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Notch1 and Notch3 Instructively Restrict bFGF-Responsive Multipotent Neural Progenitor Cells to an Astroglial Fate

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Summary

Notch1 has been shown to induce glia in the peripheral nervous system. However, it has not been known whether Notch can direct commitment to glia from multipotent progenitors of the central nervous system. Here we present evidence that activated Notch1 and Notch3 promotes the differentiation of astroglia from the rat adult hippocampus-derived multipotent progenitors (AHPs). Quantitative clonal analysis indicates that the action of Notch is likely to be instructive. Transient activation of Notch can direct commitment of AHPs irreversibly to astroglia. Astroglial induction by Notch signaling was shown to be independent of STAT3, which is a key regulatory transcriptional factor when ciliary neurotrophic factor (CNTF) induces astroglia. These data suggest that Notch provides a CNTF-independent instructive signal of astroglia differentiation in CNS multipotent progenitor cells.

Introduction

It is generally acknowledged that both extrinsic and heritable intrinsic factors play pivotal roles in generating cellular diversity in the central nervous system (CNS) (Chenn and McConnell, 1995; Kornack and Rakic, 1995; Reid et al., 1995). All the neurons and macroglial cells found in the adult central nervous system are derived from precursor cells in the ventricular zone (VZ) (Sidman and Rakic, 1973; Raedler et al., 1980; Jacobson, 1991). In vivo lineage analysis using retroviral marking indicates that the majority of VZ cells are already committed to a single lineage and are no longer multipotent progenitors in neurogenesis (Parnavelas et al., 1991; Luskin, 1993; Johe et al., 1996).

By contrast, neural stem cells enriched by the mitogenic actions of either basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) from developing and adult CNS (Gage et al., 1995; Kilpatrick and Bartlett, 1995; Johe et al., 1996; Reynolds and Weiss, 1996; Temple and Qian, 1996) provide a good system to analyze the effects of extrinsic and intrinsic factors that control lineage commitment (reviewed in Gross et al., 1996; Johe et al., 1996; Sakurada et al., 1999). The adult hippocampus-derived progenitors (AHPs) are bFGF-dependent neural stem cells (Palmer et al., 1997; Takahashi et al., 1999). When grafted into the retina or the rostral migratory pathway leading to the olfactory bulb, the AHPs were well integrated and adopted the appropriate morphologies, indicating that the AHPs have the ability to differentiate appropriately according to the environmental cues (Suhonen et al., 1996; Takahashi et al., 1998).

Notch signaling is an important pathway that controls an extraordinary broad spectrum of cell fates (Muskavitch, 1994; Artavanis et al., 1999). Notch proteins have been found to function in two types of local regulation through cell–cell interaction, namely lateral inhibition and inductive signaling (Greenwald, 1994; Artavanis et al., 1995). Examples of lateral inhibition by Notch are seen in the segregation of neural and epidermal precursor cells in the ventral ectoderm of *Drosophila* embryos. A typical inductive signaling by Notch operates in the induction of *vestigial* and *eyeless*, master regulatory genes of wing and eye formation, respectively (Kim et al., 1996; Artavanis et al., 1999).

Drosophila genetic studies show that the expression of the intracellular region of Notch results in the activated Notch phenotype (Fortini et al., 1993; Rebay et al., 1993). The intracellular region of Notch (RAMIC) contains the RAM domain, which interacts with a DNA binding protein, RBP-J (Tamura et al., 1995). Ligand binding to Notch leads to a series of proteolytic cleavages and results in transport of RAMIC to the nucleus (Kopan et al., 1996; Schroeter et al., 1998; Struhl and Adachi, 1998), where Notch RAMIC transactivates expression of RBP-J binding site–containing promoters such as HES-1 (Jarriault et al., 1995; Honjo, 1996).

Previous studies had shown that Notch signaling suppresses oligodendrocyte development from oligodendroglial precursor, O2A cells, and also to inhibit neuronal differentiation of neuroepithelial cells, most of which are committed to neurons (Wang et al., 1998; Ohtsuka et al., 1999). Recently, activated Notch has been found to promote glial fates. Notch signaling enhances the generation of radial glial cells from cortical stem cells in vivo (Gaiano et al., 2000) and Muller glial cells from retinal progenitors (Furukawa et al., 2000). Radial glial cells are one of the first cell types present in the developing forebrain, which suggests the Notch activation leads to a cell fate determination rather than terminal default fates. These results, however, cannot still exclude the possibility that Notch signaling may inhibit differentiation of progenitor cells and expand the progenitor pools, because radial glia might be a kind of stem cells (Gray and Sanes, 1992) and because another second signal in addition to activated Notch might be required for the generation of radial glial cells. In contrast, in the peripheral nervous system, the glial inductive effect of Notch was shown to be instructive but not selective (Morrison et al., 2000).

In the present study, using CNS multipotent neural progenitors, AHPs, we examined whether Notch signaling instructively promotes gliogenesis from CNS neural

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stem cells or only inhibits the differentiation to other lineages. Our studies show that activated Notch1 and Notch3 both instructively promote cellular differentiation of astroglia from AHPs by the molecular mechanism different from that of ciliary neurotrophic factor (CNTF), which is known to have a strong capacity to induce astroglia. The Notch-mediated astroglial induction from CNS multipotent progenitors is associated with suppression of both neuronal and oligodendroglial lineages.

Results

Induction of Astroglia from AHPs by Activated Notch1 and Notch3

To analyze the influence of Notch signaling on lineage commitment of multipotent neural progenitor cells, we used bFGF-dependent rat AHPs (Palmer et al., 1997). The serially passaged AHPs were a homogenous population, as demonstrated by the immunoreactivity for the type IV intermediate filament protein, i.e., nestin (Lendahl et al., 1990), and by the absence of markers for the differentiated progeny: microtubule-associated protein 2 (MAP2) for neurons; glial fibrillary acidic protein (GFAP) for astrocytes and radial glia at late stage of differentiation; and galactocerebroside (GalC) for oligodendrocytes. In agreement with the previous report (Palmer et al., 1997), AHPs differentiated to neurons, astroglia, and oligodendrocytes 1 week after culturing under the differentiating conditions: bFGF withdrawal together with addition of retinoic acid and serum (data not shown).

AHPs were transduced by the LHCX vector carrying the intracellular regions (RAMIC) of murine Notch1 or Notch3 (Figure 1A). The stably transduced cells were isolated under hygromycin B selection and the expression of Notch1 or Notch3 RAMIC was checked by Western blotting (data not shown). Introduction of Notch1 and Notch3 RAMIC promoted the dramatic differentiation of AHPs to GFAP-positive astroglia under the proliferating condition (Figure 1B). When GFAP expression was quantitated using flow cytometry analysis, ~85% of activated Notch-transduced cells were GFAP positive, as compared with ~20% of vector-infected cells (Figure 1C). The introduction of Notch RAMIC led to increase in the cellular size, the GFAP expression level, and the number of outgrown processes per cell. When LHCN1- and LHCN3-infected cells were cultured under the differentiating conditions, their expression of GFAP was accelerated as well as augmented (Figure 1C). One- to three-day culture under the differentiating conditions made 2%–3% and 17%–23% of mock-transfected and RAMIC-expressing AHPs, respectively, highly GFAP-positive astroglia. Concomitantly, low GFAP-positive astroglia of RAMIC-expressing AHPs slightly decreased as compared with culturing in the presence of bFGF. The results suggest that activated Notch may make neural stem cells commit to an astroglial fate and induce immature astroglia even in the presence of bFGF.

Notch Suppresses the Oligodendroglial and Neuronal Lineage Differentiation from AHPs

Notch RAMIC-transduced AHPs were allowed to differentiate for 4 days under the differentiating conditions, and their differentiation into various lineages was assessed by immunohistochemistry using lineage-specific markers. Activated Notch signaling led to a marked reduction of the neuronal ($MAP2^+$) and oligodendroglial ($Gal\text{-}C^+$) lineage cells in parallel with an increase in the astroglial ($GFAP^+$) lineage cells (Figure 2A). Induction of the astroglial marker (GFAP) and suppression of an oligodendrocyte marker, proteolipid protein (PLP) by activated Notch1 and Notch3 were confirmed by RT–PCR assay (Figure 2B). Similar results were obtained by quantitative analyses using flow cytometry after staining with antibodies against lineage-specific markers (Figure 2C).

