SoxB1 transcription factors and Notch signaling use distinct mechanisms to regulate proneural gene function and neural progenitor differentiation

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The preservation of a pool of neural precursors is a prerequisite for proper establishment and maintenance of a functional central nervous system (CNS). Both Notch signaling and SoxB1 transcription factors have been ascribed key roles during this process, but whether these factors use common or distinct mechanisms to control progenitor maintenance is unsettled. Here, we report that the capacity of Notch to maintain neural cells in an undifferentiated state requires the activity of SoxB1 proteins, whereas the mechanism by which SoxB1 block neurogenesis is independent of Notch signaling. A common feature of Notch signaling and SoxB1 proteins is their ability to inhibit the activity of proneural bHLH proteins. Notch represses the transcription of proneural bHLH genes, while SoxB1 proteins block their neurogenic capacity. Moreover, E-proteins act as functional partners of proneural proteins and the suppression of E-protein expression is an important mechanism by which Notch counteracts neurogenesis. Interestingly, in contrast to the Hes-dependent repression of proneural genes, suppression of E-protein occurs in a Hes-independent fashion. Together, these data reveal that Notch signaling and SoxB1 transcription factors use distinct regulatory mechanisms to control proneural protein function and to preserve neural cells as undifferentiated precursors.

KEY WORDS: CNS development, Neurogenesis, Notch, Proneural bHLH proteins, Sox proteins

INTRODUCTION

In the developing central nervous system (CNS) the generation of a large number of different neuronal and glial cells from a small founding population of self-renewing stem and progenitor cells requires molecular programs acting with high spatial and temporal precision. It is well established that an evolutionary conserved program of proneural bHLH transcription factors is necessary for the progression of neurogenesis and promotes cells to leave the cell cycle, downregulate progenitor characters and upregulate the expression of neuronal markers (Bertrand et al., 2002; Guillemot, 2007). However, to avoid premature depletion of the progenitor pool, neural cells are also subjected to mechanisms that counteract neurogenesis. The Notch signaling pathway and the SoxB1 (Sox1, Sox2 and Sox3) transcription factors have key functions during neurogenesis. The Notch signaling pathway and the SoxB1 transcription factors have been ascribed key roles during this process, but whether these factors use common or distinct mechanisms to control progenitor maintenance is unsettled. Here, we report that the capacity of Notch to maintain neural cells in an undifferentiated state requires the activity of SoxB1 proteins, whereas the mechanism by which SoxB1 block neurogenesis is independent of Notch signaling. A common feature of Notch signaling and SoxB1 proteins is their ability to inhibit the activity of proneural bHLH proteins. Notch represses the transcription of proneural bHLH genes, while SoxB1 proteins block their neurogenic capacity. Moreover, E-proteins act as functional partners of proneural proteins and the suppression of E-protein expression is an important mechanism by which Notch counteracts neurogenesis. Interestingly, in contrast to the Hes-dependent repression of proneural genes, suppression of E-protein occurs in a Hes-independent fashion. Together, these data reveal that Notch signaling and SoxB1 transcription factors use distinct regulatory mechanisms to control proneural protein function and to preserve neural cells as undifferentiated precursors.

The proneural proteins are composed of a family of basic helix-loop-helix (bHLH) transcription factors, which in the vertebrate CNS includes the proteins neurogenin1/2 (Ngn1/2), Mash1 and Math1 (Bertrand et al., 2002). These proteins promote neural cells to initiate a differentiation program that ultimately leads to the formation of mature neurons. The capacity of proneural proteins to promote the progression of neurogenesis has been suggested to be determined by their expression or activity levels (Bertrand et al., 2002). Accordingly, low levels of proneural proteins are compatible with self-renewing progenitors, whereas high levels of proneural proteins are reversibly committing neural cells to differentiation. Hence, the expression or activity level of proneural proteins determines, at least partly, whether neural cells remain as progenitors or commit to neuronal differentiation.

