Developmental Biology **244,** 199–214 (2002)
doi:10.1006/dbio.2002.0568, available online at http://www.idealibrary.com on IDEAL®

Expression Mediated by Local Cell–Cell Interactions Underlie Progressively Increasing Delta Sensitivity in Neural Crest Stem Cells

Chris J. Kubu,* Kenji Orimoto,† Sean J. Morrison,‡ Gerry Weinmaster,§ David J. Anderson,† and Joseph M. Verdi*,¶,1

**Laboratory of Neural Stem Cell Biology, John P. Robarts Research Institute, 100 Perth Drive, London Ontario, Canada N6A5K8;* †*Howard Hughes Medical Institute, Division of Biology, California Institute of Technology, Pasadena, California 91125;* ‡*Howard Hughes Medical Institute and Departments of Internal Medicine and Cell and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109;* §*Department of Biological Chemistry, University of California at Los Angeles, Los Angeles, California 90095; and* ¶*Department of Physiology, Program in Neuroscience, University of Western Ontario, London Ontario, Canada N6A5K8*

Neural stem cells become progressively less neurogenic and more gliogenic with development. Here, we show that between E10.5 and E14.5, neural crest stem cells (NCSCs) become increasingly sensitive to the Notch ligand Delta-Fc, a progliogenic and anti-neurogenic signal. This transition is correlated with a 20- to 30-fold increase in the relative ratio of expression of Notch and Numb (a putative inhibitor of Notch signaling). Misexpression experiments suggest that these changes contribute causally to increased Delta sensitivity. Moreover, such changes can occur in NCSCs cultured at clonal density in the absence of other cell types. However, they require local cell–cell interactions within developing clones. Delta-Fc mimics the effect of such cell–cell interactions to increase Notch and decrease Numb expression in isolated NCSCs. Thus, Delta-mediated feedback interactions between NCSCs, coupled with positive feedback control of Notch sensitivity within individual cells, may underlie developmental changes in the ligand-sensitivity of these cells.

Key Words: **Numb; Notch; BMP; Delta; neurogenesis; neural stem cells.**

INTRODUCTION

The neurons and glia of the vertebrate nervous system arise from multipotential, self-renewing progenitors that have been described as neural stem cells (NSCs) (reviewed in Gage, 2000; Anderson, 2001). During embryonic neural development, this progenitor population progressively changes over a period of several days from producing neurons to producing glia. Whether this slow transition reflects an all-or-none, but asynchronous, switch from neurogenesis to gliogenesis within the NSC population, a gradual and synchronous change in the probability of neurogenesis vs. gliogenesis by individual NSCs, or the death of neurogenic progenitors and their replacement by glial progenitors, is not yet clear. Nor is it clear how this transition is controlled. In the central nervous system (CNS), for example, it is not known whether the transition from neurogenesis to gliogenesis reflects a cell-autonomous program acting within NSCs, or rather changes in the levels of, or sensitivity to, local environmental signals that instruct neuronal vs. glial fates.

The neural crest has provided a useful model system to study the biology of the NSCs that generate the neurons and glia of the peripheral nervous system (PNS) (Bronner-Fraser, 1992; Anderson *et al.*, 1997). Multipotent, self-renewing neural crest stem cells (NCSCs) have been isolated from embryonic day 10.5 (E10.5) rat neural tube

[1] To whom correspondence should be addressed. Fax: (519) 663-3789. E-mail: joeverdi@rri.on.ca.

0012-1606/02 $35.00

explant cultures, using monoclonal antibodies to the low-affinity neurotrophin receptor p75 ($p75^{LNTR}$) as a cell surface marker (Stemple and Anderson, 1992). Such NCSCs can differentiate to neurons, glia (Schwann cells), and smooth muscle-like myofibroblasts (MFs) in clonal culture (Stemple and Anderson, 1992; Shah *et al.,* 1996). The differentiation of NCSCs to these three cell types can be promoted by specific growth factors, in an instructive manner. Bone morphogenetic protein-2 (BMP2) promotes neuronal differentiation and TGFβs promote MF differentiation (Shah *et al.,* 1996), while Neuregulin-1 (Nrg1) or the soluble Notch ligand Delta-Fc promotes glial differentiation (Shah and Anderson, 1997; Morrison *et al.,* 2000b). Postmigratory NCSCs have also been isolated from neural crest-derived structures such as E14.5 peripheral nerve (Morrison *et al.,* 1999) and dorsal root ganglia (DRG; Hagedorn *et al.,* 1999). The differentiative behavior, self-renewal ability, and growth factor responsiveness of these cells are qualitatively similar to that of migratory NCSCs (mNCSCs) isolated from E10.5 neural tube explants. These data indicate that multipotent, self-renewing NCSCs persist in developing PNS tissues well into mid or late gestation.

In the PNS, as in the CNS, gliogenesis generally follows neurogenesis. Recently, we found that postmigratory sciatic nerve-derived NCSCs (sNCSCs) as a population display quantitatively less neurogenic activity than earlier mNCSCs, when assayed by *in vivo* xenotransplantation (White and Anderson, 1999; White *et al.,* 2001). Parallel *in vitro* clonogenic assays indicated that this reduced neurogenic activity reflects a decreased sensitivity to BMP2 between mNCSCs and sNCSCs (White *et al.,* 2001). This decreased sensitivity to BMP2 is correlated with a small but statistically significant decrease in the expression levels of the type Ia BMP receptor (BMPRIa) mRNA between the two NCSC populations, although whether this change is causative was not established. These results suggested that the decreasing neurogenic activity of PNS-NSCs through development can be explained, at least in part, by a decreasing sensitivity of the cells to an instructive neurogenic differentiation signal, BMP2. The molecular changes responsible for this decrease, and the mechanisms that control them, remain undefined.

Here, we have asked whether, conversely, NCSCs also acquire increased sensitivity to a gliogenic signal with development, and if so, how this occurs. Using *in vitro* clonogenic assays, we show that sNCSCs are quantitatively more sensitive than mNCSCs to the Notch ligand Delta-Fc, which both inhibits neurogenesis and promotes gliogenesis in these cells (Morrison *et al.,* 2000b). This increased Delta-Fc sensitivity is associated with an almost 10-fold increase in the expression of Notch1, as well as with a 3- to 4-fold decrease in the expression of Numb, an inhibitor of Notch signaling. Forced expression of Notch1 in mNCSCs increases their Delta-Fc responsiveness to a level similar to that of sNCSCs. Conversely, forced expression of Numb in sNCSCs decreases their Delta-Fc-responsiveness to a level comparable to that of mNCSCs. These data suggest that quantitative changes in the relative expression levels of Notch and Numb may play a causal role in the developmental increase in Delta-Fc sensitivity. The increased Delta-Fc sensitivity characteristic of freshly isolated sNCSCs can be acquired *in vitro* by mNCSCs cultured at clonal density in the absence of other cell types. This apparently autonomous change is, however, dependent upon local cell–cell interactions within developing mNCSC clones. Delta-Fc itself is sufficient to increase and decrease the expression of Notch1 and Numb, respectively, in isolated mNCSCs. These data suggest that Delta-mediated feedback interactions between NCSCs, coupled with positive feedback control of Notch sensitivity within individual cells, may contribute to the developmental transition from neurogenesis to gliogenesis in NCSCs.

MATERIALS AND METHODS

Isolation of mNCSCs and sNCSCs

mNCSCs were isolated by the method of Stemple (Stemple and Anderson, 1992). In short, neural tubes from E10 rat embryos were isolated between presumptive limb buds and plated on 35-mm fibronectin-coated dishes. Crest cells were allowed to migrate away from the tube overnight in a defined medium containing 10% chick embryo extract (CEE; referred to within as Stemple medium) (Stemple and Anderson, 1992). Neural tubes were then removed by using a tungsten needle, and the explant cells were trypsinized and plated at clonal density (50 cells per 35-mm dish) in a different defined medium (Morrison *et al.,* 2000a) that enhances autonomic phenotype production (referred to within as Morrison medium). To identify mNCSCs, the cells were live labeled with the 192IgG p75 antibody (Chandler *et al.,* 1984) and the $P_0$7 monoclonal antibody against the peripheral myelin protein P_0 conjugated to phycoerythrin (gift of J. Achelos, Munich). p75-positive, P_0-negative cells (migrating neural crest stem cells) were identified and used for analysis. In PCR experiments, trypsinized crest explants were plated at 500 cells per dish. They too were labeled with the p75 and P_0 antibodies. To generate as pure a mNCSCs population as possible, in both clonogenic differentiation assays and sqPCR experiments, all non-$p75^+$, P_0^- cells were removed from the dish with a tungsten needle prior to the start of the experiment.

sNCSCs were isolated and cultured according to the method of Morrison (Morrison *et al.,* 1999, 2000a). Sciatic nerves isolated from E14.5 rat pups were enzymatically dissociated and labeled with the p75 antibody conjugated to fluorescein and the $P_0$7 monoclonal antibody conjugated to phycoerythrin. Cells were sorted by using a FACSVantage flow cytometer at the Robarts Cell Sorting Facility. The $p75^+P_0^-$ fraction (sNCSCs) was plated at clonal density for clonogenic assays in Morrison medium or at higher density for RT-PCR studies.

Cell Culture and Clonogenic Assays

Clonogenic assays were performed according to the original method of Stemple (Stemple and Anderson, 1992). Briefly, mNCSCs or sNCSCs were plated at clonal density and cultured for up to 14 days in a growth factor-rich medium containing 15% CEE (Morrison medium). The resulting clones were fixed in acid ethanol and stained with antibodies against peripherin (Chemicon AB1530, Temecula, CA) to detect neurons (N), smooth muscle actin (Sigma

A-2547, St. Louis, MO) to detect myofibroblasts (M), and GFAP to detect Schwann cells (S) (Chemicon AB5040). Clones were scored based on the appearance of just one phenotypic representative. The number and percentage of clones containing neurons only (N), Schwann cells only (S), Myofibroblasts only (M), N + S, N + M, N + S + M, and S + M were scored at day 14 unless stated otherwise. Also scored was the production of each representative phenotype in each individual clone to complement our clonal population assay.

