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Glycoprotein 130 Signaling Regulates *Notch1* Expression and Activation in the Self-Renewal of Mammalian Forebrain Neural Stem Cells

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Glycoprotein130 (gp130) and Notch signaling are thought to participate in neural stem cell (NSC) self-renewal. We asked whether gp130 regulates Notch activity in forebrain epidermal growth factor (EGF)-responsive NSCs. Disruption of *Notch1* using antisense or a γ-secretase inhibitor demonstrated a requirement for *Notch1* in the maintenance and proliferation of NSCs. Ciliary neurotrophic factor (CNTF) activation of gp130 in NSCs rapidly increased *Notch1* expression. NOTCH1 activation, indicated by tumor necrosis factor α-converting enzyme (TACE)- and *presenilin*-mediated processing, also increased. Infusion of EGF+CNTF into adult forebrain lateral ventricles increased periventricular NOTCH1 compared with EGF alone. Neither *Hes1* (*hairy* and *enhancer* of *split*) nor *Hes5* appeared to mediate gp130-enhanced NOTCH1 signaling that regulates NSC maintenance. This is the first example of a link between gp130 signaling and NOTCH1 in regulating NSC self-renewal.

Key words: **notch; gp130; CNTF; stem cell; delta; self-renewal**

Introduction

Two principal characteristics of neural stem cells (NSCs) are multipotency and self-renewal, the ability to maintain this multipotency after repeated rounds of proliferation (for review, see Gage, 2000; Alvarez-Buylla et al., 2000). In the adult mammalian CNS, a population of NSCs reside in the periventricular area of the forebrain lateral ventricles (Reynolds and Weiss, 1992; Morshead et al., 1994) and contribute neurons to the olfactory bulb throughout adulthood (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996). It is likely that epidermal growth factor (EGF) or transforming growth factor (TGF) α is the *in vivo* mitogen for adult forebrain NSCs (Reynolds et al., 1992; Morshead et al., 1994; Tropepe et al., 1997; Doetsch et al., 1999). On the other hand, little is known about the epigenetic regulation of NSC self-renewal.

Glycoprotein130 (gp130) mediates signaling initiated by the cytokine class of secreted factors, which include leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin M, and interleukin-6 (IL-6), among others (for review, see Turnley and Bartlett, 2000). LIF signals through the dimerization of its cognitive receptor, LIF receptor β (LIFRβ), with gp130, whereas CNTF signaling is mediated by a heterotrimeric complex consisting of CNTF receptor α (CNTFRα), LIFRβ, and gp130 subunits. The long-term maintenance of embryonic stem (ES) cells and NSCs requires the presence of LIF or CNTF (Smith et al., 1988; Williams et al., 1988; Conover et al., 1993; Carpenter et al., 1999; Shimazaki et al., 2001). We recently reported that CNTF signaling (through the CNTF/LIF/gp130 receptor complex) acts to maintain embryonic and adult NSCs in an undifferentiated state by blocking NSC differentiation to restricted glial precursors, with no action on stem cell survival or proliferation (Shimazaki et al., 2001). The mechanisms underlying the actions of CNTF on NSC self-renewal are not understood.

Notch signaling has also been implicated in NSC maintenance (for review, see Artavanis-Tsakonas et al., 1999). Deletion of the basic helix-loop-helix (bHLH) transcriptional repressor *Hes1* (*hairy* and *enhancer* of *split*), a known mediator of Notch signaling, causes premature neuronal progenitor cell differentiation and a reduction in the self-renewal capacity of embryonic forebrain NSCs (Nakamura et al., 2000). Overexpression of activated NOTCH1 in the embryonic cortex results in an increase of radial glial cells (Gaiano et al., 2000), which have been implicated in neurogenesis (for review, see Alvarez-Buylla et al., 2001). Recently, Hitoshi et al. (2002) demonstrated that Notch signaling was required for the maintenance of NSCs but not their generation; however, Notch signaling appears to be context dependent and can also promote glial cell fate (Morrison et al., 2000; Chambers et al., 2001).

Given the context-dependent nature of Notch signaling, we first tested whether it functioned in the maintenance of NSCs derived from the basal forebrain. We then tested the hypothesis that gp130 signaling regulates NSC self-renewal by regulating Notch signaling. Our *in vitro* and *in vivo* data support the conclusions that NOTCH1

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signaling functions in NSC maintenance and proliferation and that activation of gp130 leads to an increase in NOTCH1 signaling.

Materials and Methods

Animals and genotyping. Breeding and genotyping of LIFR mice has been described previously (Shimazaki et al., 2001). CD1 mice were obtained from the University of Calgary Animal Resources Center (Calgary, Alberta, Canada).

Cell culture. Generation of primary and secondary embryonic day 14 (E14) striatal neurospheres was performed as described previously (Shimazaki et al., 2001). Briefly, dissociated primary neurospheres were cultured at a density of 0.05×10^6 cells/ml in culture flasks containing either EGF alone or EGF and rat CNTF [20 ng/ml; Peprotech (Rocky Hill, NJ) and gift from Dr. Rob Dunn (McGill University), respectively], unless stated otherwise. Additionally, IL-6 and soluble IL-6 receptor (sIL6R) (both from R&D, Minneapolis, MN) were used at 20 and 25 ng/ml, respectively. Cells were cultured for a maximum of 7 d *in vitro* (DIV) and harvested for various molecular and biochemical analyses stated below. For NOTCH1 immunoreactive cell counts, primary neurospheres were dissociated and cultured at 50,000 cells/ml for 6 hr in either EGF or EGF+CNTF on poly-L-ornithine-coated coverslips and processed for NOTCH1 immunocytochemistry as stated below.

RT-PCR-Southern blot. Total RNA was isolated from neurospheres using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized using Superscript RT (Invitrogen) at an incubation time of 75–90 min at 42°C. RT-PCR analysis was used to establish the presence of *Notch1*, *-2*, *-3*, *-4*, *Delta1* and *-3*, *Jagged1* and *-2*, *Hes1* and *-5*, and *Mash1* in EGF-derived stem cell progenies using the conditions stated in Table 1. Each product was amplified by denaturation (94°C, 45 sec), primer annealing (45 sec), and extension (72°C 45 sec; *Jagged 2*, *Notch1*, and *Notch4* for 1 min) with the exception of *Delta1*, which was a two-step PCR (94°C for 45 sec denaturation and anneal at 72°C for 1 min). Identity of amplified products was established by Southern blot analysis using *Notch1*, *Delta3*, *Jagged1*, *Jagged2*, and *Delta1* (kindly provided by Dr. Domingos Henrique, Lisbon Medical School) cDNA probes or by PCR-based direct cloning and sequencing of *Notch2*, *-3*, *-4*, *Jagged1*, *Hes1*, *Hes5*, and *Mash1*. PCR products were purified using the Geneclean II kit (BIO 101) and ligated into pGEM-T vector plasmids (Promega, Madison, WI). Sequencing identified correct plasmid clones. Southern blot analysis was performed as described previously (Shimazaki et al., 1999). Experiments were performed at least three times with the exception of RT-PCR analyses, which were performed twice. Pictures were taken on a Kodak DC120 (Rochester, NY) and densitometric analysis was done using Kodak Digital Science 1D software.

Western blotting. Cultured cells were processed for Western blot analysis as described previously (Shimazaki et al., 1999). NucliePURE prep kit (Sigma, St. Louis, MO) was used for the isolation of nuclear proteins as per the manufacturer's instructions. Nitrocellulose membranes were incubated with the 93-4 rabbit α mouse NOTCH1 primary antibody (1:10,000), or affinity-purified (AFP) goat α mouse NOTCH1 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), or mouse α mouse MASH1 (1:25; gift from Dr. David Anderson, California Institute of Technology), and/or AFP goat α ACTIN (1:100; Santa Cruz Biotechnology) mouse overnight in the blocking buffer at 4°C, washed with Tris-buffered saline (0.1% Tween 20), and then incubated with blocking buffer plus the appropriate secondary antibody conjugated to horseradish peroxidase (Chemicon, Temecula, CA). Blots were developed using Enhanced Chemiluminescence and Hyperfilm (both from Amersham Biosciences, Baie d'Urfé, Quebec). Pictures and analysis were done as above.

Immunohistochemistry. Mice were processed for immunohistochemistry as described previously (Shimazaki et al., 2001). Coronal cryosections (8 μm) of mouse forebrain were double stained for Notch1 and CNTFR as described below. Embryonic sections were postfixed with 100% acetone for 30 sec, preblocked with rabbit IgG Fab2 fragment (Jackson ImmunoResearch, West Grove, PA; 1:100 in 10% normal donkey serum, 0.4% Triton X-100, PBS, pH 7.5) for 2 hr at room temperature, incubated overnight at 4°C with goat anti-rat CNTFRα IgG (1:100; Santa Cruz Biotechnology), washed with PBS, incubated for 1 hr at room temperature with biotin-conjugated donkey α goat IgG secondary (1:200; Jackson ImmunoResearch), followed by a 1 hr incubation in streptavidin-Cy3 (1:1000; Jackson ImmunoResearch). Sections were then washed with PBS and incubated overnight at 4°C with rabbit 93-4 anti-rat NOTCH1 (1:25). Sections were then washed and incubated with Hoechst 33258 and goat anti-rabbit fragment crystallisable-specific (1:100; FITC conjugated) secondary antibody for 30 min at 37°C, washed, and mounted with FluorSave (Calbiochem, San Diego, CA). Adult sections were incubated overnight at 4°C with rabbit anti-mouse NOTCH1EC (1:50; EC indicates extracellular antibody directed against the extracellular portion of NOTCH1 as named by Dr. Lendahl; gift from Dr. Urban Lendahl, Karolinska Institute) in 5% NGS, washed with PBS, incubated with biotin-conjugated goat anti-rabbit IgG for 1 hr at room temperature (1:200; Jackson ImmunoResearch), followed by a wash with PBS, incubated with streptavidin-Cy3 as above. Images were taken on a Photometrics Quantix camera (Tucson, AZ) mounted on a Zeiss Axioplan2 (Thornwood, NY) or with a Cohu CCD (San Diego, CA) mounted on a Zeiss Axiovert (for time-lapse images).

