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Activated *Notch2* Signaling Inhibits Differentiation of Cerebellar Granule Neuron Precursors by Maintaining Proliferation

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Summary

In the developing cerebellar cortex, granule neuron precursors (GNPs) proliferate and commence differentiation in a superficial zone, the external granule layer (EGL). The molecular basis of the transition from proliferating precursors to immature differentiating neurons remains unknown. Notch signaling is an evolutionarily conserved pathway regulating the differentiation of precursor cells of many lineages. *Notch2* is specifically expressed in proliferating GNPs in the EGL. Treatment of GNPs with soluble Notch ligand Jagged1, or overexpression of activated *Notch2* or its downstream target *HES1*, maintains precursor proliferation. The addition of GNP mitogens Jagged1 or Sonic Hedgehog (Shh) upregulates the expression of *HES1*, suggesting a role for *HES1* in maintaining precursor proliferation.

Introduction

The cerebellar cortex provides a unique system for studying central nervous system (CNS) cell specification and development. For nearly a century, all of the cerebellar cell types and the pattern of their synaptic connections have been known (Hays et al., 1999). The cerebellar granule neuron, the most abundant neuron of the CNS, has facilitated analyses of the molecular and cellular mechanisms that underlie the basic steps in neuronal differentiation. Granule neuron precursors (GNPs) arise in a superficial germinal zone, the external granule layer (EGL). After birth, rapid proliferation in the EGL generates the millions of granule neurons in the cerebellar cortex. Proliferating GNPs located in the outer layer of the EGL express a number of genes that mark the granule neuron lineage, including transcription factors such as *Math1* (Akazawa et al., 1995; Ben-Arie et al., 1997; Helms and Johnson, 1998), *RU49* (Yang et al., 1996), *Zic1, Zic2* (Aruga et al., 1994, 1998; Nagai et al., 1997), and genes involved in cell cycle progression and proliferation, including *CylinD2* (Ross et al., 1996), *Patched* (*Ptc*), *Smoothened* (*Smo*), *Gli1*, and *Gli2* (Traiffort et al., 1999; Wechsler-Reya and Scott, 1999). Newly postmitotic granule neurons located in the inner EGL express *NeuroD* (Miyata et al., 1999), the axonal glycoprotein *TAG-1* (Furley et al., 1990), neuron-specific class III β tubulin, and *p27/KIP* cell cycle inhibitor (Miyazawa et al., 2000), all of which are markers for granule neurons that have entered the early stages of terminal differentiation.

The balance between proliferation and the programmed withdrawal of cells from the cell cycle is critical to the differentiation of GNPs, and the molecules that regulate this balance are not yet understood. The Sonic Hedgehog (Shh) signaling pathway has been suggested to control the number of GNPs by stimulating the proliferation of this cell type (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Shh protein, produced by Purkinje cells, is a potent mitogen stimulating the proliferation of GNPs (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). GNPs that respond to Shh in vitro remain undifferentiated and continue to express precursor-like markers.

Homotypic cell to cell contacts between GNPs may also be critical for the maintenance of a dividing population of cells within the EGL. In vitro experiments show that purified postnatal granule cells, containing a mixture of mitotic GNPs and postmitotic granule neurons, cultured as cellular reaggregates proliferate at a rate that is ten times higher than cells plated at densities with sparse cell contact (Gao et al., 1991). Reaggregate cultures contain an expanded population of 5-bromo-2′-deoxyuridine (BrDU)-positive cells, suggesting that cell contact may play a role in maintaining GNPs in the proliferative state in vivo. The molecules that regulate proliferation within reaggregate cultures are not yet known.

Notch signaling via cell to cell interactions is a key regulator of cell fate decisions in many cell lineages of both invertebrate and vertebrate organisms (reviewed in Artavanis-Tsakonas et al., 1999; Lewis, 1996; Weinmaster, 2000). In the *Drosophila* nervous system, Notch regulates a process of lateral inhibition, whereby a single neuron differentiates within a field of roughly similar precursor cells. This process is controlled by expression of the Notch ligands *Delta* and *Serrate*. Several vertebrate Notch receptors have been identified, including *Notch1*, *Notch2*, *Notch3*, and *Notch4*, which bind to *Delta*- or *Serrate*-like ligands (*Delta-like 1* and *Delta-like 3* or *Jagged1* and *Jagged2*). Ligand binding of Notch receptors leads to the proteolytic processing and nuclear translocation of the Notch intracellular domain (ICD). When the ICD translocates to the nucleus, it forms a complex with the *CBF1* DNA binding protein. This complex then activates transcription of Notch effector genes, such as the *Hairy* and *Enhancer of Split* homologs (*HES1* or *HES5*). These genes act as transcriptional repressors and are thought to antagonize the action of bHLH transcription factors that are required for the progression of a program of neuronal differentiation (reviewed in Kageyama and Nakanishi, 1997; Lewis, 1996). In agreement with this model is the finding that precocious neuronal differentiation is observed in mice with null mutations in either the *Notch1* or *HES1* genes, suggesting that the Notch signaling pathway is important in maintaining the neural precursor population in the embryo (Cau et al., 2000; de la Pompa et al., 1997; Ishibashi et al., 1995).

Given the role of cell to cell contact in maintaining GNP populations, we have examined whether Notch

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signaling in the EGL influences the differentiation or proliferation of GNPs. Mouse *Notch2* is temporally expressed in proliferating GNPs prior to differentiation and migration. These studies show that overexpression of constitutively active *Notch2* or the *HES1* transcription repressor inhibits GNP differentiation in vitro and in cerebellar slice cultures, as evidenced by the failure to extend neurites or express early differentiation markers. Activation of Notch2 by the addition of its ligand Jagged1 protein leads to an increase in GNP proliferation. In addition, a subpopulation of GNPs responds directly to overexpression of *Notch2* ICD or *HES1* by remaining in the proliferative state. Treatment of granule cell cultures with Jagged1 or Shh proteins leads to the activation of *HES1* mRNA, and the *HES1* promoter is transactivated by both *Notch2* ICD and Shh overexpression in a heterologous system. Thus, *HES1* may represent a point of crosstalk between the Notch2 and Shh signaling cascades.

Results

During early postnatal development, cerebellar GNPs undergo massive proliferation in the EGL to expand this population into a layer of cells six to eight cells thick at postnatal day 6 (P6). After P6, GNPs progressively exit the cell cycle, differentiate, and migrate to their final destination, the internal granule layer. The mechanisms regulating the timing of the cell cycle exit and differentiation of GNPs remain poorly understood. The Notch signaling cascade has been shown to be an important determinant regulating the balance between the precursor and differentiated states in numerous lineages. Therefore, we examined the role of Notch function in the process of GNP differentiation.

Notch Pathway Genes Are Expressed in Granule Cells

To determine whether early postnatal granule cells express genes belonging to the Notch signaling cascade, we used reverse transcription-polymerase chain reaction (RT-PCR) to analyze total RNA isolated from freshly purified granule cells from P5, P6, or P7 mice. These preparations of purified postnatal granule cells contain a mixture of proliferating GNPs and differentiating granule neurons (see Experimental Procedures). Specific RT-PCR products were detected for both *mNotch1* and *mNotch2*, indicating that postnatal granule cells express these genes during the time points analyzed (Figure 1). Notch receptors bind to ligands of the *Delta-Serrate-Lag-2* (DSL) class. Purified granule cells also express the mRNAs of these Notch receptor ligands. *Jagged1* is most abundantly expressed, followed by *Delta-like 1* and *Delta-like 3*, with a small amount of *Jagged2* message detected in our experiments. Once the Notch ICD is cleaved, it translocates to the nucleus and binds to *CBF1*, an event that is essential for the propagation of the Notch signal. This complex transcriptionally activates downstream targets, including the bHLH transcriptional repressors *HES1* and *HES5*. Purified granule cells express *CBF1*, *HES1*, and *HES5* (Figure 1). Taken together, these results suggest that postnatal granule cells express all of the components necessary to transduce a Notch signal from the membrane to the nucleus.

