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Blocking HES1 Expression Initiates GABAergic Differentiation and Induces the Expression of p21$^{CIP1/WAF1}$ in Human Neural Stem Cells*

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Mammalian neural stem cells can develop into a variety of neuronal and glial cell types. This involves a highly coordinated process of differentiation in which the Notch signaling pathway and the system of helix-loop-helix (HLH) transcriptional regulators play a key role. By exercising control over proliferation, initiation of differentiation, neurite outgrowth, and synaptogenesis, the network of HLH transcription factors regulates the fate of neural stem cells and progenitors. Here we show that the HLH transcription factor HES1 regulates the proliferation of human neural stem cells and that blocking its expression stimulates the expression of cyclin-dependent kinase inhibitor p21$^{CIP1/WAF1}$. Furthermore, we demonstrate that the suppression of HES1 expression initiates differentiation of neural stem cells into neurons, the majority of which develop the GABAergic phenotype. These findings underscore the importance of the HLH network, and HES1 in particular, in guiding the phenotypic development of neural stem cells.

In the mammalian central nervous system, neuronal and glial cells develop from a single progenitor, the central nervous system stem cell. Differentiation of individual cell types is a highly coordinated process that involves proliferation of progenitors, migration of progenitors and postmitotic cells, and terminal differentiation, as well as formation of functional connections. Several signaling systems and networks of transcriptional regulators are operational during the process of neural stem cell differentiation and formation of the nervous system (1–4). Analyses of a variety of model systems, including flies and mammals, have identified helix-loop-helix (HLH)[1] transcription factors as regulators of early differentiation of neural stem cells (1, 2). HLH transcription factors form a network of positively and negatively acting transcriptional regulators that mediates and coordinates the effect of several signaling systems on a large number of target genes. HLH transcription factors participate at different stages of neural stem cell differentiation, including formation of progenitor cells, proliferation of progenitor cells, initiation of differentiation, cell fate decisions (neuron *versus* glia and different subtypes of neurons), neurite outgrowth, and synaptogenesis (1, 2, 5–10). Recently it has been shown that Notch signaling regulates the proliferation of neural stem cells (7, 11, 12). Activated Notch translocates into the nucleus and activates transcription of the HES family of HLH genes (13–15). Overexpression of HES generates more neurons, whereas lack of HES activity results in premature differentiation and lower than normal number of neurons in the developing nervous system (16–18). Also, differentiation of neural stem cells is affected by Notch signaling and expression of HES transcriptional regulators. Activation of Notch leads to glial differentiation and blocking of neuronal differentiation (19, 20). Numerous data demonstrate that the network of HLH transcription factors determines the fate of different populations of progenitor cells. In the developing spinal cord balance of Neurogenin, ATH and ASH family members of HLH genes specifie the fate of specific neuronal populations (21, 22). During the cerebellar development MATH1 regulates differentiation of granular cells and expression of NeuroD (23). Since HES1 is part of the network of HLH transcription factors, its expression and activity likely affects differentiation of specific cell types.

Here we asked two specific questions: first, how does HES affect differentiation of specific types of neurons and second, what is the mechanism of action of HES on neural stem cell proliferation. We demonstrate that blocking HES1 in human neural stem cells *in vitro* stimulates differentiation of GABAergic neurons and suppresses stem cell proliferation by stimulation of expression of cyclin-dependent Cdk kinase inhibitor p21$^{CIP1/WAF1}$.

EXPERIMENTAL PROCEDURES

Cell Culture

Human Neural Stem Cells—Resected human fetal brain tissue was placed into ice cold DMEM/F-12 with penicillin/streptomycin for further dissection. The tissue was cut into small pieces and trypsinized (0.02 mg/ml trypsin in versene (Invitrogen) at 37 °C for 10 min. After adding trypsin inhibitor mixture (Clonetics) tissue was mechanically triturated. Cell suspension was centrifuged at 400 rpm for 5 min, the pellet was washed once with DMEM/F-12 and plated at a density of 5000–10,000 viable cells/ml in the growth medium DMEM/F-12, B27 supplement (Invitrogen), 20 ng/ml of human LIF (PeproTech), 20 ng/ml of human basic fibroblast growth factor (PeproTech), penicillin/streptomycin). Stem cells were grown as neurospheres, medium changed every 3 days, spheres dissociated by mechanical trituration after every 12–15 days.