Counts of NOTCH1-immunoreactive cells in vitro *and* in vivo. Dissociated primary spheres, were exposed to either EGF or EGF+CNTF for 6 hr on poly-L-ornithine-coated coverslips. The cells were fixed for 20 min with 4% paraformaldehyde at room temperature. Cells were then preblocked in 10% NGS for 1 hr, incubated with NOTCH1EC at 1:2000 overnight at 4°C, and then incubated with rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Pictures of five random fields of each condition per independent experiment were taken on a Photometrics Quantix camera mounted on a Zeiss Axioplan2. To cap-

Table 1. Primers and PCR conditions for respective genes

Gene	Accession number and primers (5′-3′)	Annealing temperature °C	Product size (bp)	Region amplified
Delta1	X80903 cctcgttcgagacctcaagggag s tagacgtgtgggcagtgcgtgc as	72	552	1961–2467
Delta3	Y11895 cacgccattcccagacgagtgc s gcagtcgtccaggtcgtgct as	58	565	774–1295
Jagged1	L38483 cctgccagtgcctgaatggacg s ggctgtcaccaagcaacagaccc as	61	620	2913–3532
Jagged2	U70050 accgtgaccaagtgcctcagggca s gagcggagcccactggttgttgg as	59	485	4907–5322
Notch1	Z11886, S47228 gcccagccagtacaacccactacgg s ggaacggaagctggggtcctgcatc as	62	709	7029–7687
Notch2	D32210 caccttgaagctgcagacat s tggtagaccaagtctgtgatgat as	60	220	5551–5727
Notch3	X74760 atatatatggagttgctcccttcc s ggctttgagcagacaagacccctt as	63	307	7428–7685
Notch4	U43691 ggaagcgacacgtacgagtctgg s caacacccggcacatcgtaggt as	59	332	6282–6568
Hes1	D16464 aagcacctccggaacctgcagc s agtggcctgaggctctcagttcc a	65	609	1092–2278
Hes5	D32132 gatgcgtcgggaccgcatcaac s gcagcttcatctgcgtgtcgctg as	61	276	569–884
Mash1	M95603 tggagagtggagccggccag s gcgtcgtgctcgtccagcag as	67	504	93–556
β-actin	X03672 cgtgggccgccctaggcacca s ttggccttagggttcagggggg as	N/A	243	201–403

ture images of NOTCH1 immunoreactivity, all fields were exposed for 1.5 sec. Pictures were imported into Adobe Photoshop 4.0. To aid in the distinction between weakly and intensely staining NOTCH1 cells, the brightness was reduced by 150% in all cases. Blind counts were made on the percentage of intensely immunoreactive NOTCH1-positive cells out of total Hoechst 33258 positive cells. For counts of the number of NOTCH1-immunoreactive cells in EGF- and EGF+CNTF-infused animals, six sections (from three independent experiments of EGF and EGF+CNTF infusions) were randomly selected at approximately the same rostral–caudal level, and images of NOTCH1 and Hoechst immunolabeling were captured as above, imported, and pseudocolored in Photoshop 4.0. Respective NOTCH1 and Hoechst panels were overlapped, and 21.6 μm (medial–lateral) × 173 μm (dorsal–ventral) of the expanded lateral ventricle immediately below the dorsal limit was outlined and counted double blind for the number of NOTCH1-immunoreactive cells.

Notch1 antisense and γ-secretase inhibitor. Oligonucleotides were designed against a portion of the 5′ intracellular cdc10/ankyrin repeat region (CDC) as described previously (Austin et al., 1995). The CDC antisense sequence 5′-CCTCC**A**CTGCAGGAGGCAATCAT-3′ was identical to the one described previously with the exception of a G–A (in bold) switch in the mouse sequence. Antisense oligonucleotides were used in parallel with their corresponding sense oligonucleotides at 20 μM. Briefly, oligonucleotides were added to 2 million dissociated pass 1 (P1) cells in 5 ml of EGF media. Cells were triturated with a fire-polished Pasteur pipette and moved into flasks (Falcon, BDL, Franklin Lakes, NJ); 6 hr later flasks were tapped until cells lifted off the plastic surface. Twenty-four hours later, 4 ml of cells was harvested for Western blot analysis, and the remaining 1 ml was transferred to a six-well plate, and allowed to grow for 3 DIV; individual spheres were dissociated in 96-well plates and assayed for the ability to produce secondary spheres after 7 DIV. For the γ-secretase inhibitor (Calbiochem) experiments, 50 μM of the inhibitor was added to 2 million dissociated P1 cells in 5 ml of EGF media, allowed to grow for 24 hr, and then harvested for Western blot analysis. For the detection of NOTCH1-protein fragment 2 (PF2), 2 million dissociated P1 cells in 5 ml of EGF were allowed to grow for 24 hr, treated with DMSO or γ-secretase inhibitor (50 μM) for 4 hr, and then harvested for Western blot analysis. For the detection of NOTCH1-PF3, P1 cells were cultured for 3 DIV at a concentration of 400,000 cells/ml, and then DMSO or γ-secretase inhibitor II (50 μM) was added for 4 hr; the cells were then isolated for nuclear proteins as stated above. For the single-sphere dissociation experiments, γ-secretase II was added to a concentration of 30 μM, at plating, to dissociated P1 cells in a 24-well plate at a concentration of 400,000 cells/ml. Neurospheres were grown for 3 DIV, and then individual spheres were dissociated in 96-well plates and assayed for secondary neurosphere production. DMSO added to control cultures was equal to the volume of γ-secretase II inhibitor added.

In vivo *growth factor infusions. In vivo* infusion of EGF and EGF+CNTF were performed as described in Shimazaki et al. (2001).

Results

Notch1 signaling is required for the maintenance of E14 EGF-responsive NSCs

EGF-responsive NSCs of the basal forebrain proliferate to form neurospheres, which contain precursors to neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1996). The *in vitro* maintenance of an undifferentiated state by NSCs may be studied through the ability of single EGF-generated neurospheres, which are dissociated and cultured in the presence of EGF, to give rise to secondary neurospheres (Reynolds and Weiss, 1996). We recently found that the CNTFRα/LIFRβ/gp130 receptor complex operates in the maintenance of EGF-derived NSCs (Shimazaki et al., 2001). Specifically, when single P1 neurospheres (P1 neurospheres are derived from dissociated primary neurospheres, which in turn are derived from the culture of dissociated E14 striatopallidum complexes in the presence of EGF) generated in the presence of EGF+CNTF were individually dissociated and replated in EGF alone, they produced 59% more pass 2 (P2) neurospheres than equivalent-sized P1 neurospheres generated in EGF and replated in EGF. Before asking whether CNTF could modulate NOTCH1 signaling, we asked whether *Notch1* mediates, at least in part, the maintenance of EGF-responsive NSCs. We analyzed the ability of EGF-generated P1 neurospheres to produce P2 neurospheres after culturing them in the presence of a well characterized antisense to the CDC repeat portion of *Notch1* (Austin et al., 1995; Redmond et al., 2000). Exposure of dissociated primary neurospheres to *Notch1* antisense (20 μM) for their first 24 hr in culture resulted in a significant decrease in NOTCH1 expression, when compared with cells exposed to sense controls (Fig. 1*A*). Furthermore, antisense-treated P1 neuro-

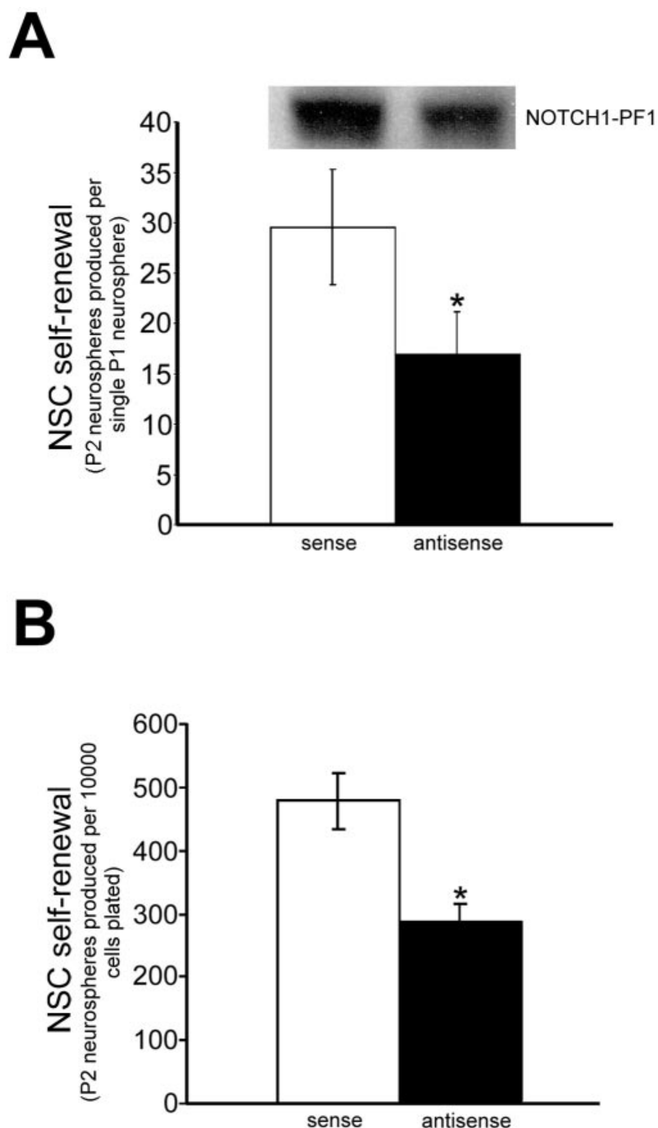

Figure 1. Notch1 antisense reduces NOTCH1 expression and NSC self-renewal. NSCs were cultured in the presence of 20 ng/ml EGF in the absence or presence of 20 μM *Notch1* antisense and harvested after 1 DIV for protein or cultured for a total of 3 DIV (to form P1 neurospheres) and assayed either by single-sphere dissociation (*A*) or batch culture (*B*) for the formation of P2 neurospheres. *A*, Western blot analysis reveals a reduction in NOTCH1-PF1 expression (*inset*; $p < 0.05$; *t* test; $n = 3$) in antisense-treated P1 neurospheres. A concomitant decrease was observed in the ability of antisense-treated, individual equivalent sized P1 neurospheres to produce P2 neurospheres (*$p < 0.05$; *t* test; $n = 3$) compared with sense treatment. *B*, Assaying for the ability of P1 neurospheres treated with *Notch1* antisense to produce P2 neurospheres by batch culture analysis also reveals a significant decrease in their ability to produce P2 neurospheres (*$p < 0.05$; *t* test; $n = 3$).

