**Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium**

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**SUMMARY**

We have characterised the functions of the bHLH transcriptional repressors HES1 and HES5 in neurogenesis, using the development of the olfactory placodes in mouse embryos as a model. Hes1 and Hes5 are expressed with distinct patterns in the olfactory placodes and are subject to different regulatory mechanisms. Hes1 is expressed in a broad placodal domain, which is maintained in absence of the neural determination gene Mash1. In contrast, expression of Hes5 is restricted to clusters of neural progenitor cells and requires Mash1 function. Mutations in Hes1 and Hes5 also have distinct consequences on olfactory placode neurogenesis. Loss of Hes1 function leads both to expression of Mash1 outside of the normal domain of neurogenesis and to increased density of MASH1-positive progenitors within this domain, and results in an excess of neurons after a delay. A mutation in Hes5 does not produce any apparent defect. However, olfactory placodes that are double mutant for Hes1 and Hes5 upregulate Ngn1, a neural bHLH gene activated downstream of Mash1, and show a strong and rapid increase in neuronal density. Together, our results suggest that Hes1 regulates Mash1 transcription in the olfactory placode in two different contexts, initially as a prepatterning gene defining the placodal domain undergoing neurogenesis and, subsequently, as a neurogenic gene controlling the density of neural progenitors in this domain. Hes5 synergises with Hes1 and regulates neurogenesis at the level of Ngn1 expression. Therefore, the olfactory sensory neuron lineage is regulated at several steps by negative signals acting through different Hes genes and targeting the expression of different proneural gene homologs.

Key words: Mouse, Olfactory placode, Mash1, Prepatter, Notch signalling, Lateral inhibition

**INTRODUCTION**

The generation of the numerous types of neurons that constitute the adult nervous system from an initially homogenous population of neuroectodermal progenitor cells is an extremely complex process, which has been thoroughly investigated, first in Drosophila (Campos-Ortega, 1993; Jan and Jan, 1994) and, more recently, in vertebrates (Tanabe and Jessell, 1996; Chan and Jan, 1999). In vertebrates, the early steps of specification and differentiation of neuronal precursors have been studied in several models, including the primary neurons of lower vertebrates (Chitnis and Kintner, 1995), the spinal cord (Tanabe and Jessell, 1996), the neural crest (Anderson et al., 1997) and the retina (Cepko, 1999). The olfactory sensory epithelium (OE) has also been a useful model to identify transcription factors and extracellular signals regulating neurogenesis in the mouse (Calof et al., 1996). The value of the OE model stems from the simple nature of this tissue, where primary olfactory sensory neurons (OSNs) are the main neuronal type, and from its capacity to renew its neuronal population during adulthood and to regenerate it after damage (Graziadei and Graziadei, 1979; Crews and Hunter, 1994). These properties have facilitated the identification and characterisation of OSN progenitors, which are present in the embryonic as well as in the adult OE. Recent evidence suggests the existence of at least two types of progenitors, representing sequential steps in the OSN lineage. A rare population of slowly dividing cells with the self-renewing capacity of stem cells has been shown to generate a much more abundant population of rapidly dividing precursors committed to neuronal differentiation (Calof and Chikaraishi, 1989; Calof et al., 1996; Mumm et al., 1996).

Several genes of the basic helix-loop-helix (bHLH) class have been implicated in neurogenesis in the olfactory placode (Cau et al., 1997). Mash1 is the first of these genes to be expressed in OSN progenitors and analysis of the OE phenotype of mice mutant for Mash1 shows that it plays a central role during early stages of the OSN lineage (Guillemot et al., 1993; Cau et al., 1997). Loss of Mash1 function results in a lack of expression of the downstream bHLH genes Neurogenin1 (Ngn1) and NeuroD, and in a failure to generate OSN precursors in the OE (Cau et al., 1997; our unpublished
data. *Mash1* has therefore a neural determination function in the OE similar to that of its *Drosophila* counterparts of the *achaete-scute* complex (AS-C) (Ghysen and Dambly-Chaudiere, 1988; Jan and Jan, 1994). A preliminary analysis of the OE phenotype of *Ngn1* mutant mice indicates that *Ngn1* is required at a later step, in the differentiation of OSNs (E.C., Q. Ma, D. Anderson and F. G., unpublished data). To get further insights into the regulation of OSN development, we have examined the function of genes that are potential regulators of *Mash1* and *Ngn1* in this tissue.

In *Drosophila* imaginal discs, expression of the AS-C proneural genes is controlled by upstream regulators, or pre-pattern genes (Ghysen and Dambly-Chaudiere, 1989; Skeath and Carroll, 1994; Simpson, 1996), whose products bind to site-specific enhancers found around the coding sequence of AS-C genes (Gomez-Skarmeta et al., 1995; Modolell, 1997). One of these upstream regulators, *hairy*, is a direct repressor of *achaete* expression in the wing and leg imaginal discs (Ohsako et al., 1994; Van Doren et al., 1994). In absence of *hairy*, the *achaete* expression domain expands and ectopic neural precursors are generated (Skeath and Carroll, 1991; Orenic et al., 1993). Proneural genes, which are initially expressed in groups of cells, called proneural clusters, become later restricted to single cells which thereby acquire a neural fate. This change in proneural gene expression involves a process of lateral inhibition mediated by the Notch receptor (Simpson, 1997). Interaction of Notch with its ligand Delta activates a signalling pathway resulting in transcriptional activation of the genes of the *Enhancer of split* (*Espl*) complex (Jennings et al., 1994; Artavanis-Tsakonas et al., 1995; Bray, 1997; Simpson, 1997). Activity of the *Espl* genes, which encode bHLH transcriptional repressors structurally related to *hairy*, inhibits neural development by antagonizing proneural gene function (Ohsako et al., 1994; Van Doren et al., 1994; Nakao and Campos-Ortega, 1996; Jennings et al., 1999).

