Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process

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SUMMARY

We examined the role of Notch signaling on the generation of neurons and glia from neural stem cells by using neurospheres that are clonally derived from neural stem cells. Neurospheres prepared from Dll1lacZ/lacZ mutant embryos segregate more neurons at the expense of both oligodendrocytes and astrocytes. This mutant phenotype could be rescued when Dll1lacZ/lacZ spheres were grown and/or differentiated in the presence of conditioned medium from wild-type neurospheres. Temporal modulation of Notch by soluble forms of ligands indicates that Notch signaling acts in two steps. Initially, it inhibits the neuronal fate while promoting the glial cell fate. In a second step, Notch promotes the differentiation of astrocytes, while inhibiting the differentiation of both neurons and oligodendrocytes.

Key words: Neural stem cells, Notch-Delta signaling, Cell fate specification, Neurospheres, Mouse

INTRODUCTION

Multipotent neural stem cells differentiate into neurons and glial cells in a progressive process, generating (first uncommitted, then gradually committed) precursors cells with restricted developmental capacities, which ultimately differentiate into neurons, astrocytes and oligodendrocytes (for a review, see Anderson, 2001).

Owing to the absence of specific markers, neural stem cells cannot be identified prospectively and their developmental behavior is therefore difficult to address in vivo.

As neurospheres are clonally derived from neural stem cells (Weiss et al., 1996), they provide a good experimental system for studying the mechanisms involved in the proliferation and differentiation of these multipotent cells in development.

The production of neurospheres relies on the selection of neural stem cells from embryonic (or adult) brain through the action of EGF. In the presence of EGF, neural stem cells proliferate and form clonally derived clusters of cells floating in the medium that are referred to as neurospheres (or spheres). Each neurosphere represents the clonal progeny of a neural stem cell; as such, it consists of a heterogeneous population of cells, including the neural stem cells themselves (representing less than 5% of the cells) and their progeny. This progeny consists of uncommitted (early progenitors) as well as committed (late) progenitor cells. These cells remain in an undifferentiated state until they are induced to differentiate by providing a solid support, on which they attach and give rise to neurons, oligodendrocytes and astrocytes in reproducible proportions (Reynolds and Weiss, 1992; Weiss et al., 1996).

The Notch signaling pathway has been shown to define a fundamental cell interaction mechanism that influences cell fate decision by interaction between cellular neighbors. The involvement of Notch signaling in neuronal development has been extensively documented in invertebrates and its action has been shown to be highly pleiotropic and indeed context dependent. During neurogenesis, Notch has been shown to inhibit neuronal differentiation in many organisms in vivo and in vitro (Fortini et al., 1993; Struhl et al., 1993; Nye et al., 1994; Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Henrique et al., 1997). Notch activation has also been shown to suppress oligodendrocyte development from oligodendrocyte precursor cells (OPCs) (Wang et al., 1998). Recently, Notch signaling has been found to trigger the differentiation of several types of glial cells, including radial glia (Gaiano et al., 2000), Schwann cells (Morrison et al., 2000), Müller cells in retina (Furukawa et al., 2000) and astrocytes (Tanigaki et al., 2001; Lütolf et al., 2002).

In the present study, we used neurospheres to examine the
involvement of Notch in the generation, the maintenance and the differentiation of neural stem cells by comparing neurospheres produced from mice embryos deficient for Delta-like gene 1 (Dll1lacZ/lacZ) (Hrabé de Angelis et al., 1997), with wild type neurospheres produced from the littermate controls. We find that the Dll1lacZ/lacZ mutation affects neither the generation nor the maintenance of neural stem cells in vitro. By contrast, the Dll1lacZ/lacZ mutation strongly affects the developmental potential of neurospheres. Neurospheres prepared from Dll1lacZ/lacZ mutant embryos display an increase in the production of neurons at the expense of both oligodendrocytes and astrocytes. This mutant phenotype could be rescued when Dll1lacZ/lacZ spheres differentiated in the presence of wild-type spheres conditioned medium. Temporal modulation of Notch activation by soluble forms of ligands indicates that Notch acts in two steps. Initially, it controls a switch between neuronal fate to glial fate, repressing neuronal fate and promoting glial fate (including both astrocytes and oligodendrocytes). In a second step, Notch affects the differentiation decisions of precursors already committed to a neuronal or a glial lineage; it promotes the differentiation of astrocytes while inhibiting the differentiation of both neurons and oligodendrocytes.

MATERIALS AND METHODS

Production and maintenance of wild-type and mutant Dll1lacZ/lacZ neurospheres

The Dll1lacZ mutant mouse line was kindly provided by Dr A. Gossler. The mutation resulted from the in frame replacement of exon1 by the lacZ gene, as described by Hrabé de Angelis et al. (Hrabé de Angelis et al., 1997). Homozygous Dll1lacZ/lacZ embryos die at embryonic day (E) 11.5, i.e. before gliogenesis begins. The homoygous embryos display a well-defined mutant phenotype with strongly hemorrhagic brain, somites boundaries not clearly defined and a kinked neural tube (Hrabé de Angelis et al., 1997).