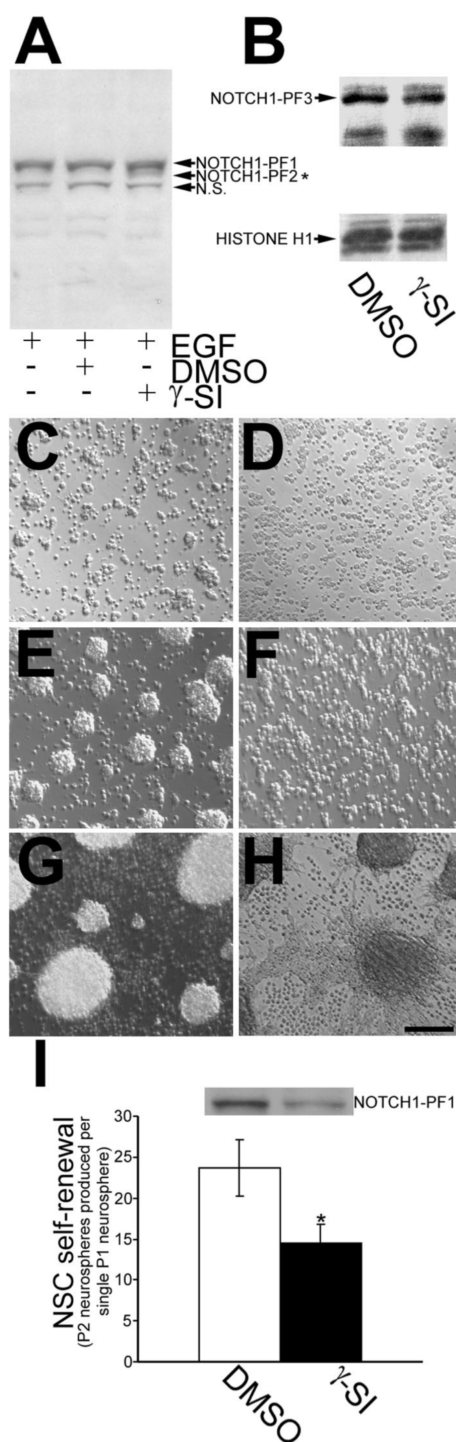

Figure 2. Disruption of NOTCH1 signaling by γ-secretase inhibitor II delays P1 neurosphere formation and reduces their ability to produce P2 neurospheres. *A*, To ensure that the γ-secretase inhibitor that we were using was effectively blocking production of NOTCH1-PF3, NSCs were cultured in 20 ng/ml EGF (20 ng/ml) for 24 hr, at which point DMSO (carrier) or γ-secretase inhibitor II (50 μM) was added, and the cells were harvested 4 hr later for total proteins and Western blot analysis. *A*, The *asterisk* indicates an increase in the P2 proteolytic product of NOTCH1, as would be expected if the γ-secretase inhibitor was effectively blocking production of NOTCH1-PF3 ($n = 3$), and identifies the upper band as furin-processed NOTCH1 or NOTCH1-PF1. *B*, Three day *in vitro* P1 neurospheres that were treated with γ-secretase inhibitor for 4 hr and harvested for nuclear proteins and Western blot analysis demonstrate a decrease in NOTCH1-PF3 compared with DMSO control. *C–H*, NSCs were cultured in EGF (20 ng/ml) and either DMSO (*C*, *E*, *G*; carrier) or γ-secretase inhibitor II (*D*, *F*, *H*; 30 μM), and digital micrographs were taken after 6 (*C*, *D*), 18 (*E*, *F*), and 88 hr (*G*, *H*). *I*, Single-sphere dissociation assay reveals a significant reduction in self-renewal capacity of P1 neurospheres generated for 3 DIV in the presence of γ-secretase inhibitor II (30 μM) compared with DMSO controls (*$p < 0.05$; *t* test; $n = 3$). *Inset* shows a reduction of NOTCH1-PF1 expression in P1 neurospheres treated for 1 DIV, from the time of plating, with 50 μM γ-secretase inhibitor II compared with the DMSO control ($p < 0.05$; *t* test; $n = 3$), indicating that constitutive inhibition of NOTCH1 activation for at least 24 hr leads to an overall decrease in NOTCH1 expression. Scale bar, 100 μm. *N.S.*, Nonspecific; *γ-SI*, γ-secretase inhibitor.

spheres had a significantly reduced capacity to produce P2 neurospheres compared with sense controls, whether assayed by single-sphere dissociation of equivalent-sized neurospheres (43%) (Fig. 1*A*) or batch culture experiments (40%) (Fig. 1*B*). These results suggest that NOTCH1 expression levels can regulate the maintenance of EGF-responsive NSCs.

We next sought to determine whether NOTCH1 cleavage/activation is necessary for the maintenance of EGF-responsive NSCs. A γ-secretase-like protease has recently been implicated in the cleavage of NOTCH1 into its active intracellular domain (De Strooper et al., 1999) and can be blocked by the peptidomimetic inhibitor γ-secretase inhibitor II (De Strooper et al., 1999; Wolfe et al., 1999). We use the nomenclature for NOTCH1 processing and products defined by Mumm et al. (2000) and Brou et al. (2000). Therefore, processing of NOTCH1 by a furin-like convertase at the S1 site produces NOTCH1-PF1 (Logeat et al., 1998), ligand-dependent processing at the S2 site by TACE produces NOTCH1-PF2, and ligand-dependent processing at the S3 site by a *presenilin*-mediated cleavage produces the active intracellular portion of NOTCH1 or NOTCH1-PF3 (De Strooper et al., 1999). Inhibition of γ-secretase should result in the accumulation of NOTCH1-PF2 if the inhibitor is effective (Brou et al., 2000; Mumm et al., 2000). Therefore, we tested the effectiveness of γ-secretase inhibitor II in preventing the production of NOTCH1-PF3 by assaying for the accumulation of NOTCH1-PF2 (which is expected because NOTCH-PF2 is the precursor for NOTCH1-PF3) and for the decrease in NOTCH1-PF3 in EGF-generated neurospheres. Western blot analysis of P1 neurospheres treated for 4 hr with γ-secretase inhibitor II (50 μM) consistently revealed the appearance of a band (NOTCH1-PF2; $n = 4$) below that of NOTCH1-PF1, compared with EGF and EGF+DMSO controls, suggesting that the inhibitor was preventing the production of NOTCH1-PF3 (Fig. 2*A*). Furthermore, nuclear protein extracts of P1 neurospheres that were treated for 4 hr with γ-secretase inhibitor II revealed a decrease (relative to HISTONE H1 expression: −34% in experiment 1, −27% in experiment 2) in NOTCH1-PF3 compared with DMSO controls, confirming that the inhibitor was preventing the production of NOTCH1-PF3 (Fig. 2*B*). Addition of γ-secretase inhibitor (30–50 μM), at plating, to a single-cell suspension derived from primary neurospheres, delayed the formation of P1 neurospheres by ~24 hr, compared with vehicle controls (Fig. 2*C–H*). Once generated, inhibitor-treated P1 neurospheres appeared more differentiated, compared with vehicle controls (Fig. 2, compare *G*, *H*). Western blot analysis revealed that 24 hr after inhibitor addition, NOTCH1-PF1 protein expression was reduced to 52% of vehicle-treated sister cultures ($n = 3$, $p < 0.005$) (Fig. 2*I*), suggesting that inhibition of NOTCH1 activation leads to an overall decrease in NOTCH1 production. After 3 DIV, γ-secretase inhibitor- and vehicle-treated P1 neurospheres of 150–200 μm in diameter were isolated, dissociated, and examined for the formation of P2 neurospheres. γ-secretase inhibitor treatment reduced P2 neurosphere formation to 61% of control ($n = 3$, $p < 0.05$) (Fig. 2*I*). These results suggest that, in addition to expres-

sion levels, NOTCH1 cleavage and signaling regulate the maintenance of EGF-responsive NSCs.

