Development/Plasticity/Repair

Identification of a Novel Basic Helix-Loop-Helix Gene, *Heslike*, and Its Role in GABAergic Neurogenesis

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Neuronal subtype specification depends on multiple transcription factors such as basic helix-loop-helix (bHLH) factors. However, transcription factor codes for most neurons remain to be determined. Here, we report identification of a novel mouse bHLH factor, termed Heslike, that has Hes1-like bHLH domain and transcriptional repressor activity. Heslike is coexpressed with the bHLH factor Mash1 in brain regions that give rise to GABAergic neurons. In the mesencephalon and the caudal diencephalon, coexpression of Heslike and Mash1 is initially restricted to small regions but expanded dorsally from embryonic day 9.5 onward, and this expansion of coexpression is followed by GABAergic neurogenesis. Misexpression of *Heslike* in mouse embryos generates ectopic GABAergic neurons only from the Mash1⁺ region. In contrast, in the mesencephalon and the caudal diencephalon of *Mash1*-null mice, GABAergic neurons are almost completely missing and, instead, other neurons are generated, although Heslike is still expressed. Furthermore, coexpression of *Heslike* and *Mash1* significantly promotes formation of GABAergic neurons, compared with each gene alone, in neural precursor cell culture. Thus, Heslike or Mash1 alone is not sufficient, but their coexpression may be important for generation of GABAergic neurons. These results suggest that combinations of distinct bHLH factors promote formation of distinct neuronal subtypes, thereby increasing neuronal diversity.

Key words: bHLH; diencephalon; GABAergic neuron; *Heslike*, *Mash1*; mesencephalon

Introduction

A wide variety of neurons is generated in a spatiotemporal-specific manner during neural development. The mechanism for generation of such neuronal diversity remains to be determined, but recent studies have revealed that transcription factors with a basic helix-loop-helix (bHLH) domain play an essential role in neurogenesis (Bertrand et al., 2002; Ross et al., 2003). Neuronal bHLH genes such as *Mash1* and *Math3* are coexpressed by subsets of cells and, in their absence, those cells that would normally differentiate into neurons adopt the glial fate, indicating that these bHLH genes cooperatively regulate neuronal versus glial fate determination (Tomita et al., 2000; Nieto et al., 2001). A neuronal bHLH gene actively inhibits glial differentiation while specifying pan-neuronal characteristics by independent mechanisms (Sun et al., 2001).

Neuronal bHLH genes such as *Mash1* and *Neurogenin2* (*Ngn2*) are expressed in a complementary manner and exhibit distinct functions. *Mash1* is primarily expressed in the ventral telencephalon and regulates formation of GABAergic interneurons, whereas *Ngn2* is expressed in the dorsal telencephalon and regulates formation of glutamatergic pyramidal neurons (Fode et al., 2000; Parras et al., 2002). Thus, bHLH genes regulate neuronal subtype identity in addition to specifying pan-neuronal characteristics. Interestingly, it was shown that combinations of distinct bHLH genes further increase the repertoire of neuronal and glial subtypes. A combination of the bHLH genes *Ngn2* and *Olig2* promotes motor neuron formation (Mizuguchi et al., 2001; Novitch et al., 2001), whereas each gene alone generates other neurons and oligodendrocytes, respectively (Lu et al., 2001, 2002; Zhou et al., 2001; Zhou and Anderson, 2002). However, the bHLH gene codes for such cell-type specification are mostly unknown.

GABAergic neurons are the principal inhibitory interneurons in brain functions. It has been shown that GABAergic neurons are born in the ventral telencephalon and migrate tangentially to the dorsal telencephalon (De Carlos et al., 1996; Anderson et al., 1997a,b; Casarosa et al., 1999; Sussel et al., 1999; Corbin et al., 2001), in contrast to the excitatory glutamatergic neurons that migrate radially along the radial fibers. In addition to the bHLH gene *Mash1*, the homeodomain genes *Nkx2.1*, *Dlx1/2*, and *Gsh2* are involved in formation of GABAergic neurons in the telencephalon (Anderson et al., 1997a,b; Casarosa et al., 1999; Sussel et al., 1999; Corbin et al., 2000; Marin et al., 2000; Toresson et al., 2000; Yun et al., 2002). Although GABAergic neurons are differentiated widely throughout the CNS, expression of *Nkx2.1* and *Dlx1/2* is restricted to the forebrain (Shimamura et al., 1995). Thus, the factors that induce GABAergic neuron formation in other brain regions remain to be determined.

