RESEARCH ARTICLE

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

Jun Hatakeyama1,*, Yoshio Wakamatsu2, Akira Nagafuchi3, Ryoichiro Kageyama4,5, Ryuichi Shigemoto6,7 and Kenji Shimamura1,*

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 differentiation of NPCs. Notch1 and its ligand Dll1 are distributed around AJs 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; 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 neurogenesis (Hatakeyama et al., 2004). Neuronal 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

Received 23 August 2013; Accepted 11 February 2014


1Department of Brain Morphogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuou-ku, Kumamoto 860-0811, Japan. 2Division of Developmental Neuroscience, Tohoku University, Graduate School of Medicine, 2-1 Seiryo-Machi, Aoba-ku, Sendai 980-8575, Japan. 3Department of Biology, Nara Medical University, 840 Shijo, Kashihara, Nara 634-8521, Japan. 4Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan. 5World Premier International Research Initiative–Institute for Integrated Cell-Material Sciences (WPI-ICeMS), Kyoto University, Kyoto 606-8501, Japan. 6Division of Cerebral Structure, National Institute for Physiological Sciences, 5-1 Higashi-yamada, Myodaiji, Okazaki 444-8787, Japan. 7Institute 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)
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),
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 forZO-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.
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 (Ohnaka 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
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...
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 AJs 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 DI1 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 DI1 were confirmed using Notch1 conditional KO (Yang et al., 2004) and DI1 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 DI1 exposed on the cell surface, live cells were stained using an antibody raised against the extracellular domain of DI1 (Wakamatsu et al., 2000). Cell-surface DI1 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 DI1, 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 DI1 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 DI1 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. 5D). 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
(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
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.
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 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...
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 endfoot 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 endfoot appears regionally different (supplementary material Fig. S1B).

**MATERIALS AND METHODS**

**Animals**

DIIH我和H 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-cN390-FLAG-pA] from Dr Masatoshi Takeichi (Riken CDB, Japan); and Nicd (pCL-FN/C), GFP (pCAGGS-GFP), DsRed (pCAGGS-dsRed2), Cre (pCAGGG-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 Notch1-cassettes of pAcCATZ (Araki et al., 1995) with GFP by blunt ligation. The Dll1 promoter (a 4.3 kb 5′ fragment of mouse Dll1 gene) was kindly provided by Dr Francois Guillemot (National Institute for Medical Research, London, UK). Dll1p-DN-cad was constructed by inserting an EcoRV-ClaI fragment of SV40 late polyA by cohesive ligation, and a PvuII-EcoRV fragment of the intron derived from the pCI (Promega) vector and a KpnI-BglII cassette of dE(HA) or dE(HA)-GFP into the EcoRV site of the Dll1 promoter by blunt ligation. Notch1-Dendra2 plasmid was constructed by inserting 690 bp of HindIII fragment of Dendra2 (Takara Bio, Shiga, Japan) into the HindIII 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-DII1 (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
Axin2 and mouseDll1 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 2×10^6 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-dDll1 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 1 min, followed by washing five times with HEPES-buffered (pH 7.5) saline containing 0.5% NP-40 and 1 mM CaCl2 with 2 mM EDTA, 50 mM NaCl and 1% Triton X-100 with protease inhibitors. The anti-ZO-1 monoclonal (Zymed) and monoclonal (Kiuchi-Saishin et al., 2002) antibodies were used at 1:200 and 1:1 dilution, 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 CaCl2 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. Kusuyu 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


DEVELOPMENT