A cardinal feature of Notch signaling during CNS development is the capacity to counteract neurogenesis and maintain neural cells in an undifferentiated state. At later stages, Notch signaling may also act instructively, to promote gliogenesis and affect functions in mature neurons (Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). Notch signaling is dependent on cell-cell communication, where the interaction between the Notch receptor and its ligands on adjacent cells induce proteolytic processing of the Notch protein. The final proteolytic cleavage is accomplished by the γ-secretase complex and results in the release and nuclear translocation of the Notch intracellular domain (NICD). In the nucleus, NICD interacts with the DNA-binding protein CSL (CBF1/Suppressor of Hairless/Lag-1), which converts CSL from a transcriptional co-repressor to an activator by a NICD-induced displacement of a transcriptional co-repressor complex (Bray, 2006). An important transcriptional output of Notch signaling is the upregulation of the bHLH transcription factors Hes1 and Hes5 (Louvi and Artavanis-Tsakonas, 2006). Hes1 and Hes5 function as classical DNA-binding repressors that antagonize the expression of proneural genes (Ohmukai et al., 1999). However, Hes transcription factors have also been suggested to oppose the progression of neurogenesis by forming non-functional pairs with proneural proteins or E-proteins (Fischer and Gessler, 2007; Sasai et al., 1992). E-proteins are ubiquitously expressed bHLH proteins, which have been proposed to function as obligatory heterodimerizing partner factors of proneural proteins. Thus, the ability of Notch signaling to maintain neural cells in an undifferentiated state appears, at least in part, to be achieved through a reduction in expression and...
activity levels of proneural proteins. However, whether this regulatory mechanism fully explains the capacity of Notch to maintain neural progenitor cells in an undifferentiated state is currently not understood.

The HMG-box transcription factors of the Sox gene family have several regulatory roles during CNS development (Wagner and Stolt, 2005). SoxB1 proteins that are expressed by a majority of neural stem and progenitor cells, both in the developing and adult CNS, counteract neuronal differentiation and maintain neural progenitor specific gene expression (Bylund et al., 2003; Graham et al., 2003). Another HMG-box protein, Sox21, has the opposite activity compared with Sox1-3, and is required for neuronal differentiation (Sandberg et al., 2005). The balance of Sox1-3 and Sox21 activities function as a determinant of whether neural cells should remain as progenitors or commit to differentiation. Interestingly, apart from counteracting the activity of Sox21, SoxB1 transcription factors also suppress neurogenesis by blocking the activity of proneural proteins (Bylund et al., 2003; Guillemot, 2007). Hence, a common feature in the ability of Notch signaling and SoxB1 proteins to regulate the commitment of progenitors to neurogenesis appears to be their capacity to modulate the activity of proneural proteins.

These findings and the functional similarities between Notch signaling and SoxB1 transcription factors evoke the question how do these proteins functionally interact to regulate neurogenesis, if at all? Here, we report that the ability of Notch to maintain neural cells in an undifferentiated state can be explained by its capacity to repress the expression of both proneural bHLH proteins and E-proteins. Notch signaling regulates the expression of these proteins by Hes-dependent and -independent mechanisms, respectively. Based on these findings, we suggest a model in which SoxB1 proteins preserve neural cells in a precursor state by maintaining the expression of progenitor properties, whereas the role of Notch is to control the balance of undifferentiated and differentiated neural cells by regulating the expression levels of proneural bHLH proteins and E-proteins.

MATERIALS AND METHODS

Expression constructs and in ovo electroporation

All cDNAs misexpressed in the chick neural tube were expressed from the CMV-IE enhancer/chick β-actin promoter in the pCAGGS vector (Niwa et al., 1991). Myc-tagged mouse N1ICD (NICD) (Kopan et al., 1996) and the R218H dominant-negative mouse CSL mutant (dnCSL) (Chung et al., 1994), generated by site-directed mutagenesis (QuickChange, Stratagene) of a wild-type CSL template, were both cloned into the pIRE5-EGFP plasmid (Clontech) prior to subcloning into pCAGGS. Hes5ΔCT-VP16 was generated by replacing the five final codons, encoding the WRPWM motif of Hes5, with the VP16 activator domain using the pSlax-VP16 shuttle vector (Bergsland et al., 2006). NICDΔCT-EnR was generated by subcloning the cDNA encoding the N-terminal part of NICD, consisting of the RAM and ankyrin repeat domains [referred to as 1100 in Beatus et al. (Beatus et al., 2001)], to the pSlax-EnR shuttle vector (Bergsland et al., 2006). Hes5ΔCT-EnR construct was a kind gift from Henrique D. (Fior and Henrique, 2005). The chick Hes1 construct was a kind gift from Dr J. Ericson (Karolinska Institute). A myc-tagged mouse E47 construct was subcloned by PCR using IMAGE: 4187386 as a template. Sox3-myc, Ngn2-myc, Sox21-myc, Sox3-EnR and 12xCSL-DsRedExpressDR have been described elsewhere (Bylund et al., 2003; Hansson et al., 2006; Sandberg et al., 2005). DNA constructs were electroporated into the neural tube of HH stage 10 chick embryos. After 5-48 hours, embryos were fixed and processed for immunohistochemistry or in situ hybridization.

