Neuron, Vol. 26, 395–404, May, 2000, Copyright ©2000 by Cell Press

Radial Glial Identity Is Promoted by Notch1 Signaling in the Murine Forebrain

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Summary

In vertebrates, Notch signaling is generally thought to inhibit neural differentiation. However, whether Notch can also promote specific early cell fates in this context is unknown. We introduced activated Notch1 (NIC) into the mouse forebrain, before the onset of neurogenesis, using a retroviral vector and ultrasound imaging. During embryogenesis, NIC-infected cells became radial glia, the first specialized cell type evident in the forebrain. Thus, rather than simply inhibiting differentiation, Notch1 signaling promoted the acquisition of an early cellular phenotype. Postnatally, many NIC-infected cells became periventricular astrocytes, cells previously shown to be neural stem cells in the adult. These results suggest that Notch1 promotes radial glial identity during embryogenesis, and that radial glia may be lineally related to stem cells in the adult nervous system.

Introduction

During mammalian development the anterior neural plate is transformed from a homogeneous sheet of neural progenitors into the brain, a highly complex structure with two primary cell types, neurons and glia (Ramon y Cajal, 1911). Studies characterizing the timing of neuronal and glial differentiation have found that, for the most part, neurogenesis precedes gliogenesis (Boulder Committee, 1970). It has long been recognized, however, that one specialized glial cell type, the radial glia, violates this temporal order and appears prior to, or concurrent with, the first neurons (Choi and Lapham, 1978; Levitt et al., 1983). Radial glia serve as the scaffold along which newborn neurons migrate, and as such are of fundamental importance during neurogenesis (Rakic, 1971, 1972, 1988; Hatten, 1990; Misson et al., 1991).

To investigate the molecular mechanisms controlling cell fate specification in the mammalian forebrain, we chose to consider the role of Notch1 signaling in this process. The Notch family of proteins includes four known vertebrate homologs and several ligands encoded by the *Delta* and *Jagged* gene families (Nye and Kopan, 1995; Weinmaster, 1997; Nye, 1999). Notch signaling has been found to influence cell fate decisions in numerous vertebrate and invertebrate model systems (Weinmaster, 1997; Artavanis-Tsakonas et al., 1999). Both *Notch1* and its ligand *Delta-like-1* (*Dll1*) are expressed in the ventricular zone (VZ) of the developing brain, consistent with a role in the generation of cell diversity in this region (Lindsell et al., 1996).

The role of Notch signaling in vertebrates has been directly addressed both through mutant analyses and gain-of-function studies (Ishibashi et al., 1994, 1995; Nye et al., 1994; Dorsky et al., 1995; Oka et al., 1995; Lardelli et al., 1996; Bao and Cepko, 1997; de la Pompa et al., 1997; Hrabe de Angelis et al., 1997). Mutations in several members of the Notch pathway in mice have found that in the absence of Notch signaling neural progenitors express early neuronal markers prematurely (de la Pompa et al., 1997). Conversely, gain-of-function studies have found that constitutive Notch signaling inhibits progenitor differentiation and may expand the progenitor pool (Ishibashi et al., 1994; Nye et al., 1994; Dorsky et al., 1995; Lardelli et al., 1996; Bao and Cepko, 1997). While informative, this body of work has not revealed whether cells in which Notch is activated may acquire a defined cellular identity other than "undifferentiated progenitor." Such a possibility is consistent with work in invertebrates suggesting that, in some cases, activation of Notch leads to defined cell fates, albeit through the inhibition of other cell fates (Artavanis-Tsakonas et al., 1999). Several lines of evidence in vertebrate systems, both in vivo and in vitro, have found that cells possessing constitutive Notch signaling can adopt glial fates (Nye et al., 1994; Dorsky et al., 1995; Bao and Cepko, 1997). However, this work has primarily concluded that Notch signaling does not inhibit these cell fates (i.e., Müller cells in the retina, astrocytes), and that they are terminal and/or default fates.

To further investigate the role of the Notch pathway in the developing telencephalon, we employed a recently developed in vivo gain-of-function approach in the mouse (Gaiano et al., 1999). Using high-titer retroviral vectors and ultrasound imaging to guide injections in utero, we introduced an activated form of mouse Notch1 (NIC) into telencephalic progenitors at embryonic day 9.5 (E9.5). We found that cells infected with NIC acquired the morphology and molecular characteristics of radial glia. As radial glia are among the first cell types present in the developing forebrain, this result demonstrates that Notch1 activation leads to the acquisition of a distinct primary cellular phenotype. Postnatally, these cells transformed into astrocytes, consistent with the previously described fate of radial glia (Schmechel and Rakic, 1979a; Voigt, 1989), and with work which has found that astrocytic differentiation is not inhibited by NIC (Nye et al., 1994) (C. Chambers, N. G., G. F., and J. S. N., submitted). In addition, NIC-infected cells were found in large clusters adjacent to the lateral ventricles. Cells in these clusters expressed glial fibrillary acidic

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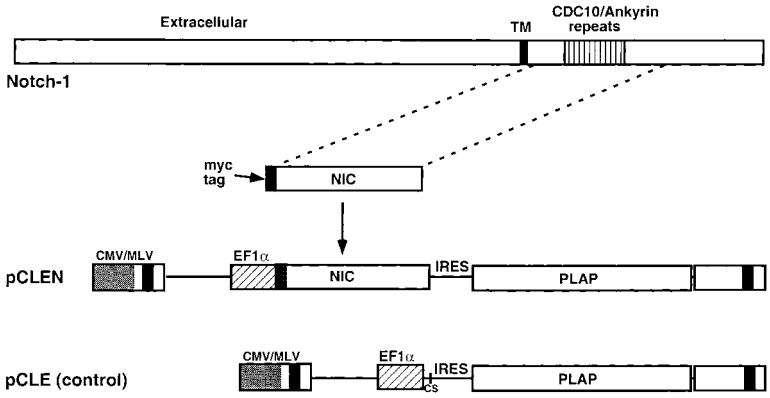

Figure 1. Retroviral Vectors and Notch

pCLE and pCLEN include a modified 5′ long terminal repeat (CMV/MLV) to obtain higher titers, as well as the *Xenopus* EF1α enhancer/promoter to improve vector expression in the brain. In addition, the vectors are dicistronic and utilize an internal ribosomal entry sequence (IRES) to allow translation of the reporter gene, human placental alkaline phosphatase (PLAP). The portion of mouse Notch1 (NIC) used (indicated with dotted lines) spans residues 1753–2185, and has a Myc tag at the N terminus. Abbreviations: cs, cloning site; TM, transmembrane.

protein (GFAP), and as such resembled recently described neural stem cells in the adult (Doetsch et al., 1999).