To characterize more fully the effects of Notch signaling on the lineage commitment of AHPs, we carried out clonal analysis. The developmental fate of individual cells was followed by infection with the low-titered replication-defective retrovirus vectors, LN1SE and LN3SE, which direct expression of Notch1 and Notch3 RAMIC, respectively, in addition to EGFP. LXSE expresses only EGFP (Figure 1A). Infected cells were identified by expression of EGFP. After 2 days of expansion in the presence of bFGF, infected AHP cells were induced to differentiate into various lineages under the differentiating conditions. Four days later the cell lineage composition of EGFP-positive clusters (colonies) derived from a single infected clone was analyzed by immunofluorescence study using antibodies against MAP2 and GFAP (Williams and Price, 1995) (Figures 3A and 3B). Colonies were categorized into three classes: neuronal ($MAP2^+GFAP^-$), mixed ($MAP2^+GFAP^+$), and astroglial ($MAP2^-GFAP^+$). As shown in Figure 3C, the distribution of colonies in these categories changed in the presence of Notch RAMIC. LXSE (control)-infected colonies were divided into 59.8% of neuronal, 24.9% of mixed, and 2.3% of astroglial. In contrast, about 20% of Notch1 and Notch3 RAMIC-expressing colonies were neuronal, and about 70% of them contained astroglia. The results clearly indicate that activated Notch facilitates differentiation of neural progenitor cells into astroglia cells and suppresses their differentiation into neurons.

The high-frequency appearance of astroglia in clones expressing activated Notch might be due to the clonal death. Since only 27% of the clones arising from LXSE infection contain astroglia in contrast to 70% from LN1SE and LN3SE (Figure 3C). About 60% of the clones should have been eliminated from LN1SE and LN3SE infectants if this difference were due to clonal death by the overexpression of Notch RAMIC. However, the numbers of EGFP-positive clones resulting from LXSE, LN1SE, and LN3SE infection were within the same range: 20.2 ± 2.9, 19.7 ± 2.6, and 21.3 ± 3.5, respectively, when we used an essentially identical number of viral particles titrated on NIH3T3 cells, and AHPs were infected in triplicates. The results indicate that negative selection against neuronal clones is unlikely to occur by activated Notch expression.

The clonal analysis also showed that activated Notch has a mitogenic effect on AHPs because the average colony size of RAMIC-positive clones was larger than that of control clones (Figure 3D). To get a clear picture of how cell numbers are influenced by Notch signaling, we classified colonies according to their composition and size. In the presence of activated Notch, 38%–45% of astroglial colonies contain more than 25 cells, but

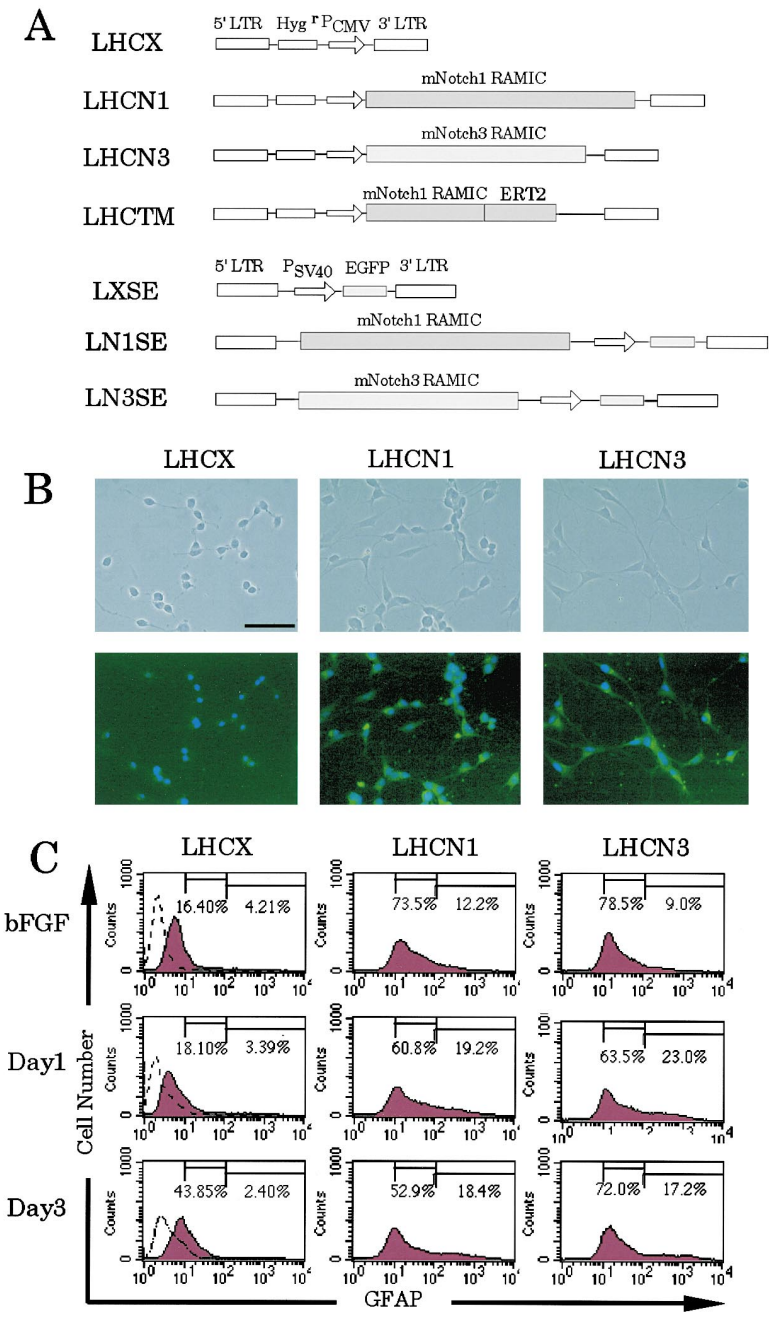

Figure 1. Activated Notch1 and Notch3 Induce Astroglia from AHPs

(A) Schematic illustration of retrovirus vectors used to transduce AHPs. LHCX and LXSE are MoMuLV-based retrovirus vectors. RAMIC encodes the entire intracellular region of murine Notch. ERT2 encodes hormone binding domain of human estrogen receptor (OHT-sensitive mutant).

(B) Immunofluorescence study of AHPs infected LHCX, LHCN1, and LHCN3. The infected AHPs were selected by hygromycin B for 2 weeks and cultured in the presence of bFGF (proliferating conditions). Cells were immunostained with antibodies against GFAP, followed by the FITC conjugated secondary antibody (green) and counterstained with Hoechst 33342 (blue). Upper panels represent phase-contrast views of the immunostained fields of lower panels. Scale bar = 100 μm. Lower panels show GFAP staining. Matched exposures were taken to permit comparison of GFAP staining intensities.

(C) Activated Notch increases both the rate and extent of astroglial differentiation. AHPs transduced with LHCX (three left histograms), LHCN1 (two middle histograms), and LHCN3 (two right histograms) were cultured in differentiating conditions for 1 or 3 days and analyzed by flow cytometry. Cells were permeabilized and stained with either the anti-GFAP (shading) or normal rabbit antibody (dashed line) plus the secondary antibody. The percentages of GFAP-positive cells are shown for indicated gates. These data are representative of four independent experiments.

none of them are larger than 25 cells in LXSE-infected clones (Table 1). By contrast, the colony sizes of neuronal and mixed type colonies were not so much affected by activated Notch. These data suggest that Notch signaling might have the mitogenic activity of astroglial progenitors. Further experiments will be required to distinguish definitely the following two possibilities: (1) Notch activation may influence the response of neural stem cells to other growth factors present in culture medium, or (2) Notch activation may result in direct promotion of cell cycle.

To examine whether the above effects of activated Notch is due to its antiapoptotic effect on selected lineages, we measured dying cells in individual colonies. Hoechst 33342 staining was used to visualize fragmented nuclei characteristic of apoptotic cells in colonies fixed every 10 hr after differentiation induction. If the effects of activated Notch are intraclonal selection of specific lineage cells, a large proportion of colonies would contain a significant number of dying cells. The percentages of the colonies containing any dying cells were similar (9.0%–21.9%) over the 90 hr period examined, regardless of astroglial induction by activated Notch (Figure 3E). In addition, dying cells constituted only a small population (1–2 cells) of each colony, even when cell death was detected. These data indicate that activated Notch does not induce preferential survival of lineage-restricted cells generated within a colony.