Here, we report identification of a novel bHLH gene, termed *Heslike*, that has Hes1-like bHLH domain and transcriptional...
repressor activity. Heslike is coexpressed with Mash1 in brain regions that give rise to GABAergic neurons. We found that these two bHLH factors cooperatively promote generation of GABAergic neurons, whereas Heslike or Mash1 alone cannot. These results suggest that combinations of distinct bHLH factors promote formation of distinct neuronal subtypes, thereby increasing neuronal subtype diversity.

Materials and Methods

Isolation and characterization of Heslike cDNA. Reverse transcriptase (RT)-mediated PCR was performed against mouse embryonic day (E) 9.5 mRNA using fully degenerate primers deduced from the amino acid sequences in the bHLH region of Hes1. A PCR clone encoding a novel bHLH amino acid sequence was selected as a probe for screening a mouse E9.5 cDNA library. A full-length cDNA clone was obtained and named...
Heslike for its high homology in the bHLH region to Hes and Hesr genes. Mouse Heslike genomic clones were also isolated and sequenced. Human, mouse, rat, zebrafish, and fugu genomic sequences were obtained from the human, mouse, rat, zebrafish, and fugu genomic databases and compared with the mouse sequence.

Luciferase assay. The reporter plasmid contained the firefly luciferase gene under the control of the thymidine kinase (TK) promoter with three repeats of the upstream activating sequence (UAS) sequences or the β-actin promoter with six repeats of the N box elements. The luciferase reporter (0.1–0.2 μg) and the expression vector for Heslike, Hes1, or the fusion of Heslike and Hesr was cotransfected into C3H10T1/2 cells with the reporter under the control of three repeats of the UAS. For Heslike or Hes1, the luciferase reporter (0.2 μg) and the expression vector for Heslike, Hes1, or the fusion of Heslike and Hesr was cotransfected into C3H10T1/2 cells with the reporter under the control of three repeats of the N box elements. Each value with an SE represents four independent experiments performed in duplicate.

Animals and genotyping. All animals used in this study were maintained and handled according to protocols approved by Kyoto University.

Antibodies. cDNA for mouse Heslike was cloned from cDNA library of mouse brain by PCR using the following primers: sense, 5′-ACCCACCACTCTGGCGACGAGAGGGATCAA-3′; and the reverse, 5′-ACCCACCACTCTGGCGACGAGAGGGATCAA-3′ and the reverse, 5′-ACCCACCACTCTGGCGACGAGAGGGATCAA-3′. These primers produced 130 and 280 bp fragments from the wild-type and mutant alleles, respectively.

Antibodies. cDNA for Heslike fused with the 6×His tag sequence at the N terminus was cloned into pMNT T7 expression vector (Hirata et al., 2001).

