Progenitors resume generating neurons after temporary inhibition of neurogenesis by Notch activation in the mammalian cerebral cortex

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Summary

The mammalian cerebral cortex comprises six layers of neurons. Cortical progenitors in the ventricular zone generate neurons specific to each layer through successive cell divisions. Neurons of layer VI are generated at an early stage, whereas later-born neurons occupy progressively upper layers. The underlying molecular mechanisms of neurogenesis, however, are relatively unknown. In this study, we devised a system where the Notch pathway was activated spatiotemporally in the cortex by in vivo electroporation and *Cre*-mediated DNA recombination. Electroporation at E13.5 transferred DNA to early progenitors that gave rise to neurons of both low and upper layers. Forced expression of a constitutively active form of Notch (caNotch) at E13.5 inhibited progenitors from generating neurons and kept progenitors as proliferating radial glial cells. After subsequent transfection at E15.5 of a Cre expression vector to remove *caNotch*, double-transfected cells, in which *caNotch* was excised, migrated into the cortical plate and differentiated into neurons specific to upper layers. Bromodeoxyuridine-labeling experiments showed that the neurons were born after *Cre* transfection. These results indicate that cortical progenitors that had been temporarily subjected to Notch activation at an early stage generated neurons at later stages, but that the generation of low-layer neurons was skipped. Moreover, the double-transfected cells gave rise to upper-layer neurons, even after their transplantation into the E13.5 brain, indicating that the developmental state of progenitors is not halted by caNotch activity.

Key words: Notch, Electroporation, Neurogenesis, Cerebral cortex, Asymmetric division

Introduction

The mammalian cerebral cortex is organized into six layers, each of which contains neurons with distinct molecular and functional properties (for reviews, see McConnell, 1995; Levitt et al., 1997). Cortical projection neurons are generated from progenitors in the ventricular zone (VZ) adjacent to the lateral ventricle (for a review, see Marín and Rubenstein, 2003). The earliest-born neurons are Cajal-Retzius (CR) cells of layer I, and then neurons of layers II to VI are generated in an 'inside-out' fashion; later-born neurons pile up over early-born low-layer neurons (for a review, see Gupta et al., 2002). Retroviral lineage analyses suggest that neurons of several layers are generated from single progenitors through asymmetric cell divisions (Luskin et al., 1988; Price and Thurlow, 1988; Walsh and Cepko, 1988; Reid et al., 1995). Asymmetric divisions of progenitors are observed in slice cultures (Chenn and McConnell, 1995; Noctor et al., 2001; Miyata et al., 2001). DNA labeling experiments suggest that progenitors execute 11 cell cycles during cortical neurogenesis (Takahashi et al., 1995), and that the laminar fate of neurons is correlated with the number of cell cycles that their progenitors have executed (Takahashi et al., 1999). Intrinsic properties of progenitors and stage-specific epigenetic cues have been implicated in the sequential generation of different types of neurons. Early progenitors, which generate low-layer neurons, are competent to generate upper-layer neurons after transplantation into older brains (McConnell et al., 1991), whereas late progenitors cannot generate low-layer neurons in younger brains (Frantz and McConnell, 1996). A recent report has indicated that an intrinsic factor, Foxg1, plays a crucial role in generational switching from CR cells to low-layer neurons (Hanashima et al., 2004). However, molecular mechanisms that regulate the laminar fate of neurons of layers II to VI are largely unknown. In *Drosophila*, the intrinsic capabilities of progenitors to generate particular types of neurons are regulated by transcription factors, which change sequentially every asymmetric division (Isshiki et al., 2001; Pearson and Doe, 2003). Asymmetric distribution of cell fate determinants, such as Numb and Pros, is required for the proper differentiation of daughter cells (Ryu et al., 1994; Ling and Vaessin, 2000). It remains to be determined whether asymmetric divisions also affect progenitors. To address these points, we have devised a system whereby neurogenesis can be modulated in the developing mammalian cerebral cortex.

Notch signaling plays a crucial role in cortical neurogenesis (for a review, see Justice and Jan, 2002). Mutations in key

components of the Notch signaling pathway have revealed that Notch signaling is required for the maintenance of self-renewal of progenitors (Nakamura et al., 2000; Hitoshi et al., 2002). Conversely, overexpression of a constitutively active form of Notch (caNotch) inhibits neurogenesis in P19 cells (Nye et al., 1994) and promotes the generation of radial glial cells (RGCs) in the embryonic brain (Gaiano et al., 2000). RGCs had been thought to be specialized cells that provide a scaffold along which newborn neurons migrate from the VZ to the cortical plate (Rakic, 1972). Recent reports have uncovered another important role of RGCs, as progenitors that generate neurons and glia (Malatesta et al., 2000; Noctor et al., 2001; Miyata et al., 2001). Although the majority of progenitors in the VZ are RGCs (Noctor et al., 2002), RGCs exhibit different neurogenic and gliogenic features, depending on their location and developmental stage (Malatesta et al., 2003; Anthony et al., 2004). Because the activation of the Notch pathway leads to gilal differentiation in various settings (for a review, see Gaiano and Fishell, 2002), cortical cells overexpressing caNotch may be fated to become gliogenic. The in vivo features of caNotch-expressing cells and their daughter cells remain to be defined. To clarify these issues, we used forced expression of *caNotch* in a spatially restricted domain using in vivo electroporation. The activity of *caNotch* was temporally controlled by the Cre recombinase-loxP system.

Materials and methods

Plasmid construction

All plasmids contained reporter genes downstream of the CAG promoter (Niwa et al., 1991). pCAG-EYFP and pCAG-DsRed-Mito carried genes encoding enhanced yellow fluorescent protein (EYFP) and a mitochondrial targeting peptide fused to DsRed, respectively (Saito and Nakatsuji, 2001). We constructed a double promoter vector, from which *EYFP* and *caNotch* were expressed in the same cells. *nlacZ*, which encoded nuclear-targeted β-galactosidase (β-gal), was expressed only after the removal of *caNotch* that was flanked by two loxP sites (Fig. 1). pCAG-EYFP-CAG-loxP-neo-loxP-nlacZ (pCAG-ExNeoZ) was constructed by inserting the end-filled *Bgl*II-*Xba*I fragment of pXneoΔSXnXZmp, carrying loxP, *neo*, loxP and *nlacZ*, into the end-filled *Eco*RI-*Hin*dIII sites downstream of the second CAG promoter of pCAG-EYFP-CAG (Saito and Nakatsuji, 2001). pXneoΔSXnXZmp was a gift from E. Mercer. To construct pCAG-ExNotchZ, *neo* of pCAG-ExNeoZ was replaced with the end-filled *Eco*RI-*Xho*I fragment encoding the FLAG peptide, and the transmembrane region, RAM domain, cdc10/ankyrin repeats and nuclear localization signal of mouse Notch1 (Saito and Nakatsuji, 2001). pCAG-ExZ, in which *caNotch* was removed, was obtained by screening clones after in vitro recombination of pCAG-ExNotchZ by Cre recombinase (Invitrogen). pXCANCre carried the nuclear-targeted *Cre* gene downstream of the CAG promoter (Kanegae et al., 1995).

In vivo electroporation

In utero surgery and electroporation was performed as described previously (Saito and Nakatsuji, 2001). ICR mice were purchased from Clea (Tokyo, Japan) and maintained on a 12-hour (8:00 am-8:00 pm) light/dark cycle. A female mouse was put in the presence of a male overnight, and the noon of a day when a vaginal plug was found was designated embryonic day (E) 0.5. The day of birth was designated postnatal day (P) 0. Animals were handled according to institutional guidelines. Two microliters of DNA solution (50 nM) was injected into the lateral ventricle, and electric pulses, at 40 V for E13.5 or 50 V for E15.5, were delivered using forceps-type electrodes. Survival and EYFP-positive ($EYFP^+$)/$DsRed^+$ rates after double electroporation at E13.5 and E15.5 were 89.9±5.1% and 65.3±7.1%, respectively (number of operated pregnant mice was 13). Each electroporation result was confirmed by using at least two independently isolated clones with the same structure and reproduced in several brains derived from at least three operated pregnant mice.