Differentiation of Human Neural Stem Cells—Neurospheres were mechanically dissociated and plated on laminin-coated (30 μl/1 ml of PBS, Roche Molecular Biochemicals) 24-well plates in differentiating medium (DMEM/F-12, B27 supplement, all-*trans*-retinoic acid (10^{-6} M) and dibutyryl cyclic AMP (Bt_2cAMP, 1 mM)).

Antisense Oligonucleotide Treatment—*S*-Oligonucleotides (phosphothioates) (Sigma Genosys) with following sequences were used: anti-Hes1-1, 5′-ACC GGG GAC GAG GAA TTT TTC; anti-Hes 1-2, 5′-CAC GGA GGT GCC GCT GTT GCT GGT GTA GAC GGG GAT GAC; control *S*-oligonucleotide contained a scrambled sequence 5′-TCG GAG ACT TTC TGT CGG GCT GAT CGG TCG GGC TGG GGA G. Oligonucleo-

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[1] The abbreviations used are: HLH, helix-loop-helix; GABA, γ-aminobutyric acid; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; Bt_2cAMP, dibutyryl cyclic AMP; BrdUrd, bromodeoxyuridine; GAD, glutamic acid decarboxylase.

tides were added to the growth or differentiation culture media at a final concentration of 5 μM. The medium with the oligonucleotides was replaced every day during the experiment.

For antisense oligonucleotide treatment of neurospheres the cells (neurospheres) were transferred into 24-well plates in growth media containing antisense or scrambled oligonucleotides for 4 days. For further analyses the cells were either plated onto poly-D-lysine coated 24-well plates or centrifuged onto glass slides and fixed in cold acetone/methanol for 2 min.

To treat differentiating cells neurospheres were mechanically dissociated and plated on laminin-coated (30 μl/1 ml of PBS, Roche Molecular Biochemicals) 24-well plates in differentiating medium (DMEM/F-12, B27 supplement, all-*trans*-retinoic acid (10^{-6} M) and Bt_2cAMP (1 mM)). Antisense or scrambled oligonucleotides were added to the differentiation media for 4 days after which the cells were used for further analyses.

Immunocytochemistry

Cells were washed with PBS (Invitrogen) and fixed for 2 min in cold acetone/methanol. After a wash in PBS, they were blocked for 30 min in 4% donkey serum. Primary antibodies against β-III-tubulin (1:100, mouse monoclonal, Chemicon); MAP-2 (1:100, mouse monoclonal, Chemicon); GFAP (undiluted, rabbit monoclonal, Roche Molecular Biochemicals); BrdUrd (1:100, mouse monoclonal, Dako); Ash1 (1:200, rabbit polyclonal CeMines), anti-Hes1 (1:1000, rabbit polyclonal, CeMines), anti-$p21^{CIP1/WAF1}$ (1:100, NeoMarkers), anti-$p27^{KIP1}$ (1:100, NeoMarkers), and GAD65&67 (1:100, rabbit polyclonal, Chemicon) were applied for 90 min. Than the cells were washed in PBS and incubated in Cy3-conjugated secondary antibodies for 30 min (Chemicon), washed in PBS, and examined under a fluorescent microscope. The cells were counterstained with DAPI (Dako) to identify nuclei.

Western Blot Analyses

Cells were washed with PBS, collected, and resuspended in Laemli sample buffer (Bio-Rad) containing β-mercaptoethanol (Sigma, 50 μl/1 ml of sample buffer). Samples were separated on SDS-PAGE gels (Bio-Rad) and transferred onto a Hybond membranes (Amersham Biosciences, Inc.). Membrane was blocked for 2 h (5% solution of dry milk and PBS with 0.05% Tween 20), washed with PBS/Tween, and incubated in primary antibody (anti-Hes1, 1:1000, rabbit polyclonal, CeMines) overnight. After washing in PBS/Tween the membrane was incubated for 30 min in horseradish peroxidase-conjugated secondary antibody anti-rabbit IgG (1:2000, Santa Cruz Biotechnology). Immunoreactivity was detected with ECL reagent (Amersham Biosciences, Inc.) and fluorography.