Neurospheres have been prepared as described by Tropepe et al. (Tropepe et al., 1999). Telencephalonos of embryos (E10.5), were dissected and mechanically dissociated in the serum-free neurosphere culture medium as described by Vescovi et al. (Vescovi et al., 1993) [DMEM:F12 (1:1) supplemented with 25 µg/ml insulin, 100 µg/ml transferrin, 20 nM progesterone, 60 µM putrescine, 30 nM selenium and 20 ng/ml EGF]. The dissociated cells were plated in 24-well plates (Nunc). After 7 days in vitro (DIV), most of the cells died, although a small percentage of cells proliferated by forming clusters of undifferentiated cells floating in the medium, referred to as the neurospheres (spheres). These primary spheres were spun down (65 g for 5 minutes) and were dissociated mechanically and chemically, making use of a ‘dissociation solution’ (Sigma) and were further expanded by transfer into fresh neurosphere culture medium in which they were cultured for generating secondary spheres. Secondary cultures were transferred into 250 ml flasks (Falcon). Culture flask were coated with poly(2-hydroxy-ethyl-methacrylate) (polyHEMA; Sigma, 1.6 mg/cm²) to prevent cell attachment. Neurospheres could be maintained for long periods of times by successive passages involving dissociation and proliferation, or were frozen in neurosphere culture medium containing 10% DMSO, when necessary. Out of seven Dll1lacZ/lacZ mutant embryos, three lines of spheres could be obtained, two of which have been maintained. We checked that these two lines behaved in the same way and showed similar differentiating potentials (see below). Many of the reported experiments have been carried out in the second line and showed similar results (data not shown).

J1EC and 3T3-conditioned medium

Conditioned medium enriched with a soluble form of human Jagged, was produced from stably transfected NIH-3T3 cells (Sestan et al., 1999). HJaggedEC-3T3 cells, and untransfected NIH-3T3 cells (used as a control) were cultured until confluence, in DMEM containing 10% FBS. When the culture became confluent, the medium was removed and replaced by the neurosphere culture medium for another 3 days. The cells were then harvested by centrifugation and the resulting conditioned supernatant was filtered through a 0.22 µm filter unit millex-GS (Millipore). This conditioned medium (referred to as J1EC) was added undiluted to the Dll1lacZ/lacZ spheres for various time windows during either the proliferation and/or the differentiation phase(s), as described in the experimental diagrams. Conditioned medium from untransfected NIH-3T3 cells (3T3) was used as a control.

Differentiation and analysis of neurospheres

After various times of proliferation, 50-100 neurospheres were plated onto polyornithine (poly-L-ornithine Sigma)-coated (14 mm) coverslips, in a 24 wells plate (Nunc) to differentiate. Differentiation of neurospheres is often described as requiring the withdrawal of EGF, which is replaced by fetal bovine serum (FBS) (Vescovi et al., 1993; Tropepe et al., 1999). In order to avoid the hazardous effect of FBS, while preserving cell survival, we reduced EGF concentration to 2 ng/ml during the differentiation phase, which is sufficient to allow cell survival but minimizes proliferation. Under these conditions, the differentiating spheres remains a dynamic structure where generation of new cells (stem cells and progenitors) is diminished but not totally abolished.

Notch activation experiments

Fully dissociated Dll1lacZ/lacZ spheres were grown in J1EC-conditioned medium, (or in NIH-3T3 control medium) in 50 ml flasks coated with polyHEMA. At various times, as indicated on the diagrams, the growing spheres were collected by centrifugation, washed out in PBS, and the incubation was continued either in 3T3 control medium or in fresh J1EC. After the proliferation phase, neurospheres were harvested and plated on polyornithine-coated coverslips either in J1EC or in the control medium. After various times of differentiation, the samples were processed for immunostaining for identification of the cell types.

Immunostaining

Neurospheres were fixed for 20 minutes in 4% paraformaldehyde in PBS (pH 7.4), washed in PBS and permeabilized 5 minutes with PBS/0.5% Triton X-100 (Sigma). The neurospheres were incubated overnight at 4°C in PBS containing 3% BSA and the appropriate mixture of antibodies. Primary antibodies used were mouse monoclonal anti-MAP2 (2a+2b) (1/400, Sigma) specific for neurons, rabbit polyclonal anti-GFAP (1/600, Dako) for astrocytes, and rabbit polyclonal PDGFRA (1/500, Santa Cruz) and mouse monoclonal anti-O4 (1/20, Boehringer) for oligodendrocytes precursors cells (OPCs) and immature oligodendrocytes, respectively. After washing in PBS, differentiating spheres were incubated for 1 hour with Cy2-, Cy3- and Cy5-conjugated secondary antibodies (1/300 Amersham) or Alexa 488-conjugated antibodies (1/1000, Molecular Probes). Preparations were counterstained with TO-PRO (1/15000, Molecular Probes), mounted in Aquamount (Polyscience) and viewed for triple immunofluorescence using a Zeiss LSM 410 confocal microscope.

Quantitative results

Data are based on three or four independent experiments in which an average of more than 10 spheres were analyzed per treatment, per experiment. Under the conditions used for cell concentration after full dissociation (1.10⁵ cells/ml) and plating (50-100 spheres/coverslip), each neurosphere may be considered as the clonal progeny of a single neural stem cell. For cell type quantitative estimation, neurospheres were chosen of approximately the same size. The confocal plane was...
at the basis of the neurosphere, i.e. where the cells differentiate. The optical slice was ≤2 μm. The results are expressed in percentage of total cell number assessed from TO-PRO staining. The graphed results are shown as means±s.e.m. Group changes were assessed using one-way ANOVA. When statistical differences were obtained between groups at $P < 0.05$, multiple pair-wise comparisons were made using the Turkey-Kramer method. In the text, $P$ indicates statistical significant differences.

