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Transient Notch Activation Initiates an Irreversible Switch from Neurogenesis to Gliogenesis by Neural Crest Stem Cells

Sean J. Morrison,‖ Sharon E. Perez,* Zhou Qiao,* Joseph M. Verdi,‡ Carol Hicks,§ Gerry Weinmaster,§ and David J. Anderson*†#

*Division of Biology
California Institute of Technology
Pasadena, California 91125
†Howard Hughes Medical Institute
‡Neurodegeneration Group
Robarts Research Institute
Department of Physiology
Program in Neuroscience
University of Western Ontario
London, Ontario N6A 5K8
Canada
§Department of Biological Chemistry
University of California Los Angeles
Los Angeles, California 90095
‖Departments of Internal Medicine and Cell and Developmental Biology
University of Michigan
Ann Arbor, Michigan 48109

Summary

The genesis of vertebrate peripheral ganglia poses the problem of how multipotent neural crest stem cells (NCSCs) can sequentially generate neurons and then glia in a local environment containing strong instructive neurogenic factors, such as BMP2. Here we show that Notch ligands, which are normally expressed on differentiating neuroblasts, can inhibit neurogenesis in NCSCs in a manner that is completely dominant to BMP2. Contrary to expectation, Notch activation did not maintain these stem cells in an uncommitted state or promote their self-renewal. Rather, even a transient activation of Notch was sufficient to cause a rapid and irreversible loss of neurogenic capacity accompanied by accelerated glial differentiation. These data suggest that Notch ligands expressed by neuroblasts may act positively to instruct a cell-heritable switch to gliogenesis in neighboring stem cells.

Introduction

Stem cells are self-renewing multipotent progenitors with the broadest developmental potential in a given tissue at a given time (Morrison et al., 1997). A great deal of attention has been focused recently on stem cells because of their therapeutic potential (Gage, 2000; Weissman, 2000). Stem cells from the mammalian neural crest provide a model system for understanding the control of self-renewal and differentiation because of their manipulability in clonogenic culture. Neural crest stem cells (NCSCs) self-renew and can generate one or more classes of neurons, glia, and myofibroblasts (also known as smooth muscle cells) in vitro (Stemple and Anderson, 1992). Moreover, the fate of NCSCs can be controlled by instructive extracellular signals such as bone morphogenetic proteins (BMPs), which promote neuronal differentiation, and Neuregulin-1 (Nrg1, glial growth factor), which promotes glial differentiation (Shah et al., 1994, 1996). Recently, prospective isolation of NCSCs from uncultured fetal nerve by flow cytometry has shown that these cells self-renew in vivo, and can generate neurons and glia when transplanted directly into the neural crest migration pathway of chick embryos (Morrison et al., 1999).

An important issue concerns the temporal switches that regulate the sequential production of different cell types by multipotent stem cells. In the embryo, neural crest cells migrate from the dorsal neural tube and aggregate to form the sensory and autonomic ganglia of the peripheral nervous system (PNS). These ganglia contain both neurons and satellite cells (glia that ensheath the cell bodies of the neurons). The first cells to differentiate become neurons in response to factors such as BMPs expressed by neighboring tissues (Reissman et al., 1996; Shah et al., 1996; Schneider et al., 1999), while glia differentiate later. These observations pose the problem of how a multipotent neural stem cell can sequentially generate neurons and glia in an environment that contains powerful instructive signals for neuronal differentiation. An attractive idea is that developing neuroblasts provide a feedback signal to neighboring undifferentiated stem cells that inhibits their further differentiation to neurons and promotes their differentiation to glia (Shah et al., 1994).

Notch ligands are obvious candidates for mediating such a feedback signal. Notch signaling has been shown to inhibit neuronal differentiation in both invertebrate and vertebrate systems (Coffman et al., 1993; Fortini et al., 1993; Struhl et al., 1993; Artavanis-Tsakonas et al., 1995, 1999; Henrique et al., 1997). *Notch* family genes are expressed by neural crest cells (Weinmaster et al., 1991; Williams et al., 1995), and Notch ligands, including *Delta* and *Jagged* are expressed by neuroblasts in developing PNS ganglia (Bettenhausen et al., 1995; Lindsell et al., 1995, 1996). Nevertheless, two issues have raised questions about whether Notch ligands could account for the switch to glial differentiation. First, it is unknown whether Notch's ability to inhibit neuronal differentiation is dominant to the powerful neurogenic influence of BMP2. Second, studies in model systems have shown that if Notch activation is only transient, then neuronal differentiation resumes upon the decay of Notch signaling (Coffman et al., 1993; Fortini et al., 1993; Struhl et al., 1993). Since *Notch* and *Delta* family genes are expressed only transiently in developing peripheral ganglia, it is difficult to see how such a system could mediate an irreversible switch from neurogenesis to gliogenesis.

To address these issues, it was necessary to study the effect of Notch activation on NCSCs under circumstances where both the duration of Notch signaling and

To whom correspondence should be addressed (e-mail: mancusog@cco.caltech.edu).

the presence or absence of competing instructive signals could be controlled. We have therefore examined the effect of a soluble form of Delta-1 on prospectively isolated NCSCs in clonal culture. Our data suggest that Notch does not maintain stem cells in an uncommitted state, but rather acts positively to promote an irreversible and cell-heritable switch from neurogenesis to gliogenesis.

Results

Notch Activation Inhibits Neuronal Differentiation by Neural Crest Cells In Vivo

To determine whether Notch signaling regulates the differentiation of neural crest cells in vivo, premigratory chick neural crest cells were electroporated in ovo at E2 with recombinant retroviral vectors carrying either constitutively active mouse Notch intracellular domain, Notch-ICD (Kopan et al., 1994), or a dominant-negative form of Suppressor of Hairless ($Su(H)^{DBM}$) (Wettstein et al., 1997) that inhibits Notch signaling. Embryos were harvested 1.5 days following electroporation, at which time neuronal differentiation had begun in the dorsal root ganglia (DRG). In animals injected with a control vector containing mouse Notch-ICD in a reverse orientation (r-Notch-ICD), 51% $\pm$ 14% (n = 5) of infected cells, identified by coexpression of the viral protein p27, coexpressed the early neuronal marker HuD (Marusich et al., 1994) (Figures 1A and 1B, arrowheads). In contrast, in animals infected with Notch-ICD, significantly fewer infected cells coexpressed HuD (11% $\pm$ 6%, n = 6) (Figures 1C and 1D, arrowheads). Conversely, in animals infected with $Su(H)^{DBM}$, significantly more infected cells coexpressed HuD than in controls (84% $\pm$ 5%, n = 6) (data not shown). Thus, constitutive Notch activation inhibited neuronal differentiation by neural crest cells in vivo, while inhibition of Notch function enhanced neurogenesis.

Determining whether Notch-ICD maintained neural crest cells in an undifferentiated state, or rather promoted gliogenesis, was confounded by a lack of definitive early markers that discriminate avian satellite glia from undifferentiated neural crest cells. The eight different peripheral glial markers that we have examined fall into one of two categories: either they are also expressed by neural crest cells, such as Sox10 (Kuhlbrodt et al., 1998), or they are expressed relatively late in development, like the myelin proteolipid protein (PLP) and the glycolipid antigen O4 (Rohrer et al., 1986; Rudel and Rohrer, 1992), and are therefore likely markers of glial maturation rather than lineage commitment (Mirsky and Jessen, 1996). Nevertheless, in several embryos, expression of constitutively activated Notch-ICD appeared to increase the number of Sox10-expressing cells in chick DRG, although it did not accelerate the expression of later markers such as PLP (data not shown). These data are consistent with the possibility that activated Notch promoted glial differentiation at the expense of neurogenesis, and that the timing of later glial maturation events is controlled by other signals (Topilko et al., 1997). However, these markers did not allow us to rigorously distinguish this effect from a strict inhibition of neurogenesis. To resolve this issue, we turned to an in vitro system in which the consequences of Notch activation in purified NCSCs could be studied in more detail.

