.* **64**, 11-20.

Takasugi, N., Takahashi, Y., Morohashi, Y., Tomita, T. and Iwatsubo, T. (2002). The mechanism of gamma-secretase activities through high molecular weight complex formation of presenilins is conserved in Drosophila melanogaster and mammals. *J. Biol. Chem.* **277**, 50198-50205.

Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-2821.

Yang, X., Klein, R., Tian, X., Cheng, H.-T., Kopan, R. and Shen, J. (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev. Biol.* **269**, 81-94.

Yokota, Y., Kim, W.-Y., Chen, Y., Wang, X., Stanco, A., Komuro, Y., Snider, W. and Anton, S. (2009). The adenomatous polyposis coli protein is an essential regulator of radial glial polarity and construction of the cerebral cortex. *Neuron* **61**, 42-56.

Zhang, J., Woodhead, G. J., Swaminathan, S. K., Noles, S. R., McQuinn, E. R., Pisarek, A. J., Stocker, A. M., Mutch, C. A., Funatsu, N. and Chenn, A. (2010). Cortical neural precursors inhibit their own differentiation via N-cadherin maintenance of beta-catenin signaling. *Dev. Cell* **18**, 472-479.

Zhu, X., Zhang, J., Tollkuhn, J., Ohsawa, R., Bresnick, E. H., Guillemot, F., Kageyama, R. and Rosenfeld, M. G. (2006). Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. *Genes Dev.* **20**, 2739-2753.