RESULTS

The generation and maintenance of neural stem cells are not affected by the Dll1\textsuperscript{lacZ/lacZ} mutation

Neural stem cells can be empirically and functionally identified by their capacity to respond to EGF by proliferating and generating neurospheres. We have prepared neurospheres from embryos homozygous for Dll1\textsuperscript{lacZ/lacZ}. The possibility of obtaining neurospheres from embryonic brains deficient for the Dll1 gene suggests that Dll1 is not essential for the generation of neural stem cells. The decrease in the number of spheres obtained from the mutant brains compared with their littermate wild-type controls could be attributed to the hemorrhagic phenotype of the mutant brains (Hrabé de Angelis et al., 1997).

The early death of Dll1\textsuperscript{lacZ/lacZ} mutant embryos (around day 12) prevented further analysis of the role of Dll1 in the maintenance of neural stem cells in vivo. In vitro, we observed no difference in the maintenance of mutant and wild-type neurospheres. Based on the neurosphere assay, the estimation of neural stem cell percentage from the dissociated neurospheres cellular population was similar in mutant and wild-type neurospheres, and was below 5% of total cell number, showing little variation through successive passages (data not shown). These observations suggest that wild type and Dll1\textsuperscript{lacZ/lacZ} neural stem cells behave approximately the same, undergoing the same number of symmetrical and asymmetrical divisions.

Dll1\textsuperscript{lacZ/lacZ} mutant spheres display an increase in neurons at the expense of glial cells

After verifying that wild-type neurospheres differentiated according to a pattern qualitatively and quantitatively reproducible under the culture conditions employed, we compared the differentiation potential of Dll1\textsuperscript{lacZ/lacZ} and wild-type neurospheres. The general experimental protocol is schematized in Fig. 1. Three-day-old spheres were plated on polyornithine in the presence of 2 ng/ml of EGF, and allowed to differentiate for various times depending on the temporal pattern of expression of the markers employed for immunocytochemistry. The percentage of each of the cell types was determined by triple immunostaining, including a combination of two specific markers (in addition to TO-PRO, a marker for nuclei), and analyzed by confocal microscopy. Cell types were identified using antibodies against MAP2 for neurons, against GFAP for astrocytes, against PDGFR for oligodendrocyte precursor cells (OPCs) (Hutchins, 1995) and against O4 for immature oligodendrocytes.

After 5 hours on polyornithine (Fig. 2A), a few cells in wild-type spheres started expressing MAP2. Low production of neurons (which do not exceed 10% of total cell number), is a characteristic of wild-type neurospheres (Fig. 2B). By contrast, GFAP-positive cells which appear after 48 hours on polyornithine, represent more than 50% of total cell number and indicate that astrocytes are the major cell type generated from wild-type neurospheres (Fig. 2A, part V). Oligodendrocytes are difficult to quantify accurately because of the aspect of the O4 marker (Fig. 2 VII); however, PDGFR-positive cells indicate that OPCs represent about 15-20% of total cell number (Fig. 2B). In keeping with the expression of PDGFR, after 6 days on polyornithine, many cells in wild-type neurospheres continue to express O4, indicative of an oligodendroglial lineage commitment. Note, however, that these O4-positive cells exhibit no processes and appear therefore as morphologically poorly differentiated (Fig. 2A, part VII).

Constrasting with wild-type spheres, Dll1\textsuperscript{lacZ/lacZ} mutant spheres show an increase in MAP2-expressing cells, which represent more than 50% of total cell number, indicating that neurons are the major cell type generated by Dll1\textsuperscript{lacZ/lacZ} mutant spheres. This increase in neurons takes place at the expense of both glial lineages as evidenced by the decrease in GFAP-positive cells (Fig. 2A, parts V, VI) as well as in PDGFR-positive cells (Fig. 2A, parts III, IV), indicating a decrease in astrocytes and OPCs, respectively. Interestingly, the few remaining O4-expressing cells exhibited many processes, suggesting that they were morphologically more differentiated than in the wild-type neurospheres (Fig. 2A, parts VII, VIII). Note that, together, neurons, oligodendrocytes and astrocytes did not amount to 100%, consistent with the observation that many cells in the core of the sphere failed to differentiate.

Fig. 1. Protocol used for analyzing the differentiation potential of neurospheres. Fully dissociated neurospheres were grown in serum-free neurosphere culture medium, containing EGF (20 ng/ml) for various times, generally not exceeding 3 days in order to minimize necrosis, which might affect the core of larger spheres, and the generation of new stem cells, which might be at the origin of a ‘subclone’ whose developmental potential could interfere with interpretation. At $t=0$, spheres (50-100) were deposited on coverslips coated with polyornithine and allowed to differentiate in neurosphere culture medium containing 2 ng/ml EGF, in order to reduce proliferation. After various times of differentiation, spheres were fixed and processed for immunocytochemistry, and analyzed by confocal microscopy (the observation plane being at the basis of the spheres where the cells differentiate).
express any marker, and were likely to correspond to early uncommitted precursors (Fig. 2B). We sought to determine whether the alteration in the differentiation potential of wild-type and Dll1lacZ/lacZ mutant spheres could be accounted for by Notch selectively acting on the different lineages by affecting their survival or proliferation.

In order to examine whether the quantitative variations between wild-type and Dll1lacZ/lacZ mutant phenotypes might be due to selective apoptosis, we searched for dying cells in individual spheres, making use of TUNEL analysis, counterstained with TO-PRO, at various times after the induction of differentiation. Up to 2 days on polyornithine, the number of apoptotic cells remained low, representing less than 5% of total cells. For longer periods of differentiation, it increased (up to 15% after 5 days on polyornithine). No significant difference was found, however, between wild-type and Dll1lacZ/lacZ neurospheres (data not shown).