Results

Notch Induces Radial Glia In Vivo

To assess the effect of constitutive Notch1 signaling on telencephalic progenitors, we designed a retroviral vector that expresses a portion of the intracellular domain of murine Notch1 (Figure 1). Expression of NIC has been shown in multiple systems to result in ligand-independent signaling (Rebay et al., 1993; Roehl and Kimble, 1993; Struhl et al., 1993; Nye et al., 1994). The retroviral vector used included the *Xenopus* EF1α enhancer/promoter, which has recently been shown to substantially improve viral expression in the brain (Gaiano et al., 1999). Concentrated stocks of control (CLE) and NIC-expressing viruses (CLEN) were injected into the telencephalic vesicles of embryonic day 9.5 (E9.5) mouse embryos, a time before the generation of the first postmitotic cells in this region. For both viruses, comparable titers of 1–3 $\times$ 10^8 cfu/ml were injected. The injection site was visualized using ultrasound backscatter microscopy as previously described (Olsson et al., 1997; Liu et al., 1998; Gaiano et al., 1999).

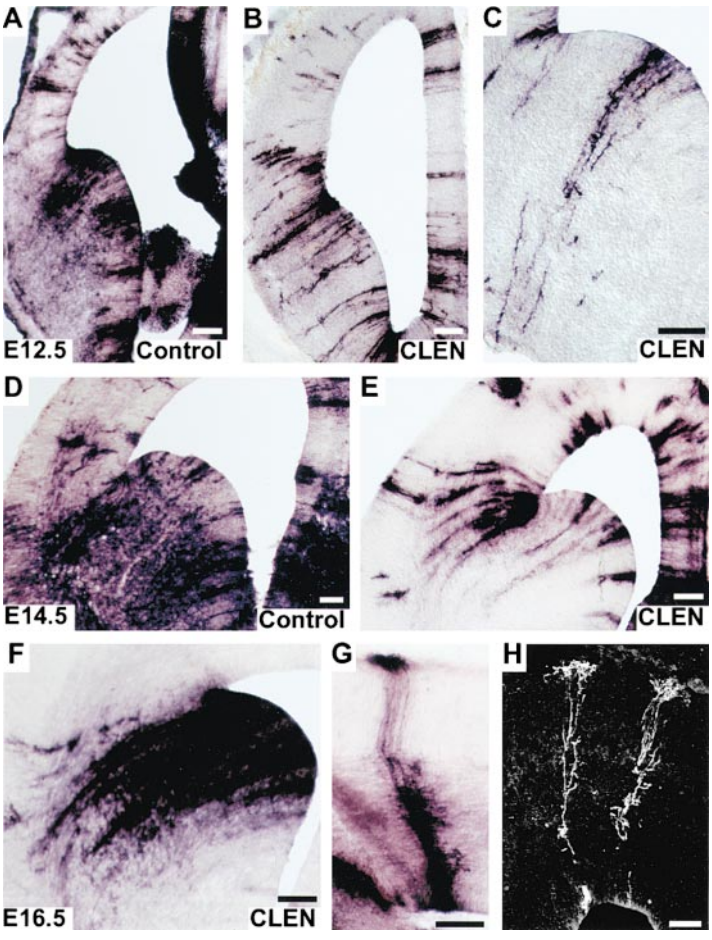

Figure 2. CLEN-Infected Cells Resemble Radial Glia In Vivo

Virally infected cells were identified histochemically by detection of the reporter PLAP at E12.5 (A–C), E14.5 (D and E), and E16.5 (F and G). In control samples (A and D) many labeled cells are dispersed throughout the tissue, while in CLEN samples (B, C, and E–G) labeled cells are typically in clusters adjacent to the ventricular surface and possess long radial processes reminiscent of radial glia. Such clusters are found both ventrally (F) and dorsally (G). Immunofluorescence to detect PLAP reveals the radial glial morphology of CLEN-infected cells in the neocortex at E16.5 (H). Scale bars: 200 μm (A–G), 50 μm (H).