Generation of Secondary mNCSCs Populations

In order to generate secondary NCSC populations, tripotential clones originating from differentiating E10 mNCSCs clones at distinct time points during differentiation (days 2, 4, 6, 8, 10, 14) were trypsinized and replated at clonal density. The resulting cells were labeled with the p75 and P0 antibodies and $p75^+P_0^-$ cells identified as putative stem cells. All non $p75^+P_0^-$ cells were removed from the dish with a tungsten needle. The cultures of secondary NCSCs were subjected to either standard clonogenic assays, as described above, or semiquantitative PR analysis.

Retroviral-Mediated Gene Transfer and Transfection Overexpression Studies

Overexpression of the genes of interest into mNCSCs or sNCSCs was achieved by retroviral-mediated gene transfer, as we have described in detail elsewhere (Verdi and Anderson, 1994; Verdi *et al.*, 1996b, 1999), or by Fugene-mediated transfection (Roche). In all overexpression studies, the gene of interest was tagged with the enhanced green or red fluorescent protein (GFP, RFP) to facilitate identification of expressing cells. Notch-ICD-GFP and NUMB-GFP were cloned into the retroviral backbone pBABE-Neo (Morgenstern and Land, 1990). Virus was produced by using the Bosc23 packing cell line (Pear *et al.*, 1993). On the day before transfection, the Bosc23 cells were split and plated at 2×10^6 cells per 60-mm dish in DMEM plus 10% FBS. Calcium chloride transfection was used to introduce pBABE constructs harboring the genes of interest into the Bosc23 packaging cell line. Twenty micrograms of precipitate per plate were added to cells for 10 h in the presence of 25 μM chloroquin. The cells were then washed with media and 2 ml of medium was added. Virus was collected for the next 72 h, at which time the virus-containing medium was divided in half and was concentrated according to the methods of Cepko (Cepko *et al.*, 1998). The resulting viral pellet was resuspended in Stemple medium minus CEE. The concentrated viral stock was added to the cultures of mNCSCs and sNCSCs in the presence of 4 μg/ml polybrene. Two 2-h rounds of infection were conducted. Thereafter, the cells were washed and split to clonal density, Morrison medium was applied, and clonogenic differentiation assays were conducted. Infection efficiencies as measured by GFP or RFP expression ranged from a low of 3% to a high of over 12%.

Overexpression of full-length Notch 1-RFP was achieved by cloning the full-length Notch 1 cDNA in-frame with an RFP cDNA under the control of a CMV promoter (Clontech). Fugene-mediated transfection was performed according to the manufacturer's instructions on explants of neural crest cultures in Stemple medium minus CEE for 6 h followed by a brief glycerol shock (10% glycerol in Stemple medium minus CEE). The resulting cells were then split to clonal density and allowed to recover for 8 h, and $p75^+$ RFP^+ cells were identified and used for the analysis. Transfection efficiencies ranged from 4 to 10%.

Preparation of Soluble Delta-Fc

Preparation of 293T-Delta-Fc and 293T-Fc has been described in detail elsewhere (Morrison *et al.*, 2000b). In short, confluent cultures of 293T Delta-Fc and 293T-Fc cells were refed with DMEM high-glucose medium and allowed to culture for an additional 5 days. The conditioned medium was concentrated by centrifugation in a Centricon biomax 30 column (Millipore, Stoughton, MA) to achieve a 50- to 100-fold total net concentration. Prior to use, the concentrated Delta-Fc-containing supernatant was incubated with a 100-fold dilution of antihuman Fc antibody (Jackson ImmunoResearch, 109-005-0098, West Grove, PA) for 30 min at room temperature to crosslink the soluble Delta-Fc. Various dilutions of concentrated Delta-Fc stock per ml of Morrison medium were then applied to the stem cell populations. Once again, a 100-fold dilution of antihuman Fc antibody was included in all cultures to ensure efficient crosslinking and activation of the Delta ligand. All experiments were performed with Delta-Fc that originated from a single isolation and concentration procedure.

Semiquantitative RT-PCR

Semiquantitative RT-PCR (sqPCR) was performed according to our published methods (Verdi and Anderson, 1994; Verdi *et al.*, 1996a). Briefly, total RNA was isolated by using the RNA microisolation kit (Stratagene, La Jolla, CA). The RNA was DNase-treated to remove potential genomic contamination and reprecipitated. The resulting RNA was oligo(dT)-primed and reverse transcribed by using Superscript II (Gibco). PCR was performed in the linear range of amplification for each primer set as empirically predetermined by using cDNA originating from 50 cell equivalents of rat brain RNA. Primers, available upon request, were chosen to have an annealing temperature of approximately 55°C and span an intron/exon border wherever possible. In order to quantify the reaction, a tracer amount of radioactive dCTP was added to each PCR. Each amplification product (ranging from 100 to 400 base pairs) was measured as a ratio relative to the amplification product of actin (350 base pairs) run in a separate reaction. Each sample was performed in triplicate using 1 of 50 μl of cDNA. All sqPCR experiments were performed at least three times from separate stem cell isolations.

RESULTS

sNCSCs Are More Sensitive than mNCSCs to Delta-Fc

In a previous study, it was shown that Delta-Fc, a soluble Notch ligand, exhibits two activities on sNCSCs: it inhibits neuronal differentiation and promotes glial differentiation (Morrison *et al.*, 2000b). That study confirmed that Delta-Fc exhibits qualitatively similar activities on mNCSCs. However, we noticed that Delta-Fc appeared more potent in its effects on sNCSCs than on mNCSCs. We therefore quantitatively compared the sensitivity of E10.5 mNCSCs and E14.5 sNCSCs (see Materials and Methods) to Delta-Fc. At a saturating concentration of the ligand (50 μl of concentrated Delta-Fc-conditioned medium; ~5 nM), the responses of both NCSC populations were qualitatively similar: neurogenesis (measured as the percentage of neuron-

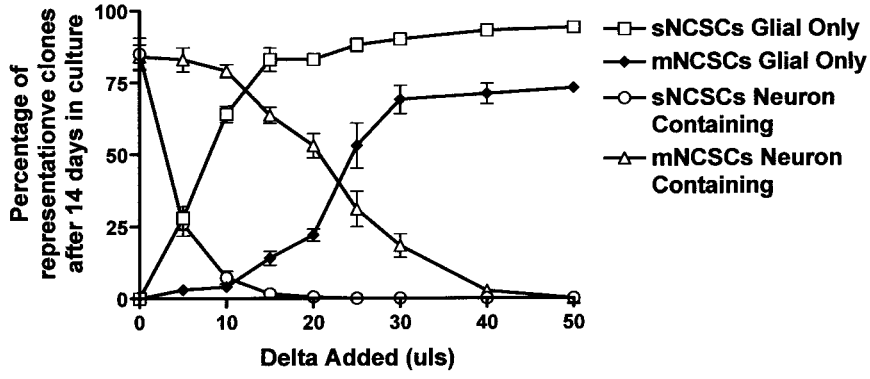


FIG. 1. sNCSCs are more responsive to the gliogenic actions of Delta than mNCSCs. Clonogenic assays of mNCSCs and sNCSCs cultured in increasing concentrations of Delta (μl Stock Delta-Fc added per ml Morrison medium) were performed. Presented are the mean ± standard deviation for at least three experiments originating from three independent stem cell isolations. Measured is the dose-response to Delta using two assays of Delta sensitivity: the percentage of glial-only clones and the percentage of neuron containing clones produced after 14 days of Delta treatment. Note, there is an approximately threefold shift to the left in the dose response curves for sNCSCs using both measures of Delta sensitivity.

containing clones) was strongly inhibited, and glial differentiation (measured as the percentage of glial-only clones) was greatly enhanced. Quantitatively, however, the proportion of glial-only clones was significantly higher in sNCSCs (92.5 ± 4.3%) than in mNCSCs (71.36 ± 4.5%, $P \leq 0.025$). The percentages of glial-only mNCSC clones at two- and threefold higher Delta concentrations were similar (data not shown), confirming that their response to Delta was indeed saturated.

To quantify the difference in the dosage sensitivity of mNCSCs and sNCSCs to Delta-Fc, we used two independent measures: (1) the promotion of glial-only clones, and (2) the inhibition of neuron-containing clones (Fig. 1). The concentration of the Notch ligand that produced a half-maximal response in sNCSCs in both assays (EC_{50} = 7 μl Delta-Fc [~0.7 nM]) was approximately threefold lower than that for mNCSCs (EC_{50} = 23 μl Delta-Fc [~2.3 nM]). Therefore, by both measures of Delta responsiveness, sNCSCs appear more sensitive than mNCSCs to Delta-Fc.

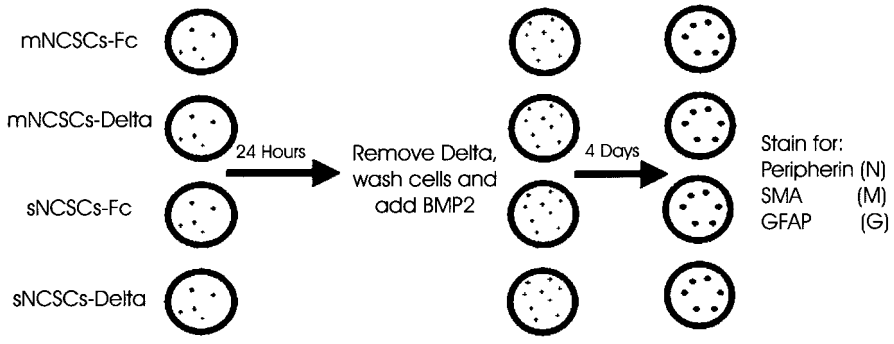
The difference in Delta-Fc sensitivity of sNCSCs and mNCSCs provided a further opportunity to determine whether the proglial differentiation effect of the Notch ligand is simply a consequence of its inhibition of alternative differentiation pathways, i.e., neuronal and/or myofibroblastic. To address this question, we compared sNCSCs and mNCSCs cultured in different concentrations of Delta-Fc, chosen so as to achieve an equal percentage of glial-only clones in the two populations (Fig. 1). For example, we chose concentrations of Delta-Fc that resulted in the production of approximately 10 and 20% of the final clones differentiating into glial-only clones (Table 1). We then compared the distribution of the remaining clone types under these conditions. Strikingly, although the percentages of glial-only and glial-containing clones were comparable between mNCSCs and sNCSCs, there were only half as many clones containing neurons and glia, but not myofibroblasts, in sNCSC cultures (Table 1, Neuron + Glia). Conversely, there was a three- to fourfold higher percentage

TABLE 1

mNCSCs Retain More Neurogenic Capacity than sNCSCs in the Presence of Subsaturating Concentrations of Delta

	Glial-containing	Glial only	Tripotential	Neuron + Glia	Myofibroblast + Glia
1.25 nM Delta-Fc mN CSCs	89.0 ± 2.8	8.8 ± 4.8	46.2 ± 7.8	21.6 ± 7.1	12.3 ± 1.4
0.3 nM Delta-Fc sNCSCs	85.9 ± 4.4	9.9 ± 4.5	32.4 ± 2.9	10.5 ± 4.3	33.1 ± 4.2c
1.75 nM Delta-Fc mNCSCs	92.1 ± 1.6	22.3 ± 6.6	44.7 ± 5.4	15.3 ± 4.6	9.8 ± 4.3
0.4 nM Delta-Fc sNCSCs	92.8 ± 4.6	21.1 ± 1.6	23.2 ± 4.2b	8.2 ± 4.9	36.6 ± 8.6c

Note. mNCSCs and sNCSCs were cultured at clonal density for 14 days in standard Morrison medium supplemented with subsaturating concentrations of Delta-Fc (Delta). Concentrations of Delta-Fc resulting in 10 and 20% of the resulting clones comprising only glial cells were used to determine the composition of the remaining differentiated clones. Presented is the mean ± standard deviation for three experiments originating from three independent stem cell isolations. Statistics within columns for each concentration were compared by two-tailed paired Student's *t*-test and significantly different statistics are followed by different letters: a = $P \leq 0.05$; b = $P \leq 0.025$; c = $P \leq 0.01$.