Neural explants

Neural tube explants were isolated from the posterior part of HH stage 10 chick embryos, embedded in collagen and cultured in F12 media (Gibco) supplemented with N2 (Gibco). The gamma secretase inhibitor DAPT (Calbiochem) was added at concentrations ranging from 0-6 μM. After culture the explants were subsequently fixed in 4% paraformaldehyde and processed for cryosectioning and antibody staining.

Immunohistochemistry and in situ hybridization

Antibody staining was performed as described previously (Tsushima et al., 1994). The following antibodies were used; rabbit anti-Sox3 (kindly provided by T. Edlund, Umeå University), rabbit anti-Sox5 (Bylund et al., 2003), rabbit anti-Ngn2 (Sandberg et al., 2005), mouse anti-NeuN (Chemicon), rabbit anti-VP16 (Abcam), mouse anti-PCNA (DAKO), mouse anti-Tuj1 (Covance), mouse FITC-anti-BrdU (Becton Dickson), mouse anti-Myc (Santa Cruz Biotech) and rabbit anti-Myc (Santa Cruz Biotech). In situ hybridization was performed as described (Tsushima et al., 1994) using chick probes for Ngn1, Ngn2, Cash1, Hes1, Hes5, E47, Notch1 and Sox3 (Jasoni et al., 1994; Kamachi et al., 1998; Perez et al., 1999). cDNAs encoding chick Hes1, Hes5, E47 and Notch1 were obtained from MRC Geneservice; clone IDs: chEST35615, chEST382121, chEST719E2 and chEST891H8.

BrdU incorporation

BrdU (100 μM) was applied to chick embryos in ovo, followed by incubation for 30 minutes at 38°C at which time the embryos were fixed.

Ngn2 and NeuroD promoter activity assay

The Ngn2 promoter construct was generated by PCR amplification of a 1.2 kb upstream sequence of the mouse Ngn2 gene. The sequence, which includes the endogenous proximal promoter together with 91bp of 5' UTR, was subcloned into the pGL3TK-Basic vector (Promega). Luciferase assays were conducted in transfected 293 HEK or P19 cells using Lipofectamine PLUS (Invitrogen). pGL3Ngn2prom1.2kb was transfected with NIDC, Hex5 or Hes5ΔCT-VP16 expression vectors. pGL3NeuroDprom1.0kb (Huang et al., 2000) was transfected with different concentrations of Ngn2, E47 and Sox3 expression vectors. As an internal transfection control an expression plasmid encoding β-gal was included in all transfections. Twenty hours after transfection, luciferase and β-galactosidase levels were determined as described previously (Castro et al., 1999).

RESULTS

Elevated levels of Notch and SoxB1 activity block neuronal differentiation

To begin to examine whether SoxB1 proteins and components of the Notch signaling pathway use distinct or similar mechanisms to regulate neurogenesis, we first compared their expression pattern, which is shown in Fig. 1. Both Notch and Sox3 block neurogenesis. (A-E) Notch1 (A), Hes5 (B), Sox3 (C), Ngn2 (D) and E47 (E) exhibited complementary expression patterns in the embryonic day 4.0 chick spinal cord. (F-H) Expression of the intracellular domain of Notch1 (NICD) (F) or Sox3 (G) for 42 hours significantly reduced the generation of Tuj1+ neurons (H). Data are represented as percentage of electroporated cells expressing Tuj1 (mean±s.e.m.). **P<0.01 relative to EGFP control transfected cells, Student's t-test. Scale bars: 50 μm.
both in relation to each other and to proneural bHLH transcription factors and E-proteins. In Hamburg-Hamilton (HH) stage 20 chick spinal cords, Notch1 and Hes5 were expressed in progenitor cells within the ventricular zone, in a pattern extensively overlapping with that of the B1-subgroup member Sox3 (Fig. 1A-C). The genes encoding the proneural bHLH factors Ngn2 and the E-protein E47 were also expressed in a pattern overlapping with that of Notch, Hes5 and Sox3, although their expression was most pronounced in progenitor cells located in the lateral margin of the ventricular zone (Fig. 1D,E).