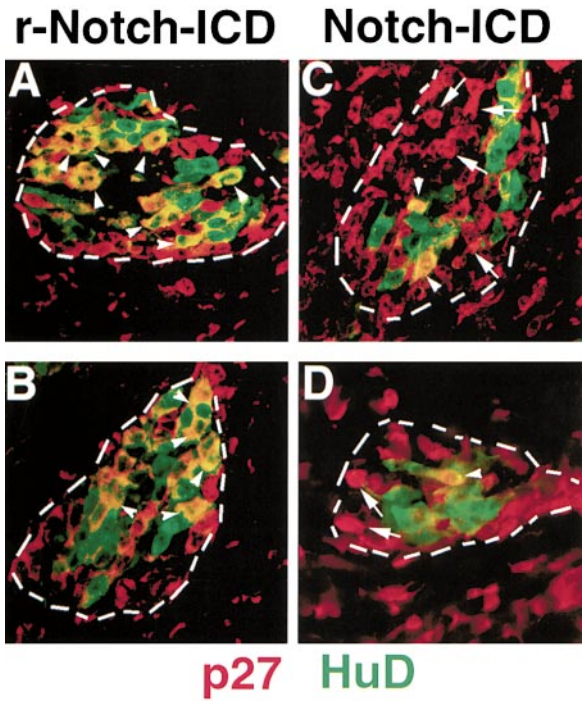


Figure 1. Constitutively Active Notch-ICD Inhibits Neuronal Differention by Chick Neural Crest Cells In Ovo

Premigratory chick neural crest cells were electroporated in ovo with replication competent RCAS vectors engineered to express either constitutively active mouse Notch intracellular domain (Notch-ICD) (Nye et al., 1994) or a control construct bearing mouse Notch-ICD in a reverse orientation (r-Notch-ICD). Embryos were incubated for 1.5 days, then sectioned and double-labeled with antibodies against a viral antigen to reveal infected cells (p27, red) and an early neuronal marker (HuD, green). A dorsal root ganglion (dotted outline) is shown in each panel.

(A and B) Many (51% $\pm$ 14%) of the infected cells from embryos injected with the r-Notch-ICD vector coexpressed the neuronal marker (yellow, arrowheads). Similar results were obtained with a different control virus lacking an insert (not shown).

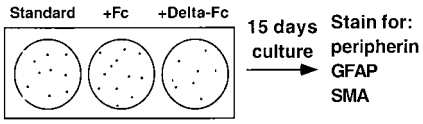
(C and D) Only a minority (11% $\pm$ 6%) of infected cells in ganglia from embryos injected with Notch-ICD coexpressed the neuronal marker (arrowheads).

Arrows point to virally infected cells that do not coexpress HuD.

Soluble Delta-Fc Permits Glial but Not Neuronal or Myofibroblast Differentiation, and Impairs the Self-Renewal of NCSCs

Self-renewing NCSCs that are capable of differentiating into neurons, glia, or myofibroblasts can be purified from E14.5 rat sciatic nerve by flow-cytometry, selecting for cells that are $p75^+$ and P_0^- (Morrison et al., 1999). To activate Notch signaling in these cells, we added conditioned medium containing soluble Delta-Fc fusion protein secreted by transfected 293 cells, clustered by addition of anti-human Fc antibody (see Experimental Procedures). As a control, we added conditioned medium from Fc-transfected cells. Consistent with our in vivo data, addition of Delta-Fc to the culture medium strongly inhibited neuronal differentiation (Table 1). In control cultures, 70%–85% of the colonies developed

Table 1. Delta-Fc Promotes Glial Differentiation by NCSCs at the Expense of Neuronal and Myofibroblast Differentiation



Medium	Plating Efficiency (%)	Frequency of Colony Types (%) N+S+M	N+S	S+M	S only	M only
Standard	48.6 ± 15.0	70.5 ± 8.4 a	1.5 ± 1.9	4.4 ± 3.8	18.8 ± 6.9 a	4.9 ± 5.7
Fc only	41.2 ± 7.8	84.3 ± 4.4 a	1.0 ± 2.1	2.7 ± 3.7	9.8 ± 4.0 a	2.1 ± 4.2
Delta-Fc	46.6 ± 6.1	0.0 ± 0.0 b	1.3 ± 2.5	6.5 ± 5.8	88.5 ± 10.6 b	3.8 ± 7.5

$p75^+P_0^-$ NCSCs were cultured at clonal density for 15 days in standard medium, or in standard medium supplemented with either Fc or Delta-Fc. Following this incubation the cultures were fixed and stained with antibodies to peripherin, GFAP, and α-SMA to detect neurons (N), Schwann cells (S), and myofibroblasts (M). Plating efficiency indicates the percentage of cells initially sorted into culture that survived and proliferated to form colonies detected 15 days later. Letters above the columns indicate colony composition: for example, N+S+M colonies contained neurons, Schwann cells, and myofibroblasts. Data are based on four independent experiments in which an average of more than 100 clones were analyzed per treatment per experiment. Statistics within columns (mean ± standard deviation) were compared by t tests and significantly different statistics are followed by different letters ($P < 0.01$).

peripherin$^+$ neurons after 15 days, while in the presence of clustered Delta-Fc less than 2% contained any neurons. Note that in these experiments, plating efficiencies were higher (41%–49%) than previously reported (25%) (Morrison et al. 1999), because the present cultures were incubated in more physiological oxygen levels (see Experimental Procedures), which promote NCSC survival (S. J. M., unpublished data). The response of NCSCs to Notch activation was not affected by oxygen level (data not shown).

The other two differentiated fates available to NCSCs are to become glia or myofibroblasts (also referred to as smooth muscle cells) (Shah et al., 1994, 1996; Morrison et al., 1999). The former can be identified by expression of glial fibrillary acidic protein (GFAP), the latter by expression of α-smooth muscle actin (SMA). Consistent with prior results (Morrison et al., 1999), triple-labeling with antibodies to peripherin, GFAP, and SMA revealed that the majority (70%–84%) of colonies grown for 15 days in either control or Fc-supplemented medium contained all three cell types (Table 1). Such multipotent colonies were characteristically large, containing tens of thousands to over a hundred thousand cells. In contrast, most (88.5% ± 10%) colonies cultured in Delta-Fc were small, containing hundreds of cells, and appeared to contain exclusively glia as determined by both antigenic phenotype and colony morphology (Table 1; see Experimental Procedures).

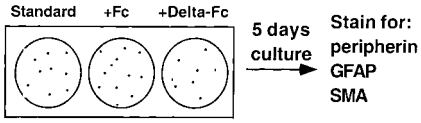
To confirm that Delta-Fc treatment promoted Schwann cell differentiation by NCSCs rather than simply impairing neuronal differentiation or promoting self-renewal, we directly examined the multipotency of NCSCs grown in Delta-Fc for 7 or 12 days by subcloning (Stemple and Anderson, 1992; Morrison et al., 1999). Under control conditions, extensive self-renewal was observed as evidenced by the ability of individual NCSCs to give rise to hundreds of multipotent daughter cells (average of 254 ± 197 multipotent subclones per founder clone, n = 10). A similarly high number of multipotent secondary colonies were obtained from founder cells grown in Fc (average of 170 ± 218 multipotent subclones per founder clone, n = 10). In contrast, founder clones grown in Delta-Fc gave rise to very few multipotent daughter cells (average of 4.6 ± 10 multipotent progeny per founder clone, n = 9 founder clones analyzed). Instead, the vast majority (86.8%) of the progeny of Delta-Fc treated cells gave rise to colonies containing only glial cells; in fact, of three founder clones subcloned after 12 days in Delta-Fc, all gave rise exclusively to Schwann-only colonies. These data confirm that growth of NCSCs in Delta-Fc does not promote or permit the self-renewal of uncommitted cells, but instead results in glial differentiation.