Condition	Percentage Of Glial Containing Clones	Percentage of Neuron Containing Clones	Glial ONLY	Neuron ONLY
mNCSCs-Fc	25.29 ± 8.42	85.47 ± 5.45	0.75 ± 1.31	62.50 ± 5.83
mNCSCs-Delta	80.58 ± 4.67	66.04 ± 2.07	9.77 ± 1.82	18.74 ± 5.07
sNCSCs-Fc	24.84 ± 8.00a	84.86 ± 3.97a	1.19 ± 2.06a	55.16 ± 8.54a
sNCSCs-Delta	83.19 ± 6.41a	43.97 ± 6 .68ab	16.94 ± 2.97ab	6.16 ± 4.22ab

FIG. 2. mNCSCs retain more neurogenic potential after transient Delta exposure. mNCSCs and sNCSCs were cultured at clonal density for 24 h in standard Morrison medium supplemented with a subsaturating concentration (EC_{50}) of Delta-Fc. After 24 h, the neurogenic potential of these transiently Delta-Fc exposed cells was challenged (see Materials and Methods) with 1 nM BMP2 for an additional 4 days. Presented is the average ± standard deviation for three experiments originating from three independent stem cell isolations. Statistics within columns were analyzed by using a pair two-tailed Student's *t*-test. The first letter represents difference between Delta-Fc-treated and Fc-treated cultures and the second letter represents differences between Delta-Fc-treated samples of mNCSCs and sNCSCs: a = $P \leq 0.01$; b = $P \leq 0.025$. Note that by two independent measures, Percentage of Neuron Containing Clones and Production of Neuron Only Clones, mNCSCs retained more neurogenic differentiation potential than sNCSCs after transient Delta exposure.

of clones containing MFs and Glia, but not neurons, in sNCSCs. These data suggest that concentrations of Delta-Fc that yield equivalent extents of glial differentiation in the two NCSC populations permit myofibroblast, but not neuronal, differentiation in sNCSCs. Thus, consistent with previous data (Morrison *et al.*, 2000b), the promotion of glial differentiation by Delta-Fc is not simply a consequence of its inhibition of all other differentiation pathways. Moreover, doses of the ligand that yield equivalent percentages of glial-only clones in the two NCSC populations more potently suppress neurogenesis in the sNCSC population, suggesting that promotion of glial differentiation and inhibition of neuronal differentiation are independent effects of Delta-Fc.

Transient Exposure to Delta-Fc Causes a More Extensive Loss of Neurogenic Capacity in sNCSCs than in mNCSCs

The foregoing experiments demonstrated that sNCSCs are more sensitive than mNCSCs to Delta-Fc, under conditions of continuous exposure to the Notch ligand. It was previously shown that transient exposure (24 h) to saturating levels of Delta-Fc promotes an irreversible loss of neurogenic capacity in most or all sNCSCs (Morrison *et al.*, 2000b). To quantitatively compare this effect of Delta-Fc on mNCSCs vs. sNCSCs, we transiently exposed these populations to subsaturating concentrations of Delta-Fc, and after 24 h removed the Notch ligand and tested the neurogenic potential of the cells by addition of BMP2. At the subsaturating concentration of Delta-Fc chosen, BMP2 was capable of inducing some neuronal differentiation in both NCSC populations. However, mNCSCs retained significantly more neurogenic activity than sNCSCs in response to BMP2, as measured by the percentage both of clones that contained any neurons and of clones comprised entirely of neurons (Neuron ONLY; Fig. 2). Thus, mNCSCs are less sensitive than sNCSCs to the ability of transient Delta-Fc exposure to promote an irreversible loss of neurogenic activity. Taken together, these experiments indicate that differences in Delta-Fc sensitivity and responsiveness exist between these two populations of NCSCs, as determined by multiple functional assays.

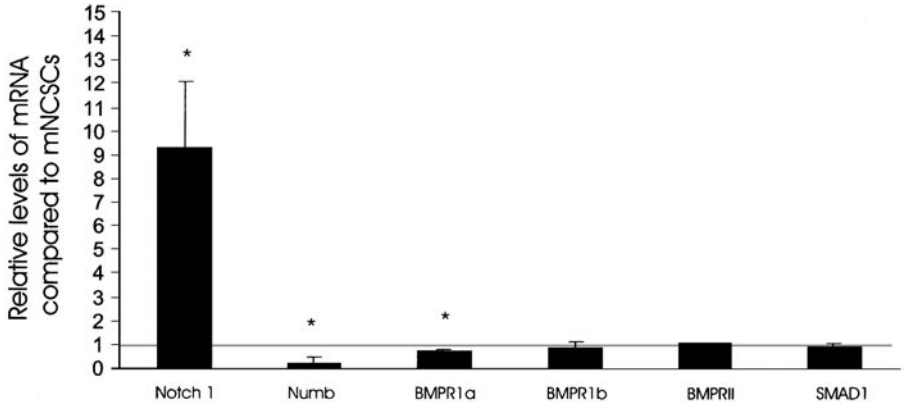


FIG. 3. sNCSCs express significantly more Notch1 mRNA and less Numb mRNA compared with mNCSCs. Semiquantitative RT-PCR was performed in triplicate on cDNAs originating from at least three independent isolations of mNCSCs and sNCSCs. Presented is the mean ± standard deviation of three sqRT-PCR experiments performed in triplicate. The numbers are presented as the relative levels of mRNA expressed in sNCSCS compared with mNCSCs. A value of 1 means no difference in the expression levels between the two populations.

Expression of Notch and Numb Correlates with Differences in Delta-Fc Responsiveness between mNCSCs and sNCSCs

The differences observed between mNCSCs and sNCSCs in their BMP2 responsiveness are correlated with small but significant differences in the expression of the BMPRIa subunit mRNA (White *et al.*, 2001). This observation, and the previous results, led us to investigate whether the differences in the extent of Delta-Fc responsiveness were, similarly, correlated with differences in the level of Notch expression between these two stem cell populations. We therefore compared the expression levels of Notch1 mRNA in freshly isolated mNCSCs and sNCSCs via sqPCR (see Materials and Methods). sNCSCs expressed more than eightfold greater levels of Notch1 mRNA than mNCSCs (Fig. 3). This difference in Notch1 expression was robust and independent of the exact isolation method or culture medium used (data not shown).

The increased sensitivity of sNCSCs to Delta-Fc could also reflect a decreased expression of negative regulators of Notch signaling. One putative negative regulator of Notch signaling is Numb (Uemura *et al.*, 1989; Verdi *et al.*, 1996b; Zhong *et al.*, 1996). Numb is a signaling adapter protein that has been shown to enhance neuronal differentiation in mNCSCs (Verdi *et al.*, 1996b, 1999; Wakamatsu *et al.*, 1999). Numb is believed to function by inhibiting the translocation of Notch to the nucleus, effectively blocking Notch signaling (Spana and Doe, 1996; Zhong *et al.*, 1996; Sestan *et al.*, 1999; Wakamatsu *et al.*, 1999; Berezovska *et al.*, 2000). There was a three- to fourfold lower level of Numb mRNA in sNCSCs compared with mNCSCs (Fig. 3). Taken together, these data indicate that there is a 24- to 32-fold increase in the relative ratio of Notch and Numb expression in NCSCs between E10.5 and E14.5.

Differences in Notch1 and Numb Expression Contribute Causatively to the Different Delta-Fc Sensitivities of sNCSCs and mNCSCs

We next sought to determine whether the correlation between the increased sNCSCs sensitivity to Delta-Fc, and their increased expression of Notch1 and decreased expression of Numb, reflected a causal relationship. To do this, we first asked whether increasing Notch1 levels in mNCSCs would make their Delta-Fc responsiveness more similar to that of sNCSCs. To this end, we overexpressed full-length Notch1 tagged with the RFP protein in freshly isolated mNCSCs. We then compared the Delta-Fc responsiveness of these cells to that of freshly isolated sNCSCs expressing RFP alone. Notch 1 overexpression in mNCSCs led to both decreased neuronal differentiation and increased production of glial-only clones, at a concentration of Delta-Fc that yielded a half-maximal response in control cells (Table 2A, "1/2 Max Delta," compare mNCSCs-RFP with mNCSCs-Notch). More importantly, in both assays, the response of mNCSCs overexpressing Notch1 was statistically indistinguishable from that of sNCSCs expressing RFP (Table 2A). Similar results were obtained at saturating concentrations of Delta as well (Table 2A), except that in this condition, no neurons were generated from either sNCSCs or mNCSCs expressing Notch1. Thus, the sensitivity to Delta-Fc of mNCSCs overexpressing Notch1 is similar to that of unmodified sNCSCs.

To ensure that the effects of Notch1 overexpression reflected its function as a receptor for Delta-Fc, we overexpressed the constitutively active intracellular domain (ICD) of Notch in mNCSCs. mNCSCs expressing Notch-ICD, and cultured in the absence of Delta-Fc, generated a similar percentage of glial-only clones (86.2 ± 4.1) as did sNCSCs expressing RFP alone and cultured in Delta-Fc (82.2 ± 9.8;

TABLE 2

The Ratio of Numb and Notch within NCSCs Plays a Causative Role in Determining Instructive Factor Sensitivity

A.