Results

Identification of a novel bHLH gene Heslike

To identify a novel bHLH gene, we performed RT-PCR using primers homologous to the bHLH domain of Hes1. We identified a bHLH gene, termed Heslike (GenBank accession number AB098077 of mouse Heslike cDNA), from cDNA library of mouse embryos at E9.5. Heslike has a high sequence homology in the bHLH domain (Fig. 1A) and a weak homology in the Orange domain (Fig. 1B) to Hes (Sasai et al., 1992) and Hesr factors (Kokubo et al., 1999; Leimeister et al., 1999; Nakagawa et al., 1999; Chien et al., 2000; Zhong et al., 2000; Iso et al., 2001). However, it lacks proline–glycine residues in the middle of the basic domain.
region, which are conserved in Hes–Hesr, respectively, and instead it has a lysine residue (Fig. 1 A, asterisk). Because the amino acid residue at this position is known to be important for specific DNA binding (Davis et al., 1990), Heslike could bind to a sequence different from the Hes–Hesr-binding sites, although Heslike protein generated in vitro can bind to the N box on gel shift assay, like Hes1 (data not shown). In addition, Heslike does not have WRPW–YRPW sequences at the carboxy terminus (Fig. 1D), which are conserved by Hes–Hesr factors, respectively. Because the WRPW sequence is known to function as a repression domain by recruiting the corepressor TLE/Grg (Paroush et al., 1994; Grbavec and Stifani, 1996), Heslike could have a transcriptional activity different from Hes. On the basis of the bHLH sequence comparison, it is likely that Heslike constitutes a related but distinct subfamily (Fig. 1C). Database analysis indicates that Heslike is conserved in other vertebrates, including human, rat, thalamus and the prethalamus (Puelles and Rubenstein, 2003), whereas the dorsal stripes end in the pretectum (Fig. 3C, D). At E10.5, the Heslike expression domain is expanded dorsally in the isthmus, the boundary between the mesencephalon and rhombencephalon (Fig. 3C, D). Ros-trally, the bilateral expression domains are split into dorsal and ventral stripes (Fig. 3C, D). The ventral stripes are extended rostrally to the zona limitans intrathalamic (ZLI), the boundary between the rostral migratory stream (Fig. 3I, J, arrowheads), which contains precursors for olfactory bulb interneurons. In addition, Heslike is expressed in the vromeronasal organ (Fig. 3K–M) but not in invertebrates such as Drosophila, ascidian, and C. elegans. In addition, using database searching, we did not find a gene more closely related to Heslike than Hesr, and Dec.

Heslike acts as a transcriptional repressor
To analyze the transcriptional activity of Heslike, we performed a transient transfection assay. We first examined the transcriptional activity of Heslike fused with the DNA-binding domain of GAL4 (GAL4BD), which binds to the UAS sequence. This fusion product efficiently represses transcription from the promoter containing the UAS sequences, whereas GAL4BD alone does not (Fig. 2A). These results indicate that Heslike has a transcriptional repressor activity. Because Heslike can bind to the N box on gel shift assay (data not shown), we next examined whether Heslike acts as an N box-dependent transcriptional repressor. As shown in Figure 2B, Heslike efficiently represses transcription from the promoter containing N box sequences, although the repression activity is weaker than Hes1. These results indicate that Heslike acts as a transcriptional repressor.

Expression pattern of Heslike
To determine the expression pattern of Heslike, we performed in situ hybridization. At E9.5, Heslike expression is first observed bilaterally in restricted regions of the rostral mesencephalon (Fig. 3A, B). At E10.5, the Heslike expression domain is expanded caudally toward the isthmus, the boundary between the mesencephalon and rhombencephalon (Fig. 3C, D). Ros-trally, the bilateral expression domains are split into dorsal and ventral stripes (Fig. 3C, D). The ventral stripes are extended rostrally to the zona limitans intrathalamic (ZLI), the boundary between the

Figure 3. In situ hybridization analysis of Heslike. A, B, At E9.5, Heslike is detectable around the alar-basal boundary of the mesencephalon. C, D, At E10.5, Heslike expression domain is expanded caudally toward the isthmus. Rostrally, the expression domain is split into two stripes. The ventral stripe is extended to the ZLI, whereas the dorsal stripe ends in the pretectum (PT). Heslike is also expressed in the olfactory placode (OP), LGE, and prethalamus (PTh). E–G, Heslike expression is upregulated during E11.5–E13.5. In addition, it is expanded dorsally in the PT and mesencephalon (Mes). The expression also occurs in the preoptic area (POA) and hypothalamus (HT). H, Transverse section of the telencephalon. Heslike is expressed in the subventricular zone (SVZ). I, J, Parasagittal sections. Heslike is expressed in the SVZ–rostral migratory stream (RMS). K–M, Transverse sections. Heslike is expressed in the sensory epithelium (S) of vomeronasal organ. Heslike domain becomes restricted to the basal layer at E13.5 (I) and later occupies the small region close to the nonsensory epithelium (NS) (M, arrowheads). Cx, Cortex; III, third ventricle; Is, isthmus; LV, lateral ventricle; OB, olfactory bulb; OE, olfactory epithelium; Th, thalamus; VNO, vromeronasal organ. Scale bars: H–J, 500 μm; K–M, 100 μm.
not in the olfactory epithelium (Fig. 3I). 