We next compared the effects on neurogenesis of increased levels of Notch or SoxB1 activity. Using chick embryo electroporation, an expression vector containing the intracellular domain of the Notch1 receptor (NICD) (Schroeter et al., 1998), together with an IRES-EGFP cassette, was transfected in the caudal part of the neural tube. Misexpression of NICD significantly decreased the number of cells expressing neuronal markers (Fig. 1F,H). In a similar manner, neural progenitor cells transfected with a myc-tagged version of Sox3 did not upregulate neuronal marker expression (N,O) or exit the cell cycle (P), regardless of the DAPT concentration. (Q) Statistical representation of NeuN-expressing cells in neural tubes transfected with EGFP, NICD, dnCsl, dnCsl/Sox3 or NICD/HMGSox3-EnR. Data are represented as means±s.e.m. **P<0.01, ***P<0.001, Student’s t-test relative to 0 μM DAPT control in O and P and relative to EGFP-transfected control cells in Q. Student’s t-test. Scale bars: 40 μm in L; 10 μm in N.

Notch-mediated control of differentiation requires Sox3 activity
To further explore whether Notch signaling and SoxB1 transcription factors use similar mechanisms to control neurogenesis, we next determined whether the capacity of Notch to control neurogenesis is dependent on the activity of SoxB1 proteins. Overexpression of NICD alone maintained the expression of progenitor characters (Fig. 2A,B), blocked the formation of neurons (Fig. 2C,Q), but did not increase the endogenous expression levels of SoxB1 proteins (Fig. 2A; and data not shown). By contrast, cells co-transfected with NICD and a dominant-negative version of Sox3 (HMGSox3-EnR) downregulated progenitor characters (Fig. 2D,E, data not shown) and instead upregulated the expression of neuronal markers (Fig. 2F,Q, data not shown). Similar results were obtained when NICD was co-electroporated with Sox21 (see Fig. S1 in the supplementary material). Thus, under conditions of blocked SoxB1 activity, NICD is unable to maintain neural cells in an undifferentiated state.
Notch, but not SoxB1 activity, represses proneural bHLH and E-protein expression

As both Notch signaling and SoxB1 transcription factors counteract neurogenesis by regulating proneural activity (Bylund et al., 2003; Ross et al., 2003), we next determined how Notch and Sox3 proteins affected the expression of the proneural factors Ngn2 and Cash1, and the genes encoding the E-proteins E47 and Tcf12. Forty-two hours after NICD electroporation, both Ngn2 and Cash1 were significantly downregulated (Fig. 3A; and data not shown), whereas these genes were induced already 10 hours after misexpression of dnCSL or NICDAC-EnR (Fig. 3C; and data not shown). Interestingly, the expression profiles of E47 and TCF12 mimicked those of the proneural genes, and were down- and upregulated in response to NICD and dnCSL misexpression, respectively (Fig. 3B,D; and data not shown). By contrast, overexpression of Sox3 did not block the expression of proneural or E-protein-coding genes (Fig. 3E,F). Hence, the expression of proneural bHLH proteins and E-proteins was efficiently downregulated by active Notch signaling, but not by Sox3, indicating that Notch and SoxB1 proteins use different strategies to control the activity of proneural bHLH factors and E-proteins.