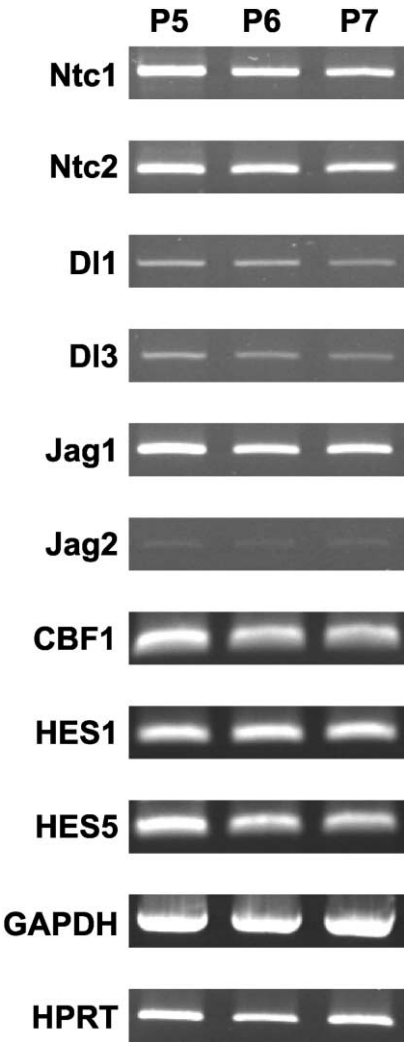

Figure 1. Genes Required for Transducing the Notch Signaling Cascade Are Expressed in Purified Cerebellar Granule Cells

Cerebellar granule cells were purified (see Experimental Procedures) from postnatal days (P) 5, 6, or 7 mice. Total RNA was isolated. Equal amounts of this RNA were subjected to reverse transcription using random hexamers as a primer for RT and then were amplified by PCR using primers specific for the Notch pathway genes. Amplification of the GAPDH and HPRT messages was used as controls for equal loading. Reactions carried out without reverse transcriptase were used as a control for genomic DNA contamination (data not shown).

Notch2 Is Highly Expressed in Proliferating GNPs in the EGL and Is Downregulated in Postmitotic Granule Neurons in the Internal Granule Layer

In vertebrates, Notch genes are known to regulate the processes of differentiation of a variety of lineages within the CNS (Bao and Cepko, 1997; Henrique et al., 1997; Myat et al., 1996; Weinmaster et al., 1991). To confirm further the expression pattern of Notch receptors in the developing cerebellum, we analyzed the pattern of *Notch2* mRNA expression by in situ hybridization on E16, P0, P6, P10, or adult mouse brains. Preliminary in situ hybridization and antibody staining analysis did not reveal significant *Notch1* RNA expression in GNPs in the EGL and, instead, suggested that the expression that

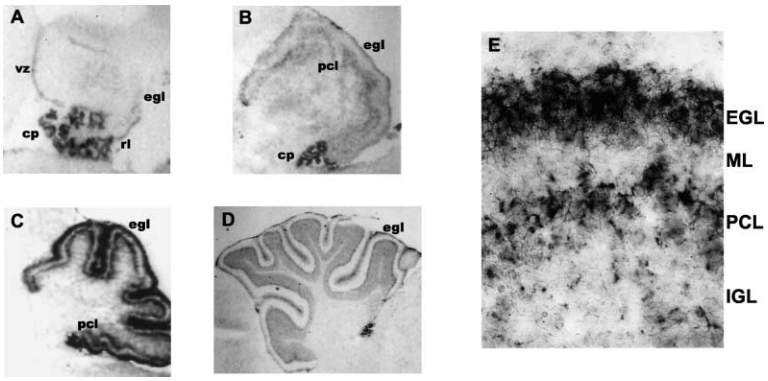

Figure 2. *Notch2* mRNA Expression in the Developing Cerebellum

Notch2 mRNA is detected in parasagital sections of developing cerebellum of E16 embryos, P0, P6, and P10 mice.

(A) At E16, *Notch2* expression is not strong in the cerebellar anlage.

(B) At P0, *Notch2* expression in the cerebellum increases and is observable in the Purkinje cell layer and in the EGL that is one to two cells thick at this stage of development.

(C) At P6, *Notch2* is expressed highly in the cerebellar EGL and is still observable in the Purkinje cell layer.

(D) At P10, *Notch2* expression is reduced in the cerebellum.

(E) A higher magnification of plate (C). *Notch2* is highly expressed in proliferating GNPs of the EGL and is downregulated in differentiating granule neurons in deeper aspects of the inner EGL. *Notch2* is not expressed in the migrating granule neurons in the molecular layer or postmigratory cells of the internal granule layer. Abbreviations are as follows: rl, rhombic lip; vz, ventricular zone; egl, external granule layer; pcl, Purkinje cell layer; ml, molecular layer; and igl, internal granule layer.

we detected by RT-PCR was specific for more mature granule cells (data not shown).

At E16, only low levels of *Notch2* expression were detected in the cerebellar anlage, but high levels were detected in the choriod plexus in the lateral and fourth ventricles at this stage (Figure 2A). At P0, *Notch2* was detected in a central layer of the cerebellar anlage, which is thought of give rise to Bergman glia, as has been reported by Tanaka et al. (1999). At P0, *Notch2* expression was also increased in GNPs of the EGL, a layer of only two cells in thickness (Figure 2B).

At P6, a time when GNP proliferation is near its peak, *Notch2* message was strongly detected in the cerebellum (Figure 2C). Under higher magnification, the message was localized to the proliferating GNPs of the EGL (Figure 2E). *Notch2* was not detected in migrating granule neurons in the molecular layer nor in the differentiated neurons already present in the nascent internal granule layer (IGL). A subpopulation of cells in the Purkinje cell layer also expresses *Notch2* mRNA. From cell body position and numbers of cell labeled, these cells are likely to represent Bergman glia. Indeed, Tanaka et al. (1999) have reported *Notch2* expression in glial cells of the cerebellum using a LacZ "knockin" into the mouse *Notch2* gene.

At P10, when a significant number of granule neurons had completed differentiation, much less *Notch2* expression was detected in the cerebellum (Figure 2D). At P20 and in adult cerebellum, *Notch2* expression is not observable in the cerebellum, a result consistent with the lack of undifferentiated GNPs at these ages (data not shown). Thus, the expression of the *Notch2* receptor is temporally regulated during the histogenesis of cerebellar development. Specifically, *Notch2* is expressed in the GNPs of the EGL, marking a stage of granule neuron development prior to the commitment of GNPs to entry into the differentiation program.

Notch2 ICD and *HES1* Overexpression Inhibits GNP Differentiation

Our expression studies indicate that *Notch2* expression declines as GNPs differentiate into postmitotic neurons. In order to analyze the role *Notch2* signaling may play in GNP differentiation, we have utilized an overexpression system. Overexpression of the ICD of Notch receptors has been shown to activate Notch signaling constitutively in a variety of systems (Weinmaster, 2000). We have therefore infected GNPs with a recombinant retrovirus containing the ICD of mouse *Notch2*. The granule cell preparations used in these studies contain a mixture of proliferating GNPs and postmitotic granule neurons. The ecotropic retroviruses used in these studies infect only proliferating cells; therefore, we specifically infect the GNPs within our purified postnatal granule cell cultures. Our retroviral constructs contain an IRES element followed by either alkaline phosphatase (AP) or the green fluorescent protein (GFP) to serve as a marker for virally infected cells. The effects of *Notch2* signaling on GNP differentiation were first determined by the length of neurites elaborated by differentiating GNPs, a morphological measure of differentiation. These assays utilized the halo of neurites that extend from a granule cell reaggregate that has been cultured on a substrate of poly-L-lysine. Under normal culture conditions, the differentiating GNPs will extend neurites 40–200 μm in length, forming a halo around the body of a reaggregate (Gao et al., 1991). Cultures that were infected with a control virus that expresses only the AP maker gene exhibit a robust degree of AP-positive neurite extension (see Figure 3A). In contrast, the halo of neurites surrounding reaggregates overexpressing the *Notch2* ICD possessed a significant reduction in the number of AP-positive neurites, indicating that overexpression of the *Notch2* ICD interferes with the ability of GNPs to differentiate and extend neurites (Figure 3B).