Condition	Glia only	Neuron-containing	Glia-containing
5 nM-Delta			
mNCSCs-RFP	68.6 ± 4.8	4.8 ± 2.4	97.2 ± 0.4
mNCSCs-Notch	86.0 ± 4.4a	0	95.3 ± 1.7
sNCSCs-RFP	88.9 ± 2.4	0	95.4 ± 2.4
½ Max Delta (2.3 nM)			
mNCSCs-RFP	32.8 ± 8.2	18.7 ± 6.4	92.2 ± 6.4
mNCSCs-Notch	60.1 ± 3.7a	1.6 ± 2.9a	93.6 ± 5.7
sNCSCs-RFP	50.2 ± 6.7	2.8 ± 4.8	87.4 ± 8.3

B.

Condition	Glia only	Neuron-containing	Glia-containing
mNCSCs-ICD-GFP	86.2 ± 4.1	0	98.0 ± 3.4
mNCSCs-GFP + Delta	69.4 ± 3.1b	1.0 ± 1.8	87.5 ± 21.6
sNCSCs-RFP + Delta	82.2 ± 9.8	0	92.7 ± 6.0
mNCSCs	0	72.9 ± 11.9c	68.8 ± 8.6a

C.

Condition	Neuron only	Glia only	Neuron-containing	Glial-containing
No Addition				
sNCSCs	0	0	62.3 ± 13.2	73.5 ± 11.2
sNCSCs-GFP	1.2 ± 2.1	0	53.0 ± 7.5a	68.6 ± 9.3
sNCSCs-Numb2-GFP	47.8 ± 10.6c	0	81.2 ± 9.7	27.2 ± 5.0c
mNCSCs	0	0	71.8 ± 7.4	72.0 ± 4.9
2.3 nM Delta-Fc				
sNCSCs	0	86.1 ± 4.1	0	94.7 ± 1.0
sNCSCs-GFP	0	83.5 ± 9.1	0	90.5 ± 5.4
sNCSCs-Numb2-GFP	0	59.6 ± 10.3b	28.7 ± 11.4c	87.6 ± 2.1
mNCSCs	0	47.9 ± 10.4	36.4 ± 6.9c	89.9 ± 8.2

Note. mNCSCs expressing full-length Notch1 or Notch ICD and sNCSCs engineered to express Numb2 were cultured at clonal density for 14 in Morrison medium or standard medium supplemented with Fc or Delta-Fc. Presented in all three panels is the mean ± standard deviations for at least three experiments originating from at least three independent stem cell isolations and overexpression paradigms. (A) Notch overexpression in mNCSCs results in significantly more glial-only and glial-containing clones compared with mNCSCs expressing RFP only. 1/2 Max Delta refers to the EC_{50} of Delta-Fc (23 μl stock or 2.3 nM) on freshly isolated mNCSCs. (B) Expression of the intracellular domain of Notch1 results in a similar bias toward gliogenesis at the expense of neurogenesis in mNCSCs. (C) Overexpression of Numb2 in sNCSCs results in a decrease in Delta effectiveness for directing glia-only and glial-containing clones. In all three panels, statistics within columns were determined by a paired two-tailed Student's *t*-test: a = $P \leq 0.05$, b = $P \leq 0.025$; c = $P \leq 0.01$.

Table 2B). The percentage of glial-only clones generated by Notch-ICD-expressing mNCSCs was also similar to that of mNCSCs expressing full-length Notch1 but cultured in saturating Delta-Fc (86 ± 4.4%; Table 2A, "Saturation-Delta, 5 nM Delta" mNCSCs-Notch). In all of these conditions, the percentage of glial-only clones was significantly higher than that generated by control GFP-expressing mNCSCs treated with Delta-Fc (69.4 ± 3.1%, $P \leq 0.015$; Table 2B, mNCSCs-GFP + Delta). Thus, either increasing the level of full-length Notch1 in mNCSCs and culturing them in Delta-Fc, or expressing a constitutively active form of Notch and culturing them in the absence of Delta-Fc, caused mNCSCs to differentiate in a manner quantitatively similar to that of sNCSCs cultured in Delta-Fc.

In order to determine, similarly, whether the increased Delta sensitivity of sNCSCs might also reflect their lower level of Numb expression, we overexpressed the Numb2 isoform (Verdi *et al.*, 1999) in sNCSCs. This isoform, which promotes neurogenesis in mNCSCs (Verdi *et al.*, 1999), also shows the strongest regulatory effect on Notch signaling in notch-specific transcriptional assays (C.J.K. and J.M.V., manuscript in preparation). sNCSCs overexpressing Numb2-GFP generated a significantly higher percentage of both neuron-only clones and neuron-containing clones, compared with control sNCSCs expressing GFP (Table 2C, No Addition). We next challenged sNCSCs overexpressing Numb2 with 2.3 nM Delta-Fc, a subsaturating concentration of the ligand (Fig. 1). Numb2-overexpressing sNCSCs

showed a marked decrease in the percentage of glial-only clones elicited by Delta-Fc treatment (59.6 ± 10.3%), compared with control GFP-expressing sNCSCs (83.5 ± 9.1%; Table 2C). This decrease in gliogenicity was also associated with an increased percentage of neuron-containing colonies (from 0% in sNCSCs-GFP to 28.7 ± 11% in sNCSCs-Numb2-GFP). The percentage of neuron-containing clones generated by Numb2-sNCSCs in 2.3 nM Delta was also statistically indistinguishable from that produced by unmodified mNCSCs (36.4 ± 6.9%) in this same condition (Table 2C, 2.3 nM Delta-Fc). Thus, increasing Numb2 expression in sNCSCs renders their Delta-Fc sensitivity more similar to that of mNCSCs. Conversely, increasing Notch1 in mNCSCs renders their Delta-Fc sensitivity more similar to that of sNCSCs (Table 2A). Taken together, these data suggest that both the increased level of endogenous Notch1 expression and the decreased level of Numb expression in sNCSCs may explain their greater sensitivity to Delta-Fc in comparison to mNCSCs.

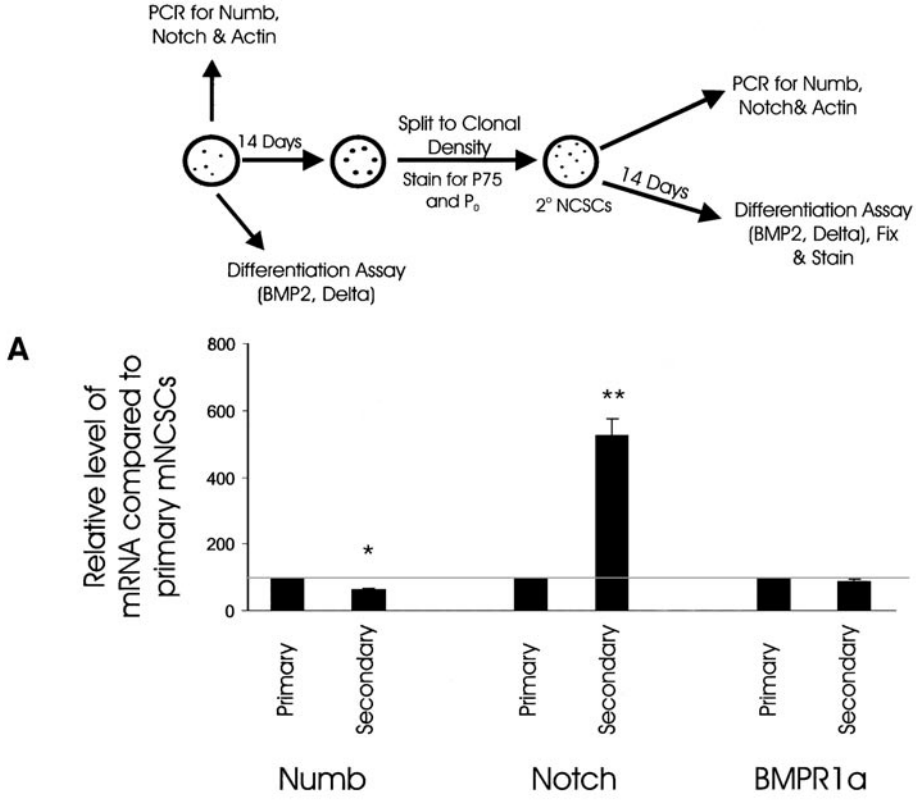
Developmental Shifts in Notch and Numb Expression and Delta and BMP2 Sensitivity Occur Spontaneously in mNCSC Cultured in Vitro

We wished to understand how the time-dependent changes in BMP2 and Delta sensitivity that occur in NCSCs *in vivo* are controlled. Do they occur cell-autonomously, or are they promoted by cell-extrinsic factors? As a first step, we asked whether mNCSCs acquire an increased Delta-Fc sensitivity when cultured at clonal density in the absence of other cell types. To this end, we recloned undifferentiated $p75^+$, P_0^- cells from 14-day mNCSC primary colonies (see Materials and Methods), and compared their differentiative behavior with that of mNCSCs freshly isolated from E10.5 neural tube explants. Such "secondary" mNCSCs were significantly more sensitive to the gliogenic action of Delta-Fc (78.7 ± 3.6% glial-only clones) than were freshly isolated mNCSCs (69.7 ± 4.4% glial-only clones; $P \leq 0.05$) (Fig. 4B). Consistent with this increased functional sensitivity to Delta-Fc, secondary mNCSCs reisolated from 14-day mNCSC colonies expressed fivefold more Notch mRNA, and 0.66-fold less Numb mRNA, than primary mNCSCs (Fig. 4A). Secondary mNCSCs isolated from 14-day clones also showed reduced neurogenic differentiation in response to BMP2 (45.1 ± 3.3% neuron-only clones in secondary mNCSC cultures vs. 67.6 ± 3.9% in primary mNCSC cultures; $P \leq 0.05$) (Fig. 4B). These data indicate that mNCSCs acquire increased Delta-Fc sensitivity, and associated changes in Notch1 and Numb expression, when cultured at clonal density. They also acquire decreased BMP2 sensitivity, as previously demonstrated for NCSCs *in vivo* (White *et al.*, 2001).