Pulses of BrdU incorporation, combined to either MAP2 or GFAP, in wild-type versus Dll1 lacZ/lacZ spheres showed no difference between the two lineages, eliminating the possibility that the higher number of neurons seen in Dll1 lacZ/lacZ mutant spheres was the result of an increased lineage specific proliferation event (data not shown).

Dll1 has a gene-dosage effect on the differentiation potential of neural stem cells

Heterozygous Dll1lacZ/+ mouse embryos are phenotypically normal (Hrabe de Angelis et al., 1997; Beckers et al., 1999). After 24 hours on polyornithine, neurospheres heterozygous for Dll1 showed a twofold increase in the number of MAP2-positive cells, but no variation in the number of GFAP-positive cells (data not shown). After 3 days of differentiation (Fig. 3A,B), the increase in neurons was confirmed (from 20.3±4.3% to 27.7±3.1%; P≤0.01) and as in homozygous Dll1lacZlacZ neurospheres, this increase seemed to take place at the expense of

Fig. 2. Compared differentiation potential of wild-type and Dll1lacZ/lacZ mutant neurospheres. (A) Kinetics of differentiation were analyzed for wild-type (I, III, V, VII) and Dll1lacZ (II, IV, VI, VIII) spheres. Three-day-old spheres were fixed after 5 hours (I, II), 10 hours (III, IV), 48 hours (V, VI) and 6 days (VII, VIII) on polyornithine. Markers used for immunostaining were: anti-MAP2 (I-VI) coupled to Cy3, for neurons (red); anti-PDGFR (I-IV) coupled to Alexa 488, for OPCs (green); anti-GFAP (V, VI) coupled to Alexa 488, for astrocytes (green); and anti-O4 coupled to Cy2 (green) (VII, VIII). In all cases, nuclei were visualized by TO-PRO (blue). (B) Approximate quantification of the results. Each of the cell type was quantified at various differentiation times: MAP2-positive cells after 48 hours; GFAP-positive cells after 48 hours; oligodendrocytes were estimated from PDGFR-positive cells after 10 hours on polyornithine. The data were cumulated and expressed as percentages of total cell number estimated from TO-PRO staining. Scale bar: 50 μm.
astrocytes (from 50.3±6.9% to 23.2±4.6%; P≤0.001), even though the increase in neurons does not quantitatively compensate for the decrease in astrocytes. This quantitative variation was accompanied by a clear morphological modification, with neurons exhibiting longer processes, whereas astrocytes appeared with thinner processes and lacking the star-like morphology characteristic of mature astrocytes.

**J1EC rescues the Dll1lacZ/lacZ mutant phenotype with thresholds different for neurons and astrocytes**

It is well established that *Drosophila* Notch is acting through interaction with membrane-bound ligands, including Delta (Vassil et al., 1985) and Serrate (Fleming et al., 1990). However, recent data have shown that under particular circumstances, invertebrate and vertebrate Notch activation can be mediated by apparently soluble forms of ligands that are found in the conditioned medium of ligand expressing cells (Klueg et al., 1998; Qi et al., 1999; Sestan et al., 1999). We found that it was indeed possible to ‘rescue’ the mutant Dll1lacZ/lacZ spheres to differentiate normally in the presence of wild-type conditioned medium (WT CM) (data not shown).

The Dll1-dependent activity present in the WT CM, provided us with a tool for temporally modulating Notch activation, making it possible to analyze the effect of Notch signaling at various steps of the complex differentiation process leading from the neural stem cell to the differentiated cell types. However, repeated experiments showed an inconsistency in the potency of the WT CM to trigger the rescue of the Dll1lacZ/lacZ mutant phenotype. We were thus obliged to resort to a more reliable source of Notch ligand.

We used NIH-3T3 cells transfected with the human jagged extracellular domain (hJagEC-3T3 cells), which was shown to be released in the medium. Conditioned medium from these cells (hereafter referred to as J1EC), has been shown capable of regulating neurite outgrowth and Notch-dependent gene expression (Sestan et al., 1999). Jagged 1 is the vertebrate counterpart of Serrate, the second Notch ligand in *Drosophila*. Jagged and soluble forms thereof, like Delta, have been shown to activate the Notch receptor (Lindsell et al., 1995; Shimizu et al., 2000a; Shimizu et al., 2000b; Solecki et al., 2001; Varnum-Finney et al., 1998).

In order to check whether a soluble form of jagged 1 product could rescue Dll1lacZ/lacZ in the same way as WT CM, 3-day-old Dll1lacZ/lacZ mutant neurospheres were exposed to dilutions (up to 20-fold) of J1EC during the differentiation period and were further analyzed by immunostaining for their differentiation capacities in comparison with mutant spheres treated with

![Fig. 3. Neurospheres differentiate in response to Notch signaling with different sensitivity for neurons and astrocytes.](image)
untransfected NIH-3T3 cell-conditioned medium (referred to as 3T3) used as a control, as described in Fig. 3C. Consistent with the effects observed with WTCM, the results (Fig. 3D,E) showed that addition of J1EC resulted in a decrease in the number of differentiated neurons (a 20-fold dilution was necessary to abolish this effect, data not shown). The decrease in neurons was accompanied by an increase in astrocytes for only higher concentrations of J1EC (Fig. 3D, compare parts II, III and IV). Quantitative data show little variation in the number of neurons and astrocytes in response to decreasing doses of J1EC (for neurons, from 36.1 to 10.8, 10.9 and 10.5 for the three dilutions of J1EC, respectively; for astrocytes, from 12.5 and 12.6 under non rescuing conditions, to 43.2 and 38.5 for higher concentrations of J1EC). These data suggest that for the tested dilutions, the response is all or nothing, with different thresholds for neurons and astrocytes. In addition, few GFAP-positive cells that were generated in response to low concentrations of J1EC (Fig. 3D, part IV), exhibited a poorly differentiated morphology, reminiscent of that observed in Dll1lacZ+ neurospheres (Fig. 3A).