Next, we examined an early marker of differentiation, TAG-1, to determine the point at which development the *Notch2* ICD overexpressing cells arrested. In these experiments, GNPs were infected with a recombinant retrovirus containing the ICD of mouse *Notch2* and plated at medium density to allow quantitation of TAG-1 expression and neurite extension. Eighty-one percent of GNPs infected with the control virus differentiated and extended neurites (Figures 4A–4C and 5A). Consistent with the morphological appearance of differentiation, all neurite-bearing cells also were positive for the TAG-1 differentiation marker. Although 18% of the GNPs in the control cultures did not extend neurites, roughly

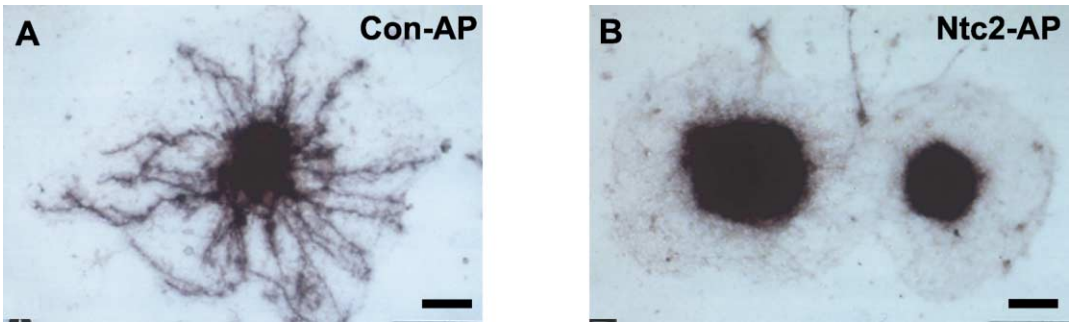

Figure 3. Overexpression of the *Notch2* ICD Blocks Neurite Extension in Cultured Granule Neurons

Purified P6 cerebellar granule cells cultured as reaggregates extend a halo of neurites when transferred to a poly-L-lysine substrate. GNPs infected with control retrovirus that expresses the AP reporter gene differentiate and possess long neurites within the halo surrounding the reaggregates (A). GNPs overexpressing the *Notch2* ICD lack neurites (B). Scale bars equal 50 μm.

70% of these cells expressed TAG-1, indicating that while they had not yet initiated neurite extension, many of these cells had entered the initial phases of differentiation marked by TAG-1 expression. These data suggest that the onset of TAG-1 expression may occur before the initiation of neurite extension. In response to overexpression of the *Notch2* ICD, only 32% of infected GNPs extended TAG-1-positive neurites, a greater than 2-fold reduction of neurite-bearing cells as compared with the control cultures (Figures 4D–4F and 5A). Only 42% of the *Notch2* ICD overexpressing the cells that lacked neurites expressed the TAG-1 differentiation marker, indicating that many of these cells did not express markers or extend processes typical of granule neuron development (Figures 4D–4F and 5B).

We have also examined the effect of overexpression of *HES1* on GNP differentiation. GNPs were infected with recombinant retroviruses that contained the cDNA of mouse *HES1*, a downstream target of Notch signaling. GNPs overexpressing *HES1* displayed a phenotype similar to that of the cells expressing the *Notch2* ICD (Figures 4G–4I). Only 28% of cells overexpressing *HES1* extended TAG-1-positive neurites (Figure 5A), whereas of the cells that lacked neurites, only 28% expressed the TAG-1 differentiation marker (Figure 5B).

A retroviral expression construct was also generated for *HES5*, the other Hairy and Enhancer of Split homolog expressed in postnatal granule cells. The results of our initial overexpression studies suggested that *HES5* did not to block the neurite extension of GNPs (data not shown); therefore, we have not further investigated the role of *HES5* in GNP differentiation. In summary, overexpression of the *Notch2* ICD and the *HES1* transcriptional repressor inhibits the onset of neurite extension and TAG-1 expression and suggests that Notch2 signaling may regulate the differentiation of GNPs.

Overexpression of *Notch2* ICD and *HES1* In Situ Inhibits GNP Differentiation

Our analysis of *Notch2* and *HES1* function suggests that these molecules may be involved in regulating the differentiation of GNPs. In order to investigate further the roles of *Notch2* and *HES1*, we examined their effects on GNP differentiation using organotypic cerebellar slice cultures. Because ecotropic retroviruses infect proliferating cells within the cerebellar slices, we could monitor the influence of *Notch2* ICD and *HES1* overexpression on GNPs before they exit the cell cycle and enter the program of differentiation. In accordance with our previous results, GNPs infected with a GFP expressing control retrovirus differentiated normally, extended long parallel fibers, and possessed either the bipolar or T-shaped morphology characteristic of granule neurons (Figure 6A) (Bhatt et al., 2000; Tomoda et al., 1999). In contrast, GNPs overexpressing *Notch2* ICD or *HES1* either lacked processes or extended significantly shortened neurites (Figures 6B and 6C). Many of the cells that lacked processes remained near the surface of the cerebellum. As GNPs differentiate, they migrate down toward the molecular layer; therefore, we also measured the degree of inward migration of infected cells away from the pial surface. Cells were placed into three depth categories: 1–20, 21–40, or 41+ μm away from the pia. The majority of control cells had migrated away from the pial surface (16% ± 0.98% within 1–20 μm versus 60% ± 2% 41+ μm) (see Figure 6E). A significant increase in the numbers of cells near the pial surface was observed with the cells overexpressing *Notch2* ICD or *HES1* (41% ± 3.6% and 50% ± 1.6% within 1–20 μm of the pia, respectively) (see Figure 6E). This increase in cells near the pial surface correlated with a reduction in cells that had migrated into deeper regions of the slice cultures (31% ± 4% and 24% ± 3.9% within 41+ μm of the pia, respectively). Therefore, cells that overexpress *Notch2* ICD or *HES1* did not undergo neurite extension or inward migration in situ.

Activation of *Notch2* Signaling Leads to the Maintenance of GNP Proliferation

To test whether activation of Notch signaling prevents the early steps of differentiation by maintaining proliferation, we incubated purified postnatal granule cell cultures in the presence or absence of soluble Jagged1 protein, a ligand for Notch receptors. ^{3}H-thymidine was added to the cultures at 48 hr, and incorporation was measured to examine GNP proliferation. Cultures treated with 100 nM Jagged1 incorporated ^{3}H-thymidine at levels 4-fold higher than control cultures, indicating that Jagged1 protein is mitogenic for GNPs (Figure 7A). We also compared the ^{3}H-thymidine incorporation elicited