Signaling through CNTFRα regulates expression of Notch1 *in vitro*

Given the similarity between the actions of NOTCH1 and gp130-mediated signaling on NSC maintenance, we asked whether gp130-mediated signaling, stimulated by CNTF, could regulate NOTCH1 expression in EGF-responsive NSCs. We first explored whether CNTFRα and NOTCH1 are coexpressed in the developing E14 basal forebrain, the origin of embryonic EGF-responsive NSCs. We found that most of the NOTCH1-expressing cells coexpress CNTFRα in the E14 basal forebrain germinal zone (Fig. 3*A–D*), consistent with the hypothesis of a link between CNTFRα and NOTCH1 signaling in forebrain NSCs. We then examined the results of gp130 activation on NOTCH1 signaling in EGF-generated neurospheres. When screening, using RT-PCR, for *Notch* gene expression, we found that P1 neurospheres expressed *Notch1* and *Notch3* but not *Notch2* or *Notch4* (data not shown). Quantitative RT-PCR Southern blot analysis was used to examine *Notch* expression in P1 neurospheres generated in EGF+CNTF (20 ng/ml) compared with those generated in EGF. *Notch1* increased approximately threefold in 1 DIV EGF+CNTF-generated P1 neurospheres compared with EGF-generated P1 neurospheres (Fig. 3*E*), whereas *Notch3* expression was unaffected (Fig. 3*F*). Because increases in mRNA expression are not always followed by concomitant increases in protein expression, we sought definitive evidence that gp130-mediated signaling could regulate NOTCH1 expression. We used two antibodies to NOTCH1 to ensure that we were in fact measuring *bona fide* NOTCH1 protein. Figure 3*G* shows that in a Western blot, both the 93-4 (Shawber et al., 1996) and Santa Cruz M-20 antibodies identify increases in NOTCH1-PF1 and NOTCH1-PF2 (more than fivefold; $p < 0.01$; $n = 5$) in 3 DIV EGF+CNTF-generated P1 neurospheres compared with EGF-generated P1 neurospheres. Furthermore, nuclear protein extracts of 3 DIV EGF+CNTF-treated P1 neurospheres demonstrate an increase in NOTCH1-PF3 compared with EGF controls as determined by Western blot analysis (Fig. 3*H*) ($p < 0.01$; *t* test; $n = 4$). Together, these findings suggest that

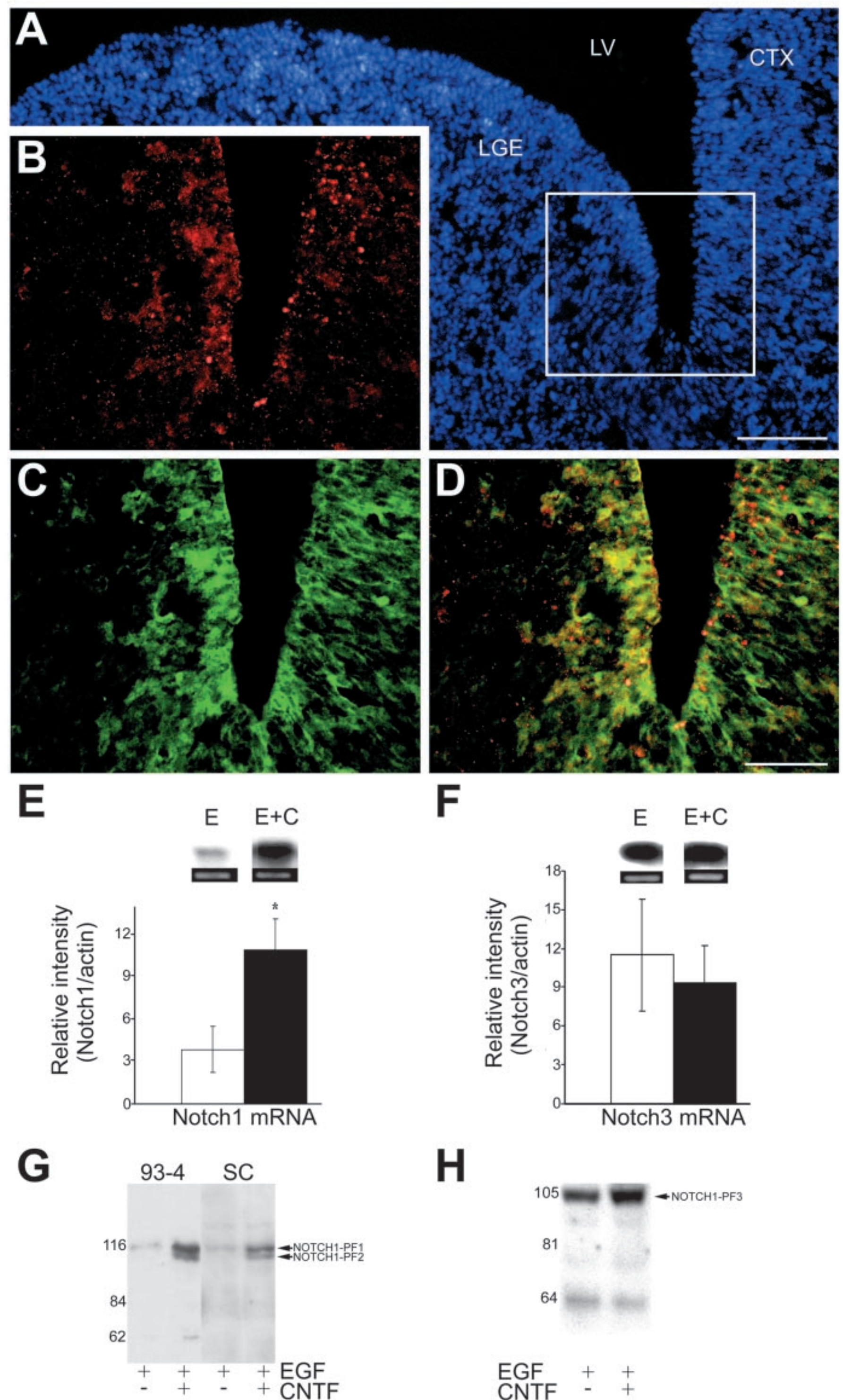

Figure 3. EGF+CNTF treatment of embryonic P1 neurospheres specifically increases *Notch1* mRNA and protein expression. *A–D*, Immunofluorescence micrographs of a coronal section (8 μm) through the forebrain of an E14 mouse embryo. *A*, Nuclei were labeled with Hoechst 33258 (*blue*). CNTFRα-immunoreactive cells in the ventricular zone were visualized with Cy3 (*B*, *red*), and *Notch1*-immunoreactive cells were labeled with FITC (*C*, *green*). *D*, A merged image of *B* and *C*, where yellow staining indicates colocalization of NOTCH1 and CNTFRα. *Box* in *A* indicates area magnified in *B–D*. *E–G*, NSCs were cultured in 20 ng/ml EGF, the absence or presence of 20 ng/ml CNTF, and harvested after 24 hr for total RNA and RT-PCR Southern blot analysis (*E*, *F*), or after 3 DIV for Western blot analysis (*G*). *Notch1* expression increased significantly (*$p < 0.05$ vs EGF; *t* test; $n = 3$) after 1 DIV of CNTF treatment (*E*) compared with no change in *Notch3* expression (*F*). Both the 93-4 and Santa Cruz intracellular NOTCH1 antibodies reveal an increase in NOTCH1-PF1 and NOTCH1-PF2 proteolytic products after 3 DIV of EGF+CNTF treatment compared with EGF alone (*G*) ($p < 0.01$; *t* test; $n = 5$). Nuclear expression of NOTCH1-PF3 increases in 3 DIV P1 neurospheres cultured constitutively in EGF+CNTF compared with EGF alone (*H*) ($p < 0.01$; *t* test; $n = 4$). Scale bars: *A*, 50 μm; *D*, 100 μm. *LGE*, Lateral ganglionic eminence; *LV*, lateral ventricle; *CTX*, cortex.

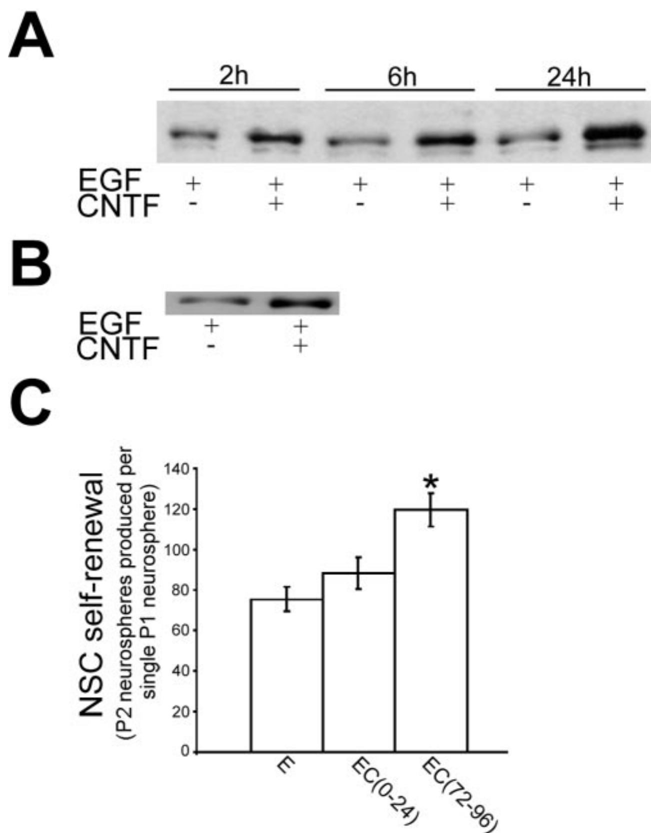

Figure 4. Cell–cell contact is not required for CNTF to increase NOTCH1 expression but is required for CNTF to increase NSC self-renewal. *A*, Western blot analysis reveals that NOTCH1-PF1 expression increases as early as 2 hr after CNTF treatment of 3 DIV EGF-derived P1 neurospheres ($n = 3$). *B*, Totally dissociated primary neurospheres were cultured in EGF+CNTF for 6 hr; Western blot analysis demonstrates a threefold increase in NOTCH1-PF1 expression ($p < 0.05$; *t* test; $n = 3$) compared with EGF controls. No increase in NOTCH1-PF2 could be detected. *C*, P1 neurospheres were generated in EGF or in EGF+CNTF in the absence (0–24 hr) or presence (72–96 hr) of cell–cell contact. After 7 DIV the three different groups were assayed for the formation of P2 neurospheres by single-sphere dissociation and culture in EGF alone (each group was washed at 24 and 96 hr). Compared with EGF, addition of CNTF for 24 hr at 3 DIV increased the formation of P2 neurospheres by 59% ($p < 0.0001$; Tukey HSD test; $n = 3$), whereas there was no difference in P2 neurosphere formation when CNTF was added for the first 24 hr ($p > 0.58$; Tukey HSD test; $n = 3$).

the increase in NOTCH1 synthesis in CNTF-stimulated P1 neurospheres further results in ligand-mediated activation of NOTCH1 signaling.