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Vertebrate Notch, Delta and Serrate/Jagged (which, like Delta, encode Notch ligands), have been implicated in processes of lateral inhibition controlling cell fate specification in numerous tissues, including the neural plate, inner ear, retina, skin and pancreas (Chitnis and Kintner, 1995; Chitnis et al., 1995; Henrique et al., 1997; Crowe et al., 1998; Landorf et al., 1999). Four genes homologous to the *Drosophila* *Hairy* and *Espl* genes, named *Hes1*-*Hes3* and *Hes5*, have been identified in mouse (Akazawa et al., 1992; Sasai et al., 1992). Genetic analysis has shown that *Hes1* and *Hes5* in particular have important roles in neurogenesis (Ishibashi et al., 1994, 1995; Tomita et al., 1996; Ohtsuka et al., 1999; reviewed in Kageyama and Nakanishi, 1997). However it has remained unclear whether these genes were regulating neural development through a process of lateral inhibition, or in a different context.

To better define the functions of *Hes1* and *Hes5* in neurogenesis, we have examined in details the regulation of expression and function of these two genes, using the development of the olfactory placode as a model. We have analysed the OE phenotype of *Hes1* null mutant mice, and we have generated mice null mutant for the gene *Hes5* to examine its function in neurogenesis and its possible synergy with *Hes1*. Our results show that *Hes1* activity in OE neurogenesis can be divided in two distinct phases. *Hes1* functions initially to delimit a domain of proneural gene expression at the onset of neurogenesis in the placode. *Hes1* subsequently functions in a process of lateral inhibition which regulates the recruitment of neural precursors in the OE. Therefore, *Hes1* appears to cumulate the functions of a prepatteren gene and of a neurogenic gene. *Mash1* is a target of *Hes1* for these two activities. In contrast, *Hes5* appears to function exclusively as a neurogenic gene and it regulates the expression of the differentiation gene *Ngn1*. Thus, development of the OSN lineage is negatively regulated by *Hes* genes at several sequential stages.

**MATERIALS AND METHODS**

**Generation of *Hes5* mutant animals**

To generate a *Hes5* targeting vector, a PGK-neo cassette was flanked by 5' and 3' arms consisting of a 3 kb *Xhol-SmaI* fragment and a 3 kb *Noll-SmaI* fragment from the mouse (129/Sv) *Hes5* locus (unpublished data), respectively. The mutation introduced by this vector is a deletion of 724 bp, including the coding region from Met1 to Ala76 and comprising the bHLH domain. The vector was electroporated in the ES cell lines H1 (from IGBMC, Strasbourg, unpublished) and R1 (Nagy et al., 1993), and selection was performed as described (Fode et al., 1998). Homologous recombination was detected by Southern blotting of ES cell genomic DNA, using a 0.9 kb *SmaI-BamHI* fragment and a 0.2 kb *DraI-Xhol* fragment as 3' and 5' external probes, respectively. Ten independent targeted ES clones were obtained out of 252 clones analysed. Three of them were used to generate chimeras by blastocyst injection. Germine transmission was obtained by crossing the chimeras with C57Bl6 females and the colony was expended on a CD1 genetic background.

**Generation of compound mutant animals for *Hes1*, *Hes5* and *Mash1***

For staging of embryos, mid-day of the vaginal plug was considered as E0.5. The *Mash1* and *Hes1* mutant mouse lines have been described in Guillemot et al. (1993) and Ishibashi et al. (1995), respectively. Compound *Hes1*:*Hes5* embryos were obtained by intercrossing *Hes1*+/−:*Hes5*+/− or *Hes1*+/−:*Hes5*−/− mice. At E10.5, the 8 expected genotypes resulting from these crosses were obtained with Mendelian ratios (see Results section).

**Whole-mount in situ hybridization**

The *Mash1*, *Ngn1* and *SCG10* probes have been described in Cau et al. (1997). The *Hes1* probe was transcribed from a 0.9 kb cDNA clone described in Ishibashi et al. (1995). The *Hes5* probe was transcribed from a 1.3 kb cDNA clone described in Akazawa et al. (1992). The protocol for whole-mount RNA in situ hybridization has been described in Cau et al. (1997). The protocol for two-colour whole-mount RNA in situ hybridization is available upon request.

**Immunohistochemistry with anti-MASH1 antibody**

Detection of the MASH1 protein was performed on 14 μm cryosections of E10.5 embryos frozen in OCT, using a monoclonal anti-MASH1 antibody (Lo et al., 1991). After postfixation in 4% paraformaldehyde at room temperature for 10 minutes, and 3 washes of 5 minutes in PBS with 0.05% Tween-20, immunohistochemistry was performed as Porteus et al. (1994).