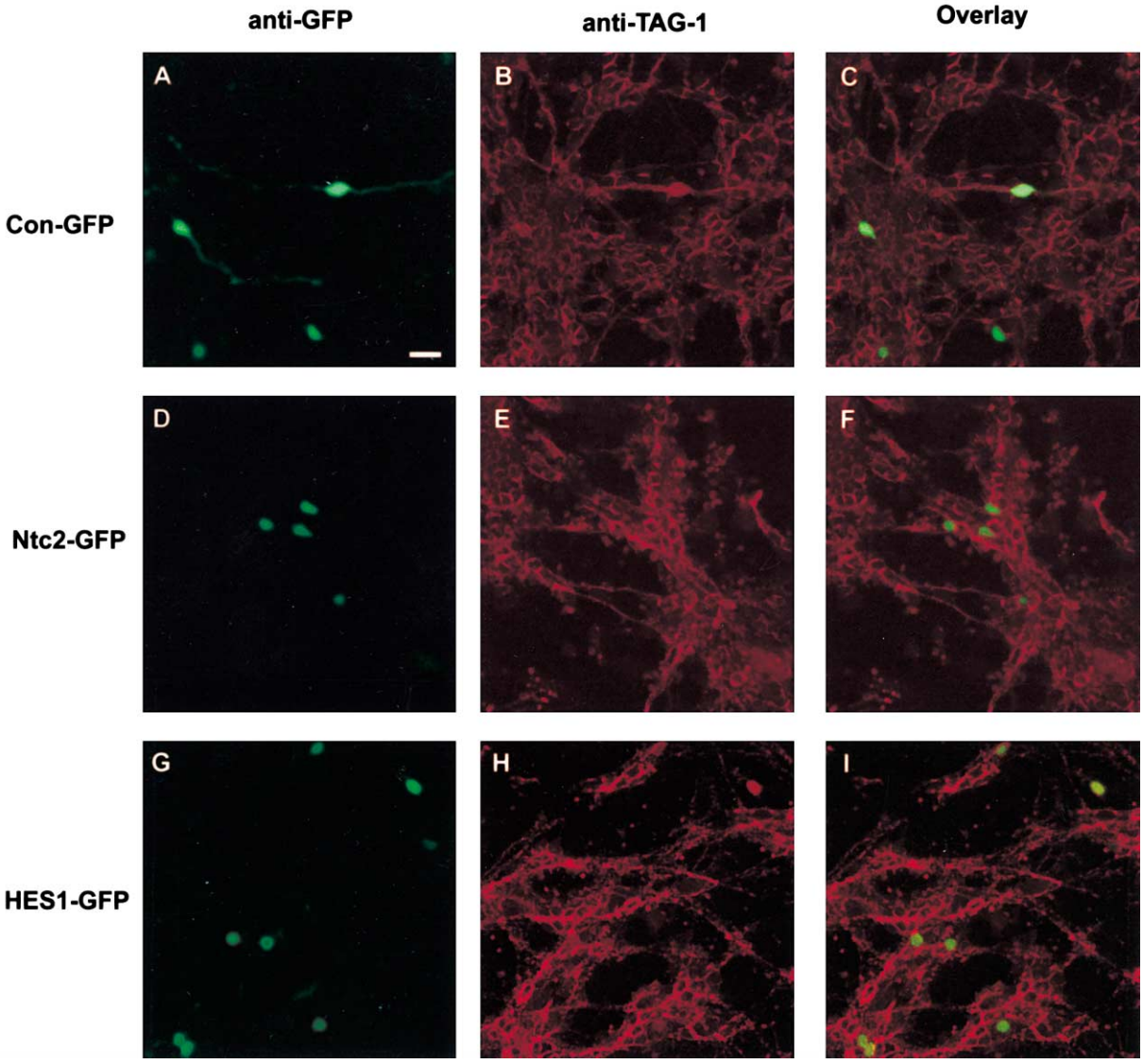

Figure 4. Overexpression of the *Notch2* ICD or *HES1* Inhibits the Onset of GNP Differentiation

Morphology and the early differentiation marker TAG-1 was used to assess the differentiation status of infected GNPs.
(A–C) GNPs differentiate normally, extend long neuritis, and express TAG-1 when infected with control retroviruses.
(D–F) A field of four cells overexpressing *Notch2* ICD that lack neurites and do not express TAG-1.
(G–I) A field of seven cells overexpressing *HES1* that lack neurites and do not express TAG-1. Scale bars equal 20 μm.

by Jagged1 to that of the known GNP mitogen Shh. Cultures treated with 100 nM Shh incorporated ^{3}H-thymidine at levels 10-fold higher than control cultures. Interestingly, at the 10 nM concentration, both factors stimulated equal levels of ^{3}H-thymidine incorporation. These results suggest that activation of Notch signaling by addition of soluble ligand stimulates GNPs in culture to proliferate as measured by ^{3}H-thymidine incorporation. Apparently, like Shh, Notch signaling in GNPs acts to maintain proliferation and inhibit early steps in differentiation.

Next, we examined whether overexpression of *Notch2* ICD or *HES1* directly maintained GNP proliferation. We pulsed cerebellar slice cultures infected with the *Notch2* ICD or *HES1* viruses with BrDU after 30 hr of culture and examined GFP expressing cells for the presence of labeled nuclei. At this time point, the majority of GNPs in the cerebellar slices have exited the cell cycle and extended parallel fibers (see Figures 6A and 7A). In accordance with the morphological appearance of differentiation, few bipolar or T-shaped granule neurons possessed BrDU-labeled nuclei in the control cultures (Figure 7B). Interestingly, a subpopulation of *Notch2* ICD or *HES1* overexpressing cells were labeled with BrDU, indicating that they remained in a proliferative state (Figure 7B). Quantitation of these results indicated that approximately 7.5% ± 0.94% of infected cells in control cultures incorporated BrDU, whereas a 2- to 3-fold increase of BrDU-positive cells was observed in infected granule neurons that overexpressed *Notch2* ICD (20.2% ±

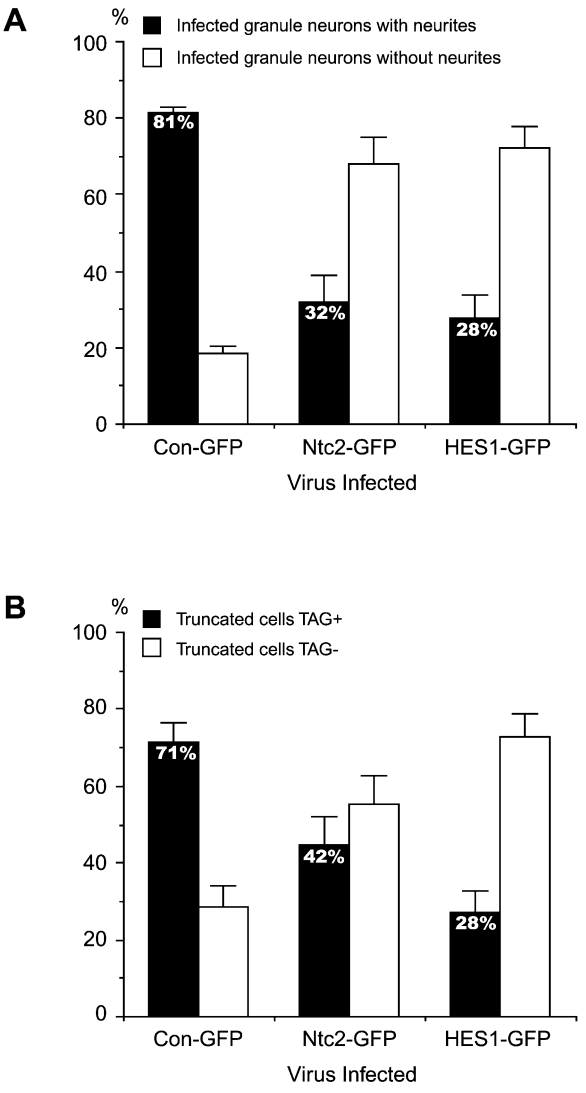

Figure 5. Quantitation of Neurite Extension and TAG-1 Expression in GNPs Overexpressing the *Notch2* ICD or *HES1*

(A) Two hundred cells were scored for the presence or absence of neurites and were counted from at least three individual experiments. Cells with processes longer than three-cell body lengths were counted as neurite-bearing cells; 81% ± 1.6% of control cells extended neuritis, whereas only 32% ± 6.8% and 28% ± 5.6% of cells overexpressing the *Notch2* ICD and *HES1* harbored neuritis, respectively.

(B) Neurite negative cells were scored for TAG-1 expression to determine their differentiation status. A cell was considered TAG-1 positive if there was any red signal associated with its cell body; 71% ± 5.3% of the control cells that lacked neuritis expressed TAG-1, indicating that although these cells did not elaborate processes, the majority of these cells had begun differentiation. A lower number of cells overexpressing the *Notch2* ICD and *HES1* were TAG-1 positive (42% ± 7.5% and 28% ± 5.6%, respectively), indicating that less of these cells had entered the differentiation cascade.

0.95%) or *HES1* (23.4% ± 2.4%, n = 4). Taken together, these results suggest that activated Notch signaling, either by exposure to Notch ligands or overexpression of *Notch2* ICD or the *HES1*, allows some GNPs to remain within the mitotic state.