**Notch activation represses both neuronal specification and differentiation**

We investigated further the effect of Notch activation on Dll1lacZ/lacZ mutant spheres by treating them with J1EC or control (3T3) for different lengths of time. The results are shown in Figs 4-6. Exposure of Dll1lacZ/lacZ mutant spheres to J1EC resulted in all cases in a decrease in the production of neurons, in comparison with Dll1lacZ/lacZ spheres treated with 3T3-conditioned medium used as a control. The strongest effect was observed when J1EC was provided during both the proliferation and differentiation phases (Fig. 4A-C, parts I,II; from 47.9±2.0% to 10.3±0.8%; \( P \leq 0.001 \)). These data are consistent with the previous observations indicating that Notch inhibits neurogenesis (Fortini et al., 1993; Struhl et al., 1993; Nye et al., 1994; Artavanis-Tsakonas et al., 1995; Henrique et al., 1997).

A decrease in the proportion of neurons was not only seen when J1EC was provided during the proliferation phase (Fig. 4A-C, parts I, III; from 47.9±2.0 to 18.8±1.8%; \( P \leq 0.001 \)), but also when it was provided transiently during the first 24 hours at the initiation of proliferation and then washed out (Fig. 4A-C, part IV; 12.7±1.7%; \( P \leq 0.001 \)). In fact, an exposure to J1EC for only 5 hours was sufficient to repress neurogenesis significantly (data not shown), whereas a treatment for 3 hours was not enough (see Fig. 6A-C, parts I, III; from 47.8±2.3% to 40.4±2.1%; n.s., \( P > 0.05 \)). These results are consistent with earlier findings in the PNS neural crest stem cells (Morrison et al., 2000) and suggest that a transient activation of Notch is capable of repressing the neurogenic potential of neural stem cells. Moreover, it appears that this inhibition was irreversible, as neurogenesis did not resume after J1EC removal.

We found that treatment of Dll1lacZ/lacZ spheres with J1EC during the differentiation phase also resulted in a decrease in neurons (Fig. 4A-C, part IV; from 47.9±2.0% to 8.0±0.7%; \( P \leq 0.001 \)). This observation suggests that precursors that were allowed to adopt a neuronal fate in response to the Notch inactivity during the proliferation phase, were prevented from further differentiating into MAP2-positive cells upon Notch activation during the differentiation phase.

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**Fig. 4.** Effect of time-dependant activation of Notch in Dll1lacZ/lacZ mutant spheres on neurons and astrocytes. Three-day-old Dll1lacZ/lacZ mutant spheres were incubated in the presence of J1EC for various time intervals, as described in the schematic protocol (A). (B) Immunocytochemical analysis. Spheres were immunostained with antibodies against MAP2 (red), GFAP (green). (C) Quantitative estimation was as described in experimental procedures. Data are representative of three independent experiments. Scale bar: 50 μm. I,II,III and IV indicate the addition of J1EC, as defined in A.
Together, these data suggest that Notch signaling inhibits neuronal differentiation not only by preventing uncommitted precursors to acquire a neuronal fate but also at a later stage, when precursors which were already committed to a neuronal fate, will take on the decision to differentiate into MAP2-positive neurons.

**Notch activation promotes the specification and accelerates the differentiation of astrocytes**

The effects of Notch activation on the production of astrocytes can be inferred from the same experiment (Fig. 4), which shows that the decrease in neurons is generally accompanied by an increase in the number of GFAP-expressing cells. This gain-of-function effect is opposite to the Dll1lacZ/lacZ phenotype, where the inability to activate the receptor (loss of function) resulted in an increase in neurons (compared with wild-type spheres) at the expense of astrocytes. Together, these observations suggest that neurons versus astrocytes define a developmental decision controlled by Notch.

However, we found that a transient exposure to J1EC for only 24 hours, even though it was reproducibly sufficient to induce a decrease in neurons, was not always accompanied by a substantial increase in astrocytes (Fig. 4A-C, part IV). Exposure to J1EC for longer times, reproducibly resulted in an increase of the production of mature astrocytes (Fig. 4A-C, parts I, II; from 11.2±1.1% to 22.2±1.9%; P≤0.001), and to a lesser extent in Fig. 4A-C, part V (from 11.2±1.1% to 17.2±1.0%; P≤0.05).