We next asked when such changes in responsiveness to instructive signals begin to emerge during the growth of primary mNCSCs colonies. To do this, primary mNCSC colonies at successive days in culture were trypsinized and replated at clonal density, and secondary mNCSCs were identified by live p75 and P_0 immunostaining and morphology. To ensure as pure a population as possible, all non-$p75^+p_0^-$ cells were physically removed from the dish (see Materials and Methods). These reisolated secondary mNCSCs ($p75^+$, P_0^- cells) were then tested by exposure to Delta-Fc (at a concentration half-maximal for freshly isolated mNCSCs; Fig. 1) or to 1 nM BMP2. Beginning on day 8, there was a significant and progressive increase in Delta-Fc responsiveness (Fig. 5A, black bars), while BMP2 responsiveness (Fig. 5A, red bars) began to decline between days 6 and 8. There was also a progressive but slight decrease in the proportion of these cells that generated neuron-containing colonies when cultured for 14 days in the absence of added factors (data not shown). Thus, the secondary mNCSCs became less neurogenic whether assayed in standard medium or in the presence of BMP2.

Does the increased gliogenicity of $p75^+P_0^-$ cells reisolated from progressively older primary mNCSC colonies reflect a change in the Delta-Fc sensitivity of multipotential cells, or rather an increased percentage of glial-restricted progenitors among the $p75^+P_0^-$ population? We calculated whether the increase in the proportion of glial-only colonies generated in Delta-Fc by such cells taken at day 10 vs. day 0, could be accounted for by the decrease in the percentage of cells with neurogenic capacity in the $p75^+P_0^-$ population, as determined by BMP2 challenge. There was a modest 1.2-fold reduction in the percentage of $p75^+P_0^-$ cells with neurogenic capacity at day 10 compared with day 0 (70 vs. 85% neuron-containing clones in BMP2, respectively). A similar fold-reduction (1.3) in the proportion of $p75^+P_0^-$ cells with neurogenic capacity was measured when the cells were allowed simply to spontaneously differentiate to neurons in the absence of exogenous BMP2, over a 14-day period (data not shown). By contrast, there was a 2.4-fold increase in the percentage of those cells that generated glial-only colonies in Delta-Fc, between days 0 (35 ± 3%) and 10 (84 ± 9%). These data suggest that the increased glial differentiation elicited by Delta-Fc from $p75^+P_0^-$ cells reisolated from day 10 colonies cannot be accounted for by a proportional increase in the percentage of glial-restricted (or nonneurogenic) progenitors. Rather, multipotent (neurogenic) mNCSCs appear to become more sensitive to Delta-Fc over time *in vitro*.

We next asked whether the onset of the change in Delta-Fc-responsiveness by secondary mNCSCs was also correlated with a change in the expression levels of Notch1 and Numb mRNAs. Secondary mNCSCs were isolated matching the time points used in the differentiation assays above (Fig. 5B). As before, after immunolabeling, the remaining $p75^-$ or P_0^+ cells were discarded from the culture with a tungsten needle and the remaining $p75^+P_0^-$ secondary mNCSCs were then pooled and lysed for RNA extraction. There were no detectable changes in the levels of Numb or Notch1 mRNA expression for the first 72 h in culture (secondary clones originating from primary clones at days 1, 2, and 3; data not shown). Beginning at day 4, a decrease in Numb mRNA expression and a corresponding



B

Ligand	Neuron Only	Glia Only	Neuron Containing	Glia Containing
No Addition				
Primary	1.59 ± 0.33	1.61 ± 1.01	67.17 ± 4.37	71.88 ± 4.84
Secondary	0.80 ± 1.37	3.14 ± 1.45a	62.10 ± 4.46	78.14 ± 2.24
Delta				
Primary	0	69.67 ± 4.44	3.55 ± 2.35	94.82 ± 1.93
Secondary	0	78.68 ± 3.62a	0	94.55 ± 8.27
BMP2				
Primary	67.59 ± 3.92	0	80.08 ± 3.59	20.15 ± 4.76
Secondary	45.10 ± 3.33a	0	80.98 ± 4.66	29.73 ± 8.79

FIG. 4. The shift from neurogenic to gliogenic bias is reconstituted *in vitro* as mNCSCS develop. Secondary NCSCs were generated from mNCSCs cultured for 14 days in standard Morrison Media (see Materials and Methods). These secondary stem cells were either prepared for semiquantitative analysis or subjected to clonogenic differentiation assays in Morrison medium supplemented with no addition, 1 nM BMP2, or 50 μl/ml Delta (~5 nM). (A) Presented is the mean ± standard deviation of three sqPCR experiments performed in triplicate. Note that the increase in Delta sensitivity and loss of neurogenic potential observed in (A) is correlated with an increase in expression of Notch1 mRNA in secondary stem cells and a decrease in Numb mRNA. *, $P \leq 0.05$; **, $P \leq 0.01$. A value of 100% (solid black line) represents no change between the two populations. (B) Presented is the mean ± standard deviation of three 14-day clonogenic assays. Note the increased Delta-Fc sensitivity as measured by an enhanced production of glial only clones when secondary stem cells were treated with saturating concentrations of Delta-Fc. Also note the reduction in the effectiveness of BMP2 to induce neuron-only clones from secondary stem cells. Statistics within columns were determined by a paired two-tailed Student's *t*-test; a = $P \leq 0.05$; b = $P \leq 0.025$.

increase in Notch1 mRNA expression were observed (Fig. 5B). These differences in Notch and Numb expression between secondary and primary stem cells became significant and accelerated after day 6. Thus, the changes in Notch1 and Numb mRNA expression occur on approximately the same schedule as the functional changes in Delta-Fc responsiveness in cultured mNCSCs. This temporal correlation is consistent with a causative role for these molecular changes, as suggested by the retroviral misexpression experiments.

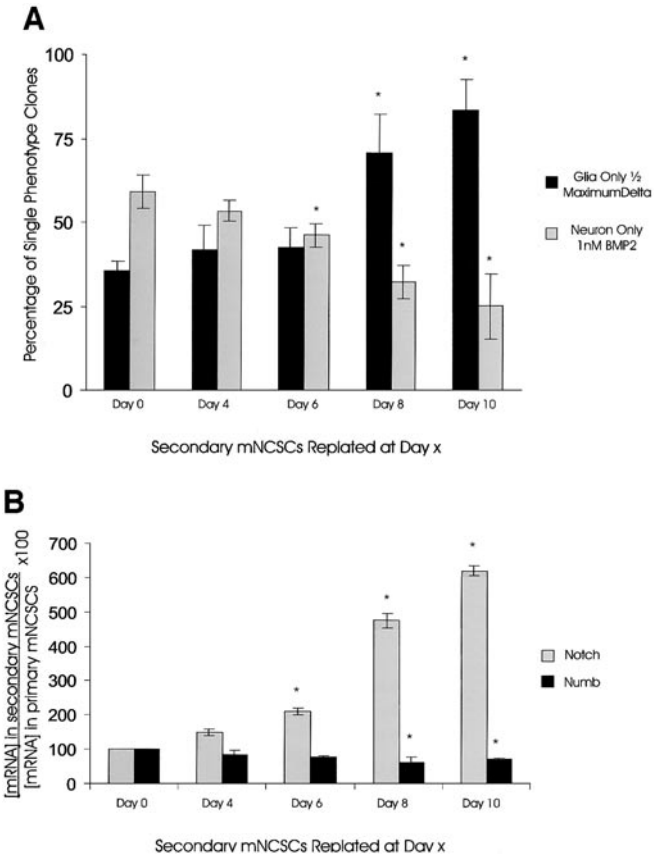


FIG. 5. Changes in BMP and Delta responsiveness during continued mNCSC self-renewal correlate with changes in Notch1 and Numb mRNA levels. Secondary NCSCs were harvested from differentiating clones at the days indicated. These secondary NCSCs were subjected to either clonogenic differentiation assays or prepared for sqPCR analyses. (A) Presented are the means ± standard deviation from three experiments originating from three independent mNCSC isolations. Black bars indicate the percentage of glial-only clones in the presence of EC_{50} Delta-Fc. Red bars indicate the percentage of neuron-only clones in the presence of 1 nM BMP2. Note the gradual decrease in BMP2 effectiveness to induce Neuron only clones and the simultaneous increase in the ability of Delta-Fc to produce glial-only clones commencing at day 8. *, $P \leq 0.05$. (B) Presented is mean ± standard deviations of three of sqPCR experiments performed in triplicate. Note that the increase in gliogenicity and decrease in neurogenicity commencing at day 6 correlates with a gradual increase in Notch mRNA expression and decrease in Numb mRNA expression. *, $P \leq 0.05$.

Changes in Delta-Fc Sensitivity Depend on Local Cell–Cell Interactions within Developing mNCSC Clones

The fact that changes in Delta-Fc sensitivity can occur in mNCSCs cultured at clonal density in the absence of other cell types raised the question of whether these changes occur by a cell-autonomous mechanism, or rather depend on local cell–cell interactions within the developing colonies. To address this question, we allowed mNCSCs to grow for 3 days (a time point at which no overt differentiation of other cell types was detected by immunohistochemical staining) and then split the forming clones to clonal density and allowed the resulting stem cells ($p75^+P_0^-$) to grow for an additional 3 days, after which we repeated this process an additional four times. This manipulation prevented the cells from reaching the high intraclonal densities that are achieved when primary colonies are allowed to grow for the same total period of time (15 days). At the end of the incubation (15 days in culture; 4 sequential replatings), we once again split the colonies to clonal density, identified $p75^+P_0^-$ cells, and examined their responsiveness to BMP2 and Delta-Fc in clonogenic assays. We also examined the levels of Numb and Notch1 mRNAs in such cells.

The successive replating of mNCSCs every 3 days abrogated the changes in both responsiveness to instructive signals and the expression of Notch1 and Numb mRNAs, that occurred when these cells were allowed to grow for the same period of time in primary colonies. For example, continuously replated or freshly isolated mNCSCs showed a similar neuronal differentiation response to BMP2 (69.1 ± 3.4 vs. 66.1 ± 9.5% neuron-only colonies), while that of mNCSCs reisolated after 15 days of growth in primary colonies was lower (41.5 ± 6.7% neuron-only colonies; Table 3). Similarly, continuously replated or freshly isolated mNCSCs generated a similar percentage of glial-only colonies in Delta-Fc (66.2 ± 5.1 vs. 69.7 ± 9.8%, respectively), and this percentage was significantly lower than the percentage generated from mNCSCs reisolated from 15-day primary colonies without continuous replating (5 nm Delta-Fc 77.6 ± 4.8%; Table 3). Finally, consistent with these functional data, there was no change in the relative levels of Numb or Notch1 mRNA expression after each successive replating (Fig. 6), even after 15 days of total time in culture. These data suggest that the changes in factor responsiveness and Notch1 and Numb expression that occur in cultured mNCSCs result from cell–cell interactions within developing colonies, rather than through a cell-autonomous mechanism, because they are abrogated by continuous replating which maintains a low cell density.