Lateral inhibition is not necessary for CNTF to increase NOTCH1 expression; however, cell–cell contact is required for CNTF to increase NSC self-renewal

To determine whether CNTF increases *Notch1* expression directly or indirectly, we examined the time course of CNTF-induced NOTCH1 expression. NOTCH1 expression increases significantly in 3 DIV neurospheres after as little as 2 hr of exposure to CNTF (Fig. 4*A*). These data suggest that *de novo* synthesis of another protein, which would then act to increase expression of NOTCH1, is unlikely. In all of the above mentioned experiments, we examined *Notch1* mRNA and protein expression in developing clusters of cells (neurospheres). Given the cell–cell contact within neurospheres, it is possible that lateral inhibition mediates the actions of CNTF on *Notch1* expression. In this case, CNTF could decrease ligand expression, which through lateral inhibition would increase *Notch1* expression in the same population of cells. To examine this possibility, we tested whether 6 hr of EGF+CNTF exposure (20 ng/ml), in comparison with EGF alone, could increase NOTCH1-PF1 or NOTCH1-PF2 expression in a completely dissociated single-cell suspension (5×10^4 cells/ml) derived from 7 DIV primary neurospheres. Western blot analysis shows that in the absence of cell–cell contact, CNTF can increase NOTCH1-PF1 expression (more than threefold; $p < 0.05$; *t* test; $n = 3$) (Fig. 4*B*). We did not detect NOTCH1-PF2 in either condition, which is what we would have predicted considering that this band should appear only in the presence of ligand-mediated activation of TACE and further indicates that we have correctly identified NOTCH1-PF2. These data suggest that the CNTF-induced increase in NOTCH1 expression is ligand independent but that NOTCH1 activation requires ligand mediated cleavage.

We then tested whether an increase in NOTCH1 expression, without its activation, was sufficient to increase the production of P2 neurospheres. Thus we treated P1 cells with EGF alone or with EGF and then added CNTF for 24 hr at plating or for 24 hr at 3 DIV. All conditions were washed at 1 and 4 DIV. After 7 DIV, we dissociated single P1 neurospheres to assay for self-renewal (by counting the numbers of P2 neurospheres per single P1 neurosphere). Figure 4*C* shows that there was no significant increase in self-renewal of P1 neurospheres that were treated with CNTF for the first 24 hr, whereas there was a significant increase in self-renewal capacity in the P1 neurospheres treated with CNTF at 3 DIV [$p < 0.0001$; Tukey honestly significant difference (HSD) test; $n = 3$]. These data suggest that cell–cell contact, which is present in 3 DIV spheres and almost entirely absent in plated cells (for the first 24 hr at this concentration), is necessary for CNTF to increase the self-renewal capacity of NSCs.

Although we had shown that CNTF could increase NOTCH1 expression in EGF-generated neurospheres, we had yet to demonstrate directly the phenomenon at the single-cell level. With this in mind and to determine whether CNTF increases expression of NOTCH1 in all EGF-generated cells or rather increases the number of NOTCH1-expressing cells, we examined the expression of NOTCH1 in dissociated primary spheres (P1 cells) treated for 6 hr with either EGF or EGF+CNTF. We observed that all of the cells, in either condition, expressed some level of immunoreactivity to NOTCH1 (data not shown). We found, however, that addition of CNTF to the culture increased the number of cells that labeled intensely (Fig. 5, *arrowheads*) (see Materials and Methods for experimental details) compared with those that labeled weakly (Fig. 5, *small arrows*) for NOTCH1 by 49% ($p < 0.003$; *t* test; $n = 3$) compared with EGF alone. Taken together, these findings suggest that CNTF directly upregulates NOTCH1 expression in EGF-generated NSC progeny.

NOTCH1 expression correlates with the capacity of P1 neurospheres to generate P2 neurospheres in LIFRβ knock-out mice

We have recently reported that adult *LIFR*β heterozygotes show a decrease in the ability to produce forebrain neurospheres (indicative of NSC number), compared with their wild-type littermates (Shimazaki et al., 2001). To determine whether gp130 regulation of NOTCH1 function is associated with changes in NSC self-renewal, we compared NOTCH1 protein expression, using Western blot analysis, in 3 DIV *LIFR*$\beta^{+/+}$ and *LIFR*$\beta^{-/-}$ embryonic P1 neurospheres treated with EGF or EGF+CNTF. Furthermore, we examined the capacity of wild-type and mutant P1 neurospheres to produce P2 neurospheres by single sphere dissociation. In wild-type (+/+) P1 neurospheres, both NOTCH1-PF1

and -P2 neurosphere production increase after CNTF addition, whereas no increase in NOTCH1-PF1 or -P2 neurosphere production was observed in $LIFR\beta^{-/-}$ P1 neurospheres cultured in EGF+CNTF (Fig. 6). Glycoprotein130 signaling through IL6R does not require LIFRβ and thus provides a means to test whether gp130 activation is sufficient for increasing NOTCH1-PF1 expression and P2 neurosphere production. Because the IL6 receptor is not expressed in EGF-generated neurospheres (T. Shimazaki and S. Weiss, unpublished observations), we generated $LIFR\beta^{-/-}$ P1 neurospheres in the presence of sIL6R+IL6. Activation of gp130 signaling in $LIFR\beta^{-/-}$ P1 neurospheres with sIL6R+IL6 was sufficient to increase their expression of NOTCH1-PF1 and -P2 neurosphere production (Fig. 6). These experiments suggest that activation of gp130 signaling is the common element in CNTFR-, LIFRβ- and IL6R-mediated increases in NOTCH1 expression of P1 neurospheres and P2 neurosphere production.

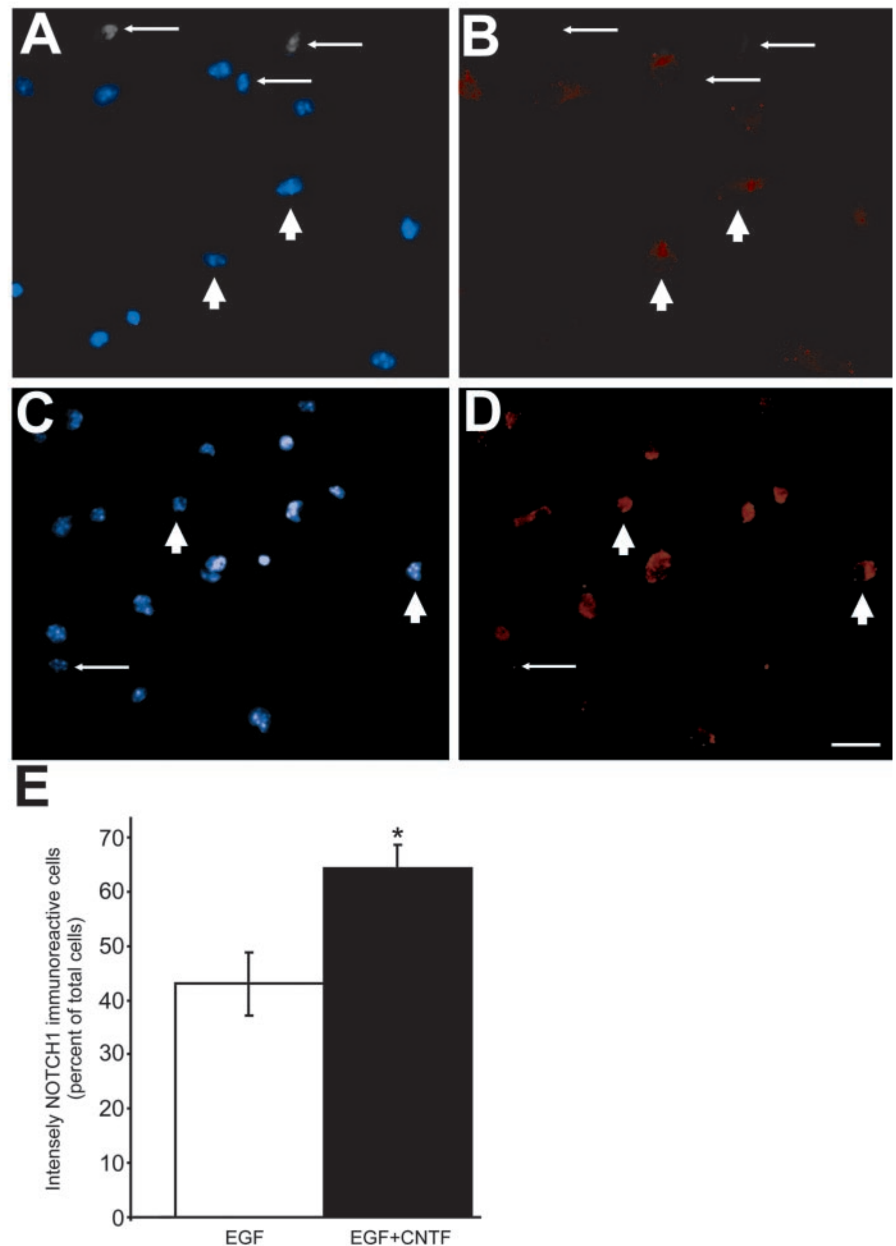

Figure 5. CNTF increases the number of intensely NOTCH1-immunoreactive cells. Primary neurospheres were dissociated and then cultured on poly-L-ornithine-coated coverslips for 6 hr in either EGF (*A*, *B*) or EGF+CNTF (*C*, *D*), and blind counts were made (as described in Materials and Methods) on the number of intensely NOTCH1-immunoreactive cells (*C*). *A*, *C*, Nuclei were labeled using Hoechst 33258 (*blue*). NOTCH1-immunoreactive cells were labeled with rhodamine (*B*, *D*, *red*). *E*, Compared with cells cultured in EGF alone, cells cultured in EGF+CNTF demonstrate a 49% increase in the number of intensely NOTCH1-immunoreactive cells (*$p < 0.003$; *t* test; $n = 3$). *Arrowheads* indicate examples of cells that stain intensely for NOTCH1, and *small arrows* indicate examples of cells that stain weakly for NOTCH1. Scale bar, 20 μm.