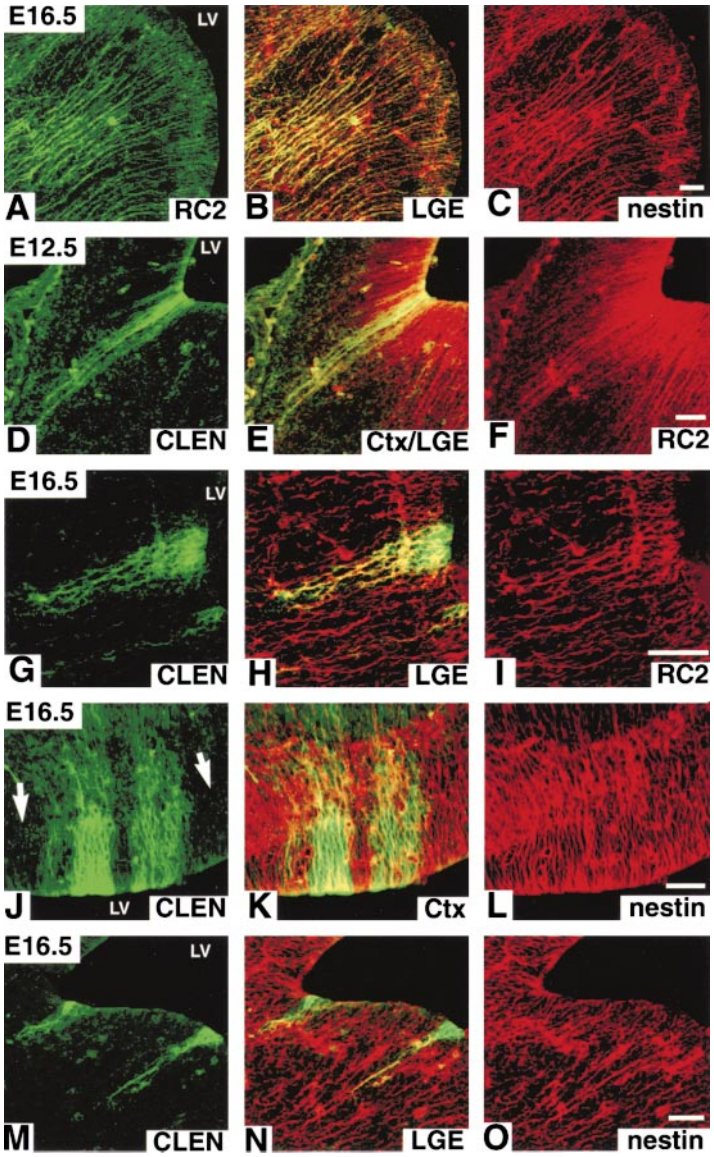

Figure 3. CLEN-Infected Cells Express RC2 and Nestin

Double immunofluorescence performed on uninfected tissue shows that RC2 and nestin are expressed in overlapping patterns in radial fibers (A–C). CLEN-infected cells were identified by immunolabeling of PLAP, and these cells were found to double label with RC2 (D–I) and nestin (J–O). In (J) the border between the ventricular and subventricular zones is indicated (arrows). Abbreviations: Ctx, neocortex; LGE, lateral ganglionic eminence; LV, lateral ventricle. Scale bars, 50 μm.

Injected embryos were sectioned at E12.5, E14.5, and E16.5, and were stained histochemically to detect the alkaline phosphatase reporter gene (PLAP) included in the vectors (Gaiano et al., 1999). At all ages examined, there was a dramatic difference in the appearance of cells infected with CLE (control) and CLEN (NIC-expressing) viruses. While CLE-infected cells were dispersed throughout the brain, CLEN-infected cells were typically found in clusters abutting the ventricular surface (Figure 2). Three days after infection (E12.5), these clusters were small and isolated cells were often evident (Figure 2B). At later times, however, these clusters appeared to increase in size and density, suggesting that they were proliferatively active (Figures 2E–2G). Strikingly, most CLEN-infected cells resembled radial glia, possessing long processes that extended toward the pial surface. Such morphologies were found in the cortex, ganglionic eminences, and septum, as well as in the diencephalon (data not shown). In CLE-infected samples, only a small subset of the total labeled population possessed radial glial morphologies (Figure 2D).

In addition to their distinctive morphology, radial glia can be identified by numerous molecular markers. These include the canonical radial glial marker RC2 (Figure 3A) (Misson et al., 1988), the intermediate filament nestin (Figure 3C) (Frederiksen et al., 1988; Lendahl et al., 1990), and brain lipid-binding protein (BLBP) (Feng et al., 1994; Feng and Heintz, 1995), a gene which is upregulated in radial glia upon neuronal contact. Furthermore, radial glia express regionally restricted markers such as Pax6 dorsally (Gotz et al., 1998) and cellular retinoid binding protein I (CRBP) ventrally (Toresson et al., 1999). CLEN-infected cells, detected by immunostaining for the PLAP reporter, were found to express RC2 (Figures 3D–3I), nestin (Figures 3J–3O), and BLBP (Figure 4). Interestingly, these cells often expressed BLBP precociously, or at higher levels than nearby uninfected cells, suggesting that Notch1 signaling actively led to the upregulation of this radial glial marker (Figure 4). CLEN-infected cells also expressed Pax6 and CRBP in patterns consistent with the sites of infection (Figures 5A–5F), suggesting that NIC does not impair the ability of infected cells to

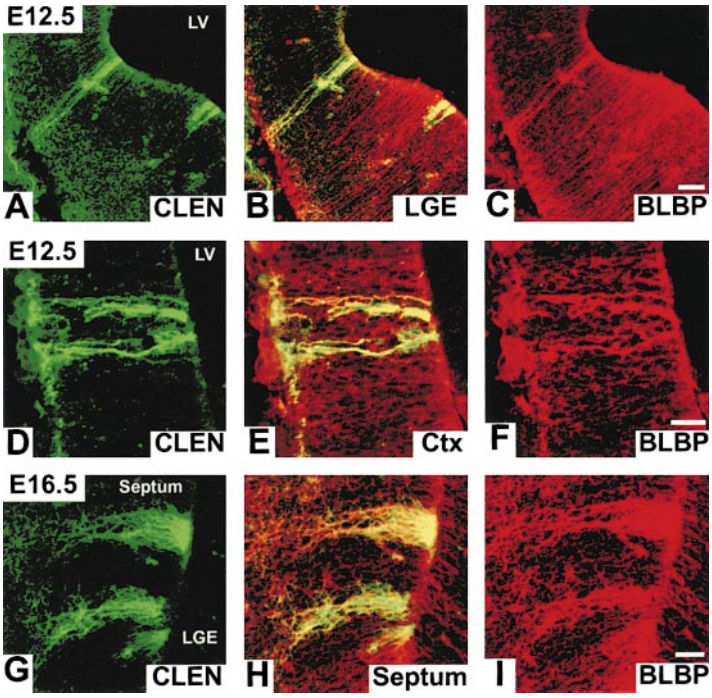

Figure 4. Upregulation of BLBP in CLEN-Infected Cells

Increased expression of BLBP occurred both dorsally (D–F) and ventrally in CLEN-infected cells (A–C and G–I). Double-labeled radial processes that span from the ventricular surface to the pia are evident (A–F). Abbreviations: Ctx, neocortex; LGE, lateral ganglionic eminence; VZ, ventricular zone; LV, lateral ventricle. Scale bars: 50 μm (A–C), 25 μm (D–I).

respond to regional cues. While CLEN-infected cells expressed the radial glial markers listed, these cells did not express the neuronal marker TuJ1 (Figures 5G–5I). Thus, marker expression, together with the morphology of CLEN-infected cells, strongly suggested that these cells have become radial glia. Control CLE-infected cells were not found to express any of these markers in unusual patterns, indicating that expression of PLAP alone does result in a prevalence of radial glial gene expression (see supplemental Figure C at http://www/neuron.org/cgi/content/full/26/2/395/DC1). This observation is consistent with previous work which has shown that many CLE-infected cells become neurons postnatally (Gaiano et al., 1999).