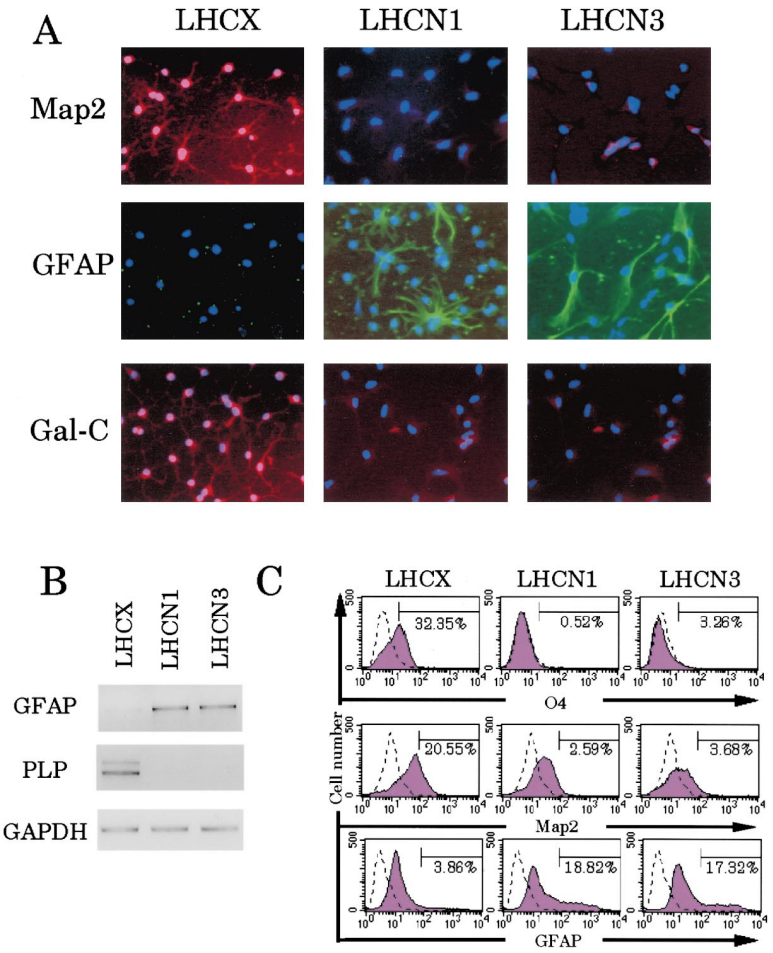

Figure 2. Activated Notch1 and Notch3 Selectively Suppress Commitment of AHPs to the Neuronal and Oligodendroglial Lineages and Induce to Differentiate into Astroglial Lineage

(A) AHPs transduced with LHCX, LHCN1, and LHCN3 were cultured in the differentiating conditions for 4 days, fixed, immunostained with antibodies against MAP2 (neuron marker, Texas red), GFAP (astrocyte marker, FITC), and Gal-C (oligodendrocyte marker, Texas red), and counterstained with Hoechst 33342 (blue). MAP2- or Gal-C-positive cells with red-stained processes were not identified in Notch RAMIC expressing AHPs.

(B) RT–PCR analysis for lineage marker expression. RNA was harvested from retrovirus-transduced AHPs cultured in the differentiating conditions for 1 day (PLP, an oligodendroglial marker).

(C) Flow cytometric analysis of AHPs that were transduced with LHCX, LHCN1, and LHCN3, and cultured in the differentiating conditions for 3 days. The staining antibodies are against MAP2, GFAP, and O4 (oligodendrocyte marker) (shaded) or normal mouse IgM, IgG, or normal rabbit IgG (dashed line). The percentages of differentiation marker-positive cells are shown for indicated gates. These data are representative of at least three independent experiments.

Transient Activation of Notch Causes an Irreversible Astroglial Commitment

We first analyzed the transactivation activity of Notch1 and Notch3 RAMIC in AHPs. To monitor RBP-J-mediated transactivation, we transiently transfected AHPs with TP-1-luc, a reporter luciferase construct under the TP-1 promoter that contains RBP-J binding sites (Kato et al., 1997). As an endogenous control, D10 cells, murine Delta1-expressing X63 cells (Kuroda et al., 1999) were cocultured with TP-1-luc-transfected AHPs, which express endogenous Notch1 on their surface (data not shown). AHPs cotransfected with TP-1-luc and Notch1 or Notch3 RAMIC showed the comparable transcription from the TP-1 promoter (Figure 4A). As expected, the level of the transactivation elicited by the Notch RAMIC construct was much higher than that of the ligand-induced transactivation by coculture with D10 cells.

To assess the effect of transient Notch activation, AHPs were transduced by the LHCX vector carrying the intracellular regions of murine Notch1 fused to the human estrogen receptor, designated as LHCTM (Figure 1A) and cloned by selection with hygromycin B. We confirmed that in the presence of 4-hydroxytamoxifen (OHT), RBP-J-dependent transcription was transactivated in LHCTM-infected clones and that the activity is reduced to background levels by passaging after treatment with OHT (Figure 4B). We cultured LHCTM-transduced AHPs for 36 hr first in the presence of OHT, passaged and then cultured in differentiating conditions in the absence of OHT for 4 days. The majority of the cells differentiate to high GFAP-positive astroglia, indicating that they had committed to an astroglial fates during the first 36 hr (Figure 4C). As positive control LHCTM-transduced AHPs were cultured for 4 days in differentiated conditions in the presence of OHT. The level of astroglial induction was similar to that of the transient exposure to OHT (Figure 4C). These data suggest that neural stem cells lose their multipotency by transient Notch activation.

Activated Notch1 and Notch3 Upregulate the Transcriptional Activity of Specific Promoters in AHPs

Activation of a well-known endogenous target of Notch signaling, namely, the HES-1 promoter, was also examined in AHPs. Recent studies show that HES-1 and HES-5 induce Muller glial cells from retinal progenitors (Furukawa et al., 2000; Hojo et al., 2000). The HES-1 expression level induced by Notch signaling oscillates by negative autoregulation, and the induction of HES-1 expression is augmented by cycloheximide treatment (Kuroda et al., 1999). In the presence of cycloheximide, Notch3 as well as Notch1 RAMIC increased the HES-1 expression level in AHPs (Figure 5A). We could not detect expression of HES-5 in the presence or absence of cycloheximide (data not shown).

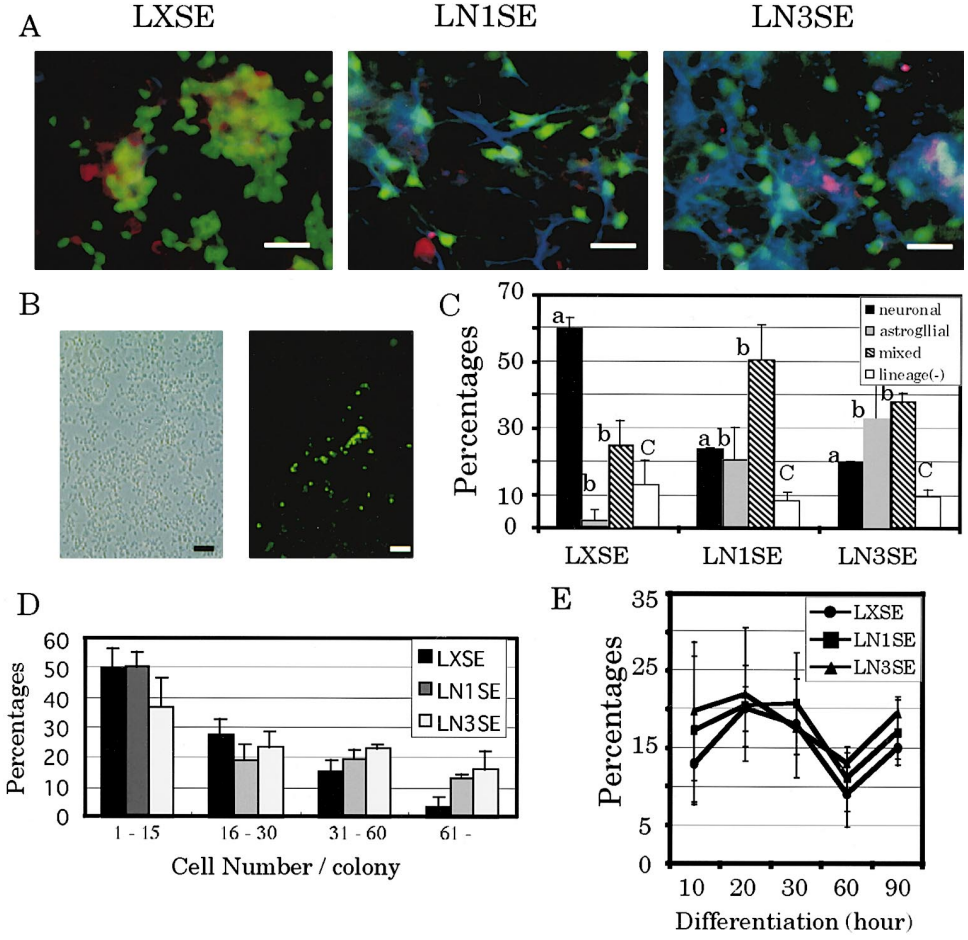

Figure 3. Activated Notch Change the Clonal Types

AHP culture was infected with low-titer retroviruses of LXSE, LN1SE, and LN3SE. After an additional 2 day expansion in the presence of bFGF, cells were differentiated under the differentiating conditions and analyzed 4 days later by double immunofluorescent staining with the antibodies against MAP2 (Texas red) and GFAP (blue, AMCA). Infected cells were identified by EGFP expression (green). Scale bar = 50 μm.