The CNTF-induced increase in NOTCH1 expression is context dependent

In vitro, one can determine the effects of factors on a population of cells one at a time or in combination. This is certainly not the case in the developing germinal zone, where progenitor cells are likely exposed to several factors at the same time. Thus, we sought to determine whether CNTF could increase NOTCH1-PF1 expression in the absence of EGF. We stimulated dissociated 7 DIV primary neurospheres (5×10^4 cells/ml) with CNTF for 6 hr, in the absence or presence of EGF (Fig. 7*A*). In the absence of EGF, CNTF failed to increase NOTCH1-PF1 expression. These data suggested that only cells receiving an EGF signal could respond to CNTF with an increase in NOTCH1 expression. An alternative possibility is that a proliferative state is necessary for CNTF to increase NOTCH1 expression in NSCs. Thus, we tested whether CNTF could increase NOTCH1-PF1 expression in the absence of EGF but in the presence of FGF2, another principal NSC mitogen. Figure 7*B* demonstrates a significant increase in NOTCH1-PF1 expression in a single-cell suspension derived from 7 DIV primary neurospheres cultured for 24 hr in FGF2+CNTF, compared with those exposed to FGF2 alone. Therefore, induction of NOTCH1 expression by CNTF appears to require that NSCs be in a proliferative state.

CNTF can upregulate NOTCH1 expression *in vivo*

Intraventricular infusion of EGF+CNTF resulted in a 50% increase in the number of neurospheres that could be derived from the periventricular area of the adult brain, compared with EGF infusion alone (Shimazaki et al., 2001). Given that CNTF could only induce the expression of NOTCH1 in the presence of either EGF or FGF2 (Fig. 7), we compared NOTCH1 expression in the forebrain periventricular area of EGF- versus EGF+CNTF-infused adult mice. Adult CD1 mice were infused with EGF or EGF+CNTF for 6 d, and the brains were processed for NOTCH1 immunohistochemistry ($n = 3$ for each treatment). The lateral aspect of the periventricular area (the specific region that is thought to be enriched in NSCs) of brains infused with EGF+CNTF exhibited a much thicker and more intense area of NOTCH1 expression compared with animals infused with EGF alone (Fig. 8, compare *A*, *B*). In particular, although NOTCH1

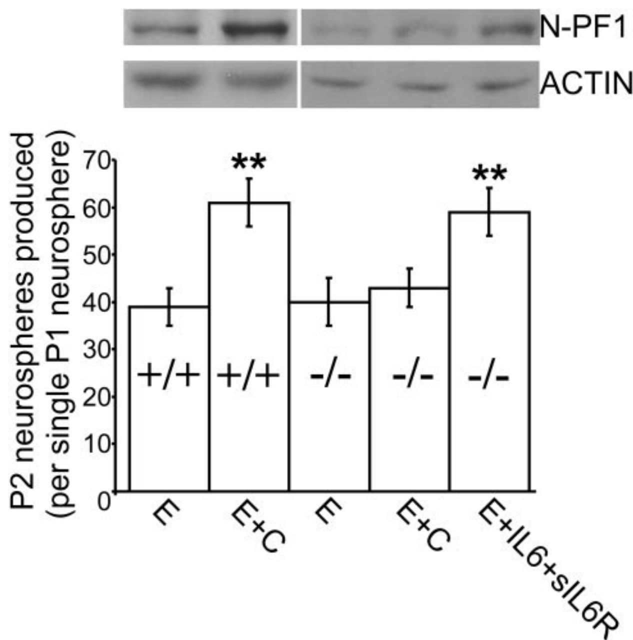

Figure 6. IL6+sIL6R increases NOTCH1 expression and P2 neurosphere production in P1 neurospheres generated from *LIFRβ*$^{-/-}$ mice. P1 neurospheres were generated from wild-type (+/+) or null mutant (−/−) *LIFRβ* littermates, in the various conditions indicated, and were then assayed after 3 DIV for NOTCH1 protein with Western blot and after 7 DIV for P2 neurosphere production by single-sphere dissociation in EGF alone. Increase in P2 neurosphere production in wild-type (+/+) EGF+CNTF generated P1 neurospheres and in *LIFRβ*$^{-/-}$ P1 neurospheres generated in EGF+IL6+sIl6R correlated with concomitant increases in NOTCH1-PF1 expression (*inset*). CNTF had no effect on P2 neurosphere production or NOTCH1-PF1 expression in *LIFRβ*$^{-/-}$ P1 neurospheres. **$p < 0.01$ versus +/+ control culture or −/− control culture; Tukey HSD test; $n = 5$. *N-PF1*, NOTCH1-PF1.

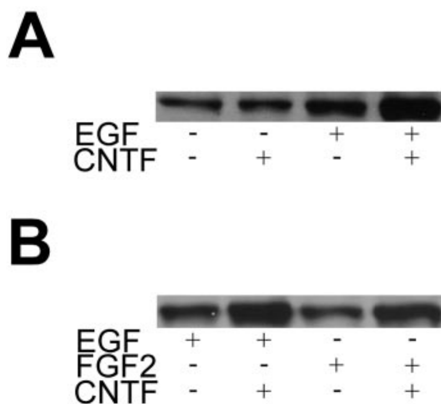

Figure 7. The CNTF-induced increase in NOTCH1 expression in dissociated primary neurospheres is dependent on either EGF or FGF2 signaling. *A*, *B*, Single-cell suspensions derived from primary neurospheres and cultured for 6 hr in the indicated conditions reveal that CNTF had no effect on NOTCH1-PF1 expression in the absence of EGF (*B*) ($n = 3$) and that CNTF can increase NOTCH1-PF1 expression in either EGF- or FGF2-containing media (*B*) ($p < 0.05$; *t* test; $n = 3$).

immunoreactivity is sporadic on the lateral aspect of the ventricle in the EGF infused brain, NOTCH1 staining appears as a thick, continuous layer in the EGF+CNTF-infused brain (Fig. 8, compare *C*, *E*). We then performed double-blind counts on the number of cells immunoreactive for NOTCH1 in the EGF- and EGF+CNTF-infused animals. Of the cells within theexpanded lateral ventricular area, 76 ± 12% expressed NOTCH1 in animals infused with EGF+CNTF compared with 39 ± 7% in the EGF-infused animals ($p < 0.026$; $n = 3$ each group; *t* test). Thus, CNTF, in the presence of EGF, can upregulate the number of NOTCH1-immunoreactive cells *in vivo*.

CNTF stimulation changes mRNA and protein expression levels of genes known to be involved in the Notch1 signaling pathway

The bHLH genes *Hes1* and *Hes5* are known mediators of Notch signaling (Kageyama and Ohtsuka, 1999) and may be involved in NSC or progenitor cell maintenance (Nakamura et al., 2000, Ohtsuka et al., 2001). In addition, HES1 can directly downregulate *Mash1* expression, a gene whose expression is an initial step in the NSC to progenitor cell transition (Chen et al., 1997; Torii et al., 1999). We thus expected that gp130-mediated signaling, initiated by CNTF, would increase the expression of *Hes1* and *Hes5*, with a concomitant decrease in *Mash1* expression. Surprisingly, 3 DIV P1 neurospheres generated in EGF+CNTF appeared to show a downregulation in *Hes1* expression, although this did not achieve statistical significance (Fig. 9*A*) ($p > 0.05$; $n = 3$). *Hes5* expression was significantly reduced in 3 DIV EGF+CNTF P1 neurospheres compared with the equivalent neurospheres generated in EGF alone (Fig. 9*A*) ($p < 0.05$; $n = 3$). We also confirmed that there were no transient increases in *Hes1* or *Hes5* expression at 2 or 6 hr after plating in EGF+CNTF compared with EGF alone (data not shown). However, as expected, CNTF markedly decreased *Mash1* expression in 3 DIV EGF+CNTF P1 neurospheres compared with EGF controls (Fig. 9*B*) ($p < 0.05$; $n = 3$). MASH1 has been reported to downregulate its own mRNA expression (Meredith and Johnson, 2000); therefore we examined MASH1 protein expression after CNTF treatment. We found that CNTF treatment significantly decreases MASH1 expression in 3 DIV neurospheres (Fig. 9*B*) ($p < 0.05$; $n = 3$).

Given the decrease in MASH1 expression and because it is a known transcriptional activator of Notch ligand expression (Casarosa et al., 1999), we examined Delta/Serrate gene expression in EGF-generated neurospheres. RT-PCR analysis revealed that *Delta1* and *-3* and *Jagged1* and *-2* were expressed in EGF-generated P1 neurospheres (data not shown). Furthermore, we found that the expression of *Delta3* decreased (Fig. 9*C*) ($p < 0.05$, $n = 3$) in 3 DIV P1 neurospheres cultured in EGF+CNTF, compared with EGF alone. The decrease in *Delta3* expression is consistent with the observed decrease in *Mash1* mRNA and protein expression in EGF+CNTF-treated P1 neurospheres and with our observation that EGF+CNTF treatment of P1 neurospheres increases *Notch1* expression.