These experiments were repeated under narrower time intervals on both Dll1lacZ/lacZ mutant and wild-type spheres (Fig. 5). The results showed that an activation time of 24 h after dissociation was sufficient to repress differentiation of neurons (from 35.2±2.2% to 9.96±1.3%; P≤0.001, Fig. 5A-C, parts II,III) but not to trigger GFAP expression (12.3±0.8% and 10.0±0.9%; not significant P>0.05). By contrast, when the activation took place later and for longer time periods (from d-1 to d+2, Fig. 5A-C, part III), GFAP-positive astrocytes increased up to more than 55% of total cell number. These results could be correlated to J1EC dilution experiments (Fig. 3C,D) and showed that the decrease in neurons was not necessarily accompanied by a correlative increase in astrocytes; these two phenomena could be disconnected by the temporal modulation of Notch function (transient activation during the proliferation...
phase), as well as by lowering the concentration of the inducing signal during the differentiation phase. We assume that these ‘disconnecting’ conditions generate non-differentiated cells which appear as immunonegative in the neurospheres and which may account for the strong decrease of the added percentage of neurons and astrocytes under the mutant and rescuing conditions (for example in Fig. 4A-C, from 59.1% in part I versus 20-30% in parts II-V). These cells are likely to undergo cell death by apoptosis as usually described for cells which were misdirected and do not differentiate properly (Lütolf et al., 2002). TUNEL analysis carried out under these experimental conditions showed an increase in TUNEL-positive cells with time (from 5.3% after 24 hours on polyornithine to 13.3% after 5 days of differentiation); however, no difference could be observed between the different experimental conditions, suggesting that apoptosis resulting from the ‘misdirection’ of cell lineage, could not be distinguished from apoptosis normally occurring in neurospheres differentiating in serum-free medium.

The early as well as late activation of Notch in wild-type spheres led to an increase in the number of GFAP-positive cells (Fig. 5A-C, parts IV, V, VI; from 47.5±4.4% to more than 80%) in both cases. In addition, GFAP-positive cells displayed a different morphology, indicative of a more mature state of development (a similar phenotype of wild-type spheres normally requires at least 5 days on polyornithine). These data suggest that the activation of Notch in wild-type spheres not only increases the number of astrocytes but also their rate of production and differentiation. We also noted that this increase in astrocytes was never accompanied by a total suppression of neurons (Fig. 5A-C, parts V, VI).

**Notch activation promotes the production of OPCs and inhibits their subsequent differentiation into mature oligodendrocytes**

Examination of the Dll1lacZ/lacZ neurospheres showed that lack of Dll1 activity resulted in a decrease in the number of PDGFR-expressing cells. Paradoxically, however, the few resultant O4-positive cells exhibited a well differentiated morphology when compared with poorly differentiated wild-type oligodendrocytes (Fig. 2A-C, parts VII, VIII). Together, these observations suggested that Notch signaling regulates the production of oligodendrocytes in two steps: first, by promoting the production of OPCs; and second, by inhibiting the differentiation of O4-expressing cells into more mature oligodendrocytes. This statement predicts that a transient treatment of Dll1lacZ/lacZ neurospheres with J1EC would result in an increase in PDGFR-positive cells and in their increased capacity to differentiate.

![Fig. 6. Effect of time-dependant activation of Notch in Dll1lacZ/lacZ mutant spheres on the production of OPCs (B, I-III) and on oligodendrocytes (B, IV-VI). (A) Experimental protocol: 24 hours (left panel) or three-day-old (right panel) Dll1lacZ/lacZ mutant spheres were incubated in the presence of J1EC for various time intervals. (B) Spheres were immunostained with anti-MAP2 (red) and anti-PDGFR (green) for OPCs production (I-III); anti-O4 (green) for oligodendrocytes production (IV-VI). (C) Quantitative estimations of neurons and OPCs (from I-III) was as described in experimental procedures. Data are representative of three independent experiments. Scale bar: 50 μm.](image-url)
The positive effect of Notch signaling on the production of OPCs was observed when Dll1lacZ/lacZ spheres were transiently exposed to \( J_1^{EC} \) during the proliferation protocol and resulted in a significant increase in PDGFR-positive cells (Fig. 6A-C, parts I, II; from 6.64±1.8\% to 25.3±2.5\%; \( P \leq 0.001 \)). When Dll1lacZ/lacZ spheres were constantly treated with \( J_1^{EC} \) during both proliferation and differentiation (Fig. 6A-C, part V), a large number of O4-expressing cells were observed (even though no precise quantitative estimation could be made), indicating that most of the PDGFR-positive cells turned into O4-positive cells, thereby suggesting that this process is not inhibited by Notch activity. However, these cells appeared as poorly differentiated, with morphology reminiscent of O4-positive cells in wild-type spheres (Fig. 2A-C, part VII). By contrast, when Dll1lacZ/lacZ neurospheres were transiently exposed to \( J_1^{EC} \) (during the proliferation phase), a large number of O4-positive cells were observed, some of which displayed a rather differentiated morphology (compare parts V and VI in Fig. 6A-C). Unfortunately, this assessment cannot be further substantiated by the use of markers for mature oligodendrocytes, such as MAG or MBP, as our cultures die before the oligodendrocytes can reach such a degree of maturation.