To determine whether Delta-Fc increased the rate as well as the extent of glial differentiation, we examined colonies after 5 days instead of 15 days. While in standard medium at this early time point, less than 18% of colonies contained any Schwann cells and less than 5% contained exclusively GFAP$^+$ cells; in Delta-Fc, over 63% of colonies contained some GFAP$^+$ cells and 27% consisted only of Schwann cells (Table 2 and Figure 2C). Few, if any, of the cells expressed either peripherin or SMA at this time under any conditions (Figures 2B and 2C and data not shown). Thus, exposure to Delta-Fc increased not only the extent of glial differentiation observed after 15 days, but also the rate, as determined by assaying earlier time points (Tables 1 and 2).

While the Fc-conditioned control medium did not inhibit neuronal differentiation, it remained possible that the active factor in Delta-Fc-conditioned medium was an unknown molecule secreted by the 293 cells in response to autocrine stimulation by Delta-Fc, rather than Delta-Fc itself. To rule out this possibility, we omitted the anti-Fc antibody used to crosslink the Delta-Fc molecules. Crosslinking of the Delta-Fc molecules at the concentrations used in these experiments is essential to detect both its binding to Notch-transfected cells as well as its ability to activate transcriptional targets of Notch signaling (C. H. and G. W., unpublished data). Omission of the crosslinking antibody completely abolished the influence of the Delta-Fc-conditioned medium, but had no effect on standard or Fc-supplemented cultures (data not shown). These data indicate that the effects of Delta-Fc are not due to other, Delta-Fc-dependent factors secreted by the 293 cells.

To confirm that clustered soluble Delta-Fc promoted

Table 2. Soluble Delta-Fc Increases the Rate as Well as the Extent of Schwann Cell Differentiation



Medium	Colony Composition (% of colonies, mean ± SD) Schwann-only	Schwann+other	No Schwann
Standard	2.1 ± 4.7 a	15.3 ± 11.9 a	82.6 ± 12.4 a
+Fc	4.2 ± 6.1 a	19.3 ± 7.6 a	76.6 ± 10.4 a
+Delta-Fc	26.9 ± 21.1 b	36.4 ± 8.6 b	36.7 ± 23.0 b

Sciatic nerve–derived $p75^{+}P_0^{-}$ stem cells were cultured for 5 days at clonal density with or without Delta-Fc, then the composition of individual colonies was analyzed as described in the legend to Table 1. "Schwann-only" colonies contained only Schwann cells. "Schwann+other" colonies contained Schwann cells as well as other cell types. "No Schwann" colonies did not contain any Schwann cells; this category includes colonies containing neurons, myofibroblasts, or undifferentiated cells. The data are based on four independent experiments in which an average of 35 clones were analyzed per treatment per experiment. Statistics within columns (mean ± standard deviation) were compared by pair-wise t tests. Significantly different statistics ($p < 0.05$) are followed by different letters. Plating efficiency varied from 31% for the Fc-only treatment to 44% for the Delta-Fc treatment, but this difference was not statistically significant.

glial differentiation by activating rather than by antagonizing Notch, NCSCs isolated from E10.5 neural tube explants (Stemple and Anderson, 1992; Shah et al., 1996) were infected with a recombinant retrovirus engineered to express the constitutively active Notch1 intracellular domain (Notch-ICD) (Nye et al., 1994). Clones that had been infected with either the *Notch-ICD* virus or a control virus were cultured for 14 days and stained for peripherin, GFAP, and SMA. *Notch-ICD* infected cultures gave rise to significantly more Schwann-only colonies (46%, $n = 133$) than did clones infected with the control virus (2%, $n = 200$; $p < 0.05$) and significantly fewer neuron-containing colonies (6%) than controls (74%). The fact that constitutively active Notch-ICD mimicked the effect of Delta-Fc indicates that the soluble ligand acts as an agonist rather than as an antagonist of Notch on NCSCs.

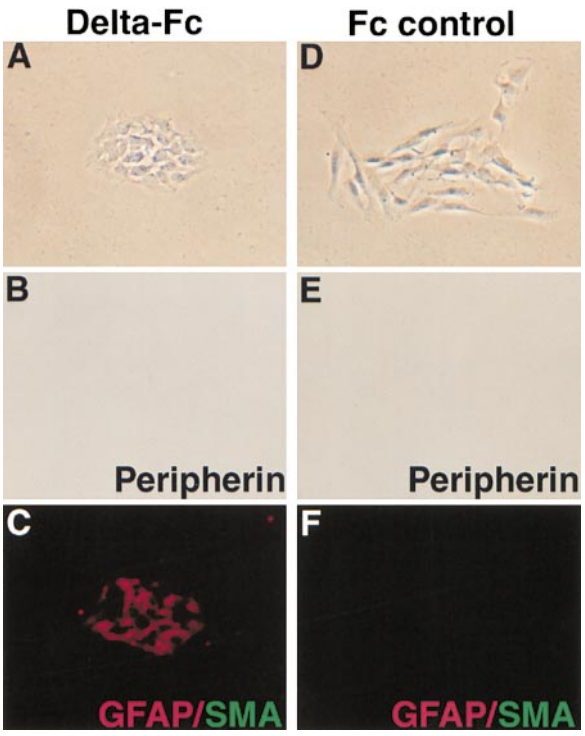


Figure 2. Delta-Fc Accelerates Glial Differentiation in Culture

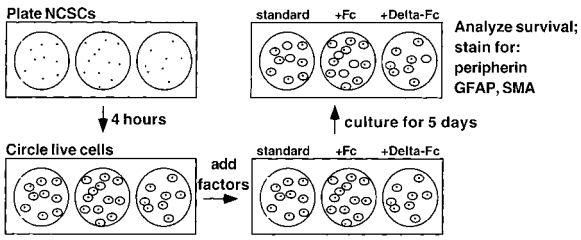
NCSCs were cultured for 5 days at clonal density in standard medium supplemented with Delta-Fc (A–C) or Fc (D–F). (A) and (D) show phase contrast images, (B) and (E) show bright-field images lacking peripherin staining, and (C) and (F) show superimposed epifluorescence images of GFAP (red) and SMA (green, negative) staining. The colony cultured in Delta-Fc contained only Schwann cells as indicated by the GFAP staining and the lack of peripherin and SMA staining. The control colony cultured in Fc supplemented medium contained only undifferentiated cells as judged by the lack of marker staining. For quantitation see Table 2.

Delta Acts Instructively to Promote Glial Differentiation

The foregoing experiments did not distinguish whether Delta-Fc instructively promoted glial differentiation by multipotent cells, or rather promoted the selective attachment or survival of contaminating glial progenitors, or conversely the death of neurogenic cells. To distinguish between these alternatives, we analyzed the survival and fate of a randomly selected population of identified founder cells to which Delta-Fc or Fc had been added after plating and attachment, rather than before (Table 3, schematic). The majority of founder cells survived in both Delta-Fc (83%) and control medium (68%) (Table 3). Yet the proportion of Schwann-containing and Schwann-only colonies observed in Delta-Fc was over 5-fold higher (73%) than that in controls (13%–14%, $p < 0.05$) (Table 3). Thus, the promotion of Schwann lineage differentiation by Delta-Fc cannot be explained by either the selective survival of subsets of restricted glial progenitors or the selective killing of progenitors lacking glial capacity, and must reflect an alteration in the differentiation program of multipotent founder cells.