Discussion

This report demonstrates, for the first time, a link between gp130 and NOTCH1 signaling pathways in the regulation of NSC maintenance. Our results show a requirement for NOTCH1 signaling in the maintenance and generation of NSCs. We find that gp130 signaling specifically increases NOTCH1 expression in EGF-responsive NSCs *in vitro* and *in vivo*. Furthermore, we find that the increase in NOTCH1 expression is followed by an increase in the ligand-mediated cleavage of NOTCH1-PF1 into NOTCH1-PF2 and further into NOTCH1-PF3. Our observed decreases in *Mash1* and *Delta3* are consistent with activation of gp130 leading to an increase in NOTCH1 signaling. However, our results and a

careful reading of related studies (see below) suggest that *Hes1* and *Hes5* may not be the critical components of NOTCH1 signaling that are involved in NSC maintenance.

Glycoprotein130 activation regulates NOTCH1signaling, which is required for the maintenance ofEGF-responsive NSCs

Recent evidence suggests that the phenotypic response to Notch signaling in developing forebrain precursors is variable and dependent on temporal and spatial cues (Chambers et al., 2001). Moreover, it appears that Notch signaling is unable to regulate the maintenance of neural crest stem cells (Morrison et al., 2000). Therefore, we first tested whether NOTCH1 expression and signaling play a role in EGF-responsive NSC maintenance. Antisense to *Notch1* reduced its expression and consistently decreased secondary neurosphere production. Furthermore, culture of neurospheres in the presence of a γ-secretase inhibitor, known to reduce production of activated NOTCH1 (De Strooper et al., 1999), also reduced the production of secondary neurospheres (Fig. 2*I*). Our results concur with the observations of Hitoshi and colleagues (2002), implicating NOTCH1 signaling in the maintenance of EGF-responsive NSCs. However, in contrast to their findings, we demonstrate that blocking the processing of NOTCH1-PF2 to NOTCH1-PF3, using a γ-secretase inhibitor, also inhibited NSCs from proliferating in response to EGF. This suggests that NOTCH1 activation is required for expansion and generation of EGF-responsive NSCs. Similarly, FGFs require an intact Notch signaling pathway to prevent the differentiation of E10.5 neuroepithelial progenitors cells into neurons (Faux et al., 2001). The conclusion by Hitoshi et al. (2002) that Notch signaling is not required for the generation of NSCs is based on their observation that *RBP-Jκ*$^{-/-}$ ES cells could give rise to the proliferation of primitive NSCs. However, the lineage relationship between these ES cell-derived NSCs and *in vivo* generated NSCs is unclear, as is the role of ES cell-derived NSCs in neurogenesis. Indeed, the fact that NSCs could not be isolatedfrom *RBP-Jκ*$^{-/-}$ or *presenilin1*$^{-/-}$/*presenilin2*$^{+/-}$ embryos concurs with our data that Notch signaling is required for the generation of NSCs.

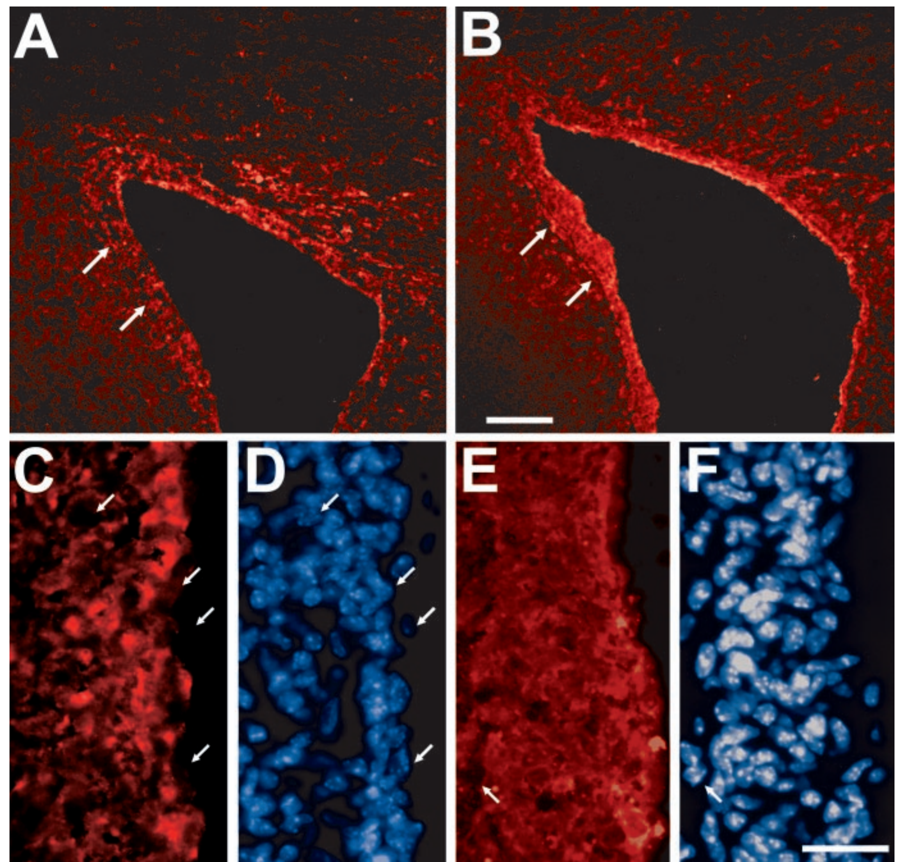

Figure 8. CNTF enhances the expression of NOTCH1 *in vivo*. Adult CD1 mice were infused with either EGF (*A, C, D*) or EGF+CNTF (*B, E, F*) for 6 d, after which brains were processed for NOTCH1 immunohistochemistry. Infusion of EGF+CNTF resulted in an overall increase in NOTCH1 staining intensity as well as a markedly thickened layer of NOTCH1 expression on the lateral aspect (*A, B*, *arrows*) of the ventricle. Furthermore, more cells in the ventricular zone labeled for NOTCH1 in EGF+CNTF (*C*, NOTCH1, 76 ± 12%; *D*, Hoechst) compared with EGF (*E*, NOTCH1, 39 ± 7%; *F*, Hoechst) infused mice ($p < 0.026$; *t* test; $n = 3$ each group). *C–F*, *Arrows* indicate NOTCH1 unlabeled cells. Scale bars: *B*, 100 μm; *F*, 25 μm.

We then tested the hypothesis that gp130-mediated signaling regulates NOTCH1 signaling in NSCs. Our results demonstrate that CNTF specifically induces *Notch1* mRNA and protein expression (no change in *Notch3* expression) in NSCs. With regards to *Notch1*, our data are consistent with that of Faux et al. (2001), who reported that LIF and members of the TGFβ family could increase the expression of *Notch1* in E10.5 neuroepithelial progenitor cells, but inconsistent in the regulation of *Notch3*, which was also upregulated by LIF and other factors in their system. Although some of these discrepancies may be attributable to the tissue or ontogenetic origin of the precursors, Faux and colleagues (2001) did not explore how growth factors regulated Notch1 signaling or its relevance to NSC function. *In vivo*, we found that CNTF, in the presence of EGF, upregulates the number of NOTCH1-expressing cells [as we reported, it increases NSC numbers (Shimazaki et al., 2001)] in the adult forebrain periventricular area, which is the location of adult NSCs. The ability of sIL6R+IL6 to enhance NOTCH1 and NSC numbers in *LIFRβ*$^{-/-}$ neurospheres demonstrates that gp130 activation mediates the increases in NOTCH1 expression and NSC maintenance. These data concur with the recent findings of Hatta et al. (2002) that signaling by gp130 keeps embryonic precursors in the stem cell state and suggests that this effect may be mediated by NOTCH1.

The observations that CNTF enhances NOTCH1-PF1 expression rapidly and in the absence of cell–cell contact suggests a direct action on NSCs that does not involve lateral inhibition. This further suggests that the decrease of *Delta3* that we observed is a result and not a cause of increased NOTCH1 signaling. On the other hand, we demonstrate that cell–cell contact is required for CNTF to increase NOTCH1-PF2 and NSC self-renewal (Fig. 4*B*, *C*).

EGF and gp130 signaling pathways cooperatively regulate NOTCH1 signaling in NSCs and may establish the pattern of cell contact-mediated signaling during development

The results of this study suggest that the gp130-mediated increase in NOTCH1 signaling is context dependent. CNTF only induced an increase in NOTCH1 expression in the presence of either EGF or FGF2. Figures 4*A–F* and 7*B* demonstrate that EGF alone can