**RESULTS**

**Expression of *Hes* genes in the embryonic olfactory epithelium**

In an effort to understand how neurogenesis is regulated in the embryonic OE, we examined the expression in this tissue of
genes belonging to the *Hes* (*Hairy and Enhancer of split*) family of transcriptional repressors. We performed this study at E12.5, a stage when the OE has begun to acquire an organisation in cell layers characteristic of the mature tissue. At this stage, the OE contains two populations of dividing progenitors, located respectively on the apical and basal sides of the epithelium. Cells dividing apically are already present in the olfactory placode, whereas basal progenitors appear in the OE only at around E12.5. As development of the OE proceeds, the proportion of basal progenitors then increases progressively, and that of apical progenitors decreases (Smart, 1971; Cuschieri and Bannister, 1975). Two genes of the *Hes* family, *Hes1* (Sasai et al., 1992) and *Hes5* (Akazawa et al., 1992), are expressed in the embryonic OE with distinct patterns. At E12.5, *Hes1* transcripts are present in most cells on the apical side of the OE and are absent from the basal side (Fig. 1G). *Hes5* in contrast is expressed in scattered cells, which are present only on the basal side of the OE (Fig. 1I). The two genes are already expressed with different patterns when neurogenesis begins in the olfactory placode. At E10.5, *Hes1* expression is widespread throughout the placode, but is highest at the periphery and lower in a large central area (Fig. 1A). The area of high *Hes1* expression abuts the more centrally located placodal territory, which contains neuronal precursors marked by expression of the neural determination gene *Mash1* (Fig. 1C; Cau et al., 1997). In contrast to the broad expression of *Hes1*, *Hes5* expression is restricted to cell clusters (Fig. 1D), which are either intermingled with *Mash1*-expressing cells or have a more central position (Fig. 1F). At this early stage, there is no distinct cell layering in the olfactory placode, and *Hes1*-positive cells and *Hes5*-positive cells are found in both apical and basal positions (data not shown). At E15.5, *Hes5* OE expression is not detectable and that of *Hes1* is low and restricted to a few apical cells (data not shown).

*Hes1* and *Hes5* have been shown to be important effectors of the Notch signalling pathway in the embryonic central nervous system (Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999). If *Hes* genes are also regulated by Notch signalling during OE neurogenesis, their expression patterns should be affected by a mutation in *Mash1*, which results in an early block in OE neurogenesis (Cau et al., 1997). Indeed, expression of *Hes1* (Fig. 1H) and *Hes5* (Fig. 1J) is absent in most of the OE of *Mash1* mutant embryos at E12.5. Therefore, activation of these genes is part of a program of neurogenesis in the OE.

Examination of *Hes1* expression in *Mash1* mutant embryos at E10.5 revealed that, in contrast with its later expression (Fig. 1H), the early expression of *Hes1* in the olfactory placode is largely unaffected by the loss of *Mash1* (Fig. 1B). This result suggests that different mechanisms regulate *Hes1* expression at the onset of neurogenesis and at later stages in the OE. As expected, expression of *Hes5* is severely reduced in the *Mash1* mutant olfactory placode (Fig. 1E), except in a small domain of the placode where neurogenesis is not affected by loss of *Mash1* (Cau et al., 1997).

![Image](2325Hes genes and olfactory epithelium neurogenesis.png)

**Fig. 1.** *Hes1* and *Hes5* expression in the olfactory epithelium requires *Mash1* function, whereas *Hes1* expression at the placodal stage is independent of *Mash1*. (A,B,D,E) Flat mounts of olfactory placodes from wild type (A,D) and *Mash1* mutant (B,E) E10.5 embryos hybridized with cRNA probes for *Hes1* (A,C) and *Hes5* (D,F). Medial is to the left, lateral to the right; dorsal is up and ventral is down. In the placode, *Hes1* is expressed broadly (A) and this pattern is not affected in a *Mash1* mutant placode (B), while *Hes5* expression is restricted to cell clusters (D) and requires *Mash1* function (E). (C,F) Flat mounts of wild-type E10.5 placodes double labelled for (C) *Mash1* (brown) and *Hes1* (blue) and (F) *Mash1* (blue) and *Hes5* (brown). The periphery of the placode is to the left, the center to the right. The domain of high *Hes1* expression is peripheral to the *Mash1*-positive cell clusters (C) and *Hes5*-positive cell clusters are either intermingled with, or more centrally located than, *Mash1*-positive cells (F). (G-J) Sections of wild type (G,I) and *Mash1* mutant (H,J) OE at E12.5 hybridized with *Hes1* (G,H) and *Hes5* (I,J) cRNA probes. At this stage, both expression of *Hes1* in apical cells (G) and of *Hes5* in basal cells (I) require *Mash1* function (H,J). *Hes1* is also expressed in the mesenchyme surrounding the OE (G,H).
Hes1 mutant phenotype in the olfactory epithelium

The distinct expression patterns of Hes1 and Hes5 in the developing OE suggested that these genes may regulate distinct steps in OE neurogenesis. To address this possibility, we examined the loss-of-function phenotypes of Hes1 and Hes5 in the OE, starting with the study of embryos carrying a null mutation in Hes1 (Ishibashi et al., 1995).