Combined expression of Ngn2 and E47 rescues Notch-induced block of neuronal differentiation

The finding that Notch suppresses the expression of both proneural and E-protein genes evokes the question is this sufficient for Notch to counteract neuronal differentiation? To examine this issue, NICD was misexpressed alone (Fig. 4A-C) or together with either Ngn2 (Fig. 4D-F) or E47 (Fig. 4G-I), or together with both Ngn2 and E47 (Fig. 4J-L). When misexpressed alone, Ngn2, and to some extent also E47, promoted cells to migrate laterally from the ventricular zone, downregulate progenitor features and upregulate the expression of neuronal markers (see Fig. 5A-F in the supplementary material). The capacity of Ngn2 or E47 to promote neurogenesis individually was, however, efficiently blocked by co-electroporated NICD (Fig. 4A-I,T) and the cells were independent of the amounts of transfected Ngn2 or E47 expression vectors (0.7 or 1.5 μg/μl) maintained as self-renewing and Sox3+ progenitor cells (Fig. 4A,B,D,E,G,H) that failed to upregulate the expression of neuronal markers (Fig. 4C,F,I,T). By contrast, the combined expression of Ngn2 and E47 efficiently rescued the NICD-induced block of neurogenesis, and the transfected cells strongly downregulated progenitor characters and instead upregulated the
expression of pan-neuronal proteins (Fig. 4J-L,T). Thus, the combination of the proneural protein Ngn2 and the E-protein E47 is sufficient to rescue the block of neurogenesis induced by NICD overexpression.

To exclude the possibility that the presence of misexpressed Ngn2 and E47 interfered with Notch-mediated downstream signaling, we monitored the activity of a 12XCSL-DsRed reporter construct, which reflects NICD-induced CSL activity (Hansson et al., 2006). When misexpressed alone, the reporter activity of 12xCSL-DsRed was low or undetectable (data not shown), in keeping with Hansson et al. (Hansson et al., 2006). However, in the presence of either co-electroporated NICD or NICD/Ngn2/E47 both progenitor cells and post-mitotic neurons expressed high levels of DsRed (Fig. 4M-R), indicating that neither Ngn2 nor E47 inhibits NICD-mediated downstream signaling.

As the combined expression of E47 and Ngn2 promoted neurogenesis also in the presence of NICD overexpression, we next examined whether these bHLH factors also could rescue the Sox3-induced block in neuronal differentiation. In contrast to Notch, Sox3 completely blocked neuronal differentiation both when misexpressed with Ngn2 or E47 alone, or in combination with both Ngn2 and E47 (Fig. 4S,T). Together these data indicate that Notch-signaling maintains neural cells in an undifferentiated state by repressing the expression of proneural bHLH and E-proteins, whereas Sox3 proteins can suppress the progression of neurogenesis by blocking the capacity of proneural bHLH and E-proteins to promote neurogenesis.

**Notch controls E47 expression in a Hes-independent manner**

As Hes genes are key components of the Notch downstream response, we next asked if the Notch-mediated repression of Ngn2 and E47 expression is achieved through the regulation of Hes gene activity. To answer this question, we first examined the expression of Hes1 and Hes5 in NICD electroporated neural cells. Misexpression of NICD for 24 hours strongly upregulated the expression of both Hes1 and Hes5 in the neural tube (Fig. 5A,B), whereas misexpression of dnCSL or NICD^ΔCT,EnR decreased the levels of both Hes1 and Hes5 (Fig. 5C,D; and data not shown). To determine whether Hes proteins could substitute for Notch in this regard, we transfected neural cells with a Hes5-IRES-EGFP expression vector (Hes5). Hes5 misexpression efficiently reduced the amount of Ngn2^+ cells (Fig. 5E,G) and also suppressed the generation of Tuj1^+ neurons (Fig. 5H,I). Interestingly, however, Hes5 did not alter the expression of E47 (Fig. 5F). Similar results were obtained with Hes1 or the combined misexpression of Hes1 and Hes5 (see Fig. S6 in the supplementary material). The finding
that Hes5 reduced expression of Ngn2 but not that of E47 prompted us to determine capacity of Ngn2 or E47 to rescue Hes5-mediated repression of neurogenesis. Cells transfected with Hes5 differentiated efficiently into neurons when co-transfected with Ngn2 (Fig. 5J-L). By contrast, neural cells transfected with Hes5 in the combination with E47 failed to upregulate the expression of Tuj1 and instead remained as Sox3+ progenitor cells (Fig. 5M-O). Similar results were obtained with E47 co-transfected with Hes1 (see Fig. S6 in the supplementary material). Hence, Notch signaling appears to control neurogenesis via the combined Hes-dependent downregulation of Ngn2 and the Hes-independent downregulation of E-proteins.