RESULTS

Blocking of HES1 Expression in Differentiating Human Neural Stem Cells Results in Neuronal Differentiation—Expression of HES1 is detectable in human neural stem cells both at the mRNA (24) and protein level (Fig. 1). We used antisense *S*-oligonucleotides (phosphothioates) to block HES1 expression in neural stem cells. Immunohistochemical and Western blot (Fig. 1) analyses results clearly demonstrate that incubation of differentiating neural stem cells with antisense oligonucleotides (5 μM) blocks HES1 protein expression. Blocking HES1 in differentiating stem cells treated with retinoic acid and Bt_2cAMP for 4 days results in massive differentiation of stem cells into β-III-tubulin and MAP2 positive cells (Fig. 2, *C* and *D*). In control cultures 5–15% of cells differentiate into neuronal cells after 3 days, whereas in antisense oligonucleotide-treated cultures ~80–90% of cells differentiate into neuronal cells that express β-III-tubulin and MAP2. Simultaneously, the number of GFAP expressing cells as well as the intensity of GFAP immunostaining was significantly reduced (Fig. 2, *E* and *F*).

GABAergic Differentiation of Neural Stem Cells—Since the network of HLH transcription factors regulates the fate of neuronal populations and blocking HES1 expression likely affects the ratios of different HLH transcription factor complexes, we analyzed the neurotransmitter phenotype of differentiating neurons. Immunohistochemical analyses using antibodies against glutamic acid decarboxylase (GAD, marker for GABAergic neurons) demonstrated that the majority of neural

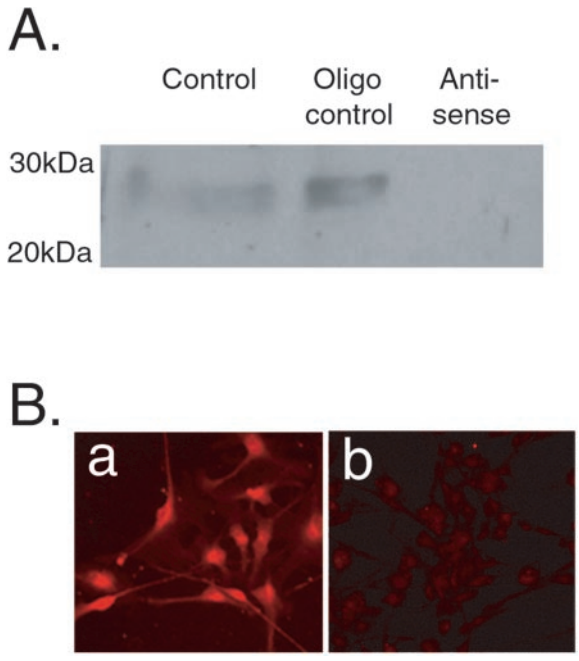

FIG. 1. **Treatment of neural stem cells with HES1 antisense oligonucleotides *in vitro* for 4 days suppresses HES1 protein expression as detected using Western blot (*A*) and immunocytochemistry (*B*).** *A*, untreated (*Control*) and control oligonucleotide (*Oligo control*)-treated cells have equal expression of HES1 protein, whereas antisense oligonucleotide treatment (antisense) blocks HES1 expression. *B*, immunocytochemical staining for HES1 protein in human neural stem cells confirmed high nuclear and cytoplasmic expression of HES1 in control cultures (*panel a*) and residual staining in antisense oligonucleotide-treated cultures (*panel b*).

cells that differentiate after blocking HES1 express GAD, suggesting that suppression of HES1 and treatment of cells with retinoic acid and Bt_2cAMP results in GABAergic differentiation. In control cultures 1–15% of neural cells express GAD after 5 days, whereas in antisense HES1-treated cultures 50–95% of neuronal cells become GABAergic (Fig. 2, *G* and *H*; Table I). Analyses of expression of choline acetyltransferase, a marker for cholinergic differentiation, did not show a difference between control and antisense HES-treated cultures.