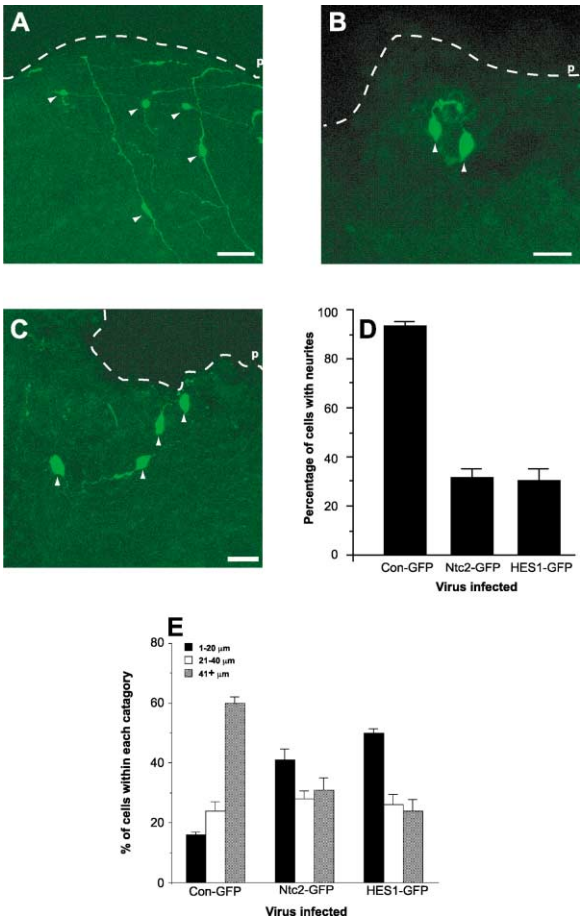

Figure 6. Overexpression of the *Notch2* ICD and *HES1* Inhibits GNP Differentiation In Situ

Organotypic slices cultures prepared from the cerebella of P6 mice were infected with either the control retrovirus expressing GFP or retroviruses expressing the *Notch2* ICD or *HES1*. After a 36 hr of incubation, retrovirally infected cells were visualized by confocal microscopy. A cluster of granule neurons (each individually marked by an arrowhead) infected with the control retrovirus exhibit either the bipolar or t-shaped morphology of differentiated granule neurons (A). Scale Bar equals 50 μm. Parallel fibers are absent from a cluster of two cells overexpressing the *Notch2* ICD (B). Scale bar equals 20 μm. Four cells overexpressing *HES1* also lack parallel fibers, a phenotype similar to that of the *Notch2* ICD overexpressing cells (C). Scale bar equals 20 μm. Note that granule neurons overexpressing the *Notch2* ICD or *HES1* remain closer to the surface of the cerebellar slice (marked by dashed line) than the control cells. Quantitation of these results revealed that overexpression of either the *Notch2* ICD or *HES1* reduced the number of cells harboring parallel fibers or t-shaped morphology by two thirds as compared with control cells expressing only GFP (D) (31.6% ± 3.6% or 30.2% ± 5% cells with neuritis, respectively, 93.4% ± 1.9% for the control). The degree of infected cell migration away from the pial surface was quantitated by measuring the location of the cell bodies of 400 infected cells for each virus construct in relation to the pial surface (E). The depths of the measured cell bodies were placed into one of three depth categories: 1–20 μm (black bars), 21–40 μm (white bars), or 41+ μm (gray bars). The majority of cells infected with the control virus were 41+ μm away from the pial surface (60% ± 2%). However, the numbers of cells overexpressing *Notch2* ICD or *HES1* within this depth category were reduced compared with control (31% ± 4% and 24% ± 3.9%, respectively), a reduction in number of cells that had migrated into deeper regions of the slices.

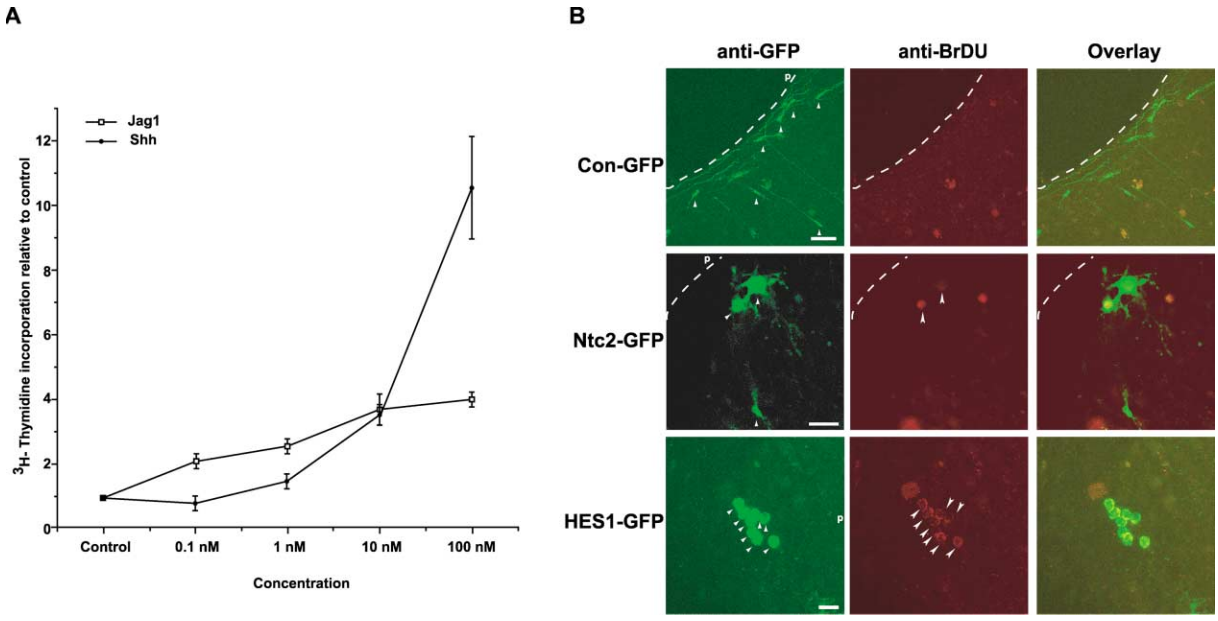

Figure 7. Activated Notch Signaling Maintains GNP Proliferation

(A) Purified postnatal granule cell cultures were incubated with 20 nM Jagged1 or 100 nM Shh. ^{3}H-thymidine was added to the cultures at 48 hr, and incorporation was measured to examine GNP proliferation. Jagged1 stimulates ^{3}H-thymidine incorporation at levels comparable to Shh, indicating that Jagged1 is a mitogen for GNPs.

(B) Organotypic slices cultures prepared from the cerebella of P6 mice were infected with the control, *Notch2* ICD, or *HES1* retroviruses and were incubated for 30 hr. BrDU was added to the culture and incubated for an additional 8–10 hr to label mitotically active granule neurons. The degree of BrDU incorporation of virally infected cells was then determined by confocal microscopy and was quantitated by the counting of at least 50–100 infected cells per experiment (n = 4). Differentiated GNPs infected with the control retrovirus (each infected cell is marked by arrowheads) exhibit few BrDU-labeled nuclei. Three cells overexpressing the *Notch2* ICD lack parallel fibers. Two of these cells appear to be mitotically active, as they possess BrDU-labeled nuclei (marked by elongated arrowheads). A cluster of eight cells overexpressing *HES1*, each of which possess BrDU-labeled nuclei, is shown.

Signals that Enhance GNP Proliferation Result in Upregulation of *HES1* Expression

As an effector of Notch signaling, *HES1* may play a significant role in regulating the differentiation of GNPs. Our overexpression experiments indicate that *HES1* inhibits neurite extension and the onset of TAG-1 expression. The results of our BrDU-labeling assays suggest that the regulation of *HES1* expression may be a key step in determining the proliferative status of GNPs. Therefore, we examined whether treating granule cell cultures with mitogens Jagged1 or Shh influenced the levels of *HES1* expression. The level of *HES1* specific product was elevated in cultures treated with Notch ligand Jagged1 and in cultures treated with 100 nM Shh (see Figure 8A).