BrdU labeling analysis

To evaluate the activity of cell proliferation, pregnant mice were injected at E14.5 intraperitoneally with 125 μg/gm body weight of bromodeoxyuridine (BrdU) (Sigma), and embryos were collected 2 hours after the injection. To examine what percentage of transfected cells in the VZ were proliferating, cumulative labeling was performed by injecting BrdU three times every 5.5 hours from E14.5, and embryos were collected 4 hours after the final injection. To determine the birth dates of neurons, P5 brains were recovered after single injection of 50 μg/gm body weight of BrdU at E14.5 or E16.5.

Immunostaining

Immunohistochemistry was performed as described (Saba et al., 2003). Fixed embryos and brains were embedded in OCT compound and sliced at 20 μm using a cryostat. Primary antibodies included mouse anti-BrdU (BD Pharmingen), rabbit anti-GFP (Molecular Probes), rabbit anti-LH2A/B, rabbit anti-ER81, goat anti-β-galactosidase (Biogenesis), mouse monoclonal anti-glial fibrillary acidic protein (GFAP, Sigma), mouse monoclonal M2 anti-FLAG (Sigma), and mouse monoclonal RC2 (Developmental Studies Hybridoma Bank, University of Iowa). The anti-LH2A/B (Liem et al., 1997) and anti-ER81 (Arber et al., 2000) antibodies were gifts from T. Jessell (Columbia University). Secondary antibodies were donkey anti-rabbit IgG conjugated with Alexa Fluor 488 or 555 (Molecular Probes), donkey anti-goat IgG conjugated with Alexa Fluor 647 (Molecular Probes), and donkey anti-mouse IgG or IgM conjugated with Cy3 (Jackson ImmunoResearch). Apoptosis was examined by using an In Situ Cell Death Detection Kit, TMR Red (Roche). Fluorescent images were analyzed using a confocal microscope LSM510 (Zeiss). Each image was the Z-projection of at least 20 optical planes through a depth of 11 μm. To quantify the intensity of immunofluorescent signals, they were recorded under the same non-saturated conditions and measured using an LSM510 software release 3.0 (Zeiss). The $BrdU^+$ cells were classified into two groups on the basis of their relative fluorescence intensity (RFI). Populations of cells that exhibited the highest fluorescence (RFI=100.0±4.3) were designated heavily labeled. Cells that showed lower fluorescence (RFI=51.1±4.3, 27.5±3.9 and 14.7±3.6) were designated lightly labeled. Each result of immunohistochemistry was replicated using several brains derived from at least three operated pregnant mice.

Transplantation

In utero transplantation was performed as described (Desai and McConnell, 2000; Wichterle et al., 2001), with modifications. pCAG-ExZ or pCAG-ExNotchZ was transfected at E13.5, and $EYFP^+$ tissue was dissected from the VZ of the somatosensory area 3 hours after transfection of *Cre* at E15.5. As a control, tissue was dissected 3 hours after transfection of pCAG-ExZ at E13.5. Cells were mechanically dissociated and rinsed twice in L-15 medium containing 10 μg/ml of DNase I (Worthington), and suspended with L-15 medium. Cell suspension (~500,000 cells/μl) was injected into the dorsal telencephalon. At P5, brains were recovered. A three-hour incubation between transfection and dissection was necessary to obtain better survival of dissociated cells. Cells prepared 3 hours after transfection showed essentially the same laminar patterns as those prepared immediately after transfection (data not shown). $EYFP^+$ cells were detected in the dorsal but not ventral telencephalon (number of examined brains=52).

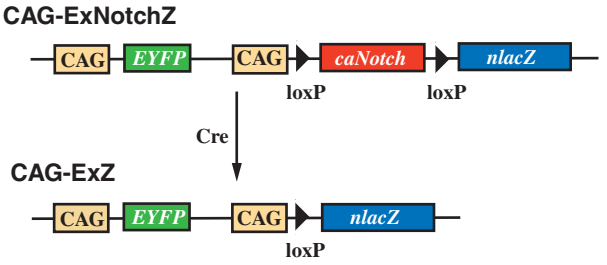

Fig. 1. Structure of a double-promoter vector, pCAG-ExNotchZ. The *caNotch* gene, which encodes the caNotch protein N-terminally tagged with the FLAG-peptide, is excised out at its flanking loxP sites by Cre recombinase to allow translation of β-gal.

Results

Stage-dependent generation of layer-specific neurons

We examined the fate of neurons labeled after electroporation at two different stages. Mouse embryonic in vivo electroporation enables us to transfect DNA into cells adjacent to the ventricle (Saito and Nakatsuji, 2001), and RGCs were transfected (see below). We focused the following analyses on the primary somatosensory area, because neurogenesis proceeds in a rostrolateral to caudomedial gradient, but not synchronously throughout the cortex (Bayer and Altman, 1991; Takahashi et al., 1999). In addition, two layer-specific markers can clearly distinguish neurons of low and upper layers in the area (see Fig. 2).

Electroporation of pCAG-ExZ (Fig. 1) at E13.5 labeled many neurons of layers II to VI (Fig. 2A-D), consistent with our previous results (Saito and Nakatsuji, 2001). EYFP and β-gal were co-expressed in the same cells (Fig. 2A,B and data not shown). β-Gal was confined in nuclei due to the nuclear localization signal, whereas EYFP spread throughout the cytoplasm. EYFP$^+$/β-gal$^+$ neurons were positive for molecular markers specific to particular layers (Fig. 2D,I-N): ER81 (Weimann et al., 1999) for layer V (Fig. 2D,I-K); and Lhx2 (Nakagawa et al., 1999) for layers II and III (Fig. 2L-N, and data not shown). Here we describe immunostaining with the anti-LH2A/B antibody, which can react with Lhx2 and Lhx9, as Lhx2 staining, because only Lhx2 is expressed in layers II and III of the area (Rétaux et al., 1999).

Contrastingly, electroporation at E15.5 labeled cells of layers II and III, which were positive for Lhx2 (Fig. 2E-H,O-Q). The laminar fate of EYFP$^+$/β-gal$^+$ neurons after electroporation at these two stages was in agreement with previous birth date analyses of neurons using nucleotide analogs (Takahashi et al., 1999). These results indicate that early progenitors transfected at E13.5 generate neurons of both low and upper layers, whereas late progenitors transfected at E15.5 generate neurons of only layers II and III.

Sequential generation of low- and upper-layer neurons

To examine whether progenitors transfected at an early stage were transfectable again at later stages, we performed double electroporation using *DsRed* and *EYFP*. Electroporation of *DsRed* at E13.5 labeled neurons of both low and upper layers (Fig. 3A), in the same fashion as that of *EYFP* (Fig. 2A).