(A) Higher magnification of representative clones of AHPs infected by LXSE, LN1SE, and LN3SE.

(B) LXSE infected AHP colony. Progeny of a single infected cell are identified as a discrete colony of EGFP-expressing cells (right).

(C) The percentages of clonal types of infected AHPs. Clonal analysis was carried out as described in Experimental Procedures. Clonal types were classified by immunostaining as follows: neuronal, MAP2-positive cells, and unstained cells; astroglial, GFAP-positive cells, and unstained cells; mixed composition of MAP2-positive, GFAP-positive, and unstained cells; lineage (−), unstained cells only. The data are based on three independent experiments in which 50 colonies in average were analyzed per experiment. Statistic significance between LXSE and LN1SE/LN3SE was compared by t tests. a, $p < 0.05$; b, $p < 0.05$; c, $p > 0.05$.

(D) The relative frequency of clones containing variable cell numbers. The results are the mean ± SD of triplicate experiments and 50 colonies in average were counted for each retrovirus infection per experiment.

(E) Analysis of cell death within clones. AHPs infected with LXSE, LN1SE, or LN3SE were cultured in the differentiating conditions. Cultures were fixed every 10 hr, stained with Hoechst 33342, and analyzed by fragmented nuclear morphology. Percentages of colonies that contained any dying cells (usually 1–2 cells) are shown. The colonies containing astroglia could be observed after 60 hr culture in differentiating conditions. The percentages of the colonies containing astroglia are as follows—60 hr: LXSE, 24.0% ± 5.7%; LN1SE, 52.0% ± 2.8%; and LN3SE, 56.0% ± 5.7%; 90 hr: LXSE, 32.0% ± 5.7%; LN1SE, 71.0% ± 1.4%; and LN3SE, 62.0% ± 11.3%. The data are based on three independent experiments and 50 colonies in average were counted and analyzed per time point. No difference was detected by t test as compared between different viruses at the same time point.

We also examined activation of the GFAP promoter in AHPs by activated Notch, although the GFAP promoter does not have RBP-J binding sites. When Notch1 and Notch3 RAMICs were cotransfected with the GFAP promoter-luc into AHPs, a reproducible transactivation of the GFAP promoter was observed (Figure 5B), and the transactivation activity of activated Notch is suppressed by R218H, a dominant–negative form of RBP-J (Figure 5C). Much weaker activities of Notch RAMIC on the GFAP promoter than on the TP1 promoter suggest that GFAP expression may be mediated through RBP-J-dependent activation of other transcription factors.

Astroglial Induction by Activated Notch Is Independent of CNTF

We then examined whether the astroglial induction by CNTF shares common mechanism with that of Notch signaling. First, immunofluorescence study and flow cytometry analysis showed that CNTF alone induced to express GFAP in AHPs, but only a small portion of the

Table 1. Colony Sizes of Various Clonal Types

Clonal Type	Colony Size	LXSE	LN1SE	LN3SE
N	<15	50.9 ± 3.4	72.2 ± 39.3	73.4 ± 9.4
		(8.4 ± 0.4)	(7.5 ± 1.6)	(6.0 ± 1.8)
	>15	49.1 ± 3.4	27.8 ± 39.3	26.7 ± 9.4
		(27.9 ± 3.4)	(22.9 ± 4.4)	(42 ± 21.2)
A	<25	100	55.6 ± 7.8	62.0 ± 28.1
		(2.5 ± 3.5)	(8.4 ± 3.0)	(13.6 ± 2.1)
	>25	0	44.5 ± 7.8	38.1 ± 28.1
			(47.5 ± 13.8)	(41.8 ± 14.5)
M	<25	42.8 ± 3.9	43.4 ± 6.6	33.3 ± 7.4
		(10.7 ± 1.3)	(11.7 ± 0.2)	(16.0 ± 2.3)
	>25	57.2 ± 3.9	56.6 ± 6.6	66.8 ± 7.4
		(47.5 ± 0.2)	(57.7 ± 5.9)	(52.0 ± 2.81)
(−)	<15	79.2 ± 5.9	60.0 ± 14.1	67.5 ± 10.6
		(4.2 ± 1.7)	(5.2 ± 0.6)	(5.5 ± 0.3)
	>15	20.9 ± 5.9	40.0 ± 14.1	32.5 ± 10.6
		(21.8 ± 6.7)	(35.5 ± 3.5)	(20.8 ± 3.9)

N, neuronal clones; A, astroglial clones; M, mixed clones; (−), lineage (−) clones. The percentage of the colonies of clonal type are indicated, and average cell numbers ± SD within a colony are shown in parentheses. The data are based on three independent experiments, and 50 colonies in average were counted for each retrovirus infection per experiment.

CNTF-treated cells increased their cell sizes and sharp process extension (Figures 6A and 6B). When AHPs expressing Notch1 RAMIC were treated with CNTF, a significantly larger proportion of cells not only increased the expression of GFAP but also became flat and polygonal, a typical morphology of astroglia. Notch signaling appears to induce an astroglial phenotype more extensively than CNTF. The additive enhancement of transcription from the GFAP promoter was shown by kinetic studies using the transient luciferase assay (Figure 6C). These data show that two different signaling pathways, CNTF and Notch, additively stimulate differentiation from the multipotent neural stem cells to astroglia.

To investigate a molecular mechanism underlying this

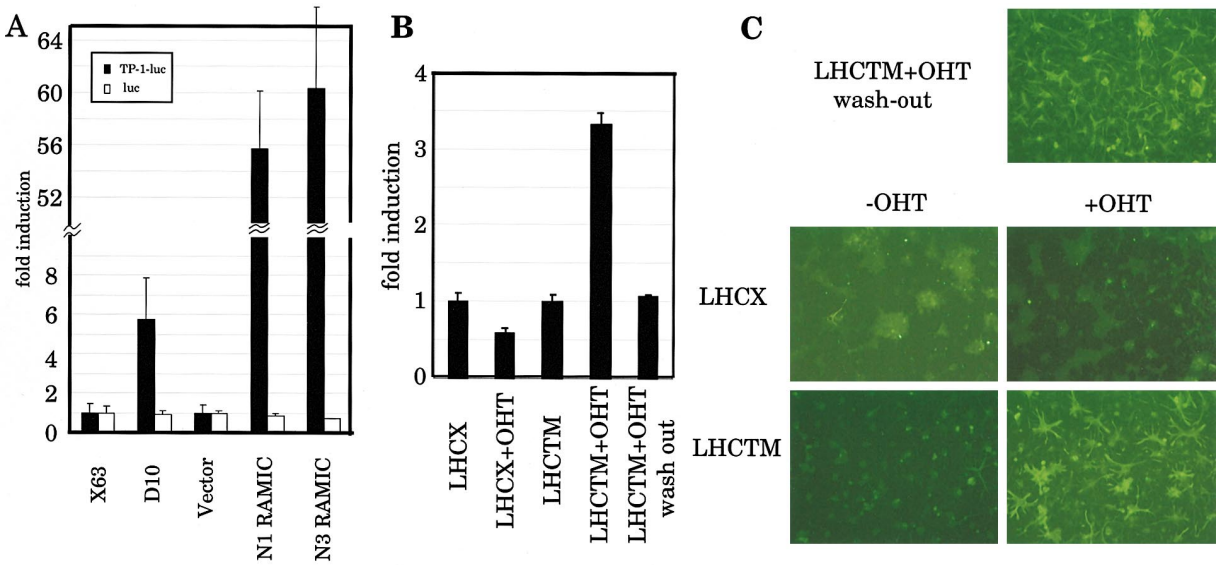

Figure 4. Transient Activation of Notch Causes Irreversible Astroglial Commitment

(A) AHPs were transfected with 500 ng of pGa50-7 (luc) or pGa981-6 (TP-1-luc) and Notch RAMIC. Alternatively, AHPs were transfected with pGa50-7 or pGa981-6 and then cocultured with D10 or X63 cells for 24 hr. Normalized luciferase activity is shown. The results are the mean ± SD of triplicate data points from a representative experiment.