Previous studies have shown that loss of Hes1 results in premature neuronal differentiation and upregulation of Mash1 in the forebrain and in morphological defects affecting with a low penetrance the cranial neural tube and the eye (Ishibashi et al., 1995; Tomita et al., 1996). The first indication that loss of Hes1 affects neurogenesis in the OE was a large increase in the number of Mash1-positive cells in the olfactory placodes of Hes1 mutant embryos. Expression of Mash1 starts in wild-type olfactory placodes at the onset of neurogenesis (E10.0 or 25 somites) and, by E10.5, Mash1 is expressed in clusters of cells located in the medial side and to a lesser extent in the lateral side of the placode (Fig. 2A; Cau et al., 1997). In Hes1 mutant placodes, ectopic Mash1 expression was observed in ventral and dorsal areas, which normally do not express the gene at this stage (Fig. 2B). There was also a marked increase in number of Mash1-positive cells in the lateral side of the placode, and scattered Mash1-positive cells in the center of the placode. This enlargement of the Mash1-positive area in Hes1 mutant placodes shows that Hes1 is involved in defining the area of the placode that activate Mash1 expression.

To determine if neurogenesis in the OE is affected in absence of Hes5, we examined the expression of Mash1 and SCG10 in the placode and OE of Hes5 mutant embryos. No difference in number and distribution of Mash1-positive OSN progenitors and SCG10-positive neurons could be detected between wild-type and mutant placode and OE (Fig. 4G,H and data not shown), suggesting that lack of Hes5 alone is not sufficient to significantly perturb neurogenesis in the OE.

Hes1 and Hes5 synergize to regulate neurogenesis in the OE

Hes1 and Hes5 have similar activities of transcriptional repressors and are both expressed in the embryonic nervous system, including the OE (Fig. 1; Akazawa et al., 1992; Sasai et al., 1992). Therefore these genes could functionally compensate for one another in animals mutant for one of the two genes. To examine this possibility, embryos carrying both Hes1 and Hes5 mutations were generated. When the progeny of Hes1+/--;Hes5−/+ and Hes1+/--;Hes5−/+ intercrosses were harvested at E10.5, the eight possible genotypes were obtained

| Table 1. Clustering of MASH1-positive cells in Hes1 mutant olfactory placodes |
|----------------------------------|----------------------|------------------|
| MASH1-positive cells             | WT (n=3)             | Hes1−/− (n=3)    |
| Cells isolated or in pairs       | 92.5±6.6             | 68.2±6.6         |
| 'small clusters' (3-4 cells)     | 7.5±6.6              | 27.1±3.7         |
| 'large clusters' (5-8 cells)     | 0.0±0.0              | 4.79±3.8         |

To quantify the clustering of MASH1-positive cells in Hes1 mutant and wild-type placodes, the percentage of MASH1-positive cells falling in the following three categories: cells isolated or in pairs, ‘small clusters’ (3-4 cells) and ‘large clusters’ (5-10 cells), are shown. In wild-type olfactory placodes, most MASH1-positive cells are either isolated or in pairs, and a small fraction is grouped in small clusters of less than 5 cells. In Hes1 mutant placodes, there is a larger percentage of cells in clusters, and the size of clusters reaches up to 8 cells. The percentages of cells are averages.d, and the numbers of animals counted are indicated in parenthesis. For each animal, three sections were counted using a minimum of 50 cells.
at low frequency neural tube defects which are never observed in homozygous mutant embryos. In addition, Hes1 and in brain morphology, as previously reported (Ishibashi et al., 1995). The embryos exhibit with a low penetrance defects in closure of the neural tube shown). In contrast, the number and density of -positive cells were found throughout wild-type placodes to determine whether OE neurogenesis was affected in regulation of neurogenesis in olfactory placodes. We first 1997; our unpublished data), whereas in Ngn1 OSNs (E. C., Q. Ma, D. J. Anderson and F. G., unpublished results suggest the following interpretation of the placode, and a higher level of expression in placodal SCG10 repressors, HES1 and HES5, in neurogenesis in the olfactory placode, and a higher level of expression in placodal SCG10 expression. At E10.0, scattered cells that normally express it. The additional results suggest the following interpretation of the placode, and a higher level of expression in placodal SCG10 repressors, HES1 and HES5, in neurogenesis in the olfactory placode, and a higher level of expression in placodal SCG10 expression. At E10.0, scattered cells that normally express it. The additional Hes genes and olfactory epithelium neurogenesis

| Table 2. Synergy between Hes1 and Hes5 in regulation of brain morphogenesis |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| WT                          | Hes1<sup>+/−</sup> | Hes1<sup>−/−</sup> | Hes5<sup>−/−</sup> |
| 0% (n=54)                   | 27.3% (n=55)                | 7.75% (n=48)                | 71.2% (n=129)               |

The numbers are percentages of embryos with neural tube defects (open anterior neural tube and reduced size of telencephalic vesicles) in the progeny of intercrossed Hes1:Hes5 double mutant animals at E10.5. Hes1<sup>−/−</sup> mutant embryos exhibit with a low penetrance defects in closure of the neural tube and in brain morphology, as previously reported (Ishibashi et al., 1995). The penetrance of these defects is strongly increased in double Hes1:Hes5 homozygous mutant embryos. In addition, Hes1<sup>−/−</sup>:Hes5<sup>−/−</sup> embryos present at low frequency neural tube defects which are never observed in Hes1<sup>−/−</sup> embryos. Numbers of embryos of each genotype are shown in parenthesis.