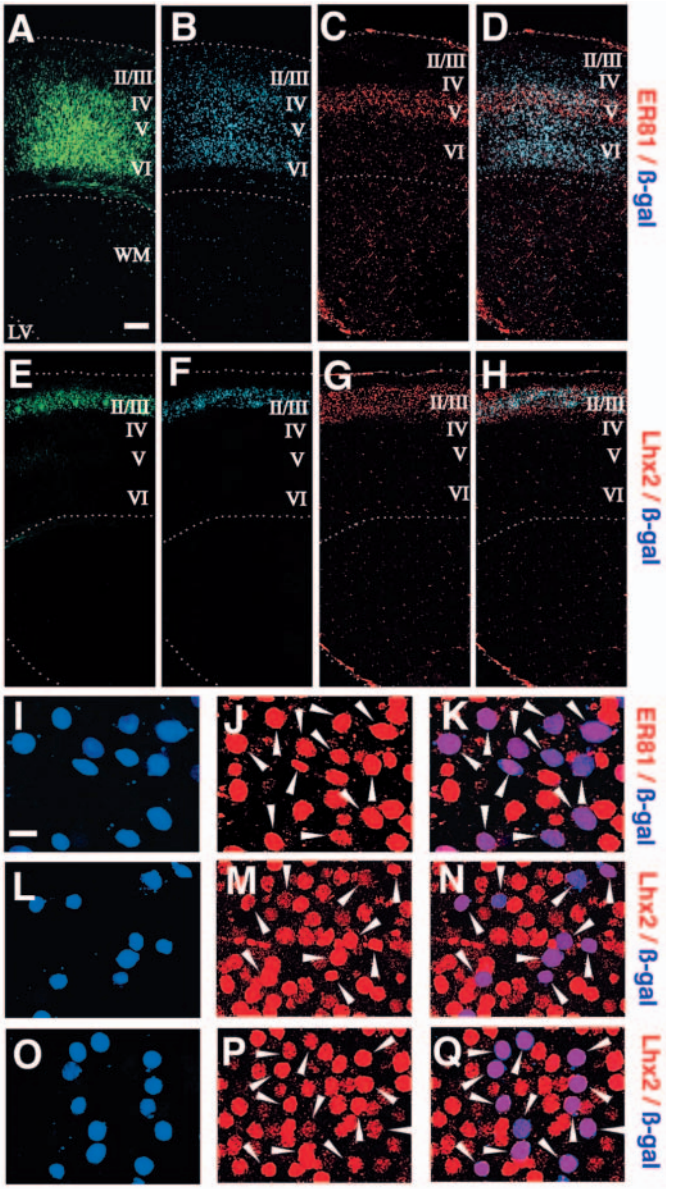

Fig. 2. Electroporation of pCAG-ExZ, carrying *EYFP* and *nlacZ*, at two developmental stages. Coronal sections of the primary somatosensory area of P5 brains after electroporation at E13.5 (A-D,I-N), and E15.5 (E-H,O-Q) are shown. Green and blue signals show EYFP fluorescence (A,E), and immunostaining with an anti-β-gal antibody (B,D,F,H,I,K,L,N,O,Q), respectively. Red signals show immunostaining with antibodies recognizing ER81 (C,D,J,K) and Lhx2 (G,H,M,N,P,Q). β-Gal$^+$ neurons of layer V, and of layers II and III, were positive for ER81 and Lhx2, respectively (arrowheads). ER81$^+$ neurons expressed ER81 relatively uniformly at high levels, whereas 79.7±2.3% of β-gal$^+$/Lhx2$^+$ neurons expressed Lhx2 at high levels. The ratio closely resembled that of strongly Lhx2-positive cells in the total population of Lhx2$^+$ cells of layers II and II (78.6±2.7%). A-D, E-H, I-K, L-N, and O-Q each show the same section; D,H,K,N and Q are merged views. WM, white matter; LV, lateral ventricle. Scale bars: 200 μm for A-H; 20 μm for I-Q.

DsRed signals were distributed as dots showing mitochondrial sites, because DsRed was fused with a mitochondrial-targeted peptide (Saito and Nakatsuji, 2001). Double electroporation of

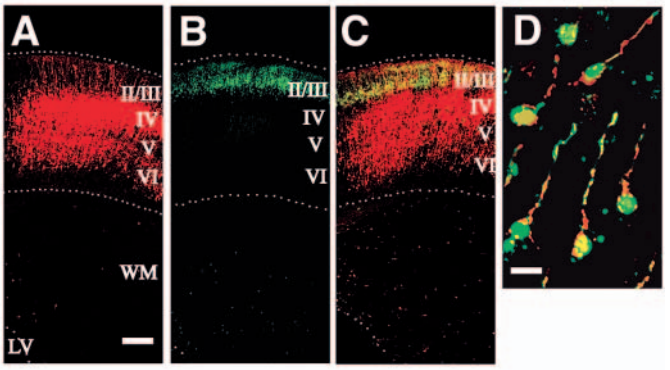

Fig. 3. Double electroporation of *DsRed* and *EYFP* at different stages. P5 brains after single electroporation of *DsRed* (red) at E13.5 (A) or *EYFP* (green) at E15.5 (B), and after double electroporation of *DsRed* at E13.5 and *EYFP* at E15.5 (C,D) are shown. (D) Magnified view of upper-layer neurons, showing co-expression of *DsRed* and *EYFP*. Scale bars: 200 μm for A-C; 10 μm for D.

DsRed at E13.5 and *EYFP* at E15.5 demonstrated DsRed$^+$ neurons in layers II to VI and EYFP$^+$ neurons in layers II and III (Fig. 3C). The laminar positions of labeled neurons were the same as those by single electroporation, showing that the laminar fate of neurons is not affected by double electroporation. Many EYFP$^+$ later-born neurons were also positive for DsRed (Fig. 3D), indicating that early progenitors transfected at E13.5 are transfectable again at E15.5.

Notch activation keeps progenitors as proliferating RGCs

We have previously shown that transfection of *caNotch* inhibits neuronal differentiation in the cerebral cortex (Saito and Nakatsuji, 2001). To assess proliferation of caNotch$^+$ cells, cortical cells were pulse-labeled with BrdU (Fig. 4A-D). One day after *EYFP* transfection at E13.5, many cells in the VZ were positive for EYFP. Numerous EYFP$^+$ differentiating cells were also observed outside the VZ (Fig. 4B), reflecting that asymmetric divisions of progenitors to generate neurons are prominent at this stage (Takahashi et al., 1996). By contrast, co-expression of EYFP and caNotch from a double promoter vector, pCAG-ExNotchZ (Fig. 1), resulted in few EYFP$^+$ cells outside the VZ (Fig. 4D), showing inhibition of neurogenesis by caNotch. BrdU signals were detected in both EYFP$^+$ and EYFP$^+$/caNotch$^+$ cells in the VZ. Transfection of *caNotch* led to a significant increase of the ratio of BrdU$^+$ cells in the total population of EYFP$^+$ cells (38.8±1.8%), compared with transfection of *EYFP* alone (21.6±2.7%, Fig. 2E). However, this increase did not simply imply that the mitotic activity of transfected cells was enhanced by caNotch, because there were few EYFP$^+$/caNotch$^+$ differentiating cells outside the VZ. The ratios of BrdU$^+$ cells in the EYFP$^+$ population of the VZ were similar (35.3±2.1% and 39.4±1.4% for *EYFP* and *EYFP/caNotch*, respectively). These results suggest that