Previous work has found that a subpopulation of radial glia are quiescent during neurogenesis (Schmechel and Rakic, 1979b). Therefore, we investigated the proliferative behavior of CLEN-infected cells in vivo. Infected embryos were saturation labeled with BrdU at E14.5 and E16.5, and sections were stained to determine whether

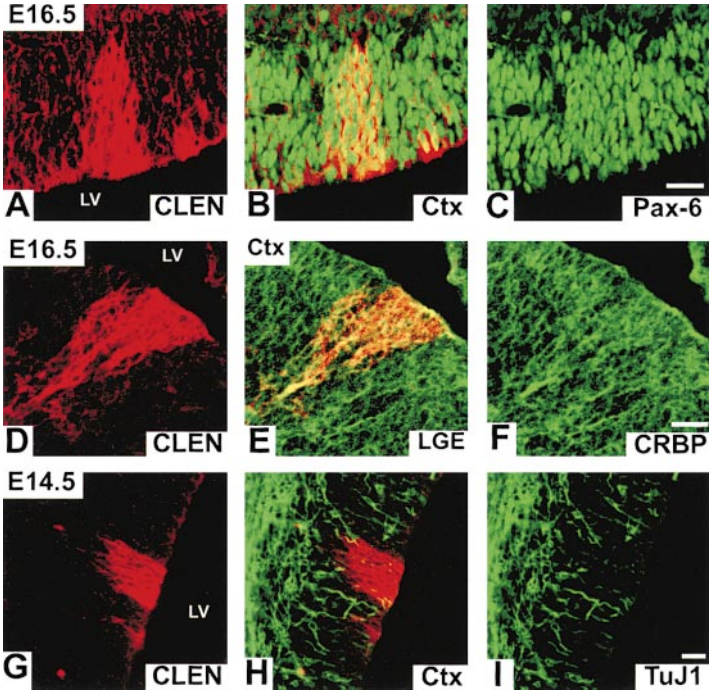

Figure 5. CLEN-Infected Cells Express Regional Telencephalic Markers but Not Neuronal Markers

Dorsal clusters of CLEN-infected cells in the telencephalon express the dorsal marker Pax6 (A–C), while ventral clusters express the ventral marker CRBP (D–F). CLEN clusters were never seen to express the neuronal marker TuJ1 (G–I). Note that in (A) through (C) and (G) through (I), as with many clusters in the rostral neocortex, the coronal plain of sectioning often obscures the radial morphology. Such clusters are clearly radial when sectioned sagittally. Abbreviations: Ctx, neocortex; LGE, lateral ganglionic eminence; LV, lateral ventricle. Scale bars, 25 μm.

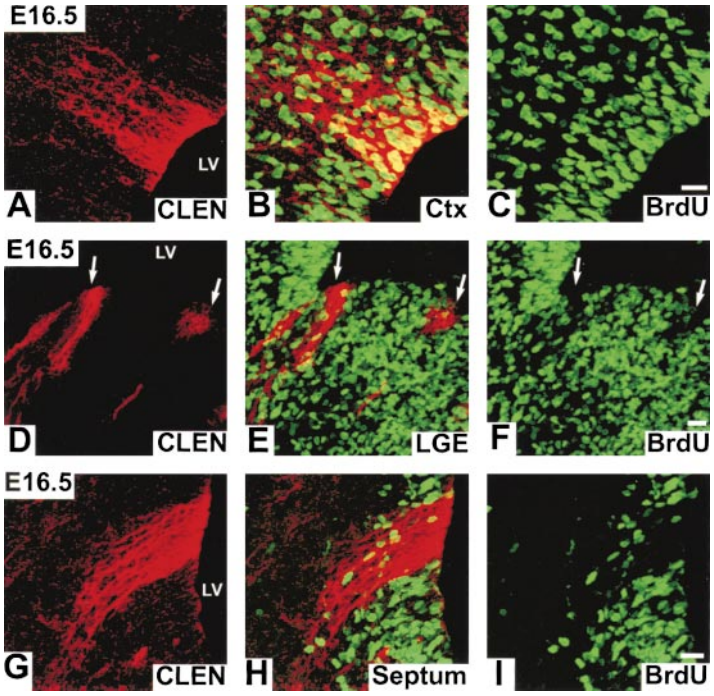

Figure 6. Proliferative Activity of CLEN-Infected Clusters

Animals saturation labeled with BrdU were double labeled to detect PLAP in infected cells (A, D, and G) and BrdU$^+$ cells (C, F, and I). While some infected clusters were found to be proliferatively active (B), others appeared to be largely quiescent (H) (arrows in [E]). Abbreviations: Ctx, neocortex; LGE, lateral ganglionic eminence; LV, lateral ventricle. Scale bars, 25 μm.

CLEN-infected clusters were BrdU$^+$. At both ages, many clusters contained large numbers of BrdU$^-$ cells (Figures 6D–6I and data not shown). This result was quite heterogeneous, however, with clusters ranging from nearly all cells being BrdU$^+$ (Figures 6A–6C) to nearly all cells being BrdU$^-$ (Figures 6D–6I). Triple labeling confirmed that these clusters expressed RC2, regardless of their proliferative status (data not shown). Therefore, the proliferative behavior of CLEN-infected cells was consistent with the previous observation that a subpopulation of radial glia are quiescent (Schmechel and Rakic, 1979b). Preliminary observations suggest that more CLEN-infected cells are proliferatively active at E14.5 than at E16.5, indicating that as neurogenesis proceeds, more of these cells are becoming quiescent (see Discussion).