It remained possible that Notch might act selectively on the progeny of multipotent founder cells within clones, by affecting their proliferation or survival. To address this, we analyzed plates cultured in Delta-Fc or in control media for the presence of apoptotic cells by DAPI staining, daily for 5 days. At all time points dead cells were rare, with most colonies containing no dead cells irrespective of treatment (20–50 colonies examined per time point per treatment in each of three independent

Table 3. Delta Acts Instructively to Promote Glial Differentiation by NCSCs



	Colony composition (% of circled clones)			
Medium	Dead	Schwann-only	Schwann+other	No Schwann
Standard	32 ± 11	4 ± 6 a	9 ± 9 a	55 ± 12 a
+Fc	39 ± 12	4 ± 4 a	10 ± 9 a	47 ± 17 a
+Delta-Fc	19 ± 7	41 ± 13 b	32 ± 6 b	8 ± 2 b

NCSCs were sorted into culture then 4 hr later, live cells that had attached to the plate were circled. After live cells were circled, Delta-Fc or Fc only were added to some cultures. Five days later, colony compositions were analyzed by triple staining (see Table 1). Statistics (mean ± standard deviation) are from three independent experiments in which a total of 141 clones were analyzed in standard medium, 135 in Fc supplemented medium, and 93 in Delta-Fc supplemented medium. Statistics within columns were compared by pair-wise t tests, significantly different statistics ($p < 0.05$) are followed by different letters.

experiments). Furthermore, colonies cultured in Delta-Fc contained the same number of, or fewer, dead cells on average than colonies cultured under control conditions. For example, on days 1 and 2 of culture, colonies were small (1–2 cells/colony) and less than 5% of colonies contained a dead cell. By day 5, colonies cultured in Delta-Fc, Fc-only, or standard medium averaged 24, 36, and 43 cells/colony, respectively, and dead cells accounted for only 0.5, 1.1, and 1.8 cells/colony, respectively (averaged across all colonies). It is therefore unlikely that Delta-Fc acted by killing nongliogenic progeny of multipotent founder cells.

We also investigated whether Delta-Fc might enhance proliferation of glial progenitors. At all time points analyzed, however, colonies cultured in Delta-Fc contained either the same number of cells, or fewer cells, than colonies cultured in control conditions. Consistent with this, when bromodeoxyuridine (BrdU) was administered to cultures for 8 hr on the fifth day, significantly less ($p < 0.01$) proliferation was observed in cultures supplemented with Delta-Fc (35% ± 27% $BrdU^+$ cells/colony) than in colonies supplemented with Fc-only (55% ± 33% $BrdU^+$ cells/colony) or standard medium (63% ± 33% $BrdU^+$ cells/colony). These data indicate that Delta-Fc could not have promoted the selective proliferation of glial precursors within colonies. Taken together, the foregoing experiments indicate that the effect of Delta-Fc to promote glial differentiation cannot be explained by either interclonal or intraclonal selection, and likely reflects instruction.

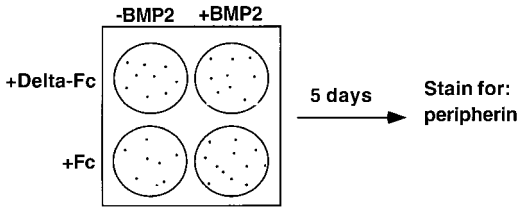
Notch Signaling Is Dominant over the Instructive Neuronal Differentiation-Promoting Signal, BMP2

We next asked whether the effect of Delta-Fc would be dominant or subordinate to that of BMP2. BMP2 both promotes and accelerates neuronal differentiation by NCSCs in an instructive manner (Shah et al., 1996), and is required for the differentiation of some crest-derived peripheral neurons in vivo (Schneider et al., 1999). Consistent with this, after 5 days of growth in medium containing control Fc protein, BMP2 induced differentiation of peripherin$^+$ neurons in more than 90% of colonies; 50% of the colonies contained only neurons (Table 4). In contrast, in medium containing Delta-Fc, BMP2 was able to induce neurogenesis in only 14% of colonies, and less than 1% of the colonies consisted only of neurons (Table 4). Moreover, staining of such cultures after 24 hr with a monoclonal antibody to MASH1 (an early marker of BMP2-induced neurogenesis) (Shah et al., 1996) indicated that the frequency of MASH1$^+$ cells was significantly lower in Delta-Fc + BMP2 (4%) than in Fc + BMP2-treated cultures (72%; more than 200 clones analyzed per treatment). These data indicate that activation of Notch signaling by Delta-Fc blocks BMP2-induced neurogenesis, at a step upstream of MASH1 induction.

Transient Exposure to Delta Causes an Irreversible Loss of Neuronal Capacity

We next sought to determine whether NCSCs would, like neural progenitors in other systems (Coffman et al., 1993; Struhl et al., 1993), remain competent to differentiate to neurons upon cessation of Notch signaling. NCSCs were incubated for 24 hr in Delta-Fc medium, washed, and then challenged by exposure for 4 days to 50 ng/ml BMP2 (Table 5, schematic). In control cultures, 60%–70% of colonies transiently incubated in either standard or Fc-containing medium contained neurons following subsequent exposure to BMP2 (Table 5, "standard" and +Fc, "Neurons"). Furthermore, only 13%–19% of such colonies contained any Schwann cells (Table 5, "Schwann-only" and "Schwann+other"). By contrast, less than 2% of colonies exposed to Delta-Fc for only 24 hr and subsequently challenged with BMP2 exhibited neuronal differentiation (Table 5, +Delta-Fc, "Neurons"). Instead, 57% of colonies pretreated with Delta-Fc contained only GFAP$^+$ Schwann cells and over 80% of colonies contained at least some GFAP$^+$ Schwann cells (Table 5 and Figures 3A–3C), despite the presence of BMP2. If anything, the intensity of GFAP expression was higher in cells transiently exposed to Delta-Fc and subsequently cultured in BMP2, than in cells simply cultured

Table 4. Delta-Fc Impairs Neuronal Differentiation Promoted by BMP2 in NCSCs



Medium Supplement	BMP2 Added	Plating Efficiency (%)	Frequency of Colony Types (%)		
			Neuron-only	Neurons+other	No neurons
Fc-only	No	27.7 ± 5.3 a	0.0 ± 0.0 a	0.0 ± 0.0 a	100.0 ± 0.0 a
Delta-Fc	No	56.2 ± 15.6 b	0.0 ± 0.0 a	0.0 ± 0.0 a	100.0 ± 0.0 a
Fc-only	Yes	41.1 ± 7.8 b	51.1 ± 23.7 b	40.9 ± 18.5 c	8.0 ± 7.4 c
Delta-Fc	Yes	47.6 ± 9.3 b	0.9 ± 1.6 a	13.0 ± 11.7 b	86.2 ± 12.4 b

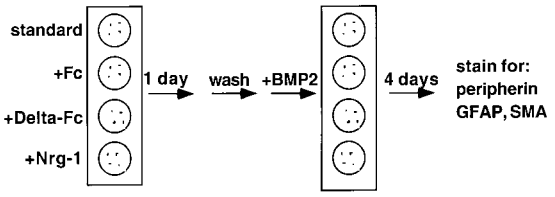
$p75^+P_0^-$ cells were sorted into culture at clonal density and cultured for 5 days followed by immunocytochemical staining; neurons were detected by expression of peripherin. The data are based on two to three independent experiments and an average of around 100 colonies were examined per treatment per experiment. Statistics within columns were compared by t tests and significantly different statistics are followed by different letters ($P < 0.01$). Cells were also cultured in unsupplemented standard medium in these experiments (data not shown), but the results did not differ from those shown in the Fc-only treatments.

continuously in Delta-Fc for the same period of time (not shown). Thus, activating Notch for only 24 hr in NCSCs caused an apparently irreversible loss of neuronal potential, and an increased frequency of glial differentiation in the face of subsequent exposure to BMP2.