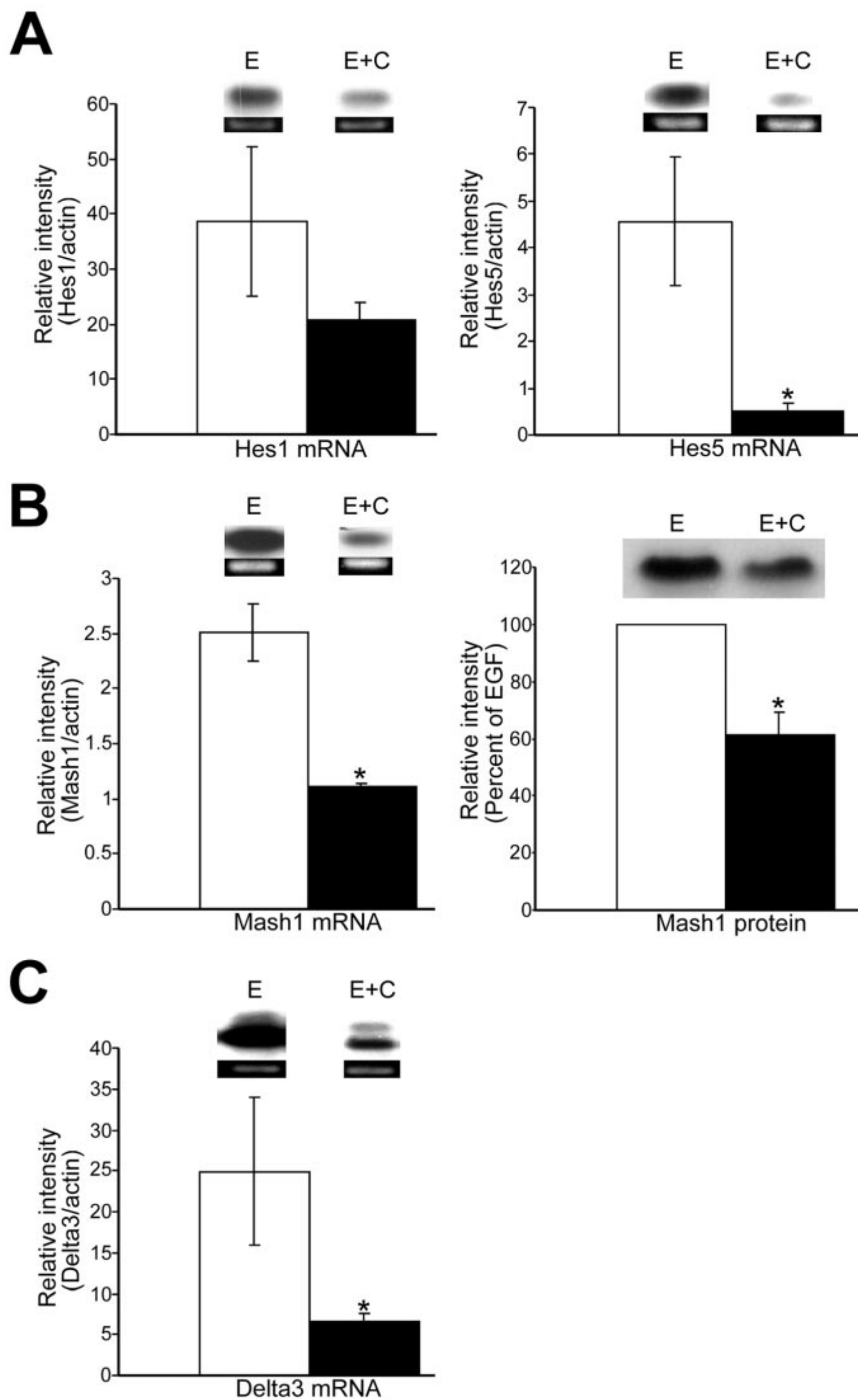

Figure 9. CNTF treatment changes the expression of genes regulated by NOTCH1 signaling. *A–C*, Primary neurospheres derived from the E14 striatum were grown in the presence of EGF for 7 DIV, dissociated, and then cultured (5×10^4 cells/ml) in either EGF or EGF+CNTF. The cells were then harvested for total RNA or protein at 3 DIV and processed for RT-PCR Southern or Western blot analyses as described in Materials and Methods. *A*, Constitutive CNTF treatment significantly decreases *Hes5* expression, whereas *Hes1* expression in CNTF-treated P1 neurospheres does not differ significantly from 3 DIV EGF-derived P1 neurospheres. *B*, *Mash1* expression, mRNA, and protein are significantly reduced in P1 neurospheres cultured for 3 DIV in the presence of EGF+CNTF compared with EGF alone. *C*, *Delta3* expression is reduced in 3 DIV EGF+CNTF P1 neurosphere cultures compared with EGF cultures. $^*p < 0.05$ versus EGF; *t* test (*A–C*; $n = 3$).

dimerized STAT proteins on serine residues, which appears to be necessary for STAT-dependent transcriptional activation (Akira, 1999). Although the upstream 5′ sequence of mouse *Notch1* is unavailable to us, we examined the genomic sequence upstream of *Drosophila Notch* for the presence of STAT binding sites. The existence of two putative STAT-element binding sites, 5′-TTCNNNGAA in *Drosophila* (Kwon et al., 2000) at −954:−962 and −1035:−1043 with respect to the *Notch* start codon (designated as 0), is highly suggestive that gp130-mediated JAK/STAT signaling may directly regulate the transcription of *Notch1* in *Drosophila* and in the mouse. Thus, it is plausible that EGF and CNTF signaling may cooperatively activate the dimerization, translocation, and activation of STAT3 proteins, which may in turn act to promote *Notch1* expression.

EGF- and gp130-mediated signaling may also cooperate to establish the pattern of NOTCH1 signaling within the developing CNS. For example, cells within the ventricular zone, exposed to high levels of CNTF/LIF and EGF signaling, would express high levels of NOTCH1 and through lateral inhibition "determine" how cell–cell contact-mediated signaling would allow distinction/separation of the adjacent progenitor cell pool in the subventricular/mantle zones. Thus cells further removed from EGF/CNTF would become progenitor cells, limited in their capacity to self-renew and more able to express genes, such as *Mash1*, involved in the determination of restricted neural progenitor cells. Indeed, EGF+CNTF decreased *Mash1* mRNA and protein expression in NSC cultures, as this model would predict. A similar concept was suggested previously by Price et al. (1997), with respect to Notch and EGFR signaling in the establishment or maintenance of posterior follicle cell fates in *Drosophila*. They provide evidence suggesting that EGFR signaling influences NOTCH signaling in posterior follicle cells and the establishment of the expression levels of DELTA ligand, which would, in turn, be maintained by lateral inhibition. Thus, as reported in other systems, our results support the contention that cell contact-mediated signaling and non-cell contact-mediated epigenetic signaling pathways are intimately linked in the establishment of neural patterning and development.

increase NOTCH1 expression *in vitro*. Both EGF and CNTF can phosphorylate tyrosine residues on STAT3, which is necessary for the dimerization of STAT3 and its translocation to the nucleus (for review, see Akira, 1999; Turnley and Bartlett, 2000). Additionally, it has been reported that MAP kinase can phosphorylate

Mediation of NSC maintenance and proliferation by NOTCH1 signaling may be independent of Hes1 and Hes5

In this study, we found that neurospheres cultured for 3 DIV in EGF+CNTF demonstrate a fivefold increase in NOTCH1 expression compared with EGF controls. This increase in NOTCH1 ex-

pression is concomitant with a significant decrease in *Hes5* expression and a trend toward a decrease (did not achieve statistical significance) in *Hes1* expression. Given the decrease in the progenitor determination gene *Mash1* (Fig. 9*B*), a gene whose transcription is repressed by *Hes1* (Chen et al., 1997), it is surprising that there was no increase in *Hes* gene expression in the CNTF treated-cultures. However, these observations are not unlike those made by Shawber et al. (1996) whereby NOTCH1 activation by JAGGED1, which kept C2C12 myoblasts from differentiating, did not result in the upregulation of *Hes1*. Stable transfection of C2C12 myoblasts with *Hes1* was also unable to inhibit their differentiation. Additionally, Furukawa et al. (2000) reported that overexpression of activated *Notch1* in the retina increased clone size, whereas overexpression of *Hes1* did not. Finally, there was no decrease found in the expression of *Hes1* in *presenilin1*$^{-/-}$ brains (Handler et al., 2000) or in *RBP-J*$\kappa^{-/-}$ ES cell sphere colonies (Hitoshi et al., 2002) where there were decreases in the self-renewal of isolated NSCs. These studies are consistent with the notion that *Hes1* is not necessarily involved in promoting an undifferentiated state.

The suggestion that *Hes* genes do not function primarily in NSC maintenance is in apparent contrast to the studies by Nakamura et al. (2000) and Ohtsuka et al. (2001), where neurospheres could be generated from embryos mutant for *Hes1* and/or *Hes5*, yet a role for these factors in NSC maintenance was suggested. In both studies, single or double mutant neurospheres were on average smaller; however, the double mutant primary neurospheres could still produce P1 neurospheres (demonstrating self-renewal). In fact, when neurospheres were normalized for total cell number, Ohtsuka et al. (2001) found that single mutants did not show a reduced number of secondary neurospheres. In our previous study (Shimazaki et al., 2001) and in the current report, reduction in self-renewal is defined as a reduced number of P2 neurospheres from single equivalent sized P1 neuropheres, or as a population normalized for cell number. The observations of smaller primary or secondary neurospheres, reported by Nakamura et al. (2000) and Ohtsuka et al. (2001), could be as readily interpreted as *Hes1* and *Hes5* functioning primarily in the maintenance of neural progenitor cells, indeed as suggested by Nakamura et al. (2000), with regard to *Hes1*. Furthermore, the observation that no neurospheres could be obtained from *presenilin1*$^{-/-}$/*presenilin2*$^{+/-}$ mice (Hitoshi et al., 2002), in contrast to *Hes1*/*Hes5* double mutants, strongly supports the contention that other factors can mediate NOTCH1 signaling actions on NSC self-renewal. Kondo and Raff (2000) reported that both *Mash1* and *Hes5* are expressed in oligodendrocyte progenitors and may mediate their differentiation. Additionally, *Hes5* appears to function in later progenitors of the olfactory neuroepithelium (Cau et al., 2000). Therefore, the marked decrease in *Hes5* expression observed in 3 DIV EGF+CNTF compared with EGF cultures may be the result of an increase in NSCs at the expense of progenitor cells with a limited self-renewal capacity, consistent with our previous findings (Shimazaki et al., 2001) that CNTF supports the maintenance of NSCs by suppressing their restriction to glial progenitors.

Considering that two new *Hes* genes, *Hes6* and *Hes7* (Bae et al., 2000; Bessho et al., 2001), have been discovered recently, it is plausible that an as yet unidentified *Hes* gene mediates the increase in NOTCH1 signaling stimulated by CNTF. Given their observation of neurosphere formation in the *Hes1/Hes5* double mutants, Ohtsuka and colleagues (2001) suggest that a splice variant of *Hes3* (*Hes3b*) may contribute to embryonal NSC maintenance. It is also possible that a *SuH/RBP-J*κ-independent pathway, which may not require *Hes*, mediates the CNTF-induced increase in NOTCH1 signaling (Shawber et al., 1996; Matsuno et al., 1997; Ordentlich et al., 1998). Future studies of gp130 regulation of *Hes* genes will likely serve to identify the family member that mediates NOTCH1 regulation of NSC maintenance and self-renewal.

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