Both the marker expression and BrdU labeling presented above support the hypothesis that Notch signaling plays a role in promoting radial glial fate. To confirm that Notch1 protein is endogenously expressed in radial glia, we triple labeled uninfected samples with antibodies to Notch1, RC2, and nestin. Notch1 protein was

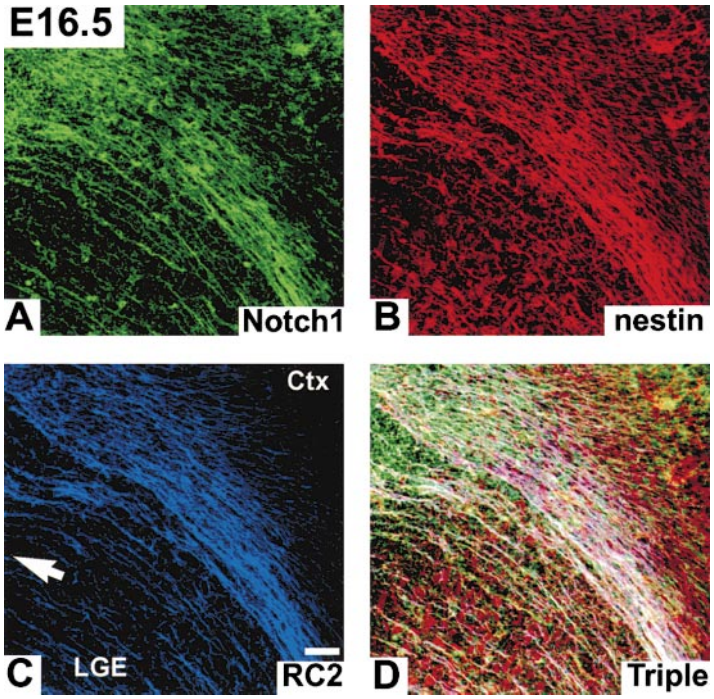

Figure 7. Endogenous Notch1 Protein Is Expressed in Radial Glial Fibers

The glial palisade between the lateral ganglionic eminence (LGE) and the neocortex (Ctx) in an uninfected brain is shown. The location of the lateral ventricle with respect to the field shown is indicated (arrow in [C]). Radial fibers expressing Notch1 are evident (A), and these fibers express both nestin (B) and RC2 (C). Triple-labeled fibers appear white when overlaid (D). Scale bar, 50 μm.

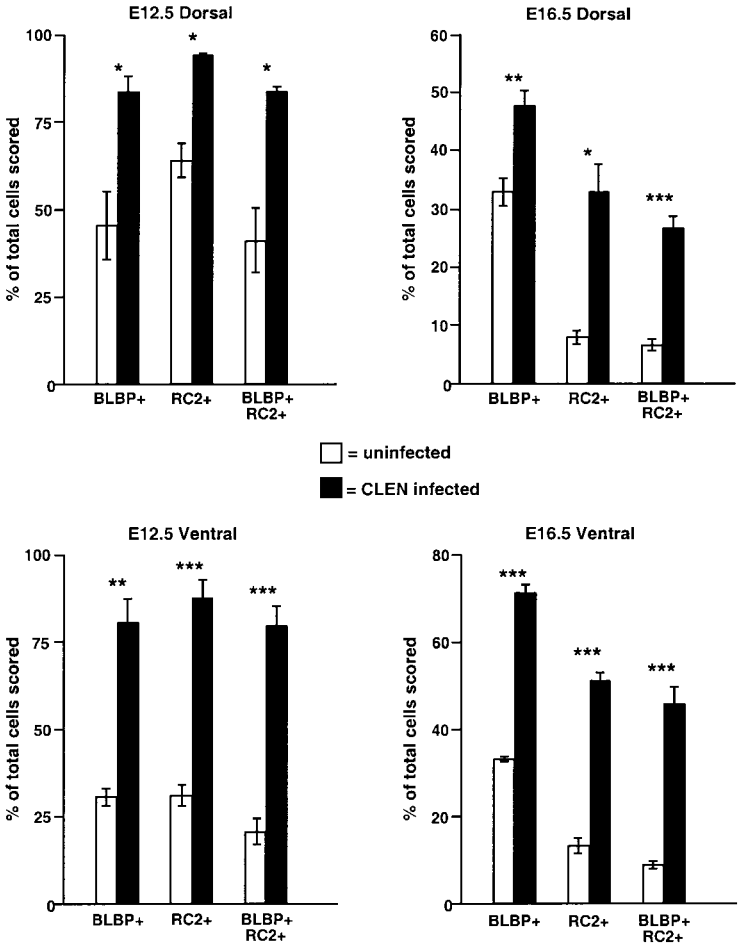

Figure 8. Quantification of Marker Expression In Vitro

A comparison of RC2 and BLBP expression in uninfected (see Results) and CLEN-infected cells shows that at both E12.5 and E16.5, both dorsally and ventrally, a significantly higher percentage of CLEN-infected cells expressed these markers. In all cases $n \geq 3$ independent populations scored. Error bars show SEM. A one-tailed heteroscedastic Student's t test was used to evaluate the significance of each difference (*$p < 0.03$, **$p < 0.01$, ***$p < 0.005$).

detected in long radial processes (Figure 7), as well as in the VZ and postmitotic area (data not shown). These processes also expressed RC2 and nestin, indicating that the labeled cells were radial glia.

Quantitation of CLEN-Infected Cells

The qualitative in vivo analysis presented above suggests that activated Notch1 promotes radial glial identity among telencephalic progenitors. As a result of the density of the labeled clusters in vivo, however, quantitation was not possible in that context. To quantitate the observed effects, we dissociated infected samples and performed immunolabeling studies on sparsely plated cells in vitro. This approach greatly facilitates colocalization analysis as has been shown by others (Gotz et al., 1998). Animals were saturation labeled with BrdU prior to being killed to permit identification of mitotically active cells.