The fact that a transient, 24 hr exposure of NCSCs to Delta-Fc was effective also provides further evidence that Notch acted instructively rather than selectively in this system. At the time that Delta-Fc was washed out of the medium, the vast majority of colonies consisted of only a single cell (on average 1.1 cells/colony). Yet, such cells still went on to develop significantly more Schwann cells and significantly fewer neurons than controls, even when they were subsequently challenged by exposure to BMP2. The fact that Delta-Fc can exert its influence on founder cells before they have a chance to divide further indicates that it cannot be acting by intraclonal selection. Furthermore, since the average colony contained about 90 cells at the end of the incubation, these data also suggest that the effect of Delta-Fc on NCSCs is cell heritable.

In these experiments, the culture medium contained chick embryo extract (CEE). Although CEE promotes NCSC survival, self-renewal, and multilineage differentiation under standard conditions, it was possible that in the presence of activated Notch some unknown factor in CEE was required in order to observe glial differentiation. However, exposure to Delta-Fc in CEE-free medium for 24 hr followed by 4 days of BMP2 exposure in either the continued absence or presence of CEE, still resulted in significantly decreased neurogenesis and increased gliogenesis (data not shown). Similarly, when clones were cultured in retinoic acid–free medium, the ability

Table 5. Delta Causes Irreversible Loss of Neurogenic Capacity in Less Than 24 Hours, before a Response Can Be Detected to Neuregulin (Nrg-1)



Initial medium	Colonies that Contain the Indicated Cell Types (% of Colonies, mean ± SD)				
	Schwann-only	Schwann+other	No Schwann	Neurons	Myofibroblasts
Standard	0.0 ± 0.0 a	13.7 ± 9.6 a	86.3 ± 9.6 a	69.4 ± 7.2 a	15.2 ± 3.5 a
+Fc	3.2 ± 6.7 a	16.3 ± 17.8 a	80.4 ± 23.8 a	61.9 ± 27.0 a	15.9 ± 11.8 a
+Delta-Fc	57.3 ± 23.0 b	25.3 ± 16.0 a	17.5 ± 11.0 b	1.8 ± 3.6 b	8.5 ± 5.1 a
+Nrg-1+Fc	1.0 ± 1.1 a	13.3 ± 10.5 a	85.7 ± 10.9 a	78.6 ± 8.5 a	13.1 ± 6.2 a

Sciatic nerve $p75^+P_0^-$ stem cells were cultured for 1 day in standard, Fc, Delta-Fc, or Nrg-1 supplemented medium. Then the medium was changed to standard medium plus BMP2 (50 ng/ml) for a final 4 days to test neuronal potential before fixation and staining (see Table 1). Note that the "Schwann-only", "Schwann+other", and "No Schwann" categories add to 100%, but there is overlap between these latter two categories and the "Neuron", and "Myofibroblast" categories since some colonies contained various combinations of Schwann cells, neurons, myofibroblasts, and undifferentiated cells. The data are based on two to four independent experiments in which an average of more than 60 clones were analyzed per treatment per experiment. Statistics (mean ± standard deviation) within columns were compared by pair-wise t tests. Significantly different statistics ($p < 0.05$) are followed by different letters.

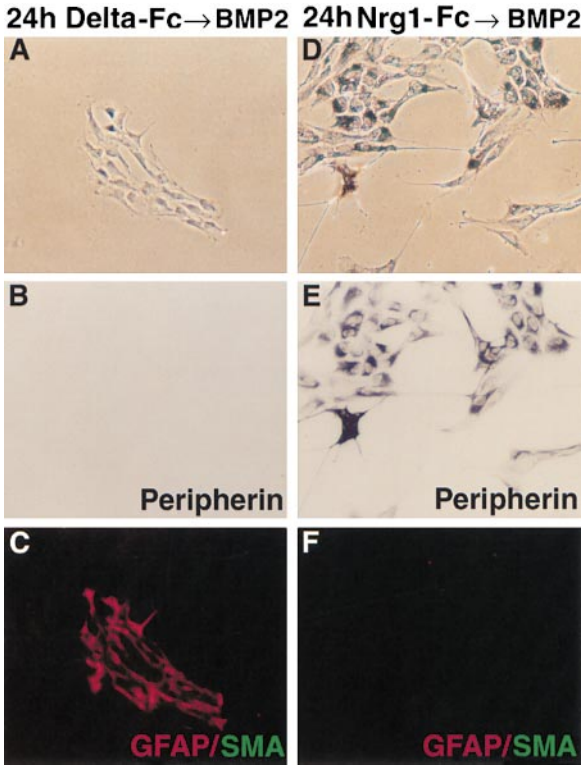


Figure 3. Delta-Fc Causes Glial Determination after Only 1 Day in Culture

Cultures of NCSCs at clonal density were supplemented either with Delta-Fc (A–C) or with Nrg-1 plus Fc (D–F). After only 24 hr, the cultures were washed into standard medium supplemented with BMP2 (50 ng/ml) and grown for 4 more days to test neuronal potential. (A) and (D) show phase contrast images, (B) and (E) show bright field images of peripherin staining, and (C) and (F) show superimposed epifluorescence images of GFAP (red) and SMA (green, negative) staining. The control colony preincubated in Nrg-1 plus Fc (D–F) contained mostly neurons and neuronal precursors induced by the BMP2 treatment, as judged by the peripherin staining and the lack of GFAP and SMA staining. In contrast, the colony preincubated in Delta-Fc (A–D) contained only Schwann cells (red) despite the BMP2 treatment, as indicated by the GFAP staining and the lack of peripherin and SMA staining. Thus, Delta-Fc acts more rapidly than Nrg-1 to instruct gliogenesis. For quantitation see Table 5.

of Delta-Fc to cause glial commitment and differentiation was undiminished (data not shown). These data suggest that the effect of Delta-Fc is not dependent on additives such as CEE and retinoic acid. Nonethless, we cannot rule out that some other component of the culture medium might promote glial differentiation in the presence but not the absence of activated Notch.

Delta-Fc Acts More Rapidly than Nrg-1 (GGF-2), Another Instructive Glial Differentiation Factor

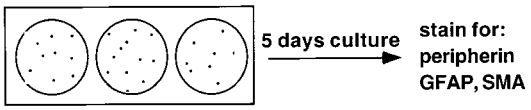
If glial differentiation were simply a consequence of inhibiting neurogenesis in NCSCs, then other instructive glial differentiation factors should act with the same kinetics as Delta-Fc. One such factor is Nrg-1 (GGF-2). Previous studies have shown that when NCSCs are grown for 12–14 days in recombinant Nrg-1, Schwann cell differentiation is promoted at the expense of neuronal and myofibroblast differentiation (Shah et al., 1994; Morrison et al., 1999). This result is therefore indistinguishable from that obtained when NCSCs are grown for similar periods of time in Delta-Fc (Table 1). It was therefore of interest to compare the kinetics of glial lineage determination in Delta-Fc to that in Nrg-1.