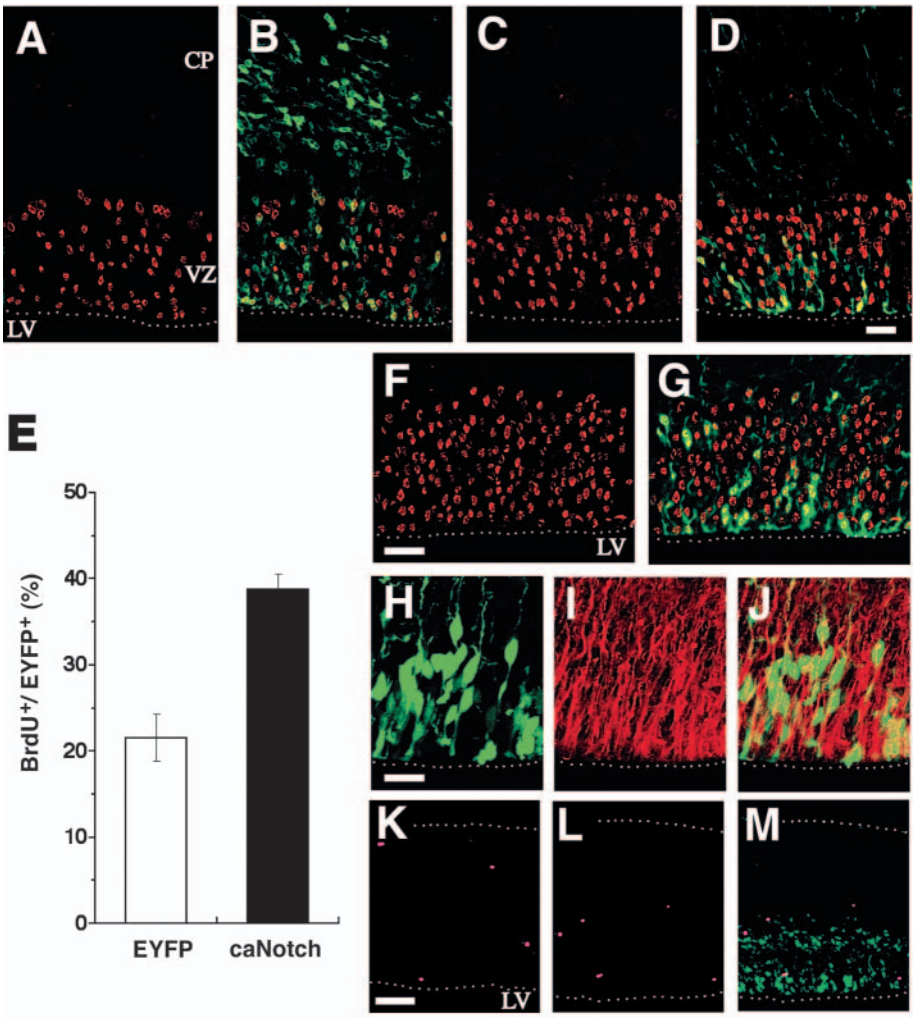

Fig. 4. Effects of caNotch on cortical progenitors. pCAG-EYFP (A,B) or pCAG-ExNotchZ (C,D,F-J,L,M) was transfected at E13.5. (A-D,F,G) Embryonic brains were labeled at E14.5 for 2 hours (A-D) or 15 hours (F,G) with BrdU. Green and red signals show immunostaining with anti-GFP and anti-BrdU antibodies, respectively. (E) The ratio of BrdU$^+$ cells in the total population of EYFP$^+$ cells or EYFP$^+$/CaNotch$^+$ cells of 2-hour-labeled brains. Each ratio was calculated from three brains. (H-J) An E15.5 brain, 2 days after transfection. Green and red signals show EYFP fluorescence and immunostaining with the RC2 antibody, respectively. The ratio of RC2$^+$ cells in the total population of EYFP$^+$ cells was calculated from three brains. (K-M) TUNEL assay performed on the E15.5 brain. The areas of the transfected hemisphere (L,M) and the opposite non-transfected hemisphere (K) of an electroporated brain are shown. Green signals show EYFP fluorescence. Transfected green cells were not detected in the non-transfected hemisphere (K). A,B; C,D; F,G; H-J; and K-M each show the same section. B,D,G,J,K and M are merged views. CP, cortical plate; LV, lateral ventricle; VZ, ventricular zone. Scale bars: 50 μm for A-D,F,G; 20 μm for H-J; 100 μm for K-M.

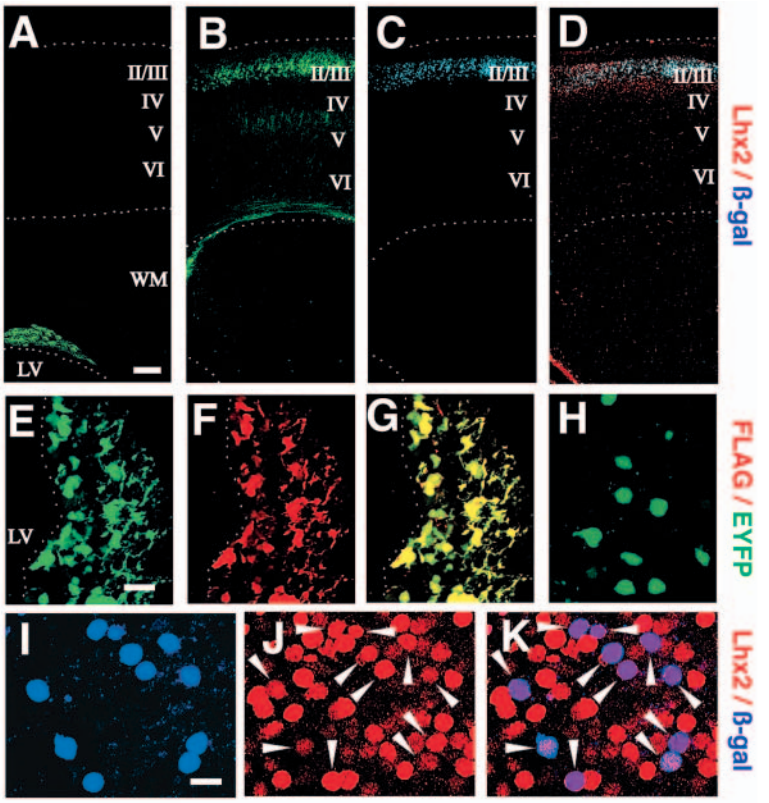

Fig. 5. Neurogenesis resumes after the removal of *caNotch*. P5 brains after single electroporation of pCAG-ExNotchZ at E13.5 (A,E-G), and after double electroporation of pCAG-ExNotchZ at E13.5 and pXCANCre at E15.5 (B-D,H-K). (E-G) Magnified view of an EYFP$^+$ region close to the lateral ventricle (LV). (H-K) Magnified views of layers II and III. Green and blue signals show EYFP fluorescence (A,B,E,G,H), and immunostaining with the anti-β-gal antibody (C,D,I,K), respectively. Red signals show immunostaining with antibodies recognizing FLAG (F-H) and Lhx2 (D,J,K). B-D, E-G, and I-K each show the same section; D,G,H and K are merged views. Note, EYFP$^+$ neurons only signal green, no yellow colouration was observed even after the merge of two colors (H) because of a lack of FLAG signals. All β-gal$^+$ neurons of layers II and III were positive for Lhx2. 80.5±2.0% of β-gal$^+$/Lhx2$^+$ neurons expressed Lhx2 at high levels. The ratio was almost identical to that of strongly Lhx2-positive cells in the total Lhx2$^+$ population of layers II and III (79.6±2.45%). A similar distribution of cells was also confirmed in five brains by using EYFP fluorescence and X-gal staining (data not shown). Scale bars: 200 μm for A-D; 20 μm for E-H,I-K.

caNotch$^+$ cells continue proliferating in the VZ without exiting the cell cycle and migrating away.

To determine whether all of the caNotch$^+$ cells were proliferating, we performed cumulative labeling of cells by exposing embryos to BrdU for 15 hours (Fig. 4F,G), which was long enough for the majority of progenitors to execute at least one cell cycle (Takahashi et al., 1995). The vast majority (90.1±2.6%) of the EYFP$^+$/caNotch$^+$ cells in the VZ were labeled with BrdU, indicating that nearly all of the caNotch$^+$ cells were proliferating.

The caNotch$^+$ cells exhibited morphologies typical of RGCs, which extended a long thin process to the pial surface (Fig. 4H). Almost all (94.7±1.3%) of the caNotch$^+$ cells were also immunoreactive with the RC2 antibody, a molecular marker for RGCs (Fig. 4I,J), confirming that the caNotch$^+$ cells were RGCs. EYFP$^+$ RGCs were also observed after transfection of *EYFP* alone (data not shown), but the ratio of RC2$^+$ cells in the EYFP$^+$ population was lower (71.8±2.9%), consistent with the generation of differentiating EYFP$^+$ cells from progenitors. Taken together with the above BrdU-labeling data, these findings further suggest that caNotch keeps progenitors proliferating, and suggest that the caNotch$^+$ cells may divide symmetrically without generating neurons, although we can not completely rule out the possibility that the caNotch$^+$ cells generate two types of progenitors.

To confirm that the increment of the BrdU$^+$ and RC2$^+$ ratios by caNotch was not caused by cell death of a subset of cells, we performed TUNEL assays (Fig. 4K-M). No significant increase in cell death was detected by *caNotch* transfection, confirming the maintenance of proliferative progenitors by caNotch.

Neurons are generated after the removal of caNotch activity

After *caNotch* transfection at E13.5, EYFP$^+$ cells remained in a thin area adjacent to the ventricle in the P5 cortex (Fig. 5A). At P15, EYFP$^+$ cells were positive for GFAP in layers adjacent to the ventricle (see Fig. S1 in the supplementary material), consistent with previous results obtained by retroviral expression of caNotch (Gaiano et al., 2000). Persistent expression of caNotch in the EYFP$^+$ cells was confirmed by immunostaining using an antibody against the FLAG peptide, which was fused to the caNotch protein (Fig. 5E-G). Subsequent transfection of a *Cre* expression vector at E15.5 generated EYFP$^+$/β-gal$^+$ neurons accumulating in layers II and III (Fig. 5B,C). No β-gal$^+$ cells were detected in brains transfected with pCAG-ExNotchZ alone (data not shown). The EYFP$^+$/β-gal$^+$ neurons were not stained with the anti-FLAG antibody (Fig. 5H), confirming that β-gal was expressed in caNotch-negative (caNotch$^-$) cells after the excision of *caNotch*. These results suggest that neurogenesis resumed after the removal of *caNotch*. The EYFP$^+$/β-gal$^+$ neurons were positive for Lhx2 (Fig. 5D,I-K), but not for ER81 (data not shown), indicating that the neurons acquired the specificity of layers II and III, with respect to not only laminar positions but also molecular markers. Similarly, transfection of pCAG-ExZ at E15.5 labeled Lhx2$^+$ neurons of layers II and III (Fig. 2). Therefore, these results suggest that progenitors skipped the generation of early-born neurons during Notch activation and started generating later-born neurons in accordance with the embryonic stage after the removal of caNotch activity.