Heslike is not expressed in the regions caudal to the isthmus (data not shown).

To examine Heslike expression in more detail, we generated an antibody (Ab) specific to the Heslike protein and performed immunohistochemistry. This Ab stains the nucleus, and all regions that are reactive to this Ab express Heslike mRNA (data not shown). Heslike+ cells are detectable in the ventricular zone of the ventral mesencephalon as early as E9.5 (Fig. 4I, arrowhead) and in number at E10.5 (Fig. 4A,B,E,F). All of the Heslike+ cells coexpress Ki67, an antigen detected in proliferating cells in all phases of the cell cycle (Fig. 4B–D) (Kill, 1996). In addition, some Heslike+ cells coexpress phosphorylated histone H3, an M phase-specific marker (Fig. 4F–H, arrowheads). Thus, Heslike is specifically expressed by proliferating ventricular cells.

To define the Heslike expression domain, we next compared it with the expression of the homeobox factor Nkx2.2. At E9.5, Heslike expression overlaps around the alar-basal boundary with the Nkx2.2+ domain (Fig. 4I, arrowhead), which extends from the alar-basal boundary into the ventral region of the mesencephalon. At E10.5, the Nkx2.2+ domain is restricted to the alar-basal boundary region (Fig. 4J) (Shimamura et al., 1995), whereas Heslike expression is expanded and includes the Nkx2.2+ domain (Fig. 4J). At approximately E10.5–E11.5, a new Nkx2.2+ domain appears dorsally, and the Heslike+ domain overlaps with both regions (Fig. 4K). By E12.5, the Heslike+ domain is further expanded dorsally, nearly reaching the roof plate (Fig. 4L). However, Heslike expression is mostly absent from the ventral mesencephalon. At E13.5, Heslike expression is downregulated and disappears at E15.5 from the Nkx2.2+ domains (data not shown). These results indicate that Heslike is expressed mostly by the mitotic cells of the dorsal mesencephalon.

**Figure 4.** Heslike expression in proliferating cells. A–H. Transverse sections of E10.5 mesencephalon. A higher magnification of the indicated region in A and E is shown in B–D and F–H, respectively. At E10.5, all Heslike+ cells express Ki67, an antigen detected in proliferating cells B–D. Depending on the phases of the cell cycle, Ki67 expression is observed as dots or diffuse expression (C,D). Some Heslike+ cells express phosphorylated histone H3, an M phase-specific marker (F–H, arrowheads). I–L, Comparison of Heslike and Nkx2.2 expression domains. At E9.5, Heslike expression overlaps with the Nkx2.2+ domain (I, arrowhead), which extends from the alar-basal boundary into the ventral region of the mesencephalon. At E10.5, the Nkx2.2+ domain is restricted to the alar-basal boundary region (J), whereas Heslike expression is expanded and includes the Nkx2.2+ domain (J). At approximately E10.5–E11.5, a new Nkx2.2+ domain appears dorsally, and the Heslike+ domain overlaps with both regions (K). By E12.5, the Heslike+ domain is further expanded dorsally, nearly reaching the roof plate (L) and still overlaps with both Nkx2.2+ domains. Scale bars: A, E, I–L, 200 μm; B–D, F–H, 100 μm.

**Coexpression of Heslike and Mash1 in the ventricular zone for GABAergic neurogenesis**

Because Heslike expression domains in the mesencephalon as well as in other regions such as the LGE, prethalamus, rostral migratory stream, and vomeronasal organ are similar to the regions for GABAergic neurogenesis (Wray et al., 1996; Katarova et al., 2000), we next examined the relationship between Heslike expression and markers for GABAergic neurons. We used antibodies to GABA and GAD65, a biosynthetic enzyme for GABA, to detect GABAergic neurons. At E9.5, when Heslike+ cells appear (Fig. 5B, arrowhead), there are no GABAergic neurons (GABA+ GAD65+) in the mesencephalon, although neurons (TuJ1+) are generated (Fig. 5A,B). At E10.5, GABAergic neurons are differentiated bilaterally in the mantle layer of the ventral mesencephalon (Fig. 5D,E,E, green staining), which are located just outside the Heslike+ domains (Fig. 5E,E, red staining). After this stage, as the Heslike+ domains in the ventricular zone are expanded dorsally, GABAergic neurons also appear dorsally in the mantle layer (Fig. 5G,G’,H, H’,J’,K’,L’,K’). Thus, expansion of GABAergic neurogenesis follows that of Heslike expression. These results suggest that onset of Heslike expression in ventricular cells induces differentiation of GABAergic neurons.