Uninfected cells and CLEN-infected cells were labeled using immunofluorescence to detect radial glial markers (see supplemental Figures A and B at http://www.neuron.org/cgi/content/full/26/2/395/DC1). While CLE-infected cells were the more appropriate control population, for unknown reasons we could not detect the PLAP reporter in sparsely plated cells by immunostaining. Therefore, while CLEN-infected cells were identified by immunostaining for the Myc tag on NIC (see Figure 1), we were not able to easily identify CLE-infected cells in this context. Since at the time of infection (E9.5) all telencephalic cells are mitotically active and thereby subject to retroviral infection, it seemed likely that the CLE-infected population would closely resemble the uninfected population. A comparison of the percentage of $BrdU^+$ cells at E12.5 and E16.5 among uninfected and CLE-infected cells (identified by histochemical staining for PLAP) indicated that these populations have similar proliferative profiles (data not shown). Furthermore, as mentioned above, in vivo stainings revealed no unusual colocalization of radial glial markers with the PLAP in CLE-infected cells (see supplemental Figure C at http://www.neuron.org/cgi/content/full/26/2/395/DC1), suggesting that these cells are representative of the uninfected population.

Consistent with the in vivo data presented above, RC2 and BLBP were each found to be expressed in a higher percentage of CLEN-infected cells as compared to control cells (Figure 8). Indeed, many CLEN-infected cells double labeled with both RC2 and BLBP, demonstrating that these markers are expressed in overlapping populations (see also supplemental Figures A and B at http://www.neuron.org/cgi/content/full/26/2/395/DC1). Thus, activation of Notch1, in addition to promoting radial glial morphology in vivo, leads to a quantitative increase in the percentage of cells expressing radial glial markers.

Based upon both the in vivo and in vitro labelings presented above, a large proportion of CLEN-infected cells coexpressed the radial glial markers RC2 and BLBP. Furthermore, in vivo labeling demonstrated that many of these cells were $BrdU^-$ and were likely to represent quiescent radial glia. To quantitate this effect in vitro, we compared the overall percentage of $RC2^+/BLBP^+/BrdU^-$ cells among both uninfected and CLEN-infected cells at E16.5. Among uninfected cells, both dorsally and ventrally, roughly 2% of the cells scored were $RC2^+/BLBP^+/BrdU^-$ (dorsal, 1.8% $\pm$ 0.2%; ventral, 2.6 $\pm$ 1.2%; in both cases, n = 3 independent populations scored). Among CLEN-infected cells there was a substantial increase in the percentage of $RC2^+/BLBP^+/BrdU^-$ cells. Dorsally, 15% of the cells fit this profile (n = 132 cells, $p < 0.001$, modified χ^2 test, see Experimental Procedures), while ventrally 12% did (n = 108 cells, $p < 0.01$).

Postnatal Phenotype of Activated Notch1

Previous studies have suggested that radial glia transform into astrocytes postnatally (Schmechel and Rakic, 1979a; Voigt, 1989). To investigate the postnatal fate of CLEN-infected cells, we examined infected animals at P3 (postnatal day 3), P21, and P42. Morphological criteria for determining cell types using PLAP histochemistry were previously established (Gaiano et al., 1999). At postnatal ages, histochemical staining for the reporter PLAP revealed that many infected cells were dispersed throughout the brain and possessed astrocytic morphologies (Figure 9C and data not shown), consistent with the predicted fate of radial glia. Furthermore, in CLEN-infected samples there appeared to be a dramatic reduction in the number of labeled oligodendrocytes and neurons present compared to controls (N. G. and G. F., unpublished data). These observations are consistent with previous work which found that Notch1 signaling can inhibit both oligodendrocyte (Wang et al., 1998) and neuronal differentiation (Nye et al., 1994; Dorsky et al., 1995; Bao and Cepko, 1997) but not astrocytic development (Nye et al., 1994) (C. Chambers, N. G., G. F., and J. S. N., submitted).

In addition to becoming dispersed astrocytes postnatally, dense clusters of CLEN-infected cells were routinely present in the periventricular area (Figures 9A, 9B, 9D, 9G, and 9J). In control-infected samples, very few infected cells were present in this region (data not shown). Unlike CLEN-infected clusters during embryogenesis, those present at P3 had at most short processes, and appeared to be losing radial glial morphology. At P21 and P42, no radial morphologies were evident.

Recent reports have found that in the adult rodent forebrain the periventricular area contains cells with multipotent stem cell character in vitro (Chiasson et al., 1999; Doetsch et al., 1999; Johansson et al., 1999) One such study identified cells expressing GFAP in this region as stem cells (Doetsch et al., 1999). Using double immunofluorescence, we found that at P21 and P42 CLEN-infected clusters adjacent to the lateral ventricles were $GFAP^+$ (Figures 9D–9L). These result indicates that in addition to becoming dispersed astrocytes postnatally, CLEN-infected cells may also give rise to neural stem cells in the adult. Previous reports have suggested that such stem cells are present either in the ependymal layer (Johansson et al., 1999) or in the subependymal layer (Chiasson et al., 1999; Doetsch et al., 1999). In the present study, periventricular CLEN-infected cells were found predominantly in the subependymal layer, although some were also observed in the ependymal layer. Notably, these ependymal cells were sometimes found to express GFAP (Figures 9G–9I).