| Table 3. Increase in number and clustering of Ngn1-positive cells in Hes1 and Hes1:Hes5 mutant olfactory placodes. |
|-----------------|-----------------|-----------------|
| Ngn1-positive cells | WT              | Hes1<sup>−/−</sup> |
| n=4             | n=5             | n=5             |
| Isolated cells  | 49.5±5.4        | 33.6±3.68       | 17.8±3.1        |
| 'small clusters' (2 to 5 cells) | 50.6±5.4       | 56.2±4.3       | 49.2±4.1       |
| 'large clusters' (6 to 20 cells) | 0±0.0          | 10.2±2.67      | 33.0±2.6       |

To quantify the clustering of Ngn1-positive cells in Hes1 mutant placodes, the percentages of Ngn1-positive cells falling in the following three categories: ‘isolated cells’ (1 cell), ‘small clusters’ (2 to 5 cells) and ‘large clusters’ (6 to 20 cells), are shown. In wild-type placodes, a similar number of Ngn1-positive cells is found in small clusters and no cluster contains more than 5 cells. In Hes1 single mutants, the proportion of cells in small clusters is the same but there are less isolated cells and larger clusters are found. In Hes1:Hes5 double mutant placodes, the number of cells in small clusters is again the same, but there are even less isolated cells and more cells in large clusters. The percentages of cells are averages d., and the numbers of embryos analysed are in parenthesis. For each animal, three sections at different levels of the placode (rostral, medial and caudal) were analysed, and at least 50 cells were counted per section.

We next examined whether Hes1 and Hes5 also synergize in the regulation of neurogenesis in olfactory placodes. We first examined the distribution of Mash1 transcripts and protein in the placodes of Hes1 and Hes5 single and double mutant embryos. As described above, Mash1 expression was not affected in Hes5 single mutant placodes (data not shown), and there was a clear increase in number of Mash1-positive cells, detectable at both RNA and protein levels, in Hes1 mutant placodes (Fig. 2A-D). In double mutant Hes1<sup>−/−</sup>:Hes5<sup>−/−</sup> placodes, there was an increase in Mash1 expression, but it was not significantly different from that observed in Hes1<sup>−/−</sup> single mutant placodes (data not shown). This result suggests that Hes5 does not synergize with Hes1 to regulate Mash1 expression.

We have previously shown that activation of Mash1 is the earliest step in olfactory placode neurogenesis and that its expression in OE progenitors is followed by the expression of another neural bHLH gene, Ngn1 (Cau et al., 1997). The sequential activation of these two genes is reflected in their requirement at distinct steps of OE neurogenesis. In absence of Mash1, basal OE progenitors are not produced (Cau et al., 1997; our unpublished data), whereas in Ngn1 mutant OE, basal progenitors are generated but they fail to differentiate in OSNs (E. C., Q. Ma, D. J. Anderson and F. G., unpublished data). We examined Ngn1 expression in Hes1 and Hes5 mutant placodes to determine whether OE neurogenesis was affected at a step downstream of Mash1 expression. At E10.0, scattered Ngn1-positive cells were found throughout wild-type placodes (Fig. 4A) and the number and distribution of Ngn1-positive cells appeared similar in Hes5 mutant placodes (data not shown). In contrast, the number and density of Ngn1-positive cells appeared higher in Hes1 mutant placodes (Fig. 4B) and further increased in Hes1:Hes5 double mutant placodes (Fig. 4C). The clearest defect was in the size and number of clusters of Ngn1-positive cells. The size of these clusters was increased (maximum size of 5 cells in WT and Hes5 mutant placodes, of 8 cells in Hes1 mutant placodes and of 20 cells in Hes1:Hes5 mutant placodes), and a larger fraction of Ngn1-positive cells was found in these clusters rather than isolated, in Hes1 single and double mutant placodes (Table 3). These results show that there is a gradual increase in Ngn1 expression from wild type to Hes1 single to Hes1:Hes5 double mutant placodes, and suggest that Hes5 and Hes1 synergize to repress Ngn1 expression in olfactory placodes.

We next examined whether the upregulation of Ngn1 observed in Hes1:Hes5 double mutant placodes results in an increase in number of neurons in these placodes. As shown earlier, the number of SCG10-positive neurons is not significantly different in wild-type, Hes1 single and Hes5 single mutant placodes at E10.5 (Figs 2E,F, 4D-H and not shown). In contrast, there is a large increase in number of SCG10-positive cells in the placodes of Hes1:Hes5 double mutant embryos at this stage (Fig. 4E-I). Therefore, Hes1 and Hes5 synergize to regulate the number and density of neurons in olfactory placodes.