Delta-Fc Induces Up-Regulation of Notch1 and Down-Regulation of Numb mRNAs in Freshly Plated mNCSCs

The time at which changes in Delta-Fc responsiveness and Notch1 and Numb mRNA expression became significant (day 6) correlated with the time at which overt neuronal differentiation begins to occur in the primary colonies (data not shown). This suggested that developing neurons might signal to uncommitted stem cells in the colonies to increase their expression of Notch. In invertebrate systems, Notch ligand-mediated signaling from neuronal precursors to nonneuronal cells causes increased expression of Notch

TABLE 3

mNCSCs Expanded in the Absence of Differentiating Cells Do Not Show a Change in Instructive Factor Responsiveness

	Neuron only	Glia only	Neuron-containing	Glia-containing
1 nM BMP2				
Primary E10.5 mNCSCs	66.1 ± 9.5b	0	77.6 ± 5.5a	89.3 ± 4.0
Continuously replated mNCSCs	69.1 ± 3.4b	0	83.7 ± 3.0a	90.8 ± 5.9
mNCSCs from 15-day colonies	41.5 ± 6.7	0	62.6 ± 4.4	90.6 ± 1.04
5 nM Delta				
Primary e10 mNCSCs	0	66.2 ± 5.1a	2.1 ± 2.0	89.3 ± 4.0
Continuously replated mNCSCs	0	69.7 ± 9.8a	5.7 ± 9.8	90.8 ± 5.9
mNCSCs from 15-day colonies	0	77.6 ± 4.8	1.6 ± 1.4	90.6 ± 1.04

Note. mNCSCs were grown at clonal density for 72 h prior to replating the forming clones at clonal density. mNCSCs ($p75^+P_0^-$) cells were identified and allowed to grow an additional 72 prior to repeating the process three additional times (15 days in culture total). The resulting mNCSCs at day 15 were subjected to standard clonogenic assays. Presented are the means ± standard deviation for three experiments originating from three distinct initial mNCSCs isolations. Note that there was no difference in the ability of primary E10.5 mNCSCs and NCSCs grown in the absence of any other cell types to respond to BMP2 or Delta. This is in stark contrast to differentiated clones resulting from NCSCs identified from 15-day-old differentiated mNCSC cultures. Statistics within columns were determined by a paired two-tailed Student's *t*-test: a = $P \leq 0.05$, b = $P \leq 0.025$.

in the responding cells (Wilkinson *et al.*, 1994). We therefore asked whether Delta-Fc similarly positively regulates Notch or negatively regulates Numb expression in this system. To this end, we treated cultures of primary mNCSCs with Delta-Fc and used sqPCR to examine the levels of both Notch and Numb mRNA following this treatment (Fig. 7). Strikingly, the levels of Notch1 mRNA doubled within the first 24 h of Delta-Fc exposure. Moreover, extending the time of Delta exposure to 48 h resulted in a fourfold increase in Notch message compared with freshly isolated mNCSCs cultured in standard medium. The levels of Numb mRNA were unchanged after 24 h of Delta treatment. However, a small but statistical significant

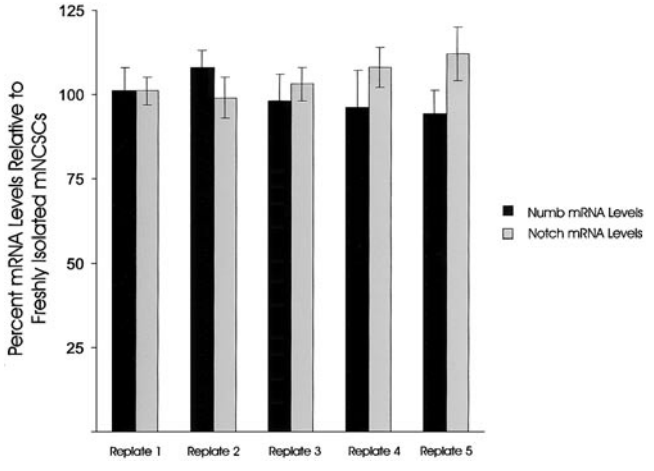


FIG. 6. Cell–cell interactions within developing NCSC clones regulate differentiation signal sensitivity. mNCSCs were plated at clonal density and cultured for 3 days prior to splitting the resulting clones back to clonal density and identifying putative NCSCs. After each successive replating, $p75^+P_0^-$ cells were subjected to semiquantitative RT-PCR to determine the endogenous levels of Numb and Notch. Presented is the means ± standard deviations for three experiments performed in triplicate originating from three independent isolations. Note there is no difference in the levels of Numb and Notch when NCSCs self renew in the absence of differentiated progeny. This is consistent with the observations that no change in the Delta and BMP sensitivity occurs when NCSCs are grown in the absence of any differentiated progeny (see Table 3).

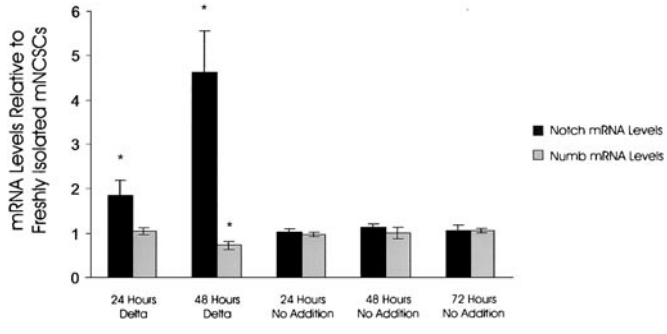


FIG. 7. Delta regulates both the induction of Notch and depression of Numb mRNA in mNCSCs. Freshly isolated mNCSCs were plated at clonal density in the presence or absence of 5 nM Delta-Fc for 24 and 48 h. The regulation of Notch and Numb mRNA was assessed by semiquantitative RT-PCR. Graphed is the percentage of Notch1 or Numb mRNA relative to actin mRNA performed in separate reactions. Presented in the mean ± standard deviations for three experiments performed in duplicate originating from three independent isolations. Note the levels of Notch increase while the levels of Numb decrease. Furthermore, the increase in Notch mRNA levels is significant ($P \leq 0.05$) after 24 h of Delta exposure ($P \leq 0.01$ at 48 h). Numb differences become significant after 48 h of Delta exposure ($P \leq 0.05$).

decrease in Numb mRNA was observed after 48 h of Delta treatment. These data indicate that the changes in Notch1 and Numb expression that occur spontaneously in developing mNCSC colonies can be mimicked by treating individual cells with Delta-Fc.

DISCUSSION

In this study, we have asked how NSCs become progressively more gliogenic and less neurogenic with development. Our results suggest that this gradual transition reflects changes in the probability of differentiation to neurons vs. glia by individual stem cells, in response to instructive signals such as BMP2 or Delta-Fc, rather than an asynchronous but all-or-none switch from generating neurons to generating glia. Thus, multipotential stem cells may undergo progressive changes in their differentiation bias, while retaining their qualitative properties of self-renewal and multipotency. The change in the propensity for neuronal vs. glial differentiation is manifested as both a decrease in sensitivity to the instructive neurogenic signal BMP2 (as shown previously; White *et al.*, 2001), as well as an increase in sensitivity to the anti-neurogenic and gliogenic signal Delta-Fc. The mechanism of this change appears to involve, at least in part, an increase in the ratio of expression of Notch1 to Numb mRNAs. These changes can occur in NCSCs cultured at clonal density in the absence of other cell types, and may be dependent on local cell–cell interactions within developing colonies that are mediated by Delta-Notch signaling itself. While it has previously been shown in invertebrate and mammalian tumor systems that such signaling can result in increased Notch expression in responding cells (Wilkinson *et al.*, 1994; Girard *et al.*, 1996), our data are the first to show that such increased expression can cause an increased functional sensitivity to Delta. In this way, Delta-to-Notch signaling between neighboring cells, coupled with positive feedback control of Notch expression within "receiving" cells, gradually causes heterogeneity in the sensitivity of different progenitor cells to Notch signaling, even before they have committed to specific fates.

Graded vs All-or-None Effects of Notch Signaling

Previously, it was shown that saturating concentrations of Delta-Fc lead to a rapid and complete loss of neurogenic capacity in sNCSCs (Morrison *et al.*, 2000b). In the present studies, however, we observe a progressive decline in the neurogenicity of mNCSCs that we attribute to Delta/Notch-mediated signaling between neighboring cells. How can Notch signaling cause such a gradual effect, if it exerts an all-or-none action to extinguish neurogenic capacity in NCSCs? One possibility is that the effect is not, in actuality, graded at the level of individual cells, but rather reflects an increasing proportion, at the population level, of cells that have irreversibly lost neurogenic potential as a consequence of the all-or-none effect of Notch to extinguish this capacity. We think this is unlikely, because our calculations suggest that the increased gliogenicity of cultured mNCSCs over time cannot be accounted for, quantitatively, by the slight decrease in the proportion of cells with neurogenic capacity during the same period.

Two factors, instead, appear to contribute to the ability of Notch signaling to gradually reduce the neurogenicity of developing mNCSCs. The first is that the ability of Delta-Fc to completely extinguish neurogenic capacity is much more pronounced on sNCSCs (Morrison *et al.*, 2000a) than it is on mNCSCs (Fig. 2), reflecting the ca. 30-fold higher ratio of Notch1 to Numb mRNA expression in the older cells. Thus, Delta does not have the ability to as efficiently cause an all-or-none loss of neurogenic potential in mNCSCs, due to the reduced sensitivity of these cells to the Notch ligand. The second is that the levels of Delta to which developing mNCSCs are exposed on neighboring cells, both *in vivo* and *in vitro*, are likely much lower than the high levels of exogenous Delta-Fc necessary to fully suppress neurogenic capacity *in vitro*. Consistent with this, we have shown that, at subsaturating concentrations of Delta-Fc, only a proportion of sNCSCs irreversibly lose neurogenic capacity and the rest retain it (Fig. 2; Morrison *et al.*, 2000b). Thus, both a lower level of endogenous Notch ligand expression and an initially reduced sensitivity of mNCSCs to these ligands may explain the ability of Notch-mediated signaling to gradually increase the Delta-Fc sensitivity of NCSCs without promoting a complete loss of neurogenic capacity. Such a loss does, however, eventually occur as evidenced by the reduced frequency of neurogenic cells in late-gestational sciatic nerve (Morrison *et al.*, 1999).