To confirm that the selective formation of neurons specific to layers II and III after the removal of *caNotch* did not result from cell death of a subpopulation of cells that had expressed caNotch and Cre, TUNEL assays were performed as above. Apoptotic cells were not increased significantly after transfection of *caNotch* and *Cre* (Fig. 6), excluding the possibility that some types of cells were selectively eliminated. In addition, simultaneous transfection of *Cre* and pCAG-ExZ showed the same patterns of EYFP$^+$/β-gal$^+$ neuron distribution as did transfection of pCAG-ExZ (see Fig. S2 in the

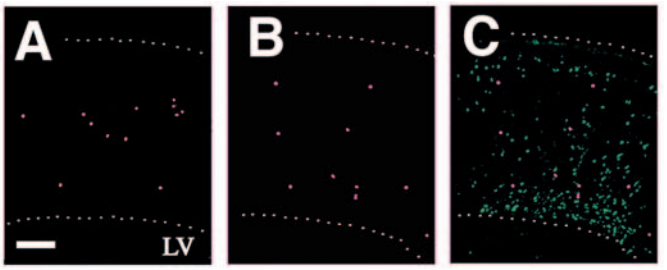

Fig. 6. Apoptosis is not enhanced by caNotch and Cre. We performed TUNEL assays for the E17.5 brain after double electroporation of pCAG-ExNotchZ at E13.5 and pXCANCre at E15.5. The areas of the double-transfected hemisphere (B,C) and the opposite non-transfected hemisphere (A) of an electroporated brain are shown. Green signals show EYFP fluorescence. All three panels show the same section; A and C are merged views. Similarly, no increase of apoptosis was observed at E16.5 (data not shown). Scale bar: 200 μm.

supplementary material), indicating that Cre expression alone did not affect the laminar fate.

Cotransfection of pCAG-ExNotchZ with *Cre* showed a small shift of EYFP$^+$/β-gal$^+$ neurons to upper layers (see Fig. S3 in the supplementary material), presumably because of transient activity of caNotch, which could be expressed until *caNotch* was excised by Cre and *caNotch* mRNA was degraded. Transfection of pCAG-ExZ at E17.5 labeled neurons in an upper area of layers II and III, and the number of EYFP$^+$ neurons was decreased (see Fig. S4A in the supplementary material), when compared with the transfection at earlier stages. Similarly, double transfection of pCAG-ExNotchZ at E13.5 and *Cre* at E17.5 showed a smaller number of EYFP$^+$ neurons in an upper area of layers II and III (see Fig. S4B,C in the supplementary material). These results suggest that the laminar fate of transfected neurons was affected by the length of time in which caNotch activity was present in progenitors.

Neurons of upper layers are born after *Cre* transfection

These results, taken together with the finding that the vast majority of caNotch$^+$ cells were proliferating progenitors, suggested that the Lhx2$^+$/β-gal$^+$ neurons were born after the removal of caNotch activity. However, we could not rule out the possibility that a minor population of the caNotch$^+$ cells had exited the cell cycle but did not migrate into the cortical plate until the removal of caNotch activity. To eliminate this possibility, we analyzed birth dates of the EYFP$^+$/β-gal$^+$ neurons. Cells were pulse-labeled with BrdU before and after *Cre* transfection and examined at P5.

One day before *Cre* transfection, BrdU was injected at E14.5. BrdU$^+$ cells were observed in layers II to V (Fig. 7). Neurons that were heavily-labeled with BrdU mostly occupied low layers, whereas many lightly-labeled neurons were scattered in the upper layers, consistent with the inside-out generation of neurons (Fig. 7A). Overall patterns of BrdU$^+$ cell distribution were not affected by caNotch (Fig. 7E), showing that corticogenesis was not severely perturbed by caNotch, presumably due to its restricted expression. The degree of BrdU labeling, which is revealed by fluorescence intensity, is indicative of birth dates of BrdU$^+$ cells. The cohort of cells that exit the cell cycle after incorporating BrdU will show maximum levels of fluorescence. Because the amount of BrdU incorporated in nuclei is reduced by half every cell division, successively weaker fluorescence will be detected in cells that have executed the cell cycle after BrdU labeling. Quantification of fluorescence distinguished at least two groups of BrdU-labeled cells: heavily labeled (RFI=100.0±4.3), and lightly

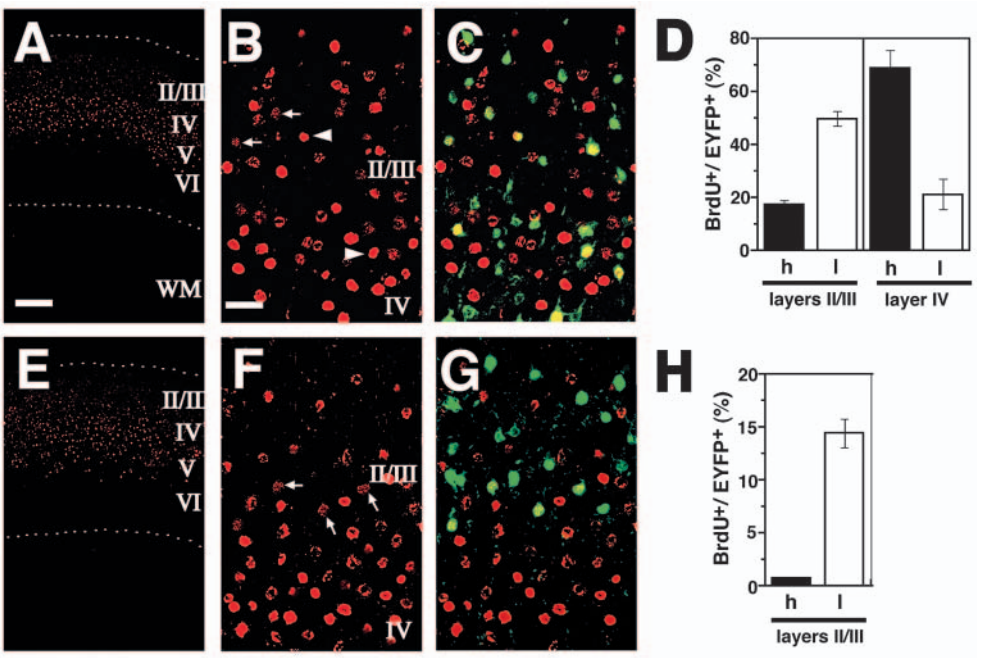

Fig. 7. Neurons of layers II and III are not born from caNotch$^+$ cells before *Cre* transfection. P5 cortices after electroporation of pCAG-EYFP (A-D) or pCAG-ExNotchZ (E-H) at E13.5, BrdU labeling at E14.5 and electroporation of pXCANCre at E15.5, are shown. Red and green signals show immunostaining with the anti-BrdU and anti-GFP antibodies, respectively. Arrowheads and arrows indicate neurons that were heavily and lightly labeled with BrdU, respectively. C and G are merged views of the sections shown in A and B, and E and F, respectively. (D,H) Ratios of neurons that were heavily (h) and lightly (l) labeled with BrdU in the EYFP$^+$ population. Each ratio was calculated from five brains. After *EYFP* transfection, layers II and III contained 17.4±1.6% heavily- and 49.8±2.9% lightly-labeled neurons, whereas layer IV contained 68.8±6.6% heavily- and 21.2±5.8% lightly-labeled neurons. *EYFP*/*caNotch* transfection showed 0.7±1.1% heavily and 14.0±1.3% lightly-labeled neurons in layers II and III. After *EYFP*/*caNotch* transfection, many EYFP$^+$ cells were not labeled with BrdU in the upper layers (85.8±1.8%), presumably because the amount of BrdU in nuclei was reduced to below the detection limit after multiple cell divisions. Scale bars: 200 μm for A,E; 20 μm for B,C,F,G.

labeled (RFI≤51.1±4.3). After *EYFP* transfection, many EYFP$^+$ neurons of layer IV were heavily labeled with BrdU (Fig. 7B-D), suggesting that these neurons were the first born cells after BrdU labeling. The ratio of EYFP$^+$ neurons that were lightly labeled with BrdU increased in upper layers, reflecting that the neurons were born after further rounds of cell divisions. By contrast, *caNotch* transfection resulted in no EYFP$^+$ neurons in layer IV, and many EYFP$^+$ neurons were lightly labeled with BrdU and localized in upper layers (Fig. 7F-H). These findings suggest that the EYFP$^+$/β-gal$^+$ neurons of layers II and III were not born from caNotch$^+$ progenitors before *Cre* transfection.