A dominant active version of Hes induces Ngn2 but not E47 expression

To further address the mechanistic role of Hes proteins both during neurogenesis and in the Notch-mediated repression of proneural proteins and E-proteins, we next explored the effects of a dominant active form of Hes5. This construct was generated by replacing the Groucho-interacting C-terminal WRPW-motif with the transactivation domain of the viral protein VP16 (Hes5ΔCT-VP16) (Berk et al., 1998). In order to verify its function as a dominant active version, 293 HEK cells were transfected with expression vectors encoding Hes5ΔCT-VP16, full-length Hes5 or NICD together with a Luc reporter, containing a 1200 bp upstream region (−1 to −1200) of the mouse Ngn2 gene (Ngn21200bp-Luc). Both NICD and Hes5 repressed the Ngn21200bp-Luc reporter, whereas the activity of this reporter was upregulated by Hes5ΔCT-VP16 (Fig. 6A). Furthermore, electroporation of Hes5ΔCT-VP16 in the neural tube rapidly induced cells to exit the cell cycle and upregulate the expression of Ngn2 (Fig. 6B,C) and the neuronal marker Tuj1 (Fig. 6E). However, misexpression of Hes5ΔCT-VP16 did not induce the expression of E47 (Fig. 6D). Thus, a dominant active version of Hes5 promotes neurogenesis and induces the expression of Ngn2, but not that of E47.

Misexpression of NICD suppressed the expression of both Ngn2 and E47, whereas Hes5ΔCT-VP16 only upregulated Ngn2 expression (Fig. 6C,D). These findings strengthen the notion that Notch represses the expression of E-proteins in a Hes-independent manner. To further examine this issue, we next analyzed the ability of Hes5ΔCT-VP16 to rescue NICD-induced block of neurogenesis. In line with the previous findings, co-electroporation of Hes5ΔCT-VP16 and NICD did not promote transfected cells to commit to neurogenesis (Fig. 6F,G,H). Neither did the combined misexpression of Hes5ΔCT-VP16, NICD and Ngn2 result in an upregulation of neuronal markers (Fig. 6F,I). By contrast, co-electroporation of Hes5ΔCT-VP16 and NICD together with E47 caused many cells to upregulate the expression of neuronal markers, 42 hours after transfection (Fig. 6F,J). Together, these findings strongly argue that Notch maintains neural cells in an undifferentiated state both by suppressing the expression of E-proteins and by activating the expression of Hes proteins that, in turn, specifically repress proneural bHLH gene expression (Fig. 6K).

DISCUSSION

In this report, we have addressed the functional relationship between Notch signaling and SoxB1 transcription factors in the regulation of vertebrate neurogenesis. Although elevated expression levels of either Notch or Sox3 leads to a block of neuronal differentiation, we demonstrate that they regulate neurogenesis by distinct mechanisms. In Fig. 6K, we propose a model for the distinct role of Notch and Sox3. This model receives from the findings that overexpression of Sox3 maintains neural cells as precursors even in the absence of Notch signaling, whereas NICD requires Sox3 activity to maintain neural cells in an undifferentiated state. Furthermore, although both Notch signaling and Sox3 proteins suppress the activity of proneural proteins, they do so at distinct regulatory levels. Although Notch signaling represses the transcription of proneural bHLH and E-proteins, SoxB1 proteins suppress their ability to promote progenitor cells to commit to a neurogenic program. Consequently, the concomitant expression of Ngn2 and E47 could rescue NICD-, but not Sox3-, induced block of neurogenesis.