**DISCUSSION**

We have examined the role of two related transcriptional repressors, HES1 and HES5, in neurogenesis in the olfactory epithelium, by examining the phenotype in this tissue of Hes1 and Hes5 single and double mutant embryos. Together, our results suggest the following interpretation of the Hes olfactory placode phenotypes. Loss of Hes1 results in an increase in number of cells expressing the determination gene Mash1 in the placode, and a higher level of Mash1 expression in placodal cells that normally express it. The additional Mash1-positive cells activate expression of the downstream differentiation gene Ngn1, with limited efficiency in absence of Hes1 only, and in more cells in absence of both Hes1 and Hes5. The
increase in Ngn1 expression then results in additional neurons in the mutant placodes. These data suggest that both Hes1 and Hes5 negatively regulate neurogenesis, but at different stages in the OSN lineage. The main level of Hes1 control is on Mash1 expression, which marks an early stage in olfactory epithelium neurogenesis. In contrast, Hes5 does not appear to regulate Mash1 expression, but it synergizes with Hes1 at a downstream step, the regulation of Ngn1 expression, to control neuronal production. The observation that Mash1 and Hes1 are both expressed in apical cells whereas Ngn1 and Hes5 are expressed in basal cells in the OE (Fig. 1; Cau et al., 1997) supports the interpretation that Hes1 and Hes5 act at distinct steps in OE neurogenesis and have distinct molecular targets.

Fig. 5 presents our current interpretation of the interactions taking place between the negative regulators Hes1 and Hes5 and the positive regulators Mash1 and Ngn1 during OE neurogenesis.

The genes of the Hes family encode bHLH transcription factors that are most closely related to two groups of negative regulators of neurogenesis in Drosophila, hairy and the products of the Enhancer of Split complex (Espl). Both hairy and Espl products have been shown to directly repress transcription of the proneural gene achaete (Ohsako et al., 1994; Van Doren et al., 1994), but their activity is required in different contexts. hairy is a prepattern gene (Skeath and Carroll, 1991, 1994; Fisher and Caudy, 1998). It is required in large areas of the wing and leg imaginal discs to prevent ectopic expression of the proneural gene achaete and the formation of ectopic bristles (Skeath and Carroll, 1991; Orenic et al., 1993; Fischer and Caudy, 1998). The genes of the Espl complex are neurogenic genes that are activated by Notch signalling in a process of lateral inhibition during embryonic and adult neurogenesis (Jennings et al., 1994). Activation of the Espl genes blocks the accumulation of high amounts of proneural protein in most cells of the proneural clusters, thereby preventing them from adopting a neural fate (Bray, 1997). In this report, we show that Hes1 regulation and mutant phenotype in the developing OE suggest that Hes1 has a dual role, acting as a prepattern (hairy-like) gene at the onset of neurogenesis in the olfactory placode and subsequently as a neurogenic (Espl-like) gene regulating Mash1 expression in OE progenitors. In addition, the regulation of Hes5 expression in the olfactory placode and the placodal phenotype of Hes1:Hes5 double mutants support a role for Hes5 as a neurogenic gene acting at a later step in the OSN lineage.

**Hes1 has characteristics of a prepattern gene in the olfactory placode**

Hes1 is expressed in a broad domain of the olfactory placode before the onset of Mash1 expression and of neurogenesis. Once Mash1 expression has been initiated (at the 24- to 26-somite stage), Hes1 expression is maintained at high level.
Hes genes and olfactory epithelium neurogenesis

throughout the periphery of the placode and is reduced in more central areas, where expression of Mash1 and other neural bHLH genes has been induced (Fig. 1; Cau et al., 1997). This early expression pattern is independent of the emergence of neural precursors, and thus of lateral inhibition, as it is unaffected in Mash1 mutant embryos in which most OSN progenitors are missing. Therefore, earlier signals involved in olfactory placode patterning must be responsible for the induction of Hes1 expression. The phenotype of the Hes1 mutant olfactory placodes, which present a vast enlargement of Mash1-expressing areas, provide further evidence that the early function of Hes1 is unrelated to Notch-mediated lateral inhibition. Together, these results support the notion that Hes1 is involved in delimiting the domains of Mash1 expression in the placode, and has therefore a function similar to that of the prepattern gene hairy (Skeath and Carroll, 1991; Orenic et al., 1993). Hairy has been shown to directly repress achaete transcription through binding to a unique CACGCG binding site in its promoter (Ohsako et al., 1994; Van Doren et al., 1994). How HES1 represses Mash1 transcription in the mouse is not known, but human HES1 has been shown to bind to a CACGCA sequence in the promoter of the Hash1 gene, and thereby to repress its expression in carcinoma cells (Chen et al., 1997), suggesting that a direct interaction between these two genes could be a conserved mechanism regulating neurogenesis in vertebrates.

**Hes1 has also characteristics of a neurogenic gene**

Several observations indicate that, after this early function as a prepattern gene, Hes1 might be transcribed in response to Notch signalling and be involved in repression of neurogenesis in a process of lateral inhibition. Activation of Notch1 has been shown to induce Hes1 expression in a variety of cell lines and in retinal explants (Jarriault et al., 1995, 1998; Ohtsuka et al., 1999). This induction of Hes1 expression requires the binding of the mouse homolog of suppressor of Hairless, RBP-J to a consensus site in the Hes1 promoter (Jarriault et al., 1995, 1998).