Conversely, to confirm that the EYFP$^+$/β-gal$^+$ neurons of layers II and III were born after *Cre* transfection, BrdU was injected at E16.5, one day after the transfection (Fig. 8). The populations of BrdU$^+$ neurons shifted to upper layers compared with those labeled by BrdU at E14.5 (Fig. 8A,E). *EYFP* transfection showed many EYFP$^+$ BrdU$^-$ neurons in layer IV, which were born before the BrdU pulse (Fig. 8C). Many EYFP$^+$ neurons of layers II and III were heavily labeled with BrdU after both transfections of *EYFP* and *EYFP*/*caNotch* (Fig. 8B-D,F-H). This result indicates that the EYFP$^+$/β-gal$^+$ neurons of layers II and III were born after *Cre* transfection. Moreover, BrdU-labeling patterns of EYFP$^+$ neurons of layers II and III were similar between transfections of *EYFP* and *EYFP*/*caNotch* (Fig. 8D,H), suggesting that progenitors double-transfected with *caNotch* and *Cre* acquired the same competence to generate neurons as normal progenitors. Taken together with the laminar fate and Lhx2 expression of the EYFP$^+$/β-gal$^+$ neurons after *Cre* transfection, this finding suggests that, after the removal of caNotch activity, the neurogenesis that resumed followed the normal time course of embryonic development.

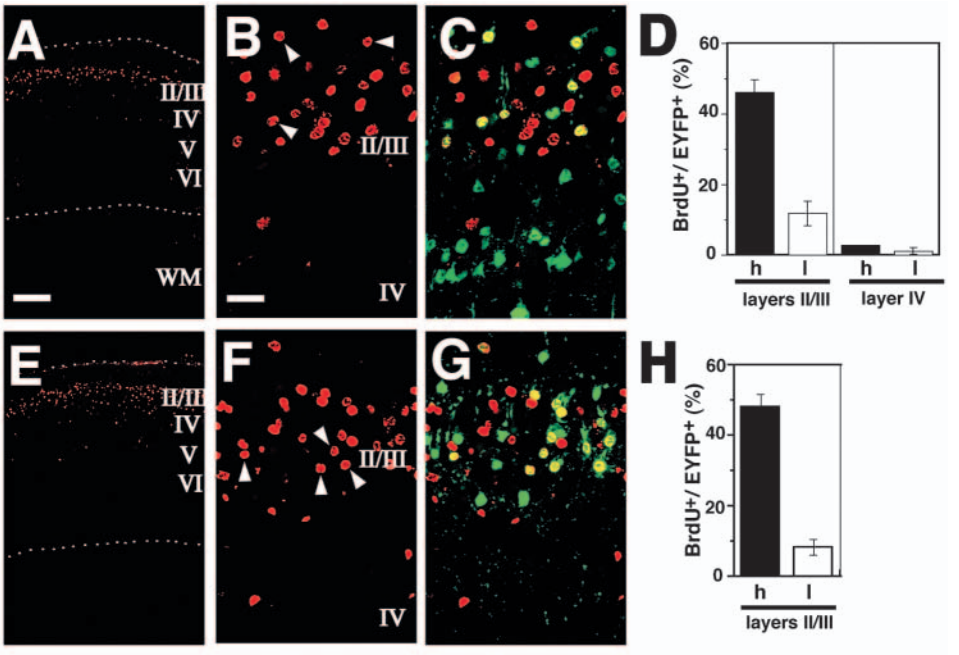

Fig. 8. EYFP$^+$/β-gal$^+$ neurons of layers II and III are born after *Cre* transfection. P5 cortices after double electroporation of pCAG-EYFP (A-D) or pCAG-ExNotchZ at E13.5 (E-H), and of pXCANCre at E15.5, and after BrdU labeling at E16.5, are shown. Red and green signals show immunostaining with the anti-BrdU and anti-GFP antibodies, respectively. Arrowheads indicate neurons that were heavily labeled with BrdU. C and G are merged views of the sections shown in A and B, and E and F, respectively. (D,H) Ratios of neurons that were heavily (h) and lightly (l) labeled with BrdU in the EYFP$^+$ population. Ratios were calculated from 5 brains. After *EYFP* transfection, layers II and III contained 46.1±3.7% heavily- and 12.0±3.4% lightly-labeled neurons, whereas layer IV contained 2.9±0.1% heavily- and 1.3±1.1% lightly-labeled neurons. *EYFP*/*caNotch* transfection showed 48.1±3.5% heavily- and 8.2±2.3% lightly-labeled neurons in layers II and III. Scale bars: 200 μm for A,E; 20 μm for B,C,F,G.

caNotch$^+$ progenitors change their potential during development

To clarify whether caNotch$^+$ progenitors lost the ability to generate low-layer neurons during development, mouse in utero transplantation was performed. We first examined the potential of E13.5 and E15.5 progenitors, by transplanting them into the E13.5 brain. Donor cells were prepared from the VZ of the somatosensory area, 3 hours or 2 days after transfection of pCAG-ExZ at E13.5 to label their progenies. E13.5 progenitors gave rise to neurons of layers II to V (Fig. 9A,E), whereas neurons from E15.5 progenitors occupied layers II and III (Fig. 9B,F). These results are in agreement with previous observations that later progenitors lose competence to generate low-layer neurons in ferret (Frantz and McConnell, 1996). Whereas transfection of pCAG-ExZ at E13.5 labeled neurons of layers II to VI (Fig. 2), transplanted E13.5 progenitors generated fewer neurons of layer VI. Neurons of layer VI, which were generated at E13.5, might be susceptible to the procedure of dissociation.

After transplantation into the E13.5 brain, progenitors double-transfected with *caNotch* at E13.5 and *Cre* at E15.5 gave rise to neurons of layers II and III (Fig. 9C,G), and the neurons were positive for Lhx2 (Fig. 9D, see also Fig. S5 in the supplementary material), suggesting that the double-transfected progenitors lost the potential to generate low-layer neurons. To confirm that the double-transfected progenitors generated neurons after transplantation, BrdU was injected at E14.5. Many β-gal$^+$ neurons were heavily labeled with BrdU (see Fig. S6 in the supplementary material), indicating that cells that were born after transplantation became upper-layer neurons. These results indicate that caNotch$^+$ progenitors lost the ability to generate low-layer neurons as embryonic development proceeded.