**DISCUSSION**

**The generation and maintenance of neural stem cells in vitro are not affected by the Dll1lacZ/lacZ mutation**

Our observations contrast with earlier data showing that the Hes1 and Hes5 mutations were accompanied by a decrease in the number of embryonic neural stem cells, which resulted in the generation of smaller and fewer neurospheres that could not be maintained over long periods of time (Ohtsuka et al., 2001) (L.G., J.B., M.R., M.H. de A., S.A.-T. and E.M., unpublished observations). Furthermore, we could not obtain neurospheres from homozygous Notch1 mutant embryos (L.G., J.B., M.R., M.H. de A., S.A.-T. and E.M., unpublished observations), consistent with the recent finding that neural stem cells were depleted in the embryonic brains of Rbp-Jk–/– and Notch1–/– mice (Hitoshi et al., 2002); based on the analysis of presenin 1 (Psen1+) mutant embryos, these authors found that Notch signaling had a role in the maintenance of neural stem cells in vivo. Owing to the early lethality of Dll1lacZ/lacZ embryos, we could not address the role of Dll1 in the maintenance of neural stem cells in vivo; however, we observed no outstanding difference in behavior between wild-type and Dll1lacZ/lacZ neurospheres in vitro, even though additional investigations are needed to characterize further the early steps of neurosphere formation and the respective contribution of symmetric and asymmetric divisions, for example.

**Neurospheres recapitulate many of the pleiotropic effects of Notch signaling on neurogenesis**

Dll1lacZ/lacZ mutant-derived neurospheres show that Notch signal modulations affect qualitatively and quantitatively the outcome of neural stem cell differentiation. The differentiation phenotype of Dll1lacZ/lacZ mutant spheres compared with that of wild-type spheres, exemplifies many earlier findings of Notch effects on neurogenesis: (1) Notch signaling inhibits neurogenesis (Fortini et al., 1993; Struhl et al., 1993; Nye et al., 1994; Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Henrique et al., 1997); (2) Notch signaling represses oligodendrocyte differentiation from OPCs (Wang et al., 1998), whereas recently it has been found to promote the differentiation of astrocytes (Tanigaki et al., 2001; Lütolf et al., 2002). Although many of these data were obtained from systems comprising essentially one single cell type, neurospheres provide a global insight of Notch function on all three major cell types comprising the CNS, in an integrated system, making possible the analysis of their interactions.

**Neural stem cells differentiate in response to Notch signaling with different sensitivity for neurons and astrocytes**

Gene-dosage effect can be inferred from the phenotype of Dll1lacZ/+ heterozygous neurospheres showing a similar (but weaker) phenotype of differentiation to that of homozygous neurospheres (Fig. 3A,B). We note, however, that the percentage of astrocytes in heterozygous spheres is strongly decreased (from 50.3\% in wild type to 23.2\%), while the increase in neurons is restrained (from 20.3\% in wild type to 27.7\% in Dll1lacZ+). This observation indicates that half a dose of Dll1 in heterozygous spheres is still sufficient to partly repress neurogenesis, although insufficient to promote astrocytic differentiation.

This statement is further corroborated by the dilution experiment (Fig. 3D,E) where all dilutions of \( J_1^{EC} \) result in the decrease in neurons, whereas the exogenous ligand triggers the appearance of GFAP-positive cells only for the highest concentrations. Together these results suggest that the inhibition of neurons is more sensitive to ligand induction than the promotion of astrocytes. The quantitative analysis of the results indicates that the response is ‘all-or-nothing’ with different thresholds for neurons and astrocytes. A molecular support of these observations has been provided with the recent finding that Ngn1, a neural bHLH gene activated downstream of MASH1, was shown to inhibit directly the transcription of the astrocyte marker gene, Gfap, in a mechanism independent of its effect to promote neuronal differentiation (Sun et al., 2001). It is thus conceivable that a decrease in ligand concentration would induce a modulation in Ngn1 production and would subsequently result in distinct and separate effects of Ngn1 in activating neuronal differentiating genes and suppressing glial-specific genes. The finding that astrocytes are requiring more ligand to achieve differentiation may account for the variation of the percentage of astrocytes from one experiment to the other (compare astrocytes in Figs 3-5) and may be attributed to the variation in the efficiency of the rescuing agent.

**Neuronal versus glial lineage defines a developmental decision controlled by the Notch pathway**

Together the analysis of the Dll1lacZ/lacZ mutant differentiation phenotype and the time-dependent modulation of the Notch pathway are consistent with the tentative model of lineage tree of neural stem cells described in Fig. 7.

We found that cells expressing neuronal markers are present in the small spheres that underwent few cells divisions.
Moreover, a few neurons were always found in differentiating neurospheres, even under conditions where Notch was overactivated by addition of exogenous J1 EC to wild-type neurospheres. Together, these observations suggest that: (1) in neurospheres, neurons are generated before glia and represent therefore the primary fate of neural stem cells; (2) cells at the origin of neurospheres maintain their multipotency even after extensive ex vivo expansion (some of our neurosphere lines are more than two years old), contrary to the progressive loss of neurogenic capacity described for neural crest stem cells (Morrison et al., 2000; Kubu et al., 2002); (3) P1 precursor, issued from the first asymmetrical division of the neural stem cell, adopts a neuronal fate and may be recalcitrant to exogenous Notch signals.

By contrast, our model predicts that Notch can be activated in P2, which, as a result, would be prevented from adopting a neuronal fate. We anticipate that P2 becomes irreversibly committed to a glial fate. The model postulates that P2 has the potential to acquire either the astrocytic or the oligodendroglial identity through a mechanism independent of Notch signaling. In a second step, Notch would affect the differentiation decision of the precursors already committed to a neuronal or a glial lineage. It would inhibit the differentiation of neurons and oligodendrocytes, while promoting the differentiation of astrocytes.