Blocking of HES1 Expression in Growing Neurospheres Suppresses Proliferation and Induces Neuronal Differentiation—The next question we asked was, does the blocking of HES1 affects proliferation and initiation of differentiation of neural stem cells *in vitro*. Some data indicate that blocking HES1 *in vivo* results in premature differentiation of precursors into neurons (16, 18) and reduction in the number of neurons in the nervous system (17). To analyze the effect of HES1 expression on proliferation and initiation of differentiation of neural stem cells *in vitro*, we blocked HES1 expression using antisense oligonucleotides in proliferating stem cells that grow as neurospheres. Neurospheres were grown in the presence of antisense oligonucleotides for 4 days, and for the last 20 h BrdUrd was added to label cells that synthesize DNA. Comparison of BrdUrd incorporation in control and antisense HES1-treated cultures showed that blocking HES1 results in significant reduction (more than 2-fold) of DNA synthesis. In control cultures 75 ± 5% of cells synthesize DNA (Fig. 3, *A* and *B*), whereas in antisense oligonucleotide-treated cultures the ratio of proliferating cells is reduced to 34 ± 4% (Fig. 3, *C* and *D*). These data are also supported by propidium iodide staining of DNA in antisense and control oligonucleotide-treated neurospheres and cell cycle analyses using flow cytometry (data not shown).

We also analyzed differentiation of stem cells grown in neuro-

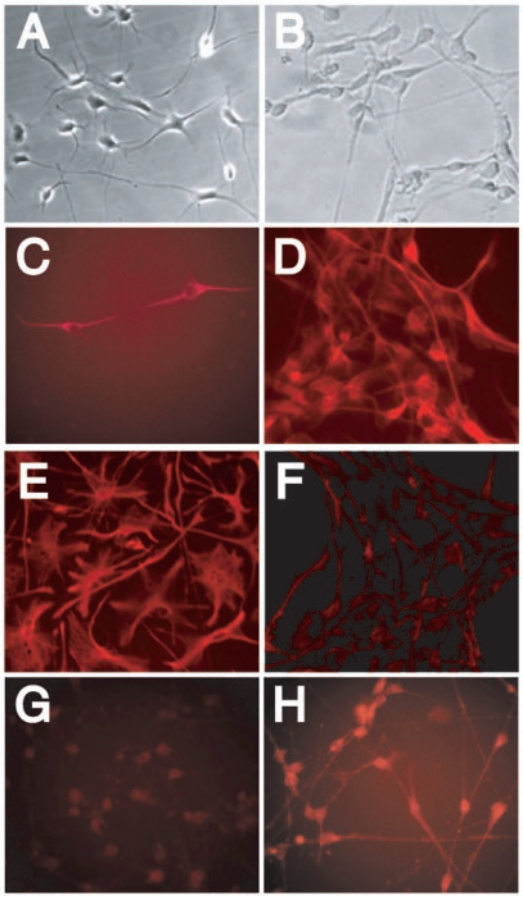

FIG. 2. **Blocking HES1 expression using antisense oligonucleotides induces neuronal and suppress glial differentiation of human neural stem cells *in vitro*.** Control oligonucleotide-treated cultures of neural stem cells differentiate into cells with glial and neuronal morphology (*A*), while a small number of cells express neuronal marker β-III-tubulin (*C*) and the majority of cells are stained for astroglial marker GFAP (*E*). Majority of antisense HES1 oligonucleotide-treated cells differentiate into bipolar cells (*B*) and express β-III-tubulin (*D*), whereas expression of GFAP was almost undetectable (*F*). A small number of cells in control oligonucleotide-treated cultures express GAD, a marker of GABAergic neurons (*G*), while GAD is expressed in the majority of cells in HES1 antisense oligonucleotide-treated cultures (*H*).

TABLE I

Suppression of HES1 in differentiating human neural stem cells induces differentiation of GABAergic neurons

Human neural stem cells were differentiated in the presence of dB-cAMP and all-*trans*-RA for 5 days. HES1 expression was inhibited using antisense *S*-oligonucleotides, and neuronal phenotype was analyzed using antibodies against β-III-tubulin (marker for neurons) and GAD (marker for GABAergic neurons).

Clone	Untreated		HES1 antisense treated	
	% neurons	% GAD + cells	% neurons	% GAD + cells
H1	3 ± 0.5	15 ± 3	87 ± 5	95 ± 6
H2	10 ± 3	9 ± 2	79 ± 6	82 ± 6
C1	4 ± 1	1 ± 1	81 ± 4	52 ± 7

spheres in the presence of antisense HES1 oligonucleotides. Differentiation of these cells was initiated by plating them onto laminin-coated surface in the presence of retinoic acid and Bt_2cAMP. These experiments show how blocking of HES1 expression affects the commitment and not the differentiation phase of neural stem cell development. In control cultures that were differentiated 4 and 7 days 5–15% and 23–25% of cells express β-III-tubulin, respectively. In antisense oligonucleotide-treated cultures 27–30% and 40–45% of cells express β-III-tubulin after 4 and 7 days, respectively.