In a side-by-side comparison, NCSCs were exposed to Delta-Fc or Nrg-1 for 24 hr, followed by washout of these factors and a subsequent 4 day challenge with BMP2. The colonies were then fixed and stained for all three markers (peripherin, GFAP, and α-SMA). As previously reported (Shah and Anderson, 1997), such a brief exposure of NCSCs to Nrg-1 was not sufficient to cause an irreversible loss of neurogenic capacity: 78.6% of colonies transiently exposed to Nrg-1 contained neurons after the subsequent challenge by BMP2 (Table 5, Nrg-1 + Fc, "Neurons" and Figures 3D and 3E), a number not significantly different from the controls (Table 5, "Standard" and +Fc). In contrast, as mentioned above, less than 2% of colonies preincubated in Delta-Fc contained neurons following the BMP2 challenge (Figures 3A and 3B). Furthermore, glial differentiation was enhanced by pretreatment in Delta-Fc compared to pretreatment in Nrg-1: 57% of cells exposed to Delta-Fc for 24 hr gave rise to colonies that contained only Schwann cells despite the subsequent BMP2 challenge, while only 1% of cells exposed to Nrg-1 did so (cf. Figure 3C versus Figure 3F and Table 5, Schwann-only). In cultures incubated for 5 days in Nrg-1, the factor did inhibit BMP2-induced neurogenesis relative to controls as previously reported (Shah and Anderson, 1997), indicating that it was active (data not shown). These data therefore indicate that glial lineage determination occurs much more rapidly in Delta-Fc than in Nrg-1, although both factors ultimately promote gliogenesis at the expense of other fates.

Delta Is Permissive for Myofibroblast Differentiation

To test whether Delta-Fc is permissive for myofibroblast differentiation, we took advantage of the fact that BMP2 promotes myofibroblast as well as neuronal differentiation in NCSCs (Shah et al., 1996; Hagedorn et al., 1999). When NCSCs were cultured for 5 days in the presence of both BMP2 and Delta-Fc, the majority (71%) of colonies contained myofibroblasts. In the presence of BMP2 but not Delta-Fc, only 20% of the colonies contained myofibroblasts, no colonies contained $GFAP^+$ glia, and about 94% contained neurons (Table 6). Myofibroblast differentiation was also promoted in the presence of BMP2 when constitutively active *Notch-ICD* was transduced into NCSCs using a retroviral vector (data not shown). These data indicate that Delta-Fc allows myofibroblast but not neuronal differentiation to be induced by BMP2 when the two factors are presented concurrently. In other experiments, NCSCs were cultured for 4 days in Delta-Fc and then challenged with serum, which also induced myofibroblast differentiation (Stemple and Anderson, 1992; Shah et al., 1996). Some cells still differentiated to myofibroblasts although many appeared committed to a Schwann cell fate (data not shown). These data suggest that Notch signaling is permissive for myofibroblast differentiation under some circumstances, and that myofibroblast potential may be lost more slowly than neuronal potential in Delta-Fc. Nevertheless, in the

Table 6. Delta-Fc Is Permissive for Myofibroblast but Not Neuronal Differentiation Induced by BMP2



	Colonies that Contained the Indicated Cell Types (mean ± SD)		
Medium	Schwann cells	Myofibroblasts	Neurons
Standard+BMP2	0.0 ± 0.0 a	19.3 ± 15.5 a	93.2 ± 6.7 a
+Fc+BMP2	0.0 ± 0.0 a	21.5 ± 16.4 a	93.9 ± 5.9 a
+Delta-Fc+BM	16.8 ± 7.6 b	71.3 ± 12.7 b	6.2 ± 7.8 b

$p75^{+}P_0^{-}$ stem cells were cultured in standard medium plus BMP2 for 5 days with or without Delta-Fc, then were fixed and stained as described in Table 1. Statistics within columns were compared by pair-wise t tests. Significantly different statistics ($p < 0.05$) are followed by different letters. Plating efficiency averaged 31% for the Fc-only treatment to 48% for the Delta-Fc treatment, but the difference was not statistically significant. The data are based on an average of 140 colonies per condition analyzed in 2 to 3 independent experiments.

absence of myofibroblast-inducing signals, Notch activation in NCSCs invariably results in glial differentiation.

Discussion

Multipotent NCSCs migrate from the neural tube and then aggregate to form PNS ganglia where they differentiate to neurons and glia. The differentiation of NCSCs to neurons is instructively promoted by local environmental signals, such as BMP2. Such a mechanism poses the problem of how a multipotent neural stem cell can generate two different cell types in an environment containing such an instructive neurogenic factor. Here we have shown that a soluble form of the Notch ligand Delta-1 can inhibit neuronal differentiation by NCSCs in a manner that is completely dominant to BMP2. Contrary to expectation, withdrawal of Delta-Fc did not allow NCSCs to resume neuronal differentiation when subsequently challenged with BMP2. Rather, transient exposure to Delta-Fc promoted a rapid and irreversible loss of neurogenic capacity accompanied by glial differentiation. These data suggest that Notch ligands expressed by differentiating neuroblasts may mediate a feedback signal that promotes a switch from neuronal to glial differentiation in neighboring stem cells. The apparently irreversible, cell-heritable nature of this switch, moreover, may allow the influence of Notch signaling to persist even after expression of Notch and its ligands is downregulated in later development.

Notch Signaling Dominantly Inhibits BMP2-Induced Neurogenesis

The fact that Notch activation is dominant to the neurogenic influence of BMP2 is surprising for two reasons. First, the only other known instructive factor for gliogenesis in this system, GGF2/Nrg1, is completely subordinate to BMP2 when the two factors are presented concurrently to NCSCs at saturating concentrations (Shah and Anderson, 1997). These results suggested that BMP2 exerts a powerful and dominant influence on the fate of NCSCs. Second, in the only other study in which cells were exposed both to Notch activation and to an instructive factor for an alternative fate (the EGF-related LIN-3 ligand in *C. elegans*), the influence of NOTCH/LIN-12 signaling was subordinate to that of the LIN-3 morphogen (Wang and Sternberg, 1999). These data suggested that the influence of morphogens and other instructive factors that promote the differentiation of "primary" fates, such as neurons, would often be dominant to the influence of Notch signaling in multipotent cells. The observation that Notch signaling is instead dominant to the neurogenic influence of BMP2 was therefore unexpected.

It may seem paradoxical that Notch signaling is dominant to the effect of BMP2 to promote neurogenesis, but not to its effect to promote myofibroblast differentiation. However, the mechanism whereby BMP2 induces both neuronal and myofibroblast differentiation in NCSCs is not yet clear. Although Notch signaling prevents MASH1 induction by BMP2, this inhibition may occur downstream of the point at which the pathways leading to neuronal and myofibroblast differentiation diverge. In this way, Notch could dominantly inhibit the neurogenic influence of BMP2 while allowing myofibroblast differentiation to occur. Clarification of this issue will require a deeper understanding of the pathways that control neuronal and myofibroblast differentiation by NCSCs in response to BMP2.

If Notch signaling inhibits BMP2-induced neurogenesis but not myofibroblast differentiation in vitro, why are myofibroblasts not observed in developing PNS ganglia in vivo? The answer may be related to the observation that when addition of BMP2 to NCSCs is delayed, the myofibroblast-promoting effect of the growth factor is lost (Hagedorn et al., 1999). Consistent with this observation, we observed that when concurrent administration of BMP2 and Delta-Fc was delayed for 24 hr after plating, gliogenesis predominated. Thus, although Notch is permissive for BMP2-induced myofibroblast differentiation at the time of plating, under most circumstances Notch activation results in glial differentiation whether or not BMP2 is present. The mechanism underlying the rapid loss of myofibroblast-inducing activity by BMP2 in vitro is not yet clear, but this phenomenon may help explain why myofibroblast differentiation has not been observed during ganglion formation in vivo.