Whether Sox3 suppresses the neurogenic activity of Ngn2 and E47 through a direct block of E47/Ngn2-protein activities or induces a molecular environment in which E47 and Ngn2 proteins are unable to promote neuronal differentiation is currently unknown. We favor the latter idea as Sox3 failed to block Ngn2/E47 proteins from transactivating the E-box containing NeuroD promoter (Huang et al., 2000) in vitro (data not shown). Furthermore, the Sox3-mediated block of neuronal differentiation could not be counteracted by high
levels of misexpressed Ngn2 and E47. In addition, blockage of Notch signaling, which induces high levels of Ngn2 and E47 expression, failed to rescue Sox3-mediated inhibition of neurogenesis. Thus, overexpression of Ngn2 and E47 is unable to rescue the block of neuronal differentiation mediated by high levels of Sox3, suggesting that Sox3 also maintains neural cells in an undifferentiated state by a Ngn2/E47 independent mechanism. It should be noted, however, that the gamma-secretase inhibitor has previously been reported to cause a subset of cultured Sox2 transduced neural cells to upregulate the expression of the pan-neuronal marker Map2 (Bani-Yaghoub et al., 2006). One possible explanation for these discrepancies is that elevated expression of proneural proteins and E-proteins induced by blocked Notch signaling can be counteracted by Sox3 proteins misexpressed at sufficient levels. Nevertheless, the mechanism by SoxB1 proteins maintain neural cells in an undifferentiated state remains to be elucidated, but it is of note that binding sites for SoxB1 proteins are frequently found in regulatory enhancer regions of genes expressed in neural progenitor cells (Bailey et al., 2006), suggesting a broader repertoire of SoxB1 downstream genes. Interestingly, misexpression of a dominant-negative version of Sox3 (HMGBox3-EnR) or Sox21, which represses genes normally activated by SoxB1 proteins (Bylund et al., 2003; Graham et al., 2003; Sandberg et al., 2005), caused neuronal cells to differentiate even in the presence of high levels of Notch signaling. One interpretation of these results is that Notch has a more defined role in balancing the maintenance versus differentiation of neural cells, by predominantly acting on Ngn2 and E47, respectively. SoxB1 transcription factors are according to this model, maintaining progenitor cells in an undifferentiated state by activating the expression of progenitor features and, in addition, blocking the activity of Ngn2 and E47. Scale bars: 20 μm in D; 50 μm in G.

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**Fig. 6. A dominant-active version of Hes5 upregulates Ngn2 but not E47 expression.** (A) NICD and Hes5 repressed the activity of the Ngn2 reporter construct in 293 HEK cells, whereas this reporter was activated by Hes5$^{Act}$-VP16. Data are represented as a logarithmic scale where mock is set to one. (B-E) Within 5 hours, Hes5$^{Act}$-VP16 transfected cells had upregulated the expression of Ngn2 (B, C) but not that of E47 (D); ectopic expression of neuronal markers could be detected 12 hours after transfection (E). (F) Quantification of electroporated cells (GFP+) in G-J expressing Tuj1. Data are represented as mean±s.e.m. *** *P<0.001, relative to NICD electroporated cells, Student's t-test. (G) Forty-two hours after electroporation, a majority of cells expressing Hes5$^{Act}$-VP16 was terminally differentiated and expressed Tuj1. (H) Cells co-transfected with NICD together with Hes5$^{Act}$-VP16 cells remained undifferentiated. (I) Similarly, cells co-transfected with Ngn2, NICD and Hes5$^{Act}$-VP16 remained undifferentiated and failed to upregulate the expression of Tuj1. (J) Misexpression of E47, Hes5$^{Act}$-VP16 and NICD promoted cells to commit to neurogenesis. (K) Proposed molecular pathway regulating neurogenesis in the vertebrate CNS. The proneural bHLH protein Ngn2 acts together with the E-protein E47 to drive the differentiation of neural progenitor cells by promoting cell cycle exit and the upregulation of neuronal protein expression. Notch signaling maintains neural cells in an undifferentiated state via the activation of CSL. Activated CSL is, in turn, inducing the expression of Hes1/5 and an alternative repressor (designated X), which subsequently represses the expression of Ngn2 and E47, respectively. SoxB1 transcription factors are according to this model, maintaining progenitor cells in an undifferentiated state by activating the expression of progenitor features and, in addition, blocking the activity of Ngn2 and E47. Scale bars: 20 μm in D; 50 μm in G.
factors. Our data show, in accordance with previous findings, that Ngn2 expression is repressed through the activation of Hes proteins (Yoon and Gaiano, 2005). By contrast, the Notch-mediated repression of E47 transcription is mediated by a Hes-independent mechanism, as supported by our findings that neither Hes5 nor its dominant active version (Hes5ΔAn-VP16) altered the expression levels of E47 or Tcf12. Moreover, the capacity of Hes5 to block neurogenesis could be counteracted by Ngn2 alone. As misexpression of a dominant-negative version of CSL (dnCSL) upregulated both the expression of Ngn2 and E47 and promoted neurogenesis, we interpret this to suggest that the repression of both Ngn2 and E47 by Notch is mediated by the activation of CSL. As Notch activation in most situations converts CSL from a repressor to an activator, it may be difficult to envisage Notch activation in most situations converts CSL from a repressor to an activator.