Because Mash1 is known to regulate differentiation of GABAergic neurons in the telencephalon (Fode et al., 2000), we next examined the relationship between Heslike and Mash1 expression patterns in the mesencephalon. At E9.5, Mash1 expression is observed in two domains: one overlaps with the Heslike+ region (Fig. 5C, insets, arrowhead), whereas the other is located in the dorsal mesencephalon (Fig. 5C). At E10.5, when the two Mash1+ domains are connected, most ventricular cells located in the alar-basal boundary regions coexpress Heslike and Mash1 (Fig. 5F,F’), whereas cells located in the dorsal mesencephalon express Mash1 only (Fig. 5F). At E11.5 and E12.5, as the Heslike+ region is gradually expanded dorsally, more cells coexpress Heslike and Mash1 (Fig. 5I,J,L,L’). Thus, most of the Heslike+ cells coexpress Mash1 in the mesencephalon, indicating that GABA+ GAD65+ cells are present in the mantle layer just outside the Heslike+ Mash1+ ventricular zone. These results raise the possibility that coexpression of Heslike and Mash1 may be involved in formation of GABAergic neurons in the mesencephalon.

We also examined the relationship between Heslike–Mash1 expression and GABAergic neurogenesis in other regions. In the pretectum, GABAergic neurons (GABA+ GAD65+) are initially differentiated in two stripes at E10.5 (Fig. 6A, arrowheads, B). Then, at E11.5 and E12.5, GABAergic neurogenesis also occurs in the dorsal region (Fig. 6E,F,I,J). At E10.5, Heslike is expressed in two bilateral stripes, which are next to the initial two stripes of...
GABAergic neurons (Fig. 6B). At this stage, Mash1 is widely expressed in the dorsal two thirds, which include the two stripes of Heslike+ domains (Fig. 6C). The dorsal stripe of the Heslike+ domain is expanded dorsally at E11.5 (Fig. 6F–H) and at E12.5 (Fig. 6J–L), whereas the ventral stripe does not show much change (Fig. 6F–H, J–L). During these stages, Heslike is coexpressed with Mash1 in the two bilateral stripes (Fig. 6G,K). Strikingly, there are many GAD65+ cells in the mantle layer just outside the Heslike+ Mash1+ ventricular zone (Fig. 6F,J). Thus, coexpression of Heslike and Mash1 correlates well to GABAergic neurogenesis in this region. We also compared expression of Heslike with that of Nkx2.2, which occurs in the alar-basal boundary region. During E10.5–E12.5, the ventral stripe of the Heslike+ domain overlaps with Nkx2.2 expression, but Heslike is not expressed ventrally to the Nkx2.2+ domain (Fig. 6D,H,L), indicating that Heslike is not expressed in the basal plate.

Heslike is also highly expressed by the cells located in a stripe caudal to the ZLI, which expresses Shh (Fig. 6P). These cells coexpress Mash1 (Fig. 6N,O, arrow), and there are many GABAergic neurons (GAD65+) outside this Heslike+ Mash1+ stripe (Fig. 6M). Altogether, these results indicate that coexpression of Heslike and Mash1 correlates well to GABAergic neurogenesis in the mesencephalon and the caudal diencephalon.

In the region rostral to the ZLI, subsets of ventricular cells in the prethalamus, LGE, CGE, and preoptic region coexpress Heslike and Mash1, but none of the cells in the MGE do (data not shown). Although GABAergic neurons are generated in the mantle layer just outside the Heslike+ Mash1+ regions, the number of Heslike+ cells is much fewer in these regions than that of GABAergic neurons, suggesting that Heslike may be involved in differentiation of only subsets of GABAergic neurons in this area (data not shown).