Notch Signaling Promotes a Rapid, Irreversible Loss of Neurogenic Capacity

As little as a 24 hr exposure to Delta-Fc virtually abolished the ability of NCSCs to differentiate to neurons when subsequently challenged by BMP2. Because the low levels of Delta-Fc bound to NCSCs immediately following its application were undetectable by immunostaining, we were unable to directly confirm that a complete washout of Delta-Fc was achieved in these experiments. Nevertheless, it is unlikely that the persistent influence of Delta-Fc is due to residual ligand associated with the cells after washout. Notch signaling involves a protease-dependent cleavage of its intracellular domain (Schroeter et al., 1998; Struhl and Greenwald, 1999; Ye et al., 1999). Thus, activation of Notch by Delta-Fc is stoichiometric rather than catalytic and receptor activation is self-terminated by such cleavage. In order for any residual Delta-Fc to continue to signal, therefore, it would have to dissociate from cleaved Notch molecules, escape endosomal degradation, and rebind to newly synthesized intact receptors on the cell surface without being diluted in the culture medium. Furthermore, even if undegraded Delta-Fc were recycled to the cell surface, it would be diluted many-fold by cell division over the ensuing culture period. During the initial 24 hr exposure to Delta-Fc, most NCSCs had not yet divided, but by the end of the subsequent incubation in BMP2, the colonies contained an average of 90 cells. Since the effects of Delta-Fc are lost after as little as a 5-fold dilution (data not shown), dilution below threshold would likely occur rapidly. Thus, the simplest interpretation is that transient exposure to Notch ligand causes an irreversible loss of neurogenic capacity in NCSCs.

The observation of such an irreversible inhibition of neurogenesis is surprising, because prior studies in *Xenopus* and *Drosophila* have suggested that neurogenic capacity can be recovered upon decay or deliberate inactivation of ectopic Notch expression (Coffman et al., 1993; Struhl et al., 1993). Our results therefore challenge the prevailing view that Notch signaling functions principally to inhibit the differentiation of progenitor cells in a reversible manner so as to maintain competence for alternative fates, although it may do so in some settings (Coffman et al., 1993; Struhl et al., 1993; Dorsky et al., 1997; Henrique et al., 1997). The molecular mechanism that underlies the apparently irreversible and cell-heritable influence of Notch activation in NCSCs will be an interesting subject for future study.

The ability of Notch activation to cause an irreversible loss of neuronal potential in NCSCs may seem inconsistent with the fact that multipotent neural crest progenitors can be isolated from tissues such as sciatic nerve (Morrison et al., 1999) and dorsal root ganglia (Hagedorn et al., 1999) where Notch ligands are expressed. However, these multipotent cells constitute a relatively small proportion ($\leq$15%) of the cells in these tissues, and are present only transiently. Nevertheless, the existence of such cells indicates that there must be mechanisms by which some neural crest progenitors can escape the influence of Notch ligands and maintain multipotency, at least temporarily. One possibility is that the cells that maintain multipotency are not in direct contact with neuroblasts that express Notch ligands. Another possibility is that cell-intrinsic inhibitors of Notch signaling, such as Numb, are differentially expressed among neural crest progenitors (Zhong et al., 1997; Verdi et al., 1999; Wakamatsu et al., 1999). Whatever the reason, the extent of glial differentiation in neural crest-derived tissues is likely regulated by factors in addition to Notch in vivo.

Notch as a Positive Differentiation Factor

Genetic studies of Notch function in binary cell fate decisions have often been interpreted to suggest that it acts negatively, to inhibit the "primary" fate so that the "secondary" fate occurs by default (reviewed in Artavanis-Tsakonas et al., 1995, 1999). However, it can be difficult to formally distinguish such a negative action from a positive effect to promote differentiation of the secondary fate. Because NCSCs have three possible fates, it cannot be the case that blocking neurogenesis simply leads to glial differentiation by default; it could just as easily have led to myofibroblast differentiation. But under most circumstances, Notch activation promoted glial but not myofibroblast differentiation. This specificity is particularly striking given that Notch signaling is permissive for induction of myofibroblast differentiation by BMP2. These observations suggest that Notch acts as a positive glial differentiation factor in this system.

Notch signaling has been shown to inhibit neurogenesis in many systems (reviewed by Artavanis-Tsakonas et al., 1995, 1999; Lewis, 1996; Harris, 1997), but has been interpreted to maintain neural precursors in an undifferentiated state rather than to promote gliogenesis. For example, expression of constitutively active Notch in the retina was interpreted to inhibit the differentiation of neurons but to be permissive for Müller glia differentiation (Bao and Cepko, 1997). Our observation that Delta-Fc increased the rate, and not just the extent, of glial differentiation by NCSCs provides further evidence for a positive effect on gliogenesis. Moreover, both Delta-Fc and Nrg1 promote glial differentiation at the expense of neuronal and myofibroblast differentiation; however, loss of neuronal potential and glial differentiation were observed much more rapidly in response to Delta-Fc. Although these observations suggest that Notch acts positively to promote gliogenesis, we cannot exclude that the rate-limiting step for gliogenesis in vitro is the loss of neurogenic capacity, and that other factors in the culture medium promote overt glial differentiation once Notch activation has accelerated this rate-limiting step.

In other vertebrate systems where Notch signaling has been shown to influence differentiation decisions, it has been difficult to distinguish between selective and instructive mechanisms. For example, in murine P19 embryonal carcinoma cells, forced expression of Notch-ICD was shown to suppress neuronal and muscle but not glial differentiation (Nye et al., 1994), but it was not determined whether Notch activation promoted gliogenesis, or simply inhibited the differentiation, proliferation, or survival of neuronal and muscle cells. Similarly, Notch has been suggested to act positively to instruct T cell subtype differentiation (Robey et al., 1996; Washburn et al., 1997). However, other observations (Linette et al.,

1994) have suggested that Notch may rather act to promote the survival of certain T cell subsets (Deftos et al., 1998; von Boehmer, 1999). Our clonal analysis firmly rules out the possibility that Notch signaling promotes glial development by selection rather than instruction in NCSCs.

Notch Ligands May Mediate a Feedback Signal that Controls a Cell-Heritable Switch from Neurogenesis to Gliogenesis

The observation that Notch activation dominantly inhibits its BMP2-induced neurogenesis in an irreversible manner and promotes gliogenesis is biologically important, because it could help to explain how multipotent NCSCs are able to generate both neurons and glia in the face of strong neurogenic inducing signals such as BMPs in vivo. Expression of Notch ligands on differentiating neuroblasts could mediate a feedback signal to neighboring uncommitted NCSCs, which would override the neurogenic influence of BMP2/4 while simultaneously promoting glial differentiation. Such a feedback mechanism could also explain why glia invariably differentiate after neurons in these ganglia. Importantly, the ability of Notch activation to cause an irreversible loss of neurogenic capacity may be important for sustaining glial differentiation by NCSCs in the face of the transient expression of Notch ligands and their receptors that occurs during peripheral ganglion formation in vivo.