In hematopoiesis, in which Notch promotes differentiation towards the T-cell lineage at the expense of B-cell differentiation, the functional role of E47 and E-proteins has not been thoroughly examined. Our data provide evidence for the regulation of E-protein expression as an important mechanism by which Notch signaling controls neural progenitor cell differentiation. The regulatory relationship between Notch and E-proteins is further underscored by observations in early hematopoiesis, in which Notch promotes differentiation towards the T-cell lineage at the expense of B-cell differentiation. In this process, instead of being transcriptionally controlled, E47 is rapidly ubiquitinated in a Notch-dependent manner and proteasomally degraded (Nie et al., 2003; Ordentlich et al., 1998). Thus, depending on the cellular context, Notch can operate both transcriptionally and post-transcriptionally to control the presence of E-proteins.

Although Hes1 and Hes5 proteins are transcriptional repressors, a number of studies based on in vitro experiments have emphasized their ability to form non-functional heterodimers with proneural proteins and E-proteins (Akazawa et al., 1992). These findings stress the capacity of Hes proteins to regulate the activity of proneural bHLH and E-proteins also at the post-transcriptional level. In this report, we have shown that misexpression of Hes5 downregulated Ngn2 and that Ngn2 was sufficient to rescue the Hes-mediated block of neuronal differentiation. Furthermore, a dominant-active version of Hes5 (Hes5ΔAn-VP16), with its putative protein dimerization domain retained, promoted neural cells to commit to differentiation. Hence, misexpression of Hes5, or derivatives of Hes5, does not block the function of proneural transcription factors or E-proteins. Together, these results argue that post-transcriptional mechanisms are unlikely to be the main mechanisms by which Hes proteins regulate neurogenesis in the developing CNS and indicate that the primary role of Hes proteins during neurogenesis is rather to act as transcriptional repressors.

Our results argue for a model in which SoxB1 transcription factors preserve the undifferentiated state by maintaining the expression of neural progenitor identities and blocking the capacity of proneural proteins to promote neurogenesis. In this model, the main role of Notch is, through the regulation of proneural- and E-protein expression, to ascertain that the correct number of cells initiate neuronal differentiation at a specific stage (Fig. 6K). This is similar to the role of Notch in Drosophila, where Notch signaling, via lateral inhibition, sorts out progenitor cells that should remain undifferentiated from those that should commit to differentiation (Chitnis, 1995). Notch, Sox and bHLH proteins are also expressed in muscle and neural crest progenitor populations, and have in these tissues been ascribed similar regulatory roles as in the developing and adult CNS (Beranger et al., 2000; Braun et al., 1990; Cheung and Briscoe, 2003; Delfini et al., 2000; Heeg-Truedell and LaBonne, 2004; Hirsinger et al., 2001; Hong and Saint-Jeannet, 2005; Lassar et al., 1991; Schmidt et al., 2003). Thus, it is likely that Notch signaling and Sox transcription factors are required for the maintenance and the coordinated differentiation of progenitors also outside the CNS. Interestingly, Notch signaling appears not to be a requirement for maintaining embryonic stem cells in a undifferentiated state, as CSL-deficient ES cells can be preserved in a self-renewing and pluripotent state (Hitoshi et al., 2002; Lowell et al., 2006). Instead ES cells rely on Sox2, which, together with the transcription factors Oct3/4, preserves cells in a self-renewing pluripotent state (Masui et al., 2007; Matoba et al., 2006). Thus, in the absence of a differentiation program, regulated by tissue-specific bHLH factors and E-proteins, Notch signaling is not a prerequisite for maintaining cells in an undifferentiated state.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/10/1843/DC1

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