**Fig. 4. Hes1 and Hes5 synergise for the regulation of neuronal differentiation in the olfactory placode.**

(A-F) E10.5 placodes from wild-type (A,D,G), Hes1−/− (B,E), Hes5−/− (H) and Hes1−/−;Hes5−/− (C,F,I) embryos hybridized with cRNA probes for Ngn1 (A-C) or SCG10 (D-I) and either sectioned (A-F) or flat mounted (G-I). Compared with wild-type placodes, Hes1 mutant placodes show an increase in both the percentage of Ngn1-positive cells found in clusters, and in the size of the clusters. This effect is more pronounced in Hes1−/−;Hes5−/− placodes (see insets in A-C; Table 3). SCG10-positive neurons are present in similar numbers in the placodes of wild-type (D,G), Hes1 mutant (E; Fig. 2J) and Hes5 mutant embryos (H). In contrast, the number and density of neurons is greatly increased in the placodes of Hes1;Hes5 double mutant embryos (F,I).

**Fig. 3. Generation of Hes5 mutant mice.**

(A) Schematic representation of the endogenous Hes5 locus (top), the targeting vector (middle) and the recombined Hes5 null allele (bottom). The genomic fragments used as 5′ and 3′ external probes are indicated at the top of the figure. (B) Analysis of the genotype of five ES cell lines, one wild-type (WT) and four with a targeted Hes5 allele (lanes 1-4). Southern blot analysis was performed on genomic ES cell DNA digested with BamHI or EcoRV and hybridized with 3′ and 5′ external probes, respectively. The 3′ probe detects a 7 kb wild-type and a 4 kb mutant band. The 5′ probe recognizes a wild-type band of more than 12 kb and a 7 kb mutant band. Restriction enzymes: RV, EcoRV; Xh, Xhol; B, BamHI; S, SmaI; N, NotI.
and thus appears to involve the same mechanism as for transcription of E(spl) genes in *Drosophila* and *Xenopus* (Artavanis-Tsakonas et al., 1995; Wettstein et al., 1997). In the OE at E12.5, we have shown that *Hes1* is expressed in apical cells and that this expression is lost in *Mash1* mutant embryos. These results suggest that *Hes1* participates in a regulatory loop between *Mash1* and a Notch-mediated lateral signalling pathway in apical cells of the OE, similar to the loop which is involved in the selection of bristle precursors in the *Drosophila* PNS (Heitzler et al., 1996).

The analysis of the *Hes1* mutant phenotype also supports the notion that *Hes1* participates in a process of lateral signalling in the OE. Loss of *Hes1* results in an increase in *Mash1* transcripts levels in areas of the placode that normally express the gene, in addition to an expansion of these areas mentioned earlier. Moreover, we have observed with an antibody that MASH1-positive precursors are found grouped in clusters of adjacent cells in *Hes1* mutant placodes, whereas they remain scattered in wild-type placodes, suggesting that *Hes1* is required to prevent adjacent placodal cells from expressing MASH1. Finally, the density of neurons in the placode is greatly increased in embryos double mutant for *Hes1* and *Hes5*. These alterations in proneural gene expression and in distribution of neurons appear similar to the phenotypes observed in the nervous system of *Drosophila* or vertebrate embryos in which Notch signalling has been disrupted (Chitnis et al., 1995; Henrique et al., 1997; Simpson, 1997).

Our results therefore support the idea that *Hes1* regulates neurogenesis in the OE in two clearly distinct contexts. Apparently conflicting results have previously been reported on whether *Hes1* expression and function depend on Notch signalling. *Hes1* has been shown to be transcribed in response to Notch activation and *Hes1* activity to be important for Notch signalling in embryonic neural tissues (Ohtsuka et al., 1999). In contrast, *Hes1* transcript levels are not detectably affected in the neural tube and other tissues of embryos mutant for *Rbp-J* or *Notch1*, suggesting on the contrary that the transcription of *Hes1* is largely independent of Notch signalling (de la Pompa et al., 1997). Therefore *Hes1* may also have both Notch-dependent and Notch-independent activities in embryonic tissues other than the OE and particularly in the neural tube.

**Hes5 is a neurogenic gene**

In contrast to the complexity of *Hes1* expression patterns and functions, our results and published data provide strong evidence that *Hes5* is transcribed in the embryonic nervous system solely in response to Notch signalling and that *Hes5* functions only in lateral inhibition. Firstly, as for *Hes1*, the *Hes5* gene contains consensus binding sites for RBP-J and Notch signalling activates the *Hes5* promoter in cell lines, and induces endogenous *Hes5* expression in retinal explants (Kageyama and Nakanishi, 1997; Ohtsuka et al., 1999).

Secondly, *Hes5* transcripts normally present in the brain and spinal cord are almost completely eliminated in *Rbp-J* mutant embryos and severely reduced in *Notch1* mutant embryos, supporting the view that *Hes5* transcription requires Notch signalling (de la Pompa et al., 1997). Similarly, *Hes5* expression in olfactory placodes is greatly reduced in *Mash1* mutant embryos and is thus likely controlled by Notch activity in this tissue as well.

Although *Hes5* mutant animals do not show any gross abnormalities, the sharp increase in the frequency of neurulation defects in double *Hes1*:*Hes5* mutant embryos, and the neurogenic phenotype of their olfactory placodes (increased size and number of *Ngf1*-positive cell clusters and increased neuronal density), argue that *Hes5* is a negative regulator of neurogenesis in the mouse embryo, and that *Hes1* and *Hes5* can to some extent functionally compensate for the loss of one another. This conclusion is strongly supported by results of experiments in which forced expression of an activated form of Notch1 was able to accelerate the differentiation of neuroepithelial cells from wild-type embryos as well as *Hes1* and *Hes5* single mutants, but not from *Hes1*:*Hes5* double mutant embryos (Ohtsuka et al., 1999). Therefore, either *Hes1* or *Hes5* appear to be required in the neural tube of mouse embryos to mediate Notch signalling. However, the neurogenic phenotype that we observe in the OE of double *Hes5* mutants appears to be only partial, since the majority of MASH1-positive cells remain isolated and only a subset of them are grouped in clusters, suggesting that other genes mediating lateral inhibition must exist in this tissue.