If Notch ligands instruct glial differentiation in developing ganglia, then what is the function of GGF2/Nrg1 in glial fate determination? Knockouts in Nrg1 and its receptors strongly reduce the number of peripheral glia (Lee et al., 1995; Meyer and Birchmeier, 1995; Riethmacher et al., 1997), but it is difficult to determine in vivo whether this reflects a role in survival, proliferation, or lineage commitment of glial progenitors: Nrg1 exhibits all of these activities in vitro (Shah et al., 1994; Dong et al., 1995; Morrison et al., 1999). One possibility is that since Nrg1 is synthesized in secreted as well as membrane-bound forms (Marchionni et al., 1993), it may provide a longer-range signal to promote glial development, e.g., in developing peripheral nerve, while Notch ligands may operate primarily at short range, e.g., in promoting the differentiation of satellite glia that envelop neuronal cell bodies. In addition, Nrg1 may be used in settings where extensive proliferation is required together with glial fate determination, as is necessary in myelinated nerves. Interestingly, in the *C. elegans* vulva, the Nrg1-homologous ligand LIN-3 acts in a concentration-dependent manner together with NOTCH/LIN-12 to promote differentiation of the 2° fate (Sternberg and Horvitz, 1989; Katz et al., 1995). Cooperation between these two signaling systems may therefore be used more generally to specify cell fates in a variety of organisms and tissues.

Experimental Procedures

Isolation of Sciatic Nerve $p75^+P_0^-$ Cells

Pregnant Sprague-Dawley rats were obtained from Simonsen (Gilroy, CA). $p75^+P_0^-$ NCSCs were isolated from E14.5 sciatic nerves as described previously (Morrison et al., 1999). In brief, nerves were dissected and dissociated by incubating in trypsin plus collagenase for 4 min at 37°C followed by mechanical trituration. The cells were stained with monoclonal antibodies against p75 (192Ig) and P_0 (P07; a gift of J. J. Archelos) and sorted using a FACSVantage flow-cytometer (Becton-Dickinson, San Jose).

Delta Conditioned Medium

293T-Delta-Fc and 293T-Fc cell lines were cultured in DMEM-high glucose with 10% fetal bovine serum and penn/strep added. 200 μg/ml of hygromycin B was added to the medium to select against loss-of-construct expression. When the cultures became confluent, the medium was replaced with DMEM-high glucose only. After 5 days of incubation, the conditioned medium was harvested. The Delta-Fc or Fc-conditioned supernatant was concentrated by centrifuging in a Centricon biomax-30 (Millipore, Stoughton, MA), then washed by adding 1 to 5 volumes of L15 medium and recentrifuging in the Centricon to achieve 50- to 100-fold total net concentration. Fifty-fold concentrated supernatant was added at a concentration of 30 μl per 1.5 ml of culture medium. A 100-fold dilution of anti-human Fc antibody (109–005–098; Jackson Immunoresearch, West Grove, PA) was included in all cultures.

Culture Conditions

Sciatic nerve progenitors were cultured at clonal density as described previously (Morrison et al., 1999). In brief, plates were coated with poly-d-lysine and human fibronectin (Biomedical Technologies, Stoughton, MA). The culture medium contained DMEM-low glucose (Gibco) with 15% chick embryo extract (Stemple and Anderson, 1992), 20 ng/ml recombinant human bFGF (R&D Systems, Minneapolis), N2 supplement (Gibco), B27 supplement (Gibco), 50 μM 2-mercaptoethanol, 35 ng/ml retinoic acid (Sigma), penicillin/streptomycin (BioWhittaker), and 10 μg/ml anti-human Fc antibody. This composition is described throughout as "standard medium". Most cultures were incubated in a reduced oxygen environment to more closely approximate physiological oxygen levels because these conditions promote the survival and multilineage differentiation of NCSCs (S. J. M., unpublished data). Tissue culture plates were inserted into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA) that were flushed with a custom gas mixture containing 1% O_2/6% CO_2/balance N_2. The incubator chambers were flushed for 1.5–2 min daily at a rate of 15 liters per min, then inserted into normal tissue culture incubators. This achieved an actual concentration inside the chamber of around 5% oxygen, as measured by microelectrode (Animus Corp., Malvern, PA).

Immunohistochemistry

For routine analysis of culture compositions, cultures were fixed in acid ethanol and stained with antibodies against peripherin (Chemicon AB1530, Temecula, CA), smooth muscle actin (Sigma A-2547), and GFAP (Sigma G-3893) as described previously (Morrison et al., 1999). In general, Schwann cells (S) were $GFAP^+$ SMA^- $peripherin^-$, myofibroblasts (M) were SMA^+ GFAP- $peripherin^-$, neurons (N) were $peripherin^+$ $GFAP^-$ SMA^-, and undifferentiated cells did not stain with any of these markers. However, in cultures that were incubated for 14 or 15 days, although multipotent colonies always contained $GFAP^+$ glia, as described previously (Morrison et al., 1999), some colonies lacking $peripherin^+$ or SMA^+ cells that otherwise appeared to consist only of glial cells did not detectably stain with GFAP. Nevertheless, the small size (typically containing hundreds of cells) and morphology of such colonies was indistinguishable from Schwann-only colonies that were $GFAP^+$ and clearly distinguishable from stem cell colonies that were typically very large (containing tens of thousands of cells). Furthermore, such colonies consistently expressed p75 and S100β, and were never observed to give rise to multipotent colonies in subcloning experiments. By these antigenic, morphologic, and functional criteria such colonies were classified as S only.

Chick embryos were fixed and processed for immunohistochemistry as previously described (Perez et al., 1999). Representative, 20 mm cryostat sections were collected from a 1 mm region of each animal at forelimb level. Infected cells were detected using a polyclonal antibody to the viral protein, p27 (SPAFAS, Charles River Farms, CT; 1/1000). Sections were counterstained with a monoclonal antibody, 16A11, to Hu neuronal antigens (gift of J. Weston; 12.5 mg/ml) (Marusich et al., 1994).

Expression of Constitutively Active Notch-ICD in Rat NCSCs

Constitutively active Notch1 (Notch-ICD) was generated by PCR (Nye et al., 1994). The cDNA was cloned into a modified pbabe-neo retroviral construct upstream of IRES driving a human alkaline phosphatase gene (Lo et al., 1997). This construct was packaged using the Bosc23 cell line (Verdi et al., 1999) and then used to infect NCSCs isolated from E10.5 neural tube explants. Transfected NCSCs were cultured in expansion medium (Stemple and Anderson, 1992) and supplemented with 500 μg/ml G418 for four days. Then the medium was removed and cells were washed in HBSS and placed in differentiation medium (Shah et al., 1994) containing 250 μg/ml G418 for 10 days. The resulting clones were fixed and stained with monoclonal antibodies to peripherin, smooth muscle actin, and GFAP.

Gain or Loss of Notch Function in Chick Embryos

Notch-ICD (Nye et al., 1994) was amplified by PCR from a plasmid provided by Jeff Nye and cloned into the replication competent retroviral vector, RCAS (Petropoulos and Hughes, 1991; Morgan and Fekete, 1996), in frame with a single myc tag. An RCAS construct carrying a dominant negative form of *Xenopus* Suppressor of Hairless, $Su(H)^{DBM}$ (Wettstein et al., 1997), was the gift of Chris Kintner. The RCAS vector carrying a backward insertion of Notch-ICD served as a control virus. Since this sequence is mouse-derived it would not be expected to anneal with endogenous chick Notch mRNA to cause antisense effects. Furthermore, results with this reverse-Notch-ICD control virus did not differ from those obtained with an empty RCAS control virus.

Chick eggs were obtained from local suppliers, incubated at 38°, and staged (Hamburger and Hamilton, 1951). Electroporation was carried out essentially as previously described (Muramatsu et al., 1997). Briefly, RCAS constructs (supercoiled plasmid at 1 mg/ml in TE) were injected into the lumen of the neural tube of animals at HH 13–15. Electrodes of 5 mm in length (Genetrodes by Genetronics, San Diego, CA) were placed on either side of the embryo (6–8 mm apart). An Electro Square Porator T820 (Genetronics) was used to deliver five 50 ms pulses of 25 volts. The animals were returned to the incubator and harvested 36 hr after electroporation, at Hamburger and Hamilton stage 22–23.

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