Discussion

The present study demonstrates that caNotch prevented cortical progenitors from generating neurons and kept progenitors proliferating. After the removal of caNotch activity,

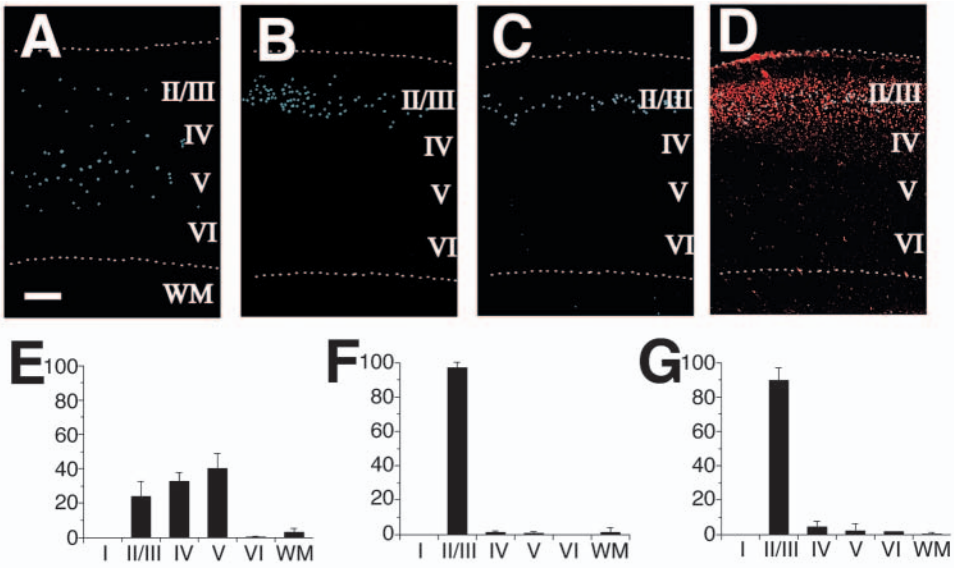

Fig. 9. caNotch$^+$ progenitors lose the ability to generate low-layer neurons. Shown are P5 cortices after transplantation at E13.5 of progenitors that had been prepared after electroporation of pCAG-ExZ at E13.5 (A,E), after double electroporation of pCAG-ExZ at E13.5 and pXCANCre at E15.5 (B,F), or after double electroporation of pCAG-ExNotchZ at E13.5 and pXCANCre at E15.5 (C,D,G). Blue and red signals show immunostaining with antibodies recognizing β-gal (A-D) and Lhx2 (D), respectively. C and D show the same section. D is a merged view. All β-gal$^+$ neurons were also positive for Lhx2. E15.5 progenitors single transfected with pCAG-ExZ at E13.5 showed the same results as did E15.5 progenitors double transfected with pCAG-ExZ at E13.5 and pXCANCre at E15.5 (data not shown), confirming that Cre did not affect the laminar fate of neurons. (E-G) Ratios of β-gal$^+$ neurons that were distributed to each layer of the cortex. Each ratio was calculated from 10 brains. Results were as follows. In E: layers II and III, 23.8±9.1%; layer IV, 32.7±5.1%; layer V, 40.1±8.7%; layer VI, 0.3±0.5%; and white matter (WM), 3.1±2.3%. In F: layers II and III, 97.1±3.5%; layer IV, 1.0±1.2%; layer V, 0.9±0.7%; and WM, 1.1±3.1%. In G: layers II and III, 89.7±7.4%; Layer IV, 4.3±3.5%; layer V, 2.0±4.2%; layer V1, 1.8±0.5%; and WM, 0.3±1.1%. Scale bar: 150 μm for A-D.

progenitors resumed generating neurons, the laminar fate of which matched the developmental stage of the embryos. These findings indicate that caNotch$^+$ progenitors maintained neurogenic potential but lost the ability to generate low-layer neurons as embryonic development proceeded. Transplantation analysis indicates that the developmental state of progenitors did not pause with caNotch activity, even though progenitors did not generate neurons.

The laminar fate of cortical neurons is determined by intrinsic properties of progenitors and extracellular signals. Previous transplantation experiments have shown that early progenitors are competent to generate both low- and upper-layer neurons, whereas late progenitors lose competence to generate low-layer neurons (McConnell and Kaznowski, 1991; Frantz and McConnell, 1996). Our results indicate that caNotch$^+$ progenitors become competent to generate upper-layer neurons after the removal of caNotch activity. Moreover, the use of molecular markers revealed that these upper-layer neurons were correctly specified as neurons of layers II and III. Although we focused on the primary somatosensory area in this study because of the availability of markers, the generation of upper-layer neurons instead of low-layer neurons after the removal of *caNotch* was also observed in other areas of the cortex (data not shown). Thus, the effect of caNotch and the resumption of neurogenesis were not specific to the primary somatosensory area. These findings corroborate that specification of laminar fate involves extracellular cues that depend on the developmental stage. In *Drosophila*, different types of neurons are generated by asymmetric divisions of neuroblasts, which alter the expression of intrinsic factors successively every division. Recently, continuous expression of *Hunchback*, which is expressed by neuroblasts at early stages, has been shown to keep neuroblasts competent to generate early-born neurons (Pearson and Doe, 2003). In early corticogenesis, Foxg1 controls production of low-layer neurons by suppressing the generation of CR cells (Hanashima et al., 2004). E15.5 progenitors lost the ability to generate low-layer neurons, suggesting that intrinsic factors in progenitors may also be important for the specification of neurons of layers II to VI.

Many cell fates are determined through asymmetric divisions, when two daughter cells with distinct fates are generated. Asymmetric distribution of cell fate determinants is crucial for proper differentiation of daughter cells. Asymmetric divisions may also affect the state of progenitors. In our present work, inhibition of neurogenesis and maintenance of proliferation of cortical progenitors by caNotch suggest that caNotch$^+$ progenitors may have undergone symmetric divisions. Although it was not possible to directly determine that the two daughter cells from a caNotch$^+$ progenitor shared the same molecular properties, neurogenesis was synchronous with embryonic development when it resumed. This also appeared to occur uniformly, further supporting symmetric divisions of caNotch$^+$ progenitors. Therefore, these results suggest that temporal inhibition of asymmetric divisions may not affect subsequent neurogenesis of cortical progenitors.

The maintenance of cortical progenitors by caNotch is consistent with previous results obtained from in vitro cell cultures using loss-of-function mutants of Hes1 and RBP-Jκ, which are downstream effectors of the Notch receptor (Nakamura et al., 2000; Hitoshi et al., 2002). The Notch pathway is activated by extracellular ligands such as Delta-like and Jagged (for a review, see Lai, 2004). Considering that a large number of neurons are generated from many progenitors simultaneously in the mammalian cerebral cortex, a flexible way to control the number of generated neurons using the balance between symmetric and asymmetric divisions of progenitors may be a more advantageous mechanism than the fixed sequential generation of neurons in *Drosophila*. β-Catenin signals have also been shown to regulate the balance

between symmetric and asymmetric divisions of cortical progenitors (Chenn and Walsh, 2002). However, some neurons still differentiate from progenitors in which β-catenin signaling is active. By contrast, few neurons were generated from caNotch$^+$ progenitors. This may suggest that the β-catenin signaling pathway is different from the Notch pathway, even if they have similar effects on progenitors. Mice carrying gain-of-function mutations in the β-catenin signaling pathway exhibit severe malformation in the cortex (Chenn and Walsh, 2002). *caNotch* transfection showed milder effects on the morphology of the cortex, presumably because of its spatiotemporally restricted expression.

The Notch pathway controls many cellular differentiation programs depending on the developmental context. One of the well-known roles of the Notch pathway is to keep progenitors in the undifferentiated state (for a review, see Artavanis-Tsakonas et al., 1999). Injection of *caNotch* mRNA into *Xenopus* embryos has suggested the maintenance of progenitors by caNotch (Coffman et al., 1993). However, control of the timing of caNotch activity has been difficult. Our experimental system using in vivo electroporation and Cre recombinase partly overcame this difficulty and revealed the resumption of neurogenesis after the removal of caNotch activity. Notch activation has been also implicated in gliogenesis (for a review, see Gaiano and Fishell, 2002). Transient Notch activation irreversibly switches neural crest stem cells to a Schwann cell fate (Morrison et al., 2000). Retroviral expression of caNotch leads to the formation of RGCs (Gaiano et al., 2000), but it was not clear whether RGCs generated by forced expression of caNotch have neurogenic potential. Our findings indicate that cortical progenitors maintain neurogenic potential even after being kept as RGCs by caNotch. This contrasts with the irreversible switching of neural crest stem cells. These different actions of the Notch pathway may be explained by the difference in cellular contexts. In *Drosophila*, the Notch pathway regulates *gcm*, which is crucial for glial differentiation, positively for subperineurial glia (Udolph et al., 2001) and negatively for peripheral sensory organs (Van De Bor and Giangrande, 2001). It will be important to identify cellular factors that are involved in different actions of the Notch pathway.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/6/1295/DC1

References

Anthony, T. E., Klein, C., Fishell, G. and Heintz, N. (2004). Radial glia serve as neuronal progenitors in all regions of the central nervous system. *Neuron* **41**, 881-890.