**Regulation of the OSN lineage by Notch signalling at several steps**

Distinct stages of OSN progenitor maturation have been defined by the sequential expression of the three neural bHLH genes *Mash1*, *Ngf1* and *NeuroD* (Cau et al., 1997). *Mash1* is involved in the generation of basal OSN progenitors (Cau et al., 1997 and our unpublished data), whereas *Ngf1* is required for their differentiation (E. C., Q. Ma, D. J. Anderson and F. G., unpublished data), and the function of *NeuroD* in this lineage has not yet been characterized. Analysis of the *Hes1* mutant phenotype demonstrates that *Hes1* regulates OSN development at the level of *Mash1* expression. Despite the enlargement of the *Mash1*-positive cell population in *Hes1* mutant olfactory placodes, there is only a relatively small increase in expression of *Ngf1*, suggesting that the step of *Ngf1* expression is also subject to negative regulation. Indeed, the phenotype of *Hes1*:*Hes5* double mutant placodes shows a further increase in number of *Ngf1*-positive cells and of *SCG10*-positive neurons without change in expression of *Mash1*, indicating that *Hes5* is likely to regulate OSN development at the level of *Ngf1* expression. Altogether, these data suggest that Notch signalling, acting through different *Hes* genes, regulates the production of OSNs by targeting the expression of two bHLH genes which control the development of the lineage at two distinct steps at least (Fig. 5). This complex negative regulation of the OSN lineage at two levels, the determination and the differentiation of basal progenitors, allows for a fine tuning of the rate of neuronal production in the OE.

The observation that Notch signalling regulates neurogenesis at several levels, proneural gene transcription and a downstream step, is not unprecedented. It has been proposed that *Drosophila* E(Spl) proteins antagonize proneural genes not only by binding to their promoter and repressing their transcription, but also by dimerizing with proneural proteins, and also possibly by competing with them for binding to the promoters of common target genes (Nakao and Campos-Ortega, 1996; Jennings et al., 1999). Similarly, HES1 represses *Mash1* transcription but also interferes with MASH1 activity.
**Fig. 5.** A model of the interactions between the negative regulators \textit{Hes1} and \textit{Hes5} and the positive regulators \textit{Mash1} and \textit{Ngn1} during OSN lineage development. The arrows represent regulations at the transcriptional level. \textit{Mash1} controls the expression of both \textit{Hes1} and \textit{Hes5} by a mechanism which, by analogy with other systems where \textit{achaete-scutep} and \textit{enhancer of split} genes interact, is likely to involve the Notch pathway. The arrow with a question mark refers to the earlier, \textit{Mash1}-independent, regulation of \textit{Hes1}. There is an interesting correlation between the stage in the OSN lineage at which positive and negative regulators act and their expression pattern in the apical-basal axis of the OE. The early activity of \textit{Mash1} in OSN precursor determination (Cau et al., 1997) and of \textit{Hes1} in \textit{Mash1} regulation (this paper) correlate with the apical expression of these two genes, while the later function of \textit{Ngn1} in OSN differentiation (our unpublished data) and of \textit{Hes5} in \textit{Ngn1} regulation (this paper) correlate with the basal expression of these two genes. This suggests a model whereby \textit{Mash1}-positive apical cells would represent an early progenitor stage in the OSN lineage, regulated by \textit{Hes1}. These cells would give rise to more mature progenitors which migrate basally, express \textit{Ngn1}, and are regulated by \textit{Hes5}. To test this model will require studying the lineal relationships between apical and basalmost cells, and between apical precursors and OSNs. Also, our analysis of the \textit{Hes1} mutant and \textit{Hes5} mutant phenotypes has not addressed whether these genes were acting in apical cells and basal cells, respectively, because it focused on placodes at E10.5, when the two populations of progenitors are not yet segregated. The synergy between \textit{Hes1} and \textit{Hes5} during \textit{Ngn1} regulation (Fig. 4; Table 3) could be due to \textit{Hes5} directly repressing \textit{Ngn1}, and \textit{Hes1} repressing the positive regulator \textit{Mash1}. Alternatively, \textit{Hes1} could also act directly on \textit{Ngn1} regulation (shown in the diagram by a dashed line). The comparison between the modest increase in \textit{Ngn1} expression and the strong increase in number of SCG10-positive neurons in \textit{Hes1}/\textit{Hes5} double mutant placodes also suggests that \textit{Hes5} could regulate a step in OSN differentiation downstream of \textit{Ngn1} expression (dashed line).

in hippocampal neuron cultures, possibly by titration of a dimerization partner of MASH1 (Castella et al., 1999). The exact mechanisms by which \textit{Hes1} and \textit{Hes5} regulate the transcription of \textit{Mash1} and \textit{Ngn1} and the activity of their products in the OSN lineage remain to be examined.

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