(B) LHCTM- or LHCX-transformed AHPs were transfected with 500 ng of pGa981-6 (TP-1-luc) and cultured for 36 hr in the presence or absence of 50 nM OHT. AHPs were cultured for 36 hr in the presence of OHT, passaged to wash out OHT, and transfected with 500 ng of pGa981-6 where indicated. Normalized luciferase activity is shown. The results are the mean ± SD of triplicate data points from a representative experiment.

(C) AHPs transduced with LHCX or LHCTM were cultured in the differentiating conditions for 4 days in the presence or absence of OHT. AHPs were cultured for 36 hr in the presence of OHT, passaged to wash out OHT, and cultured in the differentiating conditions for 4 days where indicated. After differentiation induction, they were fixed and immunostained with anti-GFAP polyclonal antibodies. Each of three clones analyzed yielded identical results, and a representative experiment is shown here.

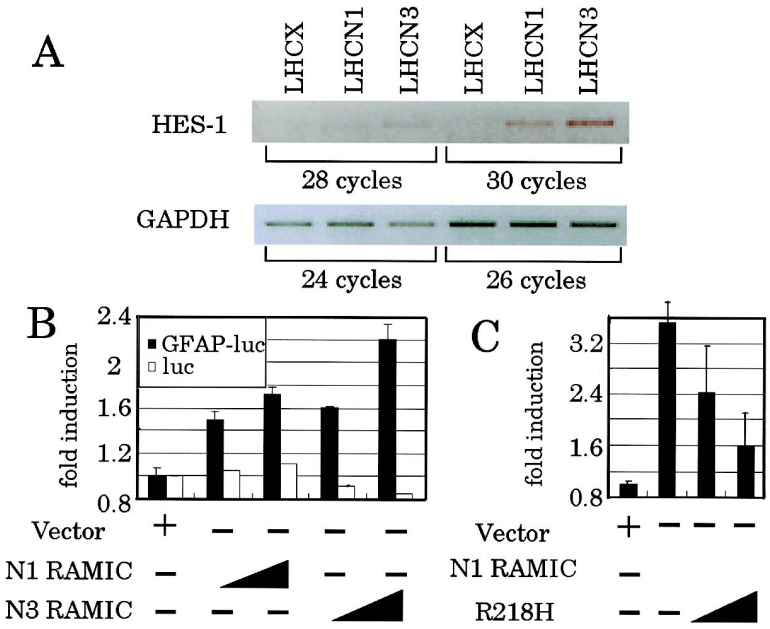

Figure 5. Notch1 and Notch3 RAMIC Upregulate Transcription from RBP-J Binding Sites Containing Promoter and GFAP Promoter

(A) Endogenous HES-1 expression increased in Notch RAMIC-expressing AHPs. RNA was isolated from AHPs infected by LHCX, LHCN1, and LHCN3 after 4 hr culture in the presence of 10 μM cycloheximide and analyzed by RT–PCR.

(B) AHPs were transfected with GFAP-luc (250 ng), and the increasing amounts of Notch1 and Notch3 RAMIC and cultured in the presence of 2 ng/ml CNTF. pRL-CMV was cotransfected as internal control. Luciferase activities are shown as Figure 4A.

(C) AHPs were transfected with GFAP-luc (250 ng), Notch1 RAMIC (100 ng), and the increasing amounts of R218H and cultured in the presence of 2 ng/ml CNTF. pRL-CMV was cotransfected as internal control. Luciferase activities are shown as Figure 4A.

additive effect, we examined phospholylation of STAT3, which is known to be directly involved in the activation of GFAP expression by CNTF (Bonni et al., 1997). There was no enhancement of STAT3 phosphorylation in Notch RAMIC-expressing AHPs, while CNTF augmented a ratio of phosphorylated STAT3 to nonphosphorylated STAT3 (Figure 6D). To examine whether STAT3 is involved in activation of the GFAP promoter by Notch, we introduced a mutation in the GFAP promoter that disrupted the binding of STAT3 (Figure 6E). Although this mutation completely abrogated the activation of the GFAP promoter by CNTF, it did not affect the transactivation activity by activated Notch in the presence or absence of CNTF, indicating that induction of GFAP transcription by activated Notch is independent of STAT3 (Figure 6F).

Discussion

We have examined the role of Notch1 and Notch3 in CNS lineage commitment, using bFGF-dependent multipotent self-renewing neural progenitor cells (AHPs). Our studies on multipotent AHPs stably expressing Notch RAMIC indicate that activated Notch1 and Notch3 behave similarly by inhibiting neuronal and oligodendroglial lineage commitment and promoting astroglial development (Figures 1 and 2). Clonal analysis indicates that Notch1 and Notch3 most likely provide an instructive regulation in the choice of the astroglial fate rather than a selective regulation in survival of astroglial precommitted progenitors (Figures 3C–3E). Furthermore, irreversible astroglial commitment takes place by transient (36 hr) activation of Notch (Figure 4). Astroglial induction by Notch is independent of STAT3 and thus mediated by a different mechanism from CNTF (Figure 6).

Instructive versus Selective Effects of Notch Signaling on the CNS Multipotent Progenitor Cells

Two models have been proposed to explain how the fate of the uncommitted progenitor cells is influenced by extrinsic and intrinsic factors. In one model, certain factors instruct multipotent progenitor cells to commit to a particular lineage. In the other model, multipotent cells choose their fate stochastically, and the proliferation or survival (or both) of specific lineage-restricted cells is supported by certain factors. It is difficult to distinguish these models experimentally, partly because of the incomplete influence of factors such as granulocyte/macrophage colony-stimulating factor on lineage commitment (Metcalf, 1980, 1991). Activated Notch1 and Notch3 increase both the rate and extent of astroglial differentiation and induce the differentiation even in the presence of a mitogen, bFGF (Figure 1). Our clonal analysis indicates that the primary action of Notch is at the level of cell lineage commitment although activated Notch has some mitogenic-survival functions on astroglial cells (Table 1; Figure 3D). Since the frequency of cell death is very low in each colony and few Notch RAMIC expressing colonies contain neurons (Figure 3E), it is unlikely that astroglial induction by Notch is apparent by preferential death of intraclonal neurons. The possibility that these effects might reflect the selection bias by hygromycin B is unlikely because astroglial induction by Notch was confirmed by the GFAP promoter luciferase assay, which was performed by transient transfection. The simplest interpretation of our data, therefore, is that activated Notch1 and Notch3 act instructively to control the cell fate determination of CNS multipotent progenitor cells, resulting in astroglial induction and neuron/oligodendrocyte suppression.