Mechanisms Underlying the Changes in Sensitivity to Delta-Fc

The difference in neuron vs. nonneuronal differentiation bias between sNCSCs and mNCSCs is correlated with reciprocal changes in Notch1 and Numb mRNA expression levels in these cells. In *Drosophila*, a loss-of-function *Numb* mutation in the sensory organ precursor lineage has the same phenotype (fewer neurons) as a gain-of-function *Notch* mutation, and conversely, a gain-of-function *Numb* mutation within this lineage results in a phenotype (more neurons) indistinguishable from a loss-of-function *Notch* mutation (Lily *et al.*, 1996). These data, along with numb overexpression studies in mammalian and avian systems have suggested that Numb functions to inhibit Notch signaling (Spana and Doe, 1996; Zhong *et al.*, 1996; Wakamatsu *et al.*, 1999; Sestan *et al.*, 1999; Imai *et al.*, 2001). Moreover, it has been shown that Numb physically interacts with the intracellular domain of Notch (Guo *et al.*, 1996; Zhong *et al.*, 1996), specifically the PEST domain of Notch1, thereby inhibiting Notch translocation into the nucleus (Wakamatsu *et al.*, 1999; Berezovska *et al.*, 2000). Our results are consistent with the idea that Numb2 negatively regulates Notch in vertebrate neural progenitors,

as overexpression of Numb2 in NCSCs has the opposite effect of overexpression of constitutively active Notch-ICD.

Our data further suggest that changes in the intrinsic balance of Numb and Notch expression can influence the differentiation bias of neural stem cells. The ratio of Notch1 mRNA to Numb mRNA expression in sNCSCs is more than 30-fold higher than in mNCSCs. Increasing the Numb2 content of sNCSCs decreases their Delta-Fc responsiveness, to a level more similar to that of mNCSCs. Conversely, increasing Notch1 expression in mNCSCs increases their Delta-Fc responsiveness, to a level more similar to that of freshly isolated sNCSCs. These data suggest that the observed changes in the ratio of Notch1 to Numb mRNA expression may contribute causatively to the difference in Delta-Fc sensitivity between mNCSCs and sNCSCs. Nevertheless, we do not feel that the difference between mNCSCs and sNCSCs is due solely to their different relative levels of Notch1 and Numb expression. Other potential differences, such as changes in BMP sensitivity (White *et al.,* 2001), or sensitivity to other as-yet unidentified factors, may also contribute.

Taken together, these data suggest that NCSCs, and perhaps CNS-NSCs, function like dynamic microprocessors: they integrate the influence of multiple, competing extracellular signals and compute an output function, which is to differentiate in one direction or another (or to self-renew) (Anderson, 2001). Our results and those of White *et al.* (2001) suggest that the cell-intrinsic integration function that computes such an output can change as a function of developmental time, leading to differences in the differentiation bias of early vs. late NSCs. The data further indicate that one component of this change in integration function may be changes in the relative expression levels of molecules mediating the influences of such extracellular signals, such as Notch1 and Numb. However, changes in the expression or function of other classes of regulatory proteins, such as transcription factors, are likely to play a role as well.

Control of Changes in Notch and Numb Expression Levels in Developing NCSCs

The developmental difference in the relative levels of Notch1 and Numb mRNA expression between freshly isolated E14.5 sNCSCs and E10.5 mNCSCs can be recapitulated, at least qualitatively, by mNCSCs grown in clonal culture in the absence of other cell types, over a 15-day period. This change does not, however, appear to reflect simply the absolute amount of time in culture, because if the cells are continuously trypsinized and replated at clonal density every 3 days over the same time period, their levels of Notch1 and Numb mRNA remain unchanged, as do their functional responses to Delta-Fc and BMP2. The simplest interpretation of these data is that local cell–cell interactions within developing mNCSC colonies cause the observed changes, and this is consistent with the ability of Delta-Fc to rapidly induce these changes in freshly isolated mNCSCs, within 24–48 h. However, we cannot exclude the possibility that the changes also or instead reflect a cell-autonomous "clock," which can somehow be "reset" by the process of replating the cells.

The fact that recombinant Delta-Fc can recapitulate the changes in Notch1 and Numb mRNA levels that occur in mNCSCs over time in clonal culture suggests that Delta expressed on differentiating neuroblasts within the colonies may cause these changes in gene expression in neighboring undifferentiated stem cells. Consistent with this hypothesis, the onset of significantly increased Notch1 mRNA and decreased Numb mRNA in developing primary mNCSC colonies occurs around day 5–6, the time at which developing neuroblasts (detected by expression of the neurogenic bHLH factor Mash1) first appear in these colonies (data not shown). This idea also fits with the fact that vertebrate proneural genes, like their *Drosophila* counterparts, are positive regulators of Notch ligand expression (Hinz *et al.,* 1994; Kunisch *et al.,* 1994).

What, then, causes the initial appearance of such neuroblasts in the developing mNCSC colonies? We have observed that such colonies express BMP2/4 mRNAs (S. Gerety and D.J.A., and independently by J.M.V. and C.J.K. unpublished observations), and that addition of noggin to mNCSC cultures significantly attenuates neuronal differentiation as well as expression of Mash1 (A. Groves, L. Lo, and D.J.A., unpublished observations). One possibility, therefore, is that autocrine or paracrine signaling by BMPs occurs within NCSC colonies, and that, as the number of cells in the colonies increases, so does the local concentration of BMPs, until it reaches a threshold necessary to trigger expression of Mash1 in at least a subset of the cells. At that point, such cells may begin to express Delta (or other Notch ligands) and signal to neighboring cells that have not yet responded to BMP2, increasing their levels of Notch1 expression and perhaps also decreasing their expression of Numb. Central to this hypothesis is the idea that, at subsaturating concentrations, BMP2 stochastically induces Mash1 expression in a subset of the cells in a colony. Dose-response experiments have provided data consistent with this assumption (Shah and Anderson, 1997). According to this view, then, developmental heterogeneity (or "symmetry-breaking") within NCSC colonies would emerge after cell division, rather than before division, as a consequence of cell-extrinsic factors acting at subthreshold levels. Consistent with this, we have not detected evidence of asymmetrical distribution of Numb in dividing cells within NCSC cultures, even when utilizing anti-Numb antibodies that do not cross-react with the nonasymmetrically distributed Numblike protein (Zhong *et al.,* 1996). We have also observed that BMP2 induces a small increase in the levels of mRNAs encoding several Numb isoforms, as well as promoting a switch to the Numb2 isoform, in NCSC cultures (C.J.K. and J.M.V., unpublished observations).

At apparent odds with this view, Weston and colleagues have observed that Numb is asymmetrically segregated in

some neural crest-derived cells undergoing mitosis *in vivo* in the chick (Wakamatsu *et al.*, 2000). Interestingly, their observations were made on precursors of sensory neurons, in forming dorsal root ganglia. By contrast, NCSCs do not generate sensory neurons in clonal culture (Greenwood *et al.*, 1999; White *et al.*, 2001), but rather generate autonomic neurons. One interesting possibility is that sensory and autonomic ganglia achieve cellular heterogeneity of Numb expression within these ganglia by different mechanisms leading to distinct roles of Numb during neurogenesis: the former by segregating Numb asymmetrically prior to cell division, the latter by stochastic induction of Numb in subsets of cells by subthreshold concentrations of inducing signals such as BMP2. Moreover, the acquired heterogeneity of Numb may serve distinct roles within distinct subset of peripheral ganglia. In support of this view, recent analysis of Numb-null mice reveals potentially different and distinct roles for Numb during neurogenesis (Zilian *et al.*, 2001). Mice lacking Numb have a dramatic blockage in sensory neuron development but show little disruption of autonomic neurogenesis. This observation, taken together with the present results, suggests that numb may regulate the Delta sensitivity of NCSCs but may not be essential for autonomic neurogenesis. Further studies will be necessary to resolve these issues.

Implications for Extended ex Vivo Expansion of NSCs: A Mechanistic Explanation for the Retention of Multipotency

Neural stem cell replacement offers potentially powerful new therapies for treating a variety of intractable neurological disorders, including neurodegenerative diseases, neurotrauma, and other neurological dysfunctions (reviewed in Horner and Gage, 2000; Svendsen and Smith, 1999). One barrier to developing such therapies is that it is not currently possible to directly isolate sufficient numbers of NSCs from uncultured tissue, especially fetal tissue. Therefore, NSCs will need to be expanded *in vitro* prior to transplantation. Although CNS-NSCs have been shown to qualitatively retain multipotency and self-renewal capacity following extended *ex vivo* expansion, either as neurospheres (Reyonds and Weiss, 1992; Reynolds *et al.*, 1992) or as monolayer cultures (Gage *et al.*, 1995), the extent to which these cells undergo progressive, quantitative decreases in neurogenic capacity such as we have described here for NCSCs has not been investigated in detail. Our data suggest that such investigations are warranted if such CNS-NSCs are to be extensively expanded for therapeutic purposes.

While this is not the first report that maintaining neural stem cells at low density helps to keep them in an undifferentiated state (Pardo and Honegger, 2000; Tsai and McKay, 2000; Temple and Davis, 1994; Tang *et al.*, 2001), the mechanism by which this occurs has not been established. The work presented here begins to provide a mechanistic basis for this phenomenon. Specifically, it involves quantitative changes in the expression levels of Notch signaling components, as a consequence of cell–cell interactions that are mediated, at least in part, by Notch signaling itself. These changes start to occur as cells within the colonies begin to differentiate. Disrupting local cell–cell interactions by dissociating the colonies may serve to abrogate the differentiation process that activates Notch signaling, Notch signaling itself, or both. Further mechanistic insights into this process may suggest alternative means of maintaining neural stem cells in their most primitive state during *ex vivo* expansion for therapeutic purposes.