Moreover, a few neurons were always found in differentiating neurospheres, even under conditions where Notch was overactivated by addition of exogenous J1 EC to wild-type neurospheres. Together, these observations suggest that: (1) in neurospheres, neurons are generated before glia and represent therefore the primary fate of neural stem cells; (2) cells at the origin of neurospheres maintain their multipotency even after extensive ex vivo expansion (some of our neurosphere lines are more than two years old), contrary to the progressive loss of neurogenic capacity described for neural crest stem cells (Morrison et al., 2000; Kubu et al., 2002); (3) P1 precursor, issued from the first asymmetrical division of the neural stem cell, adopts a neuronal fate and may be recalcitrant to exogenous Notch signals.

By contrast, our model predicts that Notch can be activated in P2, which, as a result, would be prevented from adopting a neuronal fate. We anticipate that P1, which is a neuronal precursor, is likely to express Dll1, thus providing the signal capable of activating Notch in P2, thereby suppressing its neuronal fate. In the absence of reliable markers for Notch ligands, it is difficult to further argue this hypothesis.

The observation that the transient activation of Notch in Dll1<sup>lacZ</sup>/lacZ spheres is sufficient to inhibit the production of neurons, and that this production does not resume upon removal of the ligand, suggests that Notch activation in P2 causes an apparently irreversible loss of neuronal potential. P2 is therefore committed to a glial fate instead of being maintained in an undifferentiated and multipotential state. This is consistent with the finding that in the PNS, transient activation of Notch in the neural crest stem cells was sufficient to cause an irreversible loss of neurogenic capacity accompanied by an accelerated glial differentiation (Morrison et al., 2000).

By contrast, our data are inconsistent with the recent finding that Notch signaling does not appear to have a role in the neuronal/glial fate switch (Hitoshi et al., 2002). Beside the trivial explanation that this discrepancy was due to differences in the experimental procedures, we believe it is more likely to be due to the fact that these authors were specifically addressing Notch1 behavior. This interpretation is further supported by recent experiments involving conditional ablation of Notch1 in neurospheres (V. Taylor, personal communication) and by our own results showing that neurospheres originating from embryos heterozygous for Notch1 (contrary to Dll1<sup>lacZ</sup>/spheres) show no quantitative modification in the proportion of neurons/astrocytes (J.B. and E.M., data not shown). This assumption also suggests that the manipulation of each of the ligands (Dll1 or Jagged1) we are describing in the present study is likely to affect more than the Notch1 receptor.
Notch signaling controls the differentiation decisions of precursors already committed to a neuronal or glial lineage

As a result of Notch function, precursors are generated that are fated either to a neuronal (P1) or a glial fate (P2). However, these precursors do not necessarily give rise to the more mature cell type that expresses the appropriate differentiation marker. The experimental temporal modulation of Notch activity is consistent with the notion that neuron precursors, as well as glial precursors, could be blocked in a non-differentiating state, and that their further differentiation depends on secondary Notch signaling.

Neuronal precursors that were normally generated in Dll1::LacZ mutant spheres, owing to the absence of Notch activity during the proliferation phase, do not develop into MAP2-expressing cells when Notch is activated during the differentiation phase (Fig. 4V).

On the contrary, precursors that were fated to a glial cell type upon transient activation of Notch will not differentiate into GFAP-expressing astrocytes (Fig. 4B, parts III and IV; Fig. 5B, part II) unless Notch is re-activated through the presence of soluble ligand during the differentiation phase. We assume that these cells which were blocked in a non-differentiated state, are likely to undergo cell death by apoptosis, as usually described for cells that were misdirected and do not differentiate properly (Lütolf et al., 2002).

In keeping with its role in the specification of cell types, Notch is positively acting for the differentiation of astrocytes and negatively acting for the differentiation of neurons. By contrast, Notch signaling has two contradictory effects on the production of oligodendrocytes. In a first step it acts positively to promote OPC production, whereas it negatively regulates their subsequent differentiation into oligodendrocytes; however, only the latter effect has been previously reported in other systems that were already committed to the oligodendroglial lineage (Wang et al., 1998; Kondo and Raff, 2000).

Our model postulates that P2 is restricted to a glial fate with the potential to differentiate into either astrocytes or oligodendrocytes. Owing to the absence of specific markers, P2 cannot be identified in neurospheres. The existence of such a precursor with both astrocytic and oligodendroglial potential is controversial in vivo. The OPCs (formally called 0-2A) have long been investigated and have been shown to differentiate in vitro (in the presence of 10% FBS) into both oligodendrocytes and type II astrocytes that are positive for both GFAP and A2B5. We never observed cells with characteristics of type II astrocytes. P2 is therefore different from PDGFR cells, which, as we assume, are already committed to an oligodendroglial lineage and are likely, under the conditions employed, to give rise only to 04-expressing oligodendrocytes.

Unfortunately, GFAP is likely to be a marker of astrocyte maturation rather than of lineage commitment, thereby hindering the direct comparison of OPCs with astrocyte precursors regarding Notch signaling. However, our observations show that in no case were oligodendrocytes and astrocytes mutually exclusive regarding Notch activation. We therefore conclude that the segregation between oligodendroglial and astrocyte lineages is independent of Notch signaling and might derive from another mechanism, involving for example the transcription factors OLIG1 and OLIG2 (Zhou and Anderson, 2002).

It is clear that further experimentation will be necessary to test the validity of the differentiation model we propose. It is also clear, however, that Notch signals seem to play an important role in the differentiation of the neural stem cell lineages. Further analysis of the exact role that Notch signals play in neural stem cells will not only provide insights into the biology and underlying mechanisms of these cells but also provide a potential tool for manipulating their fate for therapeutic purposes.

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