A recent work demonstrates that Notch1 promotes radial glial development in mouse embryonic brain (Gaiano et al., 2000). However, it is not clear whether this glial induction is instructive and whether radial glial cells are committed to specific lineages and have lost multipotency. In fact, retroviral lineage analysis suggests that radial glial cells might be neural stem cells (Gray and Sanes, 1992). Recent studies have shown that a subset of subventricular astrocytes and ependymal cells transformed from radial glial cells are neural stem cells in the adult (Barres, 1999). However, AHPs expressing acti-

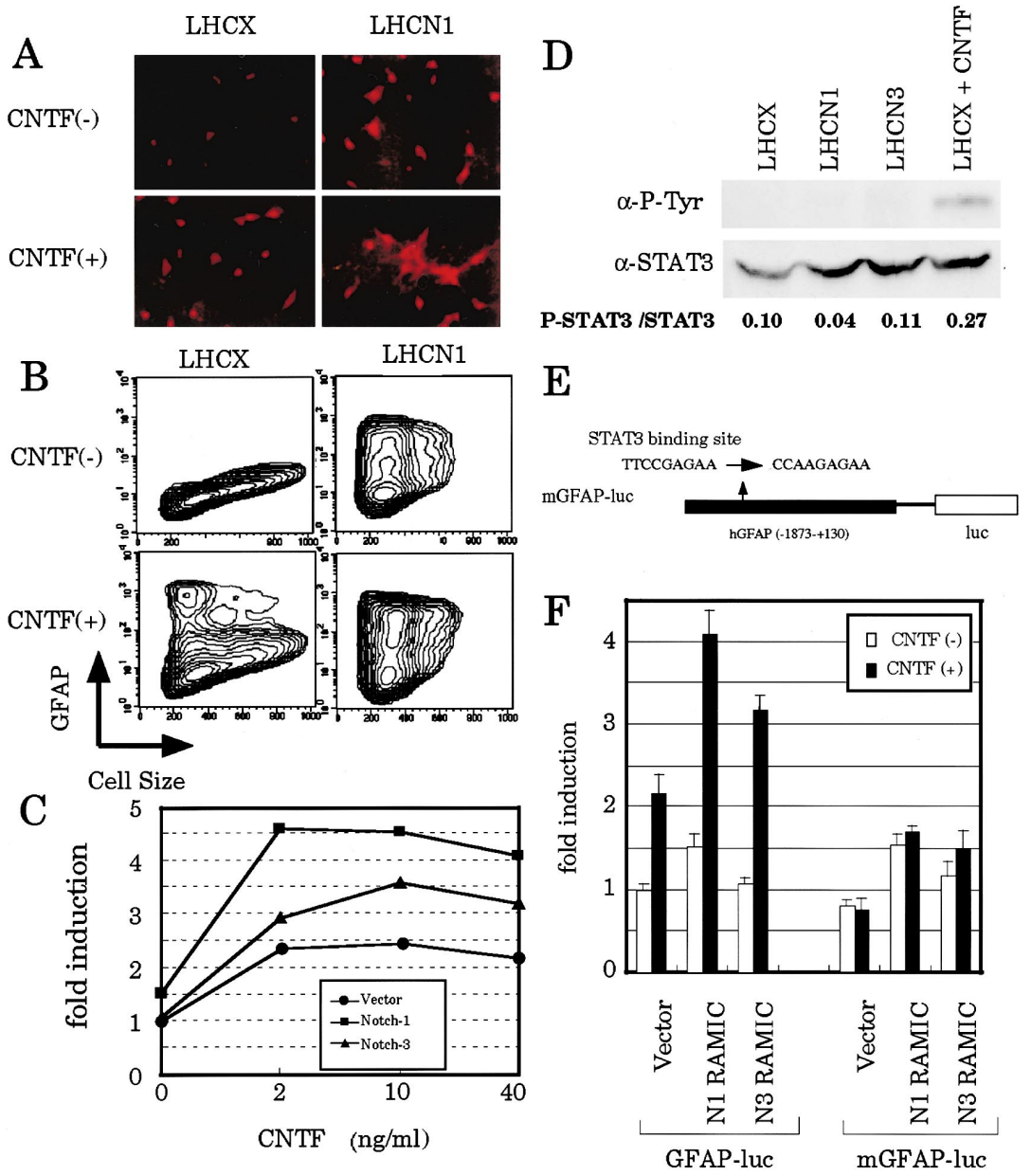

Figure 6. Additive Enhancement of Astroglial Differentiation by Activated Notch1 and CNTF and the Astroglial-Inductive Effects of Activated Notch Are Independent of STAT3

(A) AHPs cultured for 2 days either in the absence or presence of 2 ng/ml CNTF after transduction with LHCX and LHCN1 were immunostained with the anti-GFAP antibodies, followed by Texas red–conjugated anti-rabbit IgG secondary antibodies. CNTF led to the additive enhancement of the intensity of GFAP expression as well as the increase of cell size and process outgrowth.

(B) AHPs transduced with LHCX (two left panels) and LHCN1 (two right panels) were cultured in the presence or absence of CNTF in the differentiating conditions for 3 days. Cells were stained with the antibodies against GFAP and analyzed by flow cytometry.

(C) AHPs were transfected with GFAP-luc (250 ng) and Notch1 or Notch3 RAMIC (10 ng), and cultured in the absence or presence of various concentrations of CNTF. Luciferase activities were measured as described in the legend of Figure 4A.

(D) Activated Notch did not stimulate phospholylation of STAT3. AHPs were transduced with LHCX, LHCN1, and LHCN3, and cultured in the absence or presence of 2 ng/ml CNTF for 5 min. Total cell extracts were isolated and immunoprecipitated with polyclonal anti-STAT3 antibodies. Western blot filter was visualized sequentially with anti-phosphotyrosine and anti-STAT3 antibodies. The phospholylation levels were quantitated using a BAS1500 (Fuji film) and standardized by the expression level of STAT3.

(E) Schematic illustration of GFAP-luciferase constructs. Luciferase cDNA is flanked by human GFAP promoter (−1873 to 130) with or without mutated STAT3 binding motifs.

(F) Transcriptional activation from GFAP promotor by activated Notch did not require a STAT3 binding site. AHPs were transfected with the reportor plasmids shown in (E), sea urichin luciferase control vector, and Notch-1 and Notch3 RAMIC as indicated. Normalized luciferase activity is shown. The results are the mean ± SD of triplicate data from a representative experiment.

vated Notch had limited their potential to proliferate or self-renew because AHPs expressing activated Notch can proliferate immediately after infection, but after 2 months, they took terminally differentiated morphology and rapidly died out (data not shown). In addition, transient activation of Notch is sufficient for astroglial commitment of AHPs, which strongly suggests that multipotency of neural stem cells were deprived by the activation of Notch. It was difficult to see whether glial cells induced from rat AHPs by activated Notch has radial glial characteristics due to the absence of reagents for rat radial glial markers, such as RC-2 and BLBP. We observed high expression of another radial glial marker, Nestin, in GFAP-positive AHPs expressing activated Notch (unpublished data), which might suggest the possibility of radial glial cell induction by activated Notch. Further studies will be required to fully understand whether Notch really induces radial glial cells with stem cell properties.

Recent studies on neural crest stem cells and retina reached a similar conclusion to that presented here. Notch activation triggers Schwann cell generation from neural crest stem cells at the expense of neuronal differentiation and myogenesis (Morrison et al., 2000). Clonal analysis demonstrated that activated Notch instructively promotes glial differentiation. Similarly, Muller cell generation was enhanced when activated Notch, HES-1, and HES-5 were introduced to retinal progenitors (Furukawa et al., 2000; Hojo et al., 2000). Taken together with the present study, Notch signaling appears to instruct the formation of a variety of glial cells in both CNS and PNS, although the differentiation of oligodendrocytes, a kind of glia, is inhibited by Notch signaling.

Notch3 Has the Same Astroglial-Inductive Effects as Notch1

Previous studies on the function of Notch3 have shown that the intracellular region of Notch3 has a very low transactivating activity as compared to that of Notch1 and that Notch3 RAMIC can repress the transactivating activity of Notch1 RAMIC on HES-5 expression (Beatus et al., 1999). Similarly, the defects of Notch1 signaling accelerate the differentiation of pancreatic endocrine cells, while the overexpression of the intracellular region of Notch3 induces the same phenotype (Apelqvist et al., 1999). It has been thus proposed that Notch3 can function as a repressor of Notch1 by competition for binding to RBP-J or other common coactivators (Beatus et al., 1999). In contrast, our data show that both Notch1 and Notch3 have the same effects on astroglial development and HES-1 expression (Figures 1 and 5A). We also observed the strong transactivation activity of Notch3 RAMIC comparable with Notch1 RAMIC in CNS multipotent progenitor cells (Figure 4A). Recently, activated Notch3 has also shown to induce endogenous HES-1 and deltex expression in thymocytes, which indicates Notch3 has the ability to activate the RBP-J pathway in thymocytes (Bellavia et al., 2000). The difference between pancreas and thymus/CNS could be explained by the possibility that thymus/CNS multipotent progenitor cells have a different repertorie of coactivators and/or repressors from that of developing pancreatic cells. Similarly, the expression of activated Notch3 in neuroepithelial cells in vivo resulted in expansion of the ventricular zone containing undifferentiated cells, suggesting the inhibition of neural differentiation from neural-committed progenitors by activated Notch3 (Lardelli et al., 1994). These in vitro as well as in vivo studies indicate that Notch3 RAMIC has similar effects to those of Notch1 RAMIC in early neurogenesis.