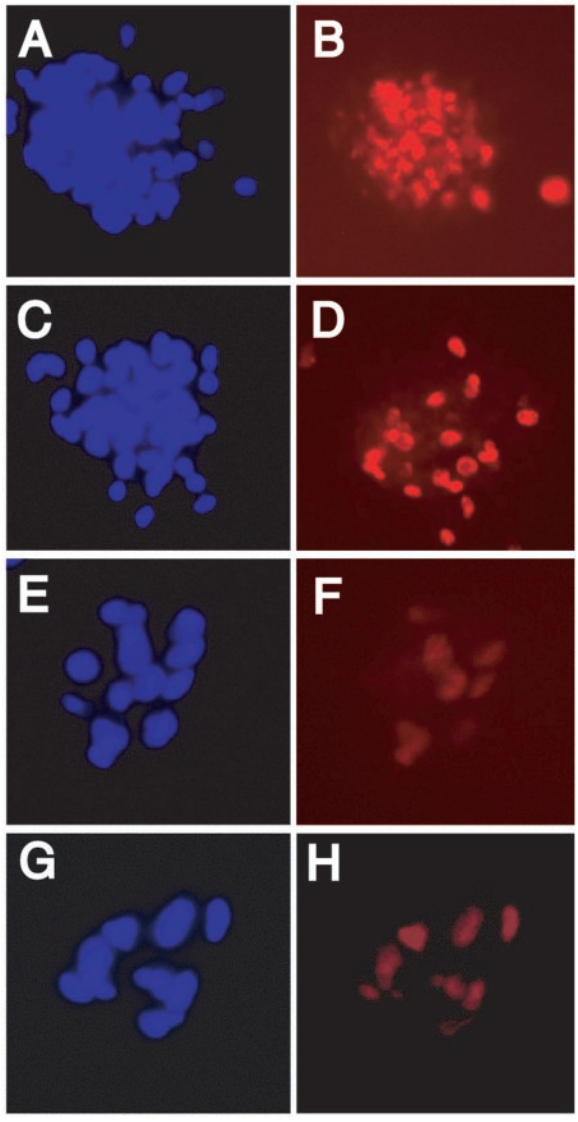

FIG. 3. **Blocking of HES1 expression in proliferating neural stem cells results in inhibition of BrdUrd incorporation (*A–D*) and stimulation of expression of $p21^{CIP1/WAF1}$ (*E–H*).** Control oligonucleotide-treated neural stem cells show a high level of DNA synthesis as it is seen from the comparison of DAPI counterstained (*A*) and BrdUrd stained nuclei (*B*). In contrast, BrdUrd incorporation is significantly lower in HES1 antisense oligonucleotide-treated neural stem cells (C-DAPI counterstaining, D-BrdUrd staining). The $p21^{CIP1/WAF1}$ is expressed at low levels in control neural stem cells (E-DAPI counterstaining, F-$p21^{CIP1/WAF1}$ staining), and its expression is induced in HES1 antisense oligonucleotide-treated cells (*G*, DAPI counterstaining; *H*, $p21^{CIP1/WAF1}$ staining).

Since blocking HES1 likely stimulates early molecular events of neuronal differentiation, we analyzed expression of basic helix-loop-helix transcription factor ASH1 in neurospheres. In control neurospheres 42 ± 6% of cells express ASH1, whereas treatment of cells for 4 days with antisense oligonucleotides doubles the number of ASH1 positive cells (95 ± 6%) (data not shown).

Blocking of HES1 Expression Induces $p21^{CIP1/WAF1}$ Expression—One potential mechanism of how blocking HES1 could affect cell proliferation is by inducing Cdk inhibitors. We used immunocytochemical staining of $p16^{INK4a}$, $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$ in control and antisense HES1 oligonucleotide-treated cells to validate this hypothesis. We were unable to detect expression of $p16^{INK4a}$ and $p57^{KIP2}$ using immunocytochemical techniques in human neural stem cells.