The enhancement of *HES1* mRNA in granule cell cultures treated with Jagged1 and Shh protein suggests that *HES1* is a transcriptional target of the Notch and Shh signaling cascades. To test this hypothesis, we performed transactivation assays using a transient transfection assay in a heterologous system using a *HES1* reporter construct. A heterologous system was utilized because cultured granule cells are not transfected by conventional methods in sufficient numbers to carry out these experiments. The C3H10 T1/2 cell line was selected because it had been previously shown to possess the basic cellular components to transduce a Notch- or Shh-based signal (Kinto et al., 1997; Nakagawa et al., 2000; Ruiz i Altaba, 1999; Zeng et al., 2001). A *HES1* promoter firefly luciferase reporter plasmid was cotransfected into C3H10 T1/2 mouse fibroblasts with expression vectors harboring either the full-length *Shh* or *Notch2* ICD cDNAs. Cotransfection of 125 ng of *Shh* expression vector led to a 28-fold activation of the *HES1* promoter compared with basal reporter activity of the *HES1* promoter in cells transfected with empty pcDNA3 vector alone (n = 3) (see Figure 8B). Cotransfection of 60 ng of the *Notch2* ICD expression vector led to 82-fold activation of *HES1* promoter activity, three times higher than the maximal level observed for *Shh*. These results indicate that the *HES-1* promoter used in these studies harbors *cis*-acting elements that render it responsive to both the Shh and Notch2 signals, although transactivation by constitutively active *Notch2* ICD is more potent. Taken together, these results suggest that *HES1* is a transcriptional target of both Shh and Notch2 signaling pathways.

Discussion

The developmental scheme used for the generation of granule neurons in the cerebellar cortex differs from the mode in other areas of the CNS. Specified GNPs at the edge of the rhombic lip steam rostrally over the roof of the cerebellar anlage until they establish a displaced germinal zone, the EGL (reviewed in Hatten and Heintz, 1995). Expansion of the GNPs within the EGL produces the vast number of granule neurons contained in the

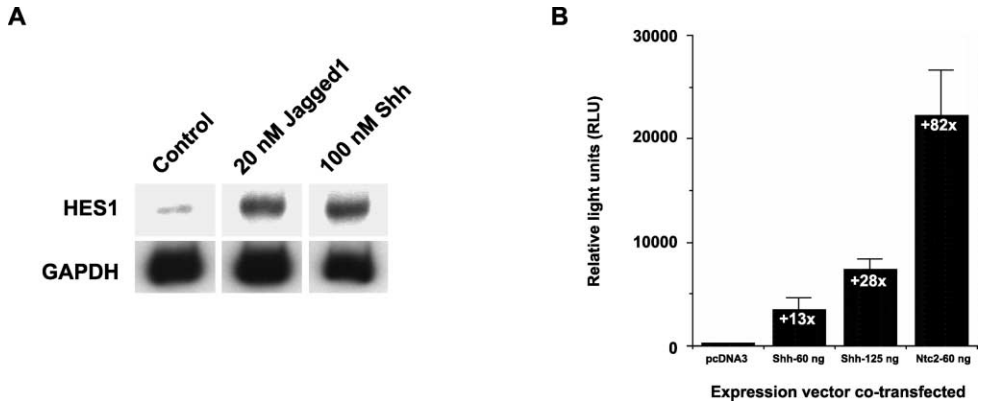

Figure 8. Notch and Shh Signaling Activates Expression of *HES1*

(A) P6 granule cells were treated with 20 nM Jagged1 or 100 nM ShhN. After 18 hr, total RNA was isolated from the cultures, and the level of *HES1* expression was examined by quantitative RT-PCR. Treatment with 20 nM Jagged1 results in a 4.5-fold enhancement in *HES1* expression, whereas treatment with 100 nM ShhN results in a 6-fold enhancement.

(B) Cotransfection of a *HES1* reporter plasmid with a *Shh* or *Notch2* ICD expression vector leads to potent stimulation of luciferase expression compared with the basal level of expression observed when empty pcDNA3 is cotransfected. Optimal *Shh* signaling leads to a 28-fold activation, whereas *Notch2* ICD activation was roughly three times more potent leading to an 82-fold activation.

adult cerebellum. Precursors isolated from the rhombic lip or E17 EGL differentiate into granule neurons when implanted into the EGL of postnatal cerebella, suggesting that precursors located in these structures are not only specified to the granule neuron fate but are competent to respond to cell-nonautonomous signals that induce terminal differentiation (Alder et al., 1996). However, during normal development, a high level of GNP proliferation occurs beyond E17 until late into postnatal development. This apparent contrast indicates that a mechanism is active in the EGL to suspend the onset of differentiation for the majority of proliferating precursors until the late postnatal period.

Expression of *Notch2*

Granule neuron differentiation can be delineated by marker gene expression into multiple stages (Kuhar et al., 1993). Transcription factors such as *Math1* (Akazawa et al., 1995; Ben-Arie et al., 1997; Helms and Johnson, 1998) and the *Zic* genes (Aruga et al., 1994, 1998; Nagai et al., 1997) mark the granule neuron lineage from the time of its specification in the rhombic lip. Intriguingly, genes such as *CyclinD2* (Ross et al., 1996) and the protein phosphatase 2A β subunit (Kuhar et al., 1993) are expressed at a low level early in the development of GNPs but are elevated as these cells take up a position in the EGL. In this article, we describe *Notch2* as a third gene that preferentially marks the proliferative GNPs residing within the EGL. *Notch2* expression is first detected in the EGL at P0. At P6, high levels of *Notch2* expression are observed within proliferating GNPs. *Notch2* expression is downregulated in the differentiating cells of the inner EGL and is undetectable in granule neurons that are migrating through the molecular layer or have already reached the IGL. The level of *Notch2* expression within GNPs peaks at P6 is still observable at P10 and then is undetectable at P20, an expression profile that tightly matches the profile of proliferation within the EGL.

Constitutive *Notch2* Signaling and Differentiation

The Notch signaling pathway represents an evolutionarily conserved system that regulates the differentiation of a variety of precursors cell types within many of the organ systems of invertebrates and vertebrates (Artavanis-Tsakonas et al., 1999; Weinmaster, 2000). The expression pattern of *Notch2* in the developing cerebellum suggested that it could regulate a critical step in the differentiation of GNPs. We have used recombinant ecotropic retroviruses to assess the effect of constitutively activating the *Notch2* pathway on GNP differentiation. GNPs within postnatal granule cell cultures quickly exit the cell cycle and differentiate. Overexpression of either *Notch2* ICD or *HES1* was able to block GNP differentiation as measured by neurite extension. Many of the cells that lacked neurites did not express the TAG-1 axonal glycoprotein, an early marker of neuronal differentiation, indicating that these cells were blocked at a stage before initiation of differentiation had commenced.

Constitutive *Notch2* Signaling and Proliferation

Some GNPs treated with soluble Jagged1 or overexpressing *Notch2* and *HES1* remained in the proliferative state, as assessed by thymidine incorporation or BrDU labeling. Although Jagged1 elicited a slightly lower amount of thymidine incorporation than Shh, its activity is significantly stronger than other known GNP mitogens such as insulin-like growth factor-1 or epidermal growth factor (Gao et al., 1991). The role of Notch proteins in regulating the proliferative status of precursors remains relatively unexplored, yet evidence is accumulating that suggests that Notch molecules may regulate proliferation directly. The overexpression of *Notch* ICD in chimeric *Drosophila* wing discs results in clones of cells with enhanced proliferation (Baonza and Garcia-Bellido, 2000). In addition, activated Notch molecules possess oncogenic potential in the *C. elegans* and mammalian systems, further suggesting that activated Notch isoforms can deregulate the normal pathways of growth control (Berry et al.,

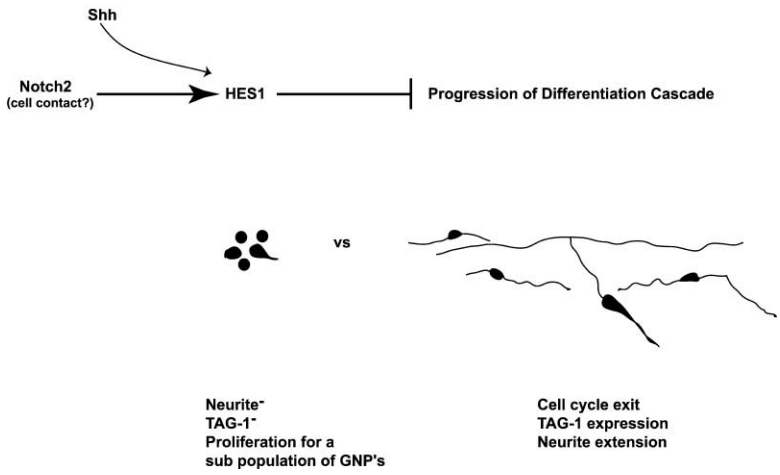

Figure 9. A Model for *Notch2* and *HES1* Function in Regulating GNP Differentiation

In this model, GNPs that are competent to differentiate are exposed to multiple signals that regulate their rate of differentiation. Notch2 and Shh mediate mitogenic signals in GNPs, thus preventing the onset of differentiation. Our data suggest that Notch and Shh signaling activates *HES1* expression. *HES1* maintains GNP proliferation and inhibits its differentiation. Therefore, regulation of *HES1* expression via these two independent signaling cascades may regulate the balance between proliferation and differentiation of GNPs.