The Pathway from Notch Activation to Astroglial Induction

Previous in vitro studies have identified several factors such as LIF, CNTF, and BMP2 that promote astroglial development (Johe et al., 1996; Richards et al., 1996; Bonni et al., 1997; Nakashima et al., 1999a). Both LIF and CNTF use gp130 in their receptor complexes as a signal-transducing component (Taga, 1996; Taga and Kishimoto, 1997). In mice deficient for gp130 or LIF receptor (LIFR), GFAP-positive astroglia are dramatically reduced, but GFAP low-positive cells are still detected. In vitro neuroepithelial cells from either gp130$^{-/-}$ or LIFR$^{-/-}$ mice can differentiate to astroglia after long passages, suggesting that other mechanisms might exist that regulate astroglial differentiation (Koblar et al., 1998; Nakashima et al., 1999b). One of the major cytoplasmic signaling molecules activated by LIF and CNTF is STAT3, and the expression of GFAP is directly enhanced by STAT3 (Bonni et al., 1997). GFAP expression by BMP2 is also dependent on STAT3 through the formation of the STAT3-Smad1 complex (Nakashima et al., 1999a). In contrast, our data show that the astroglia-inductive effect of Notch is independent of STAT3, indicating that Notch signaling promotes astroglial differentiation by a different mechanism from those of LIF, CNTF, and BMP2.

Experimental Procedures

Preparation of Retrovirus

The LHCX vector was constructed from LNCX (Clontech) by replacing the neomycin resistance gene with the hygromycin B resistance gene. To make LXSE, the hygromycin resistance gene and the CMV immediate-early promoter were replaced by the SV40 early promoter and E-GFP (Clontech) coding sequence. The fragments of mouse Notch1 (amino acid residues 1753–2531) and mouse Notch3 (amino acid residues 1663–2338) were tagged with the *myc* sequence and cloned into LHCX and LXSE. The fragments of mouse Notch1 fused to the human estrogen receptor was described previously (Schroeder and Just, 2000). The BOSC23 packaging cell line was transiently transfected with retrovirus DNA by Cell Phect (Amersham Pharmacia). The supernatant was collected 2 days later and concentrated with Centriprep 100 (Amicon).

Adult Hippocampal Progenitor Cell Culture

Neural precursors from adult rat hippocampal formations were isolated (Gage et al., 1995) and characterized extensively (Palmer et al., 1997; Takahashi et al., 1999). Primary cultures were maintained on poly-L-ornithine-laminin-coated dishes (Ray et al., 1993) in DMEM:F12 (1:1) with N2 supplement (GIBCO–BRL) and 20 ng/ml recombinant human bFGF (Genzyme) and used at passages 10 through 20.

To induce differentiation, cells were plated onto poly-L-ornithine-laminin-coated chamber slides (Nunc) at a density of 3×10^3 cells per cm^2. Cells were allowed to proliferate in N2 supplemented medium containing 20 ng/ml bFGF for 24 hr. bFGF was then withdrawn and cells were subsequently treated with N2 medium containing 0.5 μM retinoic acid and 0.5% fetal calf serum. Medium was replaced every 48 hr.

RT–PCR Analysis

Total RNA was isolated from cell culture using Trizol (GIBCO–BRL). cDNA was made using the Superscript preamplification system (GIBCO–BRL), and amplified by polymerase chain reaction using Taq polymerase. The following primers were used to amplify target cDNA: GAPDH, 5′-CTTCACCACCATGGAGAAGG-3′ and 5′-CAT GGACTGTGGTCATGAGC-3′; GFAP, 5′-CTGCGTCTGGACCAGCT TACTACC-3′ and 5′-TTACCACGATGTTCCTCTTGAGG-3′; PLP, 5′-GAGTGCTGTGCTAGATGTCTGGTAGG-3′ and 5′-TGTAAGTGGC AGCAATCATGAAGG-3′; HES-1, 5′-CGGAATTCCTCTGGGGATTG AGAAGAAAG-3′ and 5′-GCGGGATCCACGCTCGGGTCTGTGCTG AGAGC-3′. The amplified products were confirmed by DNA sequencing.

Immunofluorescent Staining

Cells were fixed in 4% paraformaldehyde and incubated with primary antibodies overnight at 4°C. Then the cells were incubated 1 hr with secondary antibodies (anti-mouse IgG Texas red and anti-rabbit IgG-FITC) (Jackson Laboratory). Primary antibodies used were monoclonal anti-MAP2 (clone HM-2, Sigma), polyclonal anti-GFAP(DAKO), and monoclonal anti-Gal-C (Boehringer Mannheim).

Clonal Analysis

Cells were plated onto poly-L-ornithine-laminin-coated chamber slides (Nunc) at a density of 3×10^3 cells per cm^2. After 24 hr in the presence of bFGF, cells were infected with low-titer retroviruses. After an additional 2 day expansion in the presence of bFGF, cells were cultured under the differentiating conditions without bFGF, and EGFP-positive colonies were analyzed 4 days later by immunofluorescent staining.

Luciferase Reportor Assay

Human GFAP promoter (−1873 to 130) was subcloned into pGL3 (Promega) by PCR. The mutation was introduced in STAT3 binding motifs by Quick Change Site-Directed Mutagenesis Kit (Stratagene). pGa981-6 and pGa50-7 were previously described (Kato et al., 1997). Reportor plasmids were cotransfected with pRL-CMV (Promega) using Lipofectamine Plus (GIBCO–BRL) . The total amount of DNA was kept constant with pGL3-basic. After 24 hr incubation, cells were subjected to luciferase assay, using a luminometer (Microplate luminometer LB96V, Berthold). Normalized luciferase activity (fire fly luciferase/sea urichin luciferase ratio) was then compared. In each experiment, samples were analyzed in triplicate, and each experiment was repeated at least three times.

Flow Cytometric Analysis

The cells were trypsinized, and 10 ml of cell suspension (DMEM:F12 with N2 supplement) was plated on a 100 mm petri dish and incubated at 37°C in a 5% CO_2 humidified atmosphere for 2–3 hr. Cells were washed and fixed, using FIX&PERM cell permeabilization kits (CALTAG). Cells were then stained using the following antibodies: monoclonal anti-MAP2 (clone HM-2, Sigma), polyclonal anti-GFAP (Dako), monoclonal anti-O4 (Chemicon), normal mouse IgM, normal mouse IgG or normal rabbit IgG (Santa Cruz), flourescein isothiocyanate-conjugated goat anti-rabbit IgG, anti-mouse IgM, or anti-mouse IgG antibody (Jackson Laboratory). Cells were washed and analyzed by a FACSCalibur with CELLQuest software version 3.1 (Becton Dickinson).

Immunoprecipitation and Immunoblotting

Cells (1×10^7) were lysed for 30 min in 1 ml of ice-cold lysis buffer containing 0.2% NP-40. After centrifugation, cell lysate was incubated with polyclonal anti-STAT3 antibodies (SantaCruz Biotechnology). The immune complex was incubated with protein G sepharose beads (Amersham Pharmacia), and the immunoprecipitate was washed and eluted in 5× SDS loading buffer by boiling. The protein was subjected to 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Hybond-P) (Amersham Pharmacia). The blot was incubated with monoclonal anti-STAT3 antibody (Zymed), monoclonal anti-phosphotyrosine antibody (PY20H) (Transduction Laboratories), and visualized by ECL detection system (Amersham Pharmacia)

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References

Apelqvist, A., Li, H., Sommer, L., Beatus, P., Anderson, D.J., Honjo, T., Hrabe, d.A.M., Lendahl, U., and Edlund, H. (1999). Notch signaling controls pancreatic cell differentiation. Nature *400*, 877–881.

Artavanis, T.S., Matsuno, K., and Fortini, M.E. (1995). Notch signaling. Science *268*, 225–232.

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

Barres, B.A. (1999). A new role for glia: generation of neurons! Cell *97*, 667–670.

Beatus, P., Lundkvist, J., Oberg, C., and Lendahl, U. (1999). The notch 3 intracellular domain represses notch 1-mediated activation through Hairy/Enhancer of split (HES) promoters. Development *126*, 3925–3935.

Bellavia, D., Alesse, E., Vacca, A., Felli, M.P., Balestri, A., Stoppacciaro, A., Tiveron, C., Tatangelo, L., Giovarelli, M., Gaetano, C., et al. (2000). Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in notch3 transgenic mice. EMBO J. *19*, 3337–3348.

Bonni, A., Sun, Y., Nadal, V.M., Bhatt, A., Frank, D.A., Rozovsky, I., Stahl, N., Yancopoulos, G.D., and Greenberg, M.E. (1997). Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. Science *278*, 477–483.