Heslike induces GABAergic neurogenesis from Mash1+ region

To characterize the function of Heslike, we generated transgenic mice misexpressing Heslike from the nestin promoter–enhancer. This promoter–enhancer induces Heslike expression widely in the ventricular zone (Fig. 7B, I), as described previously (Zimmerman et al., 1994; Isaka et al., 1999). Because these mice typically die by E12.5, we examined founder embryos of E10.5 and E11.5 (n = 5). In the mesencephalon, misexpression of Heslike induces ectopic GABAergic neurons in the regions both ventral and dorsal to the original GAD65+ domains at E10.5 (Fig. 7, compare A and B). The dorsal mesencephalon, which normally expresses only Mash1 (Fig. 7E) and does not yet give rise to any GABAergic neurons at E10.5 (Fig. 7A), prematurely generates GAD65+ cells by misexpression of Heslike (Fig. 7C; some ectopic GAD65+ cells are indicated by arrowheads). Similarly, the region just ventral to the original GAD65+ domain that normally expresses Mash1 only and does not give rise to GABAergic neurons at any stages generates ectopic GABAergic neurons by misexpression of Heslike (Fig. 7D; arrowheads). In these regions, Mash1 is also expressed (Fig. 7F–H).

In a different transgenic embryo, Heslike is ectopically expressed by subsets of ventricular cells of the mesencephalon and the caudal diencephalon at E11.5 (Fig. 7, compare J and N with I and M). In these mice, ectopic GABAergic neurons are generated in the regions both ventral and dorsal to the original GAD65+ domains (Fig. 7J,N). Again, the dorsal region, which normally expresses only Mash1 (Fig. 7I) and does not yet give rise to any GABAergic neurons at E11.5 (Fig. 7L), prematurely generates GAD65+ cells by misexpression of Heslike (Fig. 7K,L, arrow-
Mash1 is expressed (data not shown). These results suggest that the mechanism for GABAergic neurogenesis may be different between the regions rostral and caudal to the ZLI.

**Loss of GABAergic neurons in the absence of Mash1**

Because Heslike does not induce ectopic GABAergic neurons in the Mash1-negative region, we next examined the requirement of Mash1 for GABAergic neurogenesis. It was previously shown that in the absence of Mash1, although neuronal precursors are severely lost, GABAergic neurons are generated in the ventral telencephalon, suggesting that Mash1 is dispensable for GABAergic neurogenesis in the telencephalon (Casarosa et al., 1999). We thus examined other regions of Mash1-null mice. In the region between the ZLI and the isthmus of Mash1-null mice, only a very few GABAergic neurons (GABA+/GAD65−) are differentiated (Fig. 8, compare D, D′, F, and F′ with C, C′, E, and E′, respectively), even though more ventricular cells seem to express Heslike in Mash1-null mice (Fig. 8, compare B and B′ with A and A′). Thus, Heslike alone is not sufficient, but Mash1 is required for generation of most GABAergic neurons in this region. Because neurons (TuJ1+) are generated throughout Mash1-null mesencephalon (Fig. 8 F, F′) and caudal diencephalon (data not shown), it is possible that, instead of GABAergic neurons, different subtypes of neurons are generated. These results indicate that Heslike and Mash1 cooperatively specify GABAergic neurons in the region between the ZLI and the isthmus, whereas either factor alone is not sufficient for such specification.

In other regions of the nervous system of Mash1-null mice, many GABAergic neurons are still differentiated, although they are reduced in number as described previously (Casarosa et al., 1999; Parras et al., 2002; Murray et al., 2003) (data not shown). Thus, dependency on Mash1 in GABAergic neurogenesis is rather specific to the region between the ZLI and the isthmus,
sion of Heslike and Mash1 significantly increases the number of GABAergic neurons (Fig. 9A, B), compared with expression of Heslike or Mash1 alone (Fig. 9B). These results support the notion that Heslike and Mash1 cooperatively specify the GABAergic neuronal fate.