1997; Capobianco et al., 1997). Studies examining proliferative capacity of neural stem cells and immature T cells from *HES1* null mice suggest that *HES1* is required for maintenance of the proliferative state of precursor cells (Nakamura et al., 2000; Tomita et al., 1999). In addition, overexpression of *Notch1* ICD in committed glial precursors directly leads to proliferation and expansion of the precursor pool (Chambers et al., 2001). Similarly, our results establish that committed GNPs can respond to Notch signaling by remaining in the mitotic state, which is consistent with a growing body of evidence suggesting that the Notch receptors and their downstream effectors play a role in directly regulating proliferation in addition to modulating cell fate decisions.

The Notch Pathway and Shh Signaling Regulate *HES1* Expression

Jagged1 and Shh possess mitogenic activity for GNPs within granule cell cultures (Dahmane and Ruiz-i-Altaba, 1999; Gao et al., 1991; Wallace, 1999; Wechsler-Reya and Scott, 1999). We have shown that *HES1* expression is upregulated by the addition of both of these factors, suggesting that *HES1* is a transcriptional target of both signals. Consistent with other results, we have observed that only a small percentage of cells within granule cell cultures respond to mitogens like Shh (Dahmane and Ruiz-i-Altaba, 1999; Kenney and Rowitch, 2000; Wallace, 1999). Therefore, the activation of *HES1* mRNA in cultures treated with Jagged1 or Shh is likely due to a small increase in the pool of mitotic precursors. Preliminary immunostaining experiments with a HES1 antibody suggest that HES1 protein is enriched in mitotic precursors and that there is an increase in HES1-positive precursors in cultures treated with either Jagged1 or Shh (data not shown).

We also examined the transcriptional regulation of the *HES1* promoter utilizing a reporter assay in heterologous cell line. Although the *HES1* promoter might react differently in GNPs because of unique cell-type specific accessory factors, we believe that C3H10 T1/2 cells are useful for examining the general regulation of the *HES1* promoter, as they have been used by other investigators to study both Notch and Shh signaling in vitro (Kinto et al., 1997; Nakagawa et al., 2000; Ruiz i Altaba, 1999; Zeng et al., 2001). Our finding that the *HES1* promoter is responsive to both Shh and Notch2 in C3H10 T1/2 cells suggests that *HES1* is a general target of both signaling cascades. *HES1* has been previously established as a transcriptional target of the Notch signaling cascade. Our finding that Shh activates *HES1* expression in vertebrate GNPs is novel. However, a potential genetic interaction between these two factors is implied by studies examining the regulation of *Hairy* expression in the flies. In *Drosophila*, the expression of *Hairy*, the closest related bHLH factor to vertebrate *HES1*, is a downstream target of Hedgehog signaling via direct activation by the hedgehog target *Cubitus interruptus* (Hays et al., 1999).

Our findings shed light on the pathways that regulate the differentiation of granule neurons in the EGL. A possible model incorporating our observations is illustrated (Figure 9). The GNPs within the EGL that are competent to respond are exposed to environmental cues that regulate their entry into a program of differentiation. Notch2 signaling possibly mediated by homotypic cell-cell interactions and Shh block GNP differentiation. Given the finding that *HES1* overexpression inhibits differentiation and enhances proliferation, our results demonstrating that Notch2 and Shh can enhance *HES1* expression suggests that this factor may play a role in integrating some of the signals required for maintenance of the precursor state.

Conclusions

The question that remains to be addressed is how GNPs are able to disregard mitogenic signals normally and initiate differentiation. Roughly 30% of the *Notch2* ICD or *HES1* overexpressing GNP extended neurites, suggesting that a cell-autonomous mechanism exists to overcome the actions of the *Notch2* and *HES1* genes. Analysis of *Xenopus* primary neurogenesis suggests that the function of *NeuroD*, a member of the bHLH family of transcription factors, is insensitive to constitutive Notch signaling (Chitnis and Kintner, 1996). *NeuroD* is expressed in newly postmitotic granule neurons that are located in the inner EGL and is required for the survival of granule neurons progressing through the dif-

ferentiation cascade (Miyata et al., 1999). Similar to the *Xenopus* system, some of the GNPs in our experiments may already be primed to differentiate a point where Notch signaling may not be able to block differentiation.

Identifying factors that decrease GNP proliferation and allow the progression of the differentiation cascade is crucial to gaining a complete understanding of factors that regulate granule neuron differentiation in the EGL. Factors modulating the symmetry of cell division of precursors in the EGL could be an important factor for limiting the proliferative capacity of the EGL. A series of symmetric cell divisions of precursors during the early postnatal period would lead to expansion of the EGL, whereas later asymmetric divisions in which one daughter would make the transition to become a postmitotic neuron would lead to a global decrease in the number of proliferating precursor cells. The *Notch2* and *HES1* genes represent promising targets to unravel further the interactions regulating the balance between the proliferation and differentiation of GNPs in the EGL.

Experimental Procedures

RT-PCR

RT-PCR was done essentially as described (Wilson and Hemmati-Brivanlou, 1995). Briefly, total RNA was isolated from P5, P6, and P7 murine cerebellum or cultured granule neurons, and an equal amount of RNA was reverse transcribed using the Thermoscript RT-PCR system (GIBCO BRL, Grand Island, NY). Equal amounts of first strand cDNA were then amplified using primers specific for mNotch1: Ntc1 5′, AGGTGGATGCAGGCAATAAGG; Ntc1 3′, CAGT GAAGAGGTGGCCCAACCC; mNotch2: Ntc2 5′, GGCCCCTTGCCC TCTATGTAC; Ntc2 3′, CCCGCTGACCGCCTCCACCA; mDelta-like 1: Dl1 5′, TACACCTGCCATTGCCCCTTG; Dl1 3′, GTTCAGGTGGAG GCTGGTGTTTCTGTA; mDelta-like 3: Dl3 5′, AACTGAGGAGAG AAGCAGTGG; Dl3 5′, AGTCTTAGCTGTTTAGGAC; mJagged1: Jag1 5′, TGCTTGGTGACAGCCTTCTACTGG; Jag1 3′, CTCTGGGCACTT TCCAAGTC; mJagged2: Jag2 5′, CAAAGACCTGAACTACTGTGGC; Jag2 3′, GAAAAAGCATTAAGGCACGG; m*CBF1*: *CBF1* 5′, CAGAGCC AGGGCCGAGGCC; *CBF1* 3′, GTCAGTTTCAGTCCCAGCA; m*HES1*; *HES1* 5′, 5′-CAGCCAGTGTCAACACGACAC-3′; *HES1* 3′, 5′-TCGTT CATGCACTCGCTGAAG-3′; or m*HES5*: *HES5* 5′, 5′-CGCATCAAC AGCAGCATAGAG-3′; *HES5* 3′, 5′-TGGAAGTGGTAAAGCAGCTTC-3′. The cycling parameters used were as follows: 95°C for 30 s, 60°C 30 s, and 72°C for 1 min for 25 cycles. Primers specific for mGAPDH (GAPDH 5′, TGGTCTACATGTTCCAGTATG; GAPDH 3′, TCCACC ACCCTGTTGCTGTA) or mHPRT (HPRT 5′, CTGCTTTCCGGAGC GGTAGC; HPRT 3′, CAACTTGCGCTCATCTTAGG) were used as a control for equal loading of first-strand cDNA. For quantitative PCR, 1 μl of ^{32}P dATP was added to each reaction. PCR products were run on a native polyacrylamide gel electrophoresis gel, which was then dried and exposed to film.