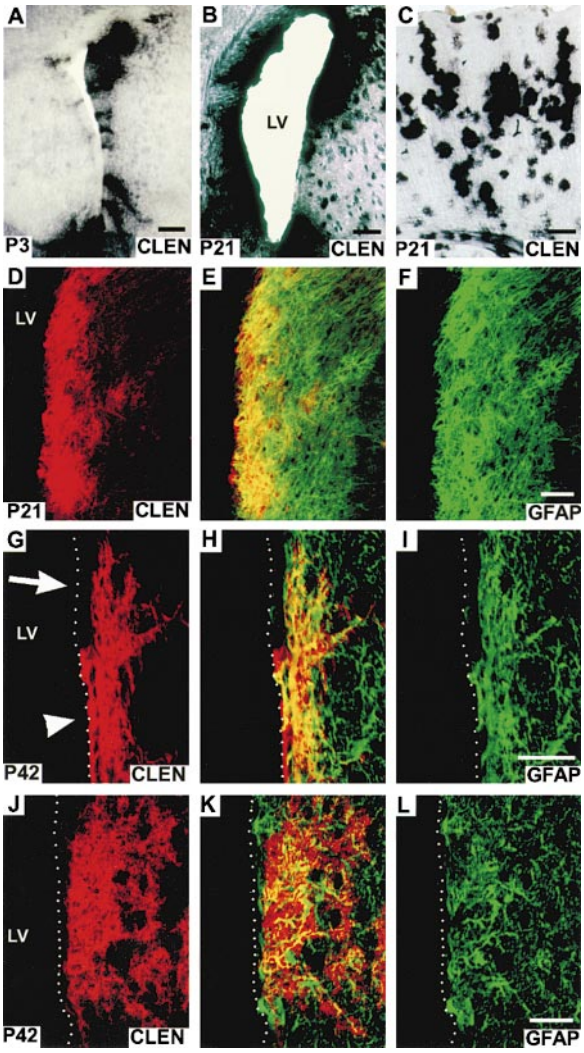

Figure 9. The Phenotype of CLEN-Infected Cells Postnatally

At P3 (A), P21 (B and D), and P42 (G and J), dense clusters of infected cells were detected adjacent to the lateral ventricles (LV). Double immunofluorescence staining at P21 and P42 revealed that these clusters, detected by PLAP expression (D, G, and J), expressed the astrocyte marker GFAP (F, I, and L). Infected cells were often present in the subependymal layer beneath apparently uninfected ependymal cells (J) (arrow in [G]). However, in some cases infected cells were also present in the ependymal layer (arrowhead in [G]). Dotted lines indicate the edge of the tissue in (G) through (L). In addition to these periventricular cells, many cells with astrocytic morphology (Gaiano et al., 1999) were present throughout the tissue (C). Scale bars: 500 μm (A), 250 μm (B and C), 50 μm (D–F), 25 μm (G–L).

Discussion

Using a recently developed gain-of-function approach, we have infected mouse forebrain progenitors at E9.5

with a virus expressing a constitutively active form of Notch1. Using this method, we obtained widespread infection of the telencephalon prior to the onset of neurogenesis. CLEN-infected cells acquired both the morphological characteristics and marker expression of radial glia. In addition, postnatally these cells became both dispersed astrocytes and GFAP$^+$ periventricular cells, the latter of which may be neural stem cells.

The observation that Notch1 promotes radial glial development is a valuable step toward understanding the mechanisms that generate cellular diversity in the brain. Given the central role of radial glia in establishing the cytoarchitecture of the brain (Rakic, 1995, 1988), a clearer understanding of the genesis of radial glia may have important ramifications for the treatment of both developmental and degenerative neural disorders. With respect to the latter, a primary problem in neuronal replacement therapies has been difficulty in obtaining integration of transplanted cells into host tissue. The ability to reestablish a radial glial migratory scaffold (which normally collapses at the end of neurogenesis) could facilitate neuronal delivery to brain regions suffering from neurodegeneration.

The finding that Notch1 promotes radial glial identity is the first example in the vertebrate nervous system of Notch activation resulting in the acquisition of an early cell type. Previous work has found that activation of Notch1 promotes Müller glial fate in the retina (Dorsky et al., 1995; Bao and Cepko, 1997). This work has suggested that Notch inhibits earlier neuronal cell fates, and that Müller glia, the last cell type to appear in the retina, is a terminal default fate. However, the fact that radial glia are among the first cell types present in the telencephalon clearly suggests that they are not a terminal default fate. Nevertheless, the manner in which Notch1 activation directs telencephalic progenitors to a radial glial fate remains unclear.

Two primary possibilities for the role of Notch1 in radial glia development remain to be distinguished: (1) Notch activation may simply inhibit neurogenesis, which via some default mechanism leads to a radial glial identity, or (2) Notch activation may result in directed changes that actively lead to radial glial identity. The precocious upregulation of BLBP seen in CLEN-infected cells supports the notion that Notch1 signaling may play an active role in promoting the radial glial fate of infected progenitors. However, further experimentation will be required to definitively distinguish between these possibilities.

Regardless of the manner in which Notch1 signaling leads to a radial glial fate, in light of the many roles played by Notch signaling throughout the embryo (Jiang et al., 1998; Lewis, 1998; Franklin et al., 1999; Sestan et al., 1999; Redmond et al., 2000), it is highly unlikely that Notch alone instructs progenitors to become this cell type. It seems more likely that Notch signaling influences the response of neural progenitors to secondary cues. Such a hypothesis is supported by recent work demonstrating that the secreted protein glial growth factor (GGF) causes elongation of radial glia and upregulation of the radial glial markers nestin and BLBP (Anton et al., 1997). Our work suggests that GGF might act on cells in which the Notch pathway has been activated. Nevertheless, we believe that the activation of Notch signaling may be an essential component of the molecular specification of radial glia.

The expression of Notch1 protein in endogenous radial glia, together with the observation that activated Notch1 promotes a radial glial phenotype, raises the question as to how Notch1 is normally activated in this cell type. The most likely explanation is that newly generated neurons, expressing high levels of a Notch ligand such as Dll1, activate Notch1 in radial glia during migration along the radial processes. This activation would allow radial glia to respond to environmental cues, such as GGF perhaps, which might then maintain their morphology and gene expression.

The traditional view of "lateral signaling" in the nervous system via the Notch pathway predicts that cells in which Notch1 is activated will remain as progenitors to participate in subsequent waves of differentiation (Lewis, 1998). Consistent with this view, in mammalian cells Notch1 signaling has been found to inhibit neuronal differentiation (Nye et al., 1994; Bao and Cepko, 1997) (C. Chambers, N. G., G. F., and J. S. N., submitted), and Notch3 signaling to result in an expansion of the progenitor pool (Lardelli et al., 1996). This previous work, taken together with our findings, both embryonically and postnatally, begs the question of whether radial glia are a subclass of progenitors and perhaps even embryonic stem cells. Historically, this possibility was considered unlikely because of their long radial processes, and the observation that during neurogenesis some radial glia are quiescent (Schmechel and Rakic, 1979b). More recently, however, several lines of evidence suggest that radial glia might be progenitors. For example, radial glia express nestin, a gene that is also expressed in multipotent neural progenitors (Lendahl et al., 1990). In addition, lineage analysis in the chick tectum found that clusters of labeled cells in vivo often contained a single radial glia in addition to other cell types including neurons (Gray and Sanes, 1992). Furthermore, in the adult avian brain the location of radial glia correlates with sites of neurogenesis (Alvarez-Buylla et al., 1990). The observation that some CLEN-infected radial glia express progenitor markers, such as RC2 and nestin, and are quiescent at E16.5 may be consistent with neural stem cell identity. Although, during neurogenesis, such cells would be expected to proliferate quite actively, by E16.5 the progenitor pool is diminishing and the bulk of VZ cells are migrating out to postmitotic areas (Takahashi et al., 1996). Those cells fated to become the "relatively quiescent" neural stem cells in the adult (Morshead et al., 1994) would likely remain in the proliferative zone and become less mitotically active as neurogenesis proceeds.