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

Fortini, M.E., Rebay, I., Caron, L.A., and Artavanis, T.S. (1993). An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye. Nature *365*, 555–557.

Furukawa, T., Mukherjee, S., Bao, Z.Z., Morrow, E.M., and Cepko, C.L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron *26*, 383–394.

Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T., and Ray, J. (1995). Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc. Natl. Acad. Sci. USA *92*, 11879–11883.

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.

Gray, G.E., and Sanes, J.R. (1992). Lineage of radial glia in the chicken optic tectum. Development *114*, 271–283.

Greenwald, I. (1994). Structure/function studies of lin-12/Notch proteins. Curr. Opin. Genet. Dev. *4*, 556–562.

Gross, R.E., Mehler, M.F., Mabie, P.C., Zang, Z., Santschi, L., and Kessler, J.A. (1996). Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. Neuron *17*, 595–606.

Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F., and Kageyama, R. (2000). Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. Development *127*, 2515–2522.

Honjo, T. (1996). The shortest path from the surface to the nucleus: RBP-J kappa/Su(H) transcription factor. Genes Cells *1*, 1–9.

Jacobson, M. (1991). Developmental Neurobiology, Third Edition (New York: Plenum Press).

Jarriault, S., Brou, C., Logeat, F., Schroeter, E.H., Kopan, R., and Israel, A. (1995). Signaling downstream of activated mammalian Notch. Nature *377*, 355–358.

Johe, K.K., Hazel, T.G., Muller, T., Dugich, D.M., and McKay, R.D. (1996). Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev. *10*, 3129–3140.

Kato, H., Taniguchi, Y., Kurooka, H., Minoguchi, S., Sakai, T., Nomura, O.S., Tamura, K., and Honjo, T. (1997). Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives. Development *124*, 4133–4141.

Kilpatrick, T.J., and Bartlett, P.F. (1995). Cloned multipotential precursors from the mouse cerebrum require FGF-2, whereas glial restricted precursors are stimulated with either FGF-2 or EGF. J. Neurosci. *15*, 3653–3661.

Kim, J., Sebring, A., Esch, J.J., Kraus, M.E., Vorwerk, K., Magee, J., and Carroll, S.B. (1996). Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene. Nature *382*, 133–138.

Koblar, S.A., Turnley, A.M., Classon, B.J., Reid, K.L., Ware, C.B., Cheema, S.S., Murphy, M., and Bartlett, P.F. (1998). Neural precursor differentiation into astrocytes requires signaling through the leukemia inhibitory factor receptor. Proc. Natl. Acad. Sci. USA *95*, 3178–3181.

Kopan, R., Schroeter, E.H., Weintraub, H., and Nye, J.S. (1996). Signal transduction by activated mNotch: importance of proteolytic processing and its regulation by the extracellular domain. Proc. Natl. Acad. Sci. USA *93*, 1683–1688.

Kornack, D.R., and Rakic, P. (1995). Radial and horizontal deployment of clonally related cells in the primate neocortex: relationship to distinct mitotic lineages. Neuron *15*, 311–321.

Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H., and Honjo, T. (1999). Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. J. Biol. Chem. *274*, 7238–7244.

Lardelli, M., Dahlstrand, J., and Lendahl, U. (1994). The novel Notch homologue mouse Notch 3 lacks specific epidermal growth factor-repeats and is expressed in proliferating neuroepithelium. Mech. Dev. *46*, 123–136.

Lendahl, U., Zimmerman, L.B., and McKay, R.D. (1990). CNS stem cells express a new class of intermediate filament protein. Cell *60*, 585–595.

Luskin, M.B. (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron *11*, 173–189.

Metcalf, D. (1980). Clonal analysis of proliferation and differentiation of paired daughter cells: action of granulocyte-macrophage colony-stimulating factor on granulocyte-macrophage precursors. Proc. Natl. Acad. Sci. USA *77*, 5327–5330.

Metcalf, D. (1991). Lineage commitment of hemopoietic progenitor cells in developing blast cell colonies: influence of colony-stimulating factors. Proc. Natl. Acad. Sci. USA *88*, 11310–11314.

Morrison, S.J., 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.

Muskavitch, M.A. (1994). Delta-notch signaling and Drosophila cell fate choice. Dev. Biol. *166*, 415–430.

Nakashima, K., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Kawabata, M., Miyazono, K., and Taga, T. (1999a). Synergistic signaling in fetal brain by STAT3-Smad1 complex bridged by p300. Science *284*, 479–482.

Nakashima, K., Wiese, S., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Yoshida, K., Kishimoto, T., Sendtner, M., and Taga, T. (1999b). Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. J. Neurosci. *19*, 5429–5434.

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

Palmer, T.D., Takahashi, J., and Gage, F.H. (1997). The adult rat hippocampus contains primordial neural stem cells. Mol. Cell. Neurosci. *8*, 389–404.

Parnavelas, J.G., Barfield, J.A., Franke, E., and Luskin, M.B. (1991). Separate progenitor cells give rise to pyramidal and nonpyramidal neurons in the rat telencephalon. Cereb. Cortex *1*, 463–468.

Ray, J., Peterson, D.A., Schinstine, M., and Gage, F.H. (1993). Proliferation, differentiation, and long-term culture of primary hippocampal neurons. Proc. Natl. Acad. Sci. USA *90*, 3602–3606.

Raedler, E., Raedler, A., and Feldhaus, S. (1980). Dynamical aspects of neocortical histogenesis in the rat. Anat. Embryol. Berl. *158*, 253–269.

Rebay, I., Fehon, R.G., and Artavanis, T.S. (1993). Specific truncations of Drosophila Notch define dominant activated and dominant negative forms of the receptor. Cell *74*, 319–329.

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

Reynolds, B.A., and Weiss, S. (1996). Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev. Biol. *175*, 1–13.

Richards, L.J., Kilpatrick, T.J., Dutton, R., Tan, S.S., Gearing, D.P., Bartlett, P.F., and Murphy, M. (1996). Leukaemia inhibitory factor or related factors promote the differentiation of neuronal and astrocytic precursors within the developing murine spinal cord. Eur. J. Neurosci. *8*, 291–299.

Sakurada, K., Ohshima-Sakurada, M., Palmer, T.D., and Gage, F.H. (1999). Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. Development *126*, 4017–4026.

Schroeder, T., and Just, U. (2000). Notch signaling via RBP-J promotes myeloid differentiation. EMBO J. *19*, 2558–2568.

Schroeter, E.H., Kisslinger, J.A., and Kopan, R. (1998). Notch-1 signaling requires ligand-induced proteolytic release of intracellular domain. Nature *393*, 382–386.

Sidman, R.L., and Rakic, P. (1973). Neuronal migration, with special reference to developing human brain: a review. Brain Res. *62*, 1–35.

Struhl, G., and Adachi, A. (1998). Nuclear access and action of notch in vivo. Cell *93*, 649–660.

Suhonen, J.O., Peterson, D.A., Ray, J., and Gage, F.H. (1996). Differentiation of adult hippocampus-derived progenitors into olfactory neurons in vivo. Nature *383*, 624–627.

Taga, T. (1996). gp130, a sgared signal transducing receptor component for hematopoietic and neuropoietic cytokines. J. Neurochem. *67*, 1–10.

Taga, T., and Kishimoto, T. (1997). gp130 and the interleukin-6 family of cytokines. Annu. Rev. Immunol. *15*, 797–819.

Takahashi, M., Palmer, T.D., Takahashi, J., and Gage, F.H. (1998). Widespread integration and survival of adult-derived neural progenitor cells in the developing optic retina. Mol. Cell. Neurosci. *12*, 340–348.

Takahashi, J., Palmer, T.D., and Gage, F.H. (1999). Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. J. Neurobiol. *38*, 65–81.

Tamura, K., Taniguchi, Y., Minoguchi, S., Sakai, T., Tun, T., Furukawa, T., and Honjo, T. (1995). Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). Curr. Biol. *5*, 1416–1423.

Temple, S., and Qian, X. (1996). Vertebrate neural progenitor cells: subtypes and regulation. Curr. Opin. Neurobiol. *6*, 11–17.

Wang, S., Sdrulla, A.D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G., and Barres, B.A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. Neuron *21*, 63–75.

Williams, B.P., and Price, J. (1995). Evidence for multiple precursor cell types in the embryonic rat cerebral cortex. Neuron *14*, 1181–1188.