In Situ Hybridization

Hybridization and detection were performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993) on fresh frozen saggital sections fixed for 30 min in 4% paraformaldehyde and infused with 30% sucrose before cryosectioning at 15 μm. Probes were prepared from portions of the *Notch2* ICD cDNA. No staining was detected with a sense probe after a prolonged incubation period.

Construction of Retroviral Vectors, Expression Vectors, and HES1 Reporter Construct

The portion of the m*Notch2* cDNA encoding amino acids 1706–2470 of full-length mNotch2 corresponding to the ICD was isolated from a random primed mouse brain cDNA library (Clontech, Palo Alto, CA). Sequence analysis of this clone showed that it was identical to that which was previously published. The m*HES1* cDNA was amplified from P6 granule cell RNA using the following primers: *HES1* ATG, AAAGAATTCATGCCAGCTGATATAATGGAG, and *HES1* TGA, CTGGAATTCTCAGTTCCGCCACGGTCTCCAC. The *mNotch2* and *mHES1* cDNAs were inserted into the EcoRI site of the retroviral expression vector (described in Tomoda et al., 1999). The retroviral expression vectors used in our overexpression studies harbor IRES-AP or IRES-GFP to allow the visualization of infected granule neurons either by simple colormetric assay or by green epifluorescence. An *mNotch2* expression vector was constructed by inserting the *Notch2* ICD cDNA into the EcoRI site of pcDNA3 (Invitrogen, Carlsbad, CA). The mShh cDNA (kindly provided by Andrew McMahon, Harvard University, Boston, MA) was excised from pKS using EcoRI and XhoI and was inserted into the EcoRI and XhoI sites of pcDNA3. The *HES1* promoter fragment corresponding to −856 to +1 was amplified from human genomic DNA based on the sequence deposited in Genbank Accession #L19314 using the following primers: HES1 Prom 5′ 5′-ACCGCTAGCCTACGGATGAAAAGGGAAAGGGT-3′ and HES1 Prom 3′ 5′-ATCAGATCTGATCCCTAGGCCCTGGCGGCCTC TATATATA-3′. The amplified product was then cloned into the NheI and BglII restriction sites of the promoterless mammalian luciferase expression vector pGL2-Basic (Promega, Madison, WI). Sequence analysis of the PCR fragment revealed no changes from the previously published sequence. The *HES1* promoter fragment drove luciferase expression when transfected into mammalian cells, indicating that the *HES1* promoter was active.

Retrovirus Production

Recombinant ecotropic replication-incompetent retroviruses were produced as previously described (Tomoda et al., 1999). Briefly, 293 cells were cotransfected with a retroviral construct and pCL-Eco, an ecotropic packaging construct (IMGEN) (Naviaux et al., 1996). Twenty-four-hour posttransfection media were replaced with granule cell medium (Hatten, 1985), and the culture supernatant containing the retroviruses was harvested 24 and 48 hr later and filtered though a 0.45 μm pore. Virus was titered by infecting primary granule neurons and observing the amounts of infected cells. The amounts of cell culture supernatant used for subsequent infections were then adjusted to achieve roughly equal levels of infected cells.

Preparation and Culture of Postnatal Granule Cells

Granule cells were prepared as described (Hatten, 1985). Briefly, cerebella were dissected away from the brains of P6 mice. After the pial layer was peeled away, the tissue was treated with Trypsin and triturated into a single-cell suspension using fine bore Pasteur pipettes. The suspension was layered unto a discontinuous Percoll gradient and separated by centrifugation. The small cell fraction was isolated, and granule cells were further enriched by panning on tissue culture treated plastic dishes. The resulting cultures routinely contain greater than 95% of cells of the granule cell lineage (Hatten, 1985) but harbor a mixture of mitotic GNPs and postmitotic granule neurons. Serum-free medium was used for thymidine incorporation and RT-PCR experiments (Neurobasal medium supplemented with B27 supplement and 50 U/ml penicillin-streptomycin). For thymidine incorporation assays, 150,000 cells were added per well of a 96-well microtiter plate and were incubated in the presence or absence of the indicated amounts of Jagged1 or Shh for 50 hr. Thymidine was then added, and the cells were harvested 12 hr later to measure incorporation. For RT-PCR experiments, ten million granule cells were plated in uncoated four-well Lab-Tek glass chambers in the presence of 20 nM Jagged1 or 100 nM ShhN. After 18 hr, RNA was isolated for analysis by RT-PCR.

Infection of Primary Cerebellar Granule Cells In Vitro

Retroviral infection of postnatal granule cell cultures was performed essentially as described (Tomoda et al., 1999). In brief, dissociated granule cells purified from P6 C57BL/6J mice were plated at high density (1×10^6 cell/well) in an uncoated 16-well Lab-Tek glass chamber (Nunc, Naperville, IL) and infected with recombinant retroviruses overnight. After 18 hr, the culture was gently resuspended, replated in Lab-Tek slides coated with poly-L-lysine and Matrigel, cultured for an additional 18 hr to allow for GNP differentiation and neurite extension, and then fixed and processed for AP- or GFP-marker gene expression. The amount of virus titer routinely used for these studies yielded a 1% rate of infection.

Preparation and Infection of Organotypic Cerebellar Slices

Organotypic cerebellar slices were prepared according to Stoppini et al. (1991). To visualize parallel fibers within the slices, cerebella isolated from P6 mice were chopped coronally 250 to 300 μm in thickness by a McILWAIN Tissue Chopper (Brinkman, Westbury, NY) and maintained on a tissue culture insert (Millicell; Millipore, Bedford, MA), which was submerged in culture media (basal medium Eagle supplemented with 10 mg/ml bovine serum albumin [A-9418; Sigma, St. Louis, MO], 2 mM L-glutamine, 0.5% glucose, 1× insulin-transferrin-selenite [ITS; Sigma I-1884], and 50 U/ml penicillin-streptomycin). Two hours after dissection, virus infection was initiated; 36 to 72 hr after the start of virus infection, slices were fixed and processed for immnunostaining.

Immunocytochemistry and AP Staining

Antibody staining of primary granule cell cultures or organotypic cerebellar slices was performed essentially as described (Gao et al., 1991). Immunostaining was used to detect GFP expression due to increased sensitivity. The dilutions of antibodies used were anti-GFP antibody (polyclonal Molecular Probes, 1:2000, Eugene, OR) and anti-TAG-1 antibody (monoclonal, IgM, 4D7, 1:2). Specimens were viewed with an MRC-600 confocal microscope (Bio-Rad, Hercules, CA). For detection and morphological assessment of infected cells in culture by AP colorimetric reaction, cells were fixed with 4% paraformaldehyde and incubated at 65°C for 90 min to heat inactivate endogenous AP activity. The cells were then incubated in AP staining buffer (100 mM NaCl, 100 mM Tris-Cl [pH 9.5], 50 mM $MgCl_2$) containing 0.33 mg/ml NBT, 0.17 mg/ml BCIP, and 0.24 mg/ml levamisole at room temperature overnight.

Purification of Jagged1

Briefly, purified Jagged1 was isolated by transiently transfecting 293T cells with a soluble Jagged1 Fc expression vector (gift of Shigeru Chiba and Hisamaru Hirai, Tokyo Japan). The supernatants of transfected cells were collected, and Jagged1 Fc protein was purified using ammonium sulfate precipitation and a HiTrap Protein A column.

HES1 Reporter Assays

Transient transfections were carried out in the C3H10 T1/2 mouse fibroblast cell line using Fugene6 reagent (Roche, Indianapolis, IN) as per the manufacturer's instructions. Each transfection mixture contained 250 ng of the *HES1* reporter plasmid and either 60 or 125 ng of the pcDNA3-Shh or pcDNA3-Ntc2 expression vectors. Cotranfections were filled in with empty pcDNA3 so that a total of 250 ng of pcDNA3 backbone plasmid would be constant for each transfection. Basal reporter activity was determined by cotransfecting empty pcDNA3 vector alone. At 18 hr posttransfection, cells were harvested, and luciferase activity in the lysates of transfected cells was measured using Luciferase Assay Reagent (Promega).

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