Fully understanding the relationship between the embryonic and postnatal fates of CLEN-infected cells will require further study. Nevertheless, we have shown that the activation of Notch1 in telencephalic progenitors promotes radial glial identity during embryogenesis, and development into both dispersed astrocytes and GFAP$^+$ periventricular cells postnatally. If radial glia are in fact embryonic stem cells, their generation and isolation using activated Notch1 will provide a valuable tool in the study of neural stem cell biology.

Experimental Procedures

Animals, Virus Preparation, and Injection

Swiss Webster mice (Taconic, Germantown, New York) used in these studies were maintained according to protocols approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. Virus preparation and ultrasound surgery were both performed as previously described (Gaiano et al., 1999). Concentrated stocks of both CLE and CLEN were injected at titers of 1–3 $\times$ 10^8 cfu/ml.

Saturation BrdU Labeling In Vivo

Pregnant mothers were repeatedly injected intraperitoneally with a solution containing 20 mg/ml 5-bromo-2′-deoxyuridine (BrdU, Sigma B9285) and 2 mg/ml 5-fluoro-2′-deoxyuridine (Sigma F0503) in 0.007 N NaOH. At each time point, 1 mg of BrdU per 10 g body weight was injected. To account for the increasing cell cycle length as development proceeds (Takahashi et al., 1996), injections were spaced differently depending upon the age of the embryos. At E12.5, three injections were separated by 4 hr each. At E14.5, three injections were separated by 5.5 hr each. At E16.5, four injections were separated by 4.5 hr each. In all cases, embryos were harvested 2 hr after the final injection.

Staining of Tissue Sections

The fixation of infected samples, preparation of frozen sections, histochemical staining to detect PLAP activity, and immunofluorescence protocols have been described elsewhere (Gaiano et al., 1999). However, several modifications were made for immunofluorescent detection of BrdU during double and triple labelings. After application of both primary and secondary antibodies, for the non-BrdU antigens, the tissue was fixed in cold methanol for 5 min and rinsed in PBS. Sections were treated with 5 μg/ml of deoxyribonuclease (DNase) in TBS (50 mM Tris, 150 mM NaCl [pH 7.5]) with 10 mM $MgCl_2$ and 10 mM $MnCl_2$ for 30 min at 37°C. After several rinses in TE and PBS, sections were incubated with Sheep anti-BrdU (1:50, Biodesign International, Saco, ME) for 1 hr at room temperature. Sections were then washed three times with PBS, blocked for 30 min (PBS + 1% normal donkey serum + 0.2% Triton X-100), and incubated in secondary antibody. Other primaries used were as follows: α-PLAP (rabbit, 1:200, Accurate Chemical, Westbury, NY; sheep, 1:200, American Research Products, Belmont, MA); mouse α-RC2 (IgM, supernatant, 1:2, ascites fluid 1:150, Developmental Studies Hybridoma Bank, Iowa City, IA); mouse α-nestin (IgG_1, supernatant, 1:4, Developmental Studies Hybridoma Bank); rabbit α-BLBP (1:3000, gift of N. Heintz); goat α-Notch1 (C-20) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit α-GFAP (1:200, Accurate); rabbit α-CRBP (1:200, gift of U. Eriksson); mouse α-Pax6 (IgG_1, 1:1000, gift of A. Kawakami); mouse TuJ1 (IgG_{2a}, Berkeley Antibody Company, Richmond, CA). Most secondaries were obtained from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania) and were raised in donkies. Cy3-conjugated goat α-mouse IgG, which was Fcγ fragment specific (Jackson), was used to detect nestin and the Myc tag in vitro. Fluorescent images were acquired using a confocal microscope (Leica) and TCS NT software.

Cell Dissociation and Quantitation

Dorsal (neocortex and hippocampus) and ventral (medial and lateral ganglionic eminences) areas of E12.5 and E16.5 embryos were dissected apart and chopped into small pieces. These were treated with 0.25% trypsin for 10–15 min at 37°C, followed by addition of fetal bovine serum, and DNase. Each sample was triturated, washed with DMEM/F12 media including 2% serum, and plated onto 8-well Lab-Tek II chamber slides that had been coated with poly-D-lysine. Cells were fixed 3 hr later for 10 min with ice-cold 4% paraformaldehyde and stored in PBS with 1% serum (with or without 0.2% Triton X-100). Cells were incubated with primary antibodies for 1 hr at room temperature, washed with PBS, and then incubated with secondary antibodies for 1 hr at room temperature. To detect BrdU, cells were first treated for 30 min at room temperature in 2 μg/ml DNase. Uninfected cells were generally used as control (see Results). CLEN-infected cells were identified either by histochemical detection of PLAP after the completion of immunofluorescence scoring, or by immunolabeling markers of interest together with the Myc tag on NIC (see supplemental Figure B at http://www.neuron.org/cgi/content/full/26/2/395/DC1). To increase the stringency of the χ^2 tests performed, the expected values were set as the mean + 2 SEM.

Acknowledgments

The authors thank Daniel Turnbull for instruction on the use of the ultrasound scanner, Alexander Schier and Joshua Corbin for critical reading of the manuscript, and Muriel Cleary for exceptional technical assistance. This work was supported by grants from the National Institutes of Health to G. F. (NS32993) and J. S. N. (NS35566). N. G. was supported by a postdoctoral fellowship from the American Cancer Society (PF4473).

Received March 10, 2000; revised April 24, 2000.

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