Arber, S., Ladle, D. R., Lin, J. H., Frank, E. and Jessell, T. M. (2000). ETS gene Er81 controls the formation of functional connections between group Ia sensory afferents and motor neurons. *Cell* **101**, 485-498.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Bayer, S. A. and Altman, J. (1991). Neocortical development. New York: Raven Press.

Chenn, A. and McConnell, S. K. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82**, 631-641.

Chenn, A. and Walsh, C. (2002). Regulation of cerebral cortical size by control of cell cycle exit in neural precursors. *Science* **297**, 365-369.

Coffman, C. R., Skoglund, P., Harris, W. A. and Kintner, C. R. (1993). Expression of an extracellular deletion of *Xotch* diverts cell fate in Xenopus embryos. *Cell* **73**, 659-671.

Desai, A. R. and McConnell, S. K. (2000). Progressive restriction in fate potential by neural progenitors during cerebral cortical development. *Development* **127**, 2863-2872.

Frantz, G. D. and McConnell, S. K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron* **17**, 55-61.

Gaiano, N. and Fishell, G. (2002). The role of Notch in promoting glial and neural stem cell fates. *Annu. Rev. Neurosci.* **25**, 471-490.

Gaiano, N., Nye, J. S. and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26**, 395-404.

Gupta, A., Tsai, L. H. and Wynshaw-Boris, A. (2002). Life is a journey: a genetic look at neocortical development. *Nat. Rev. Genet.* **3**, 342-355.

Hanashima, C., Li, S. C., Shen, L., Lai, E. and Fishell, G. (2004). Foxg1 suppresses early cortical cell fate. *Science* **303**, 56-59.

Hitoshi, S., Alexson, T., Tropepe, V., Donoviel, D., Elia, A. J., Nye, J. S., Conlon, R. A., Mak, T. W., Bernstein, A. and van der Kooy, D. (2002). Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev.* **16**, 846-858.

Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* **106**, 511-521.

Justice, N. J. and Jan, Y. N. (2002). Variations on the Notch pathway in neural development. *Curr. Opin. Neurobiol.* **12**, 64-70.

Kanegae, Y., Lee, G., Sato, Y., Tanaka, M., Nakai, M., Sakaki, T., Sugano, S. and Saito, I. (1995). Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. *Nucl. Acids Res.* **23**, 3816-3821.

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-973.

Levitt, P., Barbe, M. F. and Eagleson, K. L. (1997). Patterning and specification of the cerebral cortex. *Annu. Rev. Neurosci.* **20**, 1-24.

Liem, K. F., Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFβ-related proteins in neuronal pattering in the dorsal spinal cord. *Cell* **91**, 127-138.

Ling, L. and Vaessin, H. (2000). Pan-neuronal prospero terminates cell proliferation during Drosophila neurogenesis. *Genes Dev.* **14**, 147-151.

Luskin, M. B., Pearlman, A. L. and Sanes, J. R. (1988). Cell lineage in the cerebral cortex of the mouse studied in vivo and in vitro with a recombinant retrovirus. *Neuron* **1**, 635-647.

Malatesta, P., Hartfuss, E. and Götz, M. (2000). Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. *Development* **127**, 5253-5263.

Malatesta, P., Hack, M. A., Harfuss, E., Kettenmann, H., Klinkert, W., Kirchhoff, F. and Götz, M. (2003). Neuronal or glial progeny: regional differences in radial glia fate. *Neuron* **37**, 751-764.

Marín, O. and Rubenstein, J. L. R. (2003). Cell migration in the forebrain. *Annu. Rev. Neurosci.* **26**, 441-483.

McConnell, S. K. (1995). Constructing the cerebral cortex: neurogenesis and fate determination. *Neuron* **15**, 761-768.

McConnell, S. K. and Kaznowski, C. E. (1991). Cell cycle dependence of laminar determination in developing neocortex. *Science* **254**, 282-285.

Miyata, T., Kawaguchi, A., Okano, H. and Ogawa, M. (2001). Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron* **31**, 727-741.

Morrison, S. J., Perez, S. E., Qiao, Z., Verdi, J. M., Hicks, C., Weinmaster, G. and Anderson, D. J. (2000). Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell* **101**, 499-510.

Nakagawa, Y., Johnson, J. E. and O'Leary, D. M. J. (1999). Graded and areal expression patterns of regulatory genes and cadherins in embryonic neocortex independent of thalamocortical input. *J. Neurosci.* **19**, 10877-10885.

Nakamura, Y., Sakakibara, S., Miyata, T., Ogawa, M., Shimazaki, T., Weiss, S., Kageyama, R. and Okano, H. (2000). The bHLH gene hes1 as

a repressor of the neuronal commitment of CNS stem cells. *J. Neurosci.* **20**, 283-293.

Niwa, H., Yamamura, K. and Miyazaki, J. (1991). Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-200.

Noctor, S. C., Flint, A. C., Weissman, T. A., Dammerman, R. S. and Kriegstein, A. R. (2001). Neurons derived from radial glial cells establish radial units in neocortex. *Nature* **409**, 714-720.

Noctor, S. C., Flint, A. C., Weissman, T. A., Wong, W. S., Clinton, B. K. and Kriegstein, A. R. (2002). Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. *J. Neurosci.* **22**, 3161-3173.

Nye, J. S., Kopan, R. and Axel, R. (1994). An activated Notch suppresses neurogenesis and myogenesis but not gliogenesis in mammalian cells. *Development* **120**, 2421-2430.

Pearson, B. J. and Doe, C. Q. (2003). Regulation of neuroblast competence in *Drosophila*. *Nature* **425**, 624-628.

Price, J. and Thurlow, L. (1988). Cell lineage in the rat cerebral cortex: a study using retroviral-mediated gene transfer. *Development* **104**, 473-482.

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *J. Comp. Neurol.* **145**, 61-84.

Reid, C. B., Liang, I. and Walsh, C. (1995). Systematic widespread clonal organization in cerebral cortex. *Neuron* **15**, 299-310.

Rétaux, S., Rogard, M., Bach, I., Failli, V. and Besson, M.-J. (1999). Lhx9: a novel LIM-homeodomain gene expressed in the developing forebrain. *J. Neurosci.* **19**, 783-793.

Ryu, M. S., Jan, L. Y. and Jan, Y. N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell* **76**, 477-491.

Saba, R., Nakatsuji, N. and Saito, T. (2003). Mammalian BarH1 confers commissural neuron identity on dorsal cells in the spinal cord. *J. Neurosci.* **23**, 1987-1991.

Saito, T. and Nakatsuji, N. (2001). Efficient gene transfer into the embryonic mouse brain using in vivo electroporation. *Dev. Biol.* **240**, 237-246.

Takahashi, T., Nowakowski, R. S. and Caviness, V. S., Jr (1995). The cell cycle of the pseudostratified ventricular epithelium of the embryonic murine cerebral wall. *J. Neurosci.* **15**, 6046-6057.

Takahashi, T., Nowakowski, R. S. and Caviness, V. S., Jr (1996). The leaving or Q fraction of the murine cerebral proliferative epithelium: a general model of neocortical neurogenesis. *J. Neurosci.* **16**, 6183-6196.

Takahashi, T., Goto, T., Miyama, S., Nowakowski, R. S. and Caviness, V. S., Jr (1999). Sequence of neuron origin and neocortical laminar fate: relation to cell cycle of origin in the developing murine cerebral wall. *J. Neurosci.* **19**, 10357-10371.

Udolph, G., Rath, P. and Chia, W. (2001). A requirement for Notch in the genesis of a subset of glial cells in the Drosophila embryonic central nervous system which arise through asymmetric divisions. *Development* **128**, 1457-1466.

Van De Bor, V. and Giangrande, A. (2001). Notch signaling represses the glial fate in fly PNS. *Development* **128**, 1381-1390.

Walsh, C. and Cepko, C. L. (1988). Clonally related cortical cells show several migration patterns. *Science* **241**, 1342-1345.

Weimann, J. M., Zhang, Y. A., Levin, M. E., Devine, W. P., Brület, P. and McConnell, S. K. (1999). Cortical neurons require Otx1 for the refinement of exuberant axonal projections to subcortical targets. *Neuron* **24**, 819-831.

Wichterle, H., Turnbull, D. H., Nery, S., Fishell, G. and Alvarez-Buylla, A. (2001). In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development* **128**, 3759-3771.