ACKNOWLEDGMENTS

We would like to thank Dr. James MacDonald for their insightful and critical reading of earlier versions of this manuscript. We would also like to thank Kevin Partridge for computer assistance and figure preparation, and Martin White of the John P. Robarts Cell Sorting Facility for his expertise and time. This work was supported in part by grants from the Heart and Stroke Foundation of Ontario (NA4497), the Medical Research Council of Canada (MT-1313), and the Canadian Institute of Health Research (MOP-44035). S.J.M. is a Howard Hughes Associate Investigator, D.J.A. is a Howard Hughes Principal Investigator, and J.M.V. is a scholar of the MRC (now the CIHR) and an EJLB Foundation Scholar.

REFERENCES

Anderson, D. J. (2001). Stem cells and pattern formation in the nervous system: The possible versus the actual. *Neuron* **30,** 19–35.

Anderson, D. J., Groves, A., Lo, L., Ma, Q., Rao, M., Shah, N. M., and Sommer, L. (1997). Cell lineage determination and the control of neuronal identity in the neural crest. *Cold Spring Harbor Symp. Quant. Biol.* **62,** 493–504.

Berezovska, O., Jack, C., McLean, P., Aster, J. C., Hicks, C., Xia, W., Wolfe, M. S., Kimberly, W. T., Weinmaster, G., Selhoe, D. J., and Hyman, B. T. (2000). Aspartate mutations in presenilin and gamma-secretase inhibitors both impair proteolysis and nuclear translocation with relative preservation of notch1 signaling. *J. Neurochem.* **75,** 583–593.

Bronner-Fraser, M. (1992). Cell lineage segregation in the vertebrate neural crest. In "Determinants of Neuronal Identity" (M. Shankland and E. R. Macagno, Eds.), pp. 359–389. Academic Press, San Diego.

Cepko, C. L., Ryder, E., Austin, C., Golden, J., Fields-Berry, S., and Lin, J. (1998). Lineage analysis using retroviral vectors. *Methods* **14,** 393–406.

Chandler, C. E., Parsons, L. M., Hosang, M., and Shooter, E. M. (1984). A monoclonal antibody modulates the interaction of nerve growth factor with PC12 cells. *J. Biol. Chem.* **259,** 6882–6889.

Gage, F. H. (2000). Mammalian neural stem cells. *Science* **287,** 1433–1438.

Gage, F. H., Ray, J., and Fischer, L. J. (1995). Isolation, characterization and use of stem cells from the CNS. *Annu. Rev. Neurosci.* **18,** 159–192.

Girard, L., Hanna, Z., Beaulieu, N., Hoemann, C. D., Simard, C., Kozak, C. A., and Jolizoeur, P. (1996). Frequent provirus inser-

tional mutagenesis of Notch1 in thymomas of MMTVD/myc transgenic mice suggests a collaboration of c-myc and Notch1 oncogenesis. *Genes Dev.* **10,** 1930–1944.

Greenwood, A. L., Turner, E. E., and Anderson, D. J. (1999). Identification of dividing, determined sensory neuron precursors in the mammalian neural crest. *Development* **126,** 3545–3559.

Guo, M., Jan, L. Y., and Jan, Y. N. (1996). Cortical of daughter cell fates during asymmetric division: Interaction of numb and notch. *Neuron* **17,** 27–41.

Hagedorn, L., Suter, U., and Sommer, L. (1999). P0 and PMP22 mark a multipotential neural crest derived cell type that displays community effects in response to TGF*β* family factors. *Development* **126,** 3781–3794.

Hinz, U., Biebel, B., and Campos-Ortega, J. A. (1994). The basic-helix–loop–helix domain of *Drosophila* lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76,** 77–88.

Horner, P. J., and Gage, F. H. (2000). Regenerating the damaged central nervous system. *Science* **407,** 963–970.

Imai, T., Tokunaga, A., Yoshida, T., Hashimoto, M., Mikoshiba, K., Weinmaster, G., Nakafuku, M., and Okano, H. (2001) The neural RNA-binding protein Mushashi1 translationally regulates mammalian numb gene expression by interacting with its mRNA. *Mol. Cell. Biol.* **21,** 3888–3900.

Kunisch, M., Haenlin, M., and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the *Drosophila* neurogenic gene *Delta* is enhanced by proneural genes. *Proc. Natl. Acad. Sci. USA* **91,** 10139–10143.

Lily, M. G., Jan, L. Y., and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: Interaction of Numb and Notch. *Neuron* **17,** 27–41.

Morgenstern, J. P., and Land, H. (1990). Advanced mammalian gene transfer: High titer retroviral vectors with multiple drug selection markers and complementary helper free packing cell line. *Nucleic Acids Res.* **18,** 3587–3596.

Morrison, S. J., Csete, M., Groves, A. K., Melega, W., Wold, B., and Anderson, D. J. (2000a). Culture in reduced levels of oxygen promotes clonogenic sympathoadrenal differentiation by isolated neural crest stem cells. *J. Neurosci.* **20,** 7370–7376.

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

Morrison, S. J., White, P. M., Zock, C., and Anderson, D. J. (1999). Prospective identification and isolation by flow cytometry, and *in vivo* self-renewal of multipotent mammalian neural crest stem cells. *Cell* **96,** 737–749.

Pardo, B., and Honegger, P. (2000). Differentiation of rat striatal embryonic stem cells *in vitro*: Monolayer culture vs. three-dimensional coculture with differentiated brain cells. *J. Neurosci. Res.* **59,** 504–512.

Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993). Production of high titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA* **90,** 8392–8396.

Reynolds, B. A., and Weiss, S. (1992). Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255,** 1707–1710.

Reynolds, B. A., Tetzlaff, W., and Weiss, S. (1992). A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes. *J. Neurosci.* **12,** 4565–4574.

Sestan, N., Artavanis-Tsakonas, S., and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by Notch signaling. *Science* **286,** 741–746.

Shah, N. M., and Anderson, D. J. (1997). Integration of multiple instructive cues by neural crest stem cells reveals cell-intrinsic biases in relative growth factor responsiveness. *Proc. Natl. Acad. Sci. USA* **94,** 11369–11374.

Shah, N. M., Groves, A. K., and Anderson, D. J. (1996). Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell* **83,** 331–343.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest cells to a glial fate. *Cell* **77,** 349–360.

Spana, E. P., and Doe, C. Q. (1996). Numb antagonizes Notch signaling to specify sibling neuron cell fates. *Neuron* **17,** 21–26.

Stemple, D. L., and Anderson, D. J. (1992). Isolation of a stem cell for neurons and glia from the mammalian neural crest. *Cell* **71,** 973–985.

Svendsen, C. N., and Smith, A. G. (1999). New prospects for human stem-cell therapy in the nervous system. *Trends Neurosci.* **22,** 357–564.

Tang, D. G., Tokumoto, Y. M., Apperly, J. A., Lloyd, A. C., and Raff, M. C. (2001). Lack of replicative senescence in cultured rat oligodendrocyte precursor cells. *Science* **291,** 868–871.

Tsai, R. Y. L., and McKay, R. D. G. (2000). Cell contacts regulate fate choice by cortical stem cells. *J. Neurosci.* **20,** 3725–3725.

Temple, S., and Davis, A. A. (1994). Isolated rat cortical progenitor cells are maintained in division *in vitro* by membrane-associated factors. *Development* **120,** 999–1008.

Uemura, T., Sheperd, S., Ackerman, L., Jan, L. Y., and Yan, Y. N. (1989). Numb, a gene required in determination of cell fate during sensory organ formation in *Drosophila* embryos. *Cell* **58,** 349–360.

Verdi, J. M., and Anderson, D. J. (1994). Neurotrophins regulate sequential changes in neurotrophin receptor expression by sympathetic neuroblasts. *Neuron* **13,** 1359–1372.

Verdi, J. M., Bashirullah, A., Goldhawk, D., Meakin, S. O., and Lipshitz, H. D. (1999). Distinct human NUMB isoforms regulate differentiation versus proliferation in the neuronal lineage. *Proc. Natl. Acad. Sci USA* **96,** 10472–10476.

Verdi, J. M., Groves, A. K., Farinas, I., Jones, K., Marchionni, M., Reichardt, L. F., and Anderson, D. J. (1996a). A reciprocal cell–cell interaction mediated by neurotrophin-3 and neuregulins controls the early survival and development of sympathetic neuroblasts. *Neuron* **16,** 515–527.

Verdi, J. M., Schmandt, R., Bashirullah, A., Jacob, S., Salvino, R., Craig, C., Lipshitz, H. D., and McGlade, J. (1996b). Mammalian NUMB is an evolutionarily conserved signaling adapter protein that specifies neuronal cell fates. *Curr. Biol.* **6,** 1134–1145.

Wakamatsu, Y., Maynard, T. M., Jones, S. U., and Weston, J. A. (1999). NUMB localizes in the basal cortex of Mitotic Avian neuroepithelial cells and modulates neuronal differentiation by binding to Notch-1. *Neuron* **23,** 71–81.

Wakamatsu, Y., Maynard, T. M., and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127,** 2811–2821.

White, P. M., Morrison, S. J., Orimoto, K., Kubu, C. J., Verdi, J. M., and Anderson, D. J. (2001) Direct *in vivo* transplantation of isolated neural crest stem cells reveals cell-intrinsic, develop-

mental changes in sensitivity to instructive signals. *Neuron* **29,** 57–71.

White, P. M., and Anderson, D. J. (1999). *In vivo* transplantation of mammalian neural crest cells into chick hosts reveals a new autonomic sublineage restriction. *Development* **126,** 4351–4363.

Wilkinson, H. A., Fitzgerald, K., and Greenwald, I. (1994). Reciprocal changes in expression of the receptor lin-12 and its ligand lag-2 prior to commitment in a *C. elegans* cell fate decision. *Cell* **79,** 1187–1198.

Zhong, W., Feder, J. N., Jiang, M-M., Jan, L. Y., and Jan, Y. N. (1996). Asymmetric localization of a mammalian numb homologue during mouse cortical neurogenesis. *Neuron* **17,** 43–53.

Zilian, O., Saner, C., Hagedorn, L., Lee, H-Y., Sauberli, E., Suter, U., Sommer, L., and Aguet, M. (2001). Multiple roles of mouse numb in tuning developmental cell fates. *Curr. Biol.* **11,** 494–501.

Received for publication November 29, 2001
Accepted December 19, 2001
Published online March 6, 2002