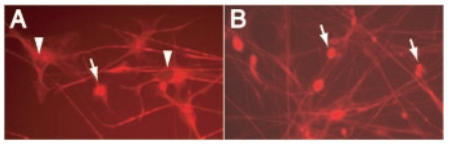

FIG. 4. **Differential expression of $p27^{KIP1}$ in glial and neuron-like cells.** In both control oligonucleotide (*A*) and Hes1 antisense oligonucleotide-treated (*B*) cultures the neuron-like cells express $p27^{KIP1}$ in their nuclei (*arrows*), whereas glial cells showed no nuclear $p27^{KIP1}$ staining (*arrowheads*).

Blocking expression of HES1 in growing neurospheres for 4 days results in significant stimulation of $p21^{CIP1/WAF1}$ expression. In control neural stem cell cultures 24 ± 7% of cells show nuclear staining of $p21^{CIP1/WAF1}$. Blocking of HES1 expression with antisense oligonucleotides for 4 days increases the number of cells that have nuclear staining of $p21^{CIP1/WAF1}$ to 50 ± 5% (Fig. 3, *E–H*). Expression of $p27^{KIP1}$ was not changed in neurospheres treated with antisense HES1 oligonucleotides. Contrary to neurosphere cultures, the ratio of $p27^{KIP1}$-expressing cells in cultures that were treated with antisense HES1 oligonucleotides during differentiation doubled. Approximately 30 ± 5% of cells show nuclear staining in differentiated control cultures, whereas in antisense HES1-treated cultures $p27^{KIP1}$ is present in 60 ± 4% of nuclei. Immunostaining results also demonstrate that $p27^{KIP1}$ is localized in the nuclei of neurons and not in glial cells (Fig. 4). Since the number of neurons is higher in antisense HES1-treated cultures compared with controls then the increase of the number of $p27^{KIP1}$-expressing cells is a reflection of changed ratio of neurons and glial cells.

DISCUSSION

Human neural stem cells differentiate into neurons, astrocytes, and oligodendrocytes when plated on supportive substrate and upon removal of growth factors such as EGF and basic fibroblast growth factor. The ratios of neurons and glial cells among differentiated cells vary depending on the stem cell isolate. On average, 2–15% of the cells differentiate into neurons and the rest develop into glial cells, whereas the majority, more than 80%, are astrocytes and less than 20% oligodendrocytes. Recently it has been reported that astroglial development requires the expression of HES transcription factors and that suppression of their expression results in premature neuronal differentiation (17, 25). Our previous data (24) show that HES1 is highly expressed in human neural stem cells grown as neurospheres *in vitro*. Here we tested the hypothesis that blocking HES1 expression in human neural stem cells stimulates specific neuronal differentiation and blocks proliferation of neural stem cells. We used two different experimental conditions to answer these questions. First, we blocked HES1 in differentiating neural stem cells that grow as a monolayer on laminin-coated surface in the absence of growth factors and presence of retinoic acid and dibutyryl cyclic AMP. Second, we analyzed the proliferation and differentiation of stem cells that were grown as neurospeheres during the blocking of HES1 expression. Our results demonstrate that blocking HES1 expression affects two different stages of neuronal differentiation: selection of neuronal *versus* glial development and differentiation of specific neuronal phenotype, GABAergic neurons. Presence and activity of HLH transcription factors determines development of neuronal lineage (8, 9). Overexpression of HES generates more neurons, whereas a lack of HES activity results in premature differentiation and lower than normal number of neurons in the developing nervous system. In contrast, overexpression of proneuronal genes from the neurogenin and ash family is essential for the development of neuronal cells and inhibition of glial differentiation (for review, see Ref. 10). HES1 as a negative regulator inhibits the activity of HLH transcription factors, which results in blocking of neuronal differentiation and either differentiation into glial lineage or continuing proliferation. Glial differentiation occurs only if appropriate signals such as CNTF and LIF (26) are present. The precise molecular mechanisms of action of HLH transcription factors, including the balance of positive and negative factors, has to be elucidated. Even less is known about the choices of specific neuronal differentiation pathways. Some data indicate that a set of specific HLH transcription factors determines the fate of progenitor cells (9, 10). HES1 as a negative regulator likely changes this delicate balance and drives progenitors into a specific type of neurons. We demonstrate that the blocking of HES1 expression induces the differentiation of neural stem cells into GABAergic neurons, and this may indicate that GABAergic differentiation is a default pathway for neuronal precursors.

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