**Discussion**

Heslike, together with Mash1, specifies the GABAergic neuronal fate

Here, we identified a novel bHLH factor, termed Heslike, which is coexpressed with Mash1 by mitotic cells in the ventricular zone of many brain regions. At E9.5, Heslike and Mash1 are coexpressed in the ventral mesencephalon and then this coexpression is expanded to other regions. Strikingly, many GABAergic neurons are formed in the mantle layer just outside the Mash1−/− ventricular zone after Heslike is coexpressed. GABAergic neurogenesis in the region between the ZLI and the isthmus always follows coexpression of Heslike and Mash1, indicating that Heslike and Mash1 cooperatively promote GABAergic neurogenesis. It is likely that Heslike regulates the timing of GABAergic neuronal differentiation from Mash1+ cells.

Immunohistochemical analysis does not show any coexpression of Heslike and GAD65−/−GABA because Heslike is expressed by proliferating cells, whereas GAD65 and GABA are expressed by postmitotic cells. Thus, it remains to be determined whether Heslike+Mash1+ cells really differentiate into GABAergic neurons. However, previous studies demonstrated that, unlike in the telencephalon, the majority of the ventricular cells in the mesencephalon migrate radially (Tan et al., 2002). Thus, it is most likely that the Heslike+Mash1+ ventricular cells differentiate into GABAergic neurons. Consistent with this notion, misexpression of Heslike in the Mash1+ region generates ectopic GABAergic neurons in the mantle layer just outside the Heslike+Mash1+ ventricular zone in the mesencephalon and the caudal diencephalon.

In Mash1-null mice, GABAergic neurons are primarily missing in the region between the ZLI and the isthmus, although Heslike is still expressed. In these mutants, TuJ1+ neurons are differentiated, suggesting that different subtypes of neurons are generated when Mash1 is absent and only Heslike is expressed. Similarly, when only Mash1 is expressed, there are no GABAergic neurons in the region between the ZLI and the isthmus, although TuJ1+ neurons are differentiated, suggesting that different subtypes of neurons are generated when Heslike is absent and only Mash1 is expressed. Thus, Heslike or Mash1 alone is not sufficient, but their coexpression may be required for GABAergic

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Figure 7. Promotion of GABAergic neurogenesis in mice misexpressing Heslike. A–T. Transgenic mice misexpressing Heslike from the nestin promoter−enhancer (B−D, F−H, J−L, N−P, S, T) and wild-type mice (A, E, I, M, O, R) were analyzed at E10.5 and E11.5 by immunohistochemistry. B−D, F−H. In this transgenic embryo, Heslike is widely expressed in the mesencephalon. Many GABAergic neurons are ectopically formed in the regions both ventral and dorsal to the original GAD65+/− region (B−D, arrowheads, compare with A). In these regions, Mash1 is coexpressed (G, H). J−L, N−P. In this transgenic embryo, Heslike is misexpressed in the mesencephalon (J−L) and the caudal diencephalon (N−P). In the dorsal region, which normally expresses only Mash1 at this stage, misexpression of Heslike prematurely generates GABAergic neurons (K, L, arrowheads). In the ventral region, which normally expresses Mash1 only and does not give rise to GABAergic neurons at any stages, misexpression of Heslike ectopically generates GABAergic neurons (GAD65+/−) (O, P, arrowheads). The original GAD65−/− domains are indicated by brackets (I, J, M, N). Q−T. In the dorsal telencephalon (Q) and the thalamus (R), Mash1 is not expressed. In these regions, misexpression of Heslike does not generate ectopic GABAergic neurons (S, T, arrowheads). Scale bars: A, B, E, F, I−T, 200 μm; C, D, G, H, 50 μm.

Co-expression of Heslike and Mash1 increases the population of GABAergic neurons in neural precursor cell culture

To examine the cooperative activities of Heslike and Mash1 in GABAergic neurogenesis, we performed neural precursor cell culture. The expression vectors for Heslike and Mash1 were transfected into E11.5 embryonal telencephalon, and neural precursor cells were prepared from the transfected brains. Coexpression of Heslike and Mash1 increases the population of GABAergic neurons in neural precursor cell culture, suggesting that in other regions, as yet unidentified factors may be involved in generation of GABAergic neurons.

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neurogenesis. However, it is also possible that in Mash1-null mice, GABAergic neurons are simply eliminated because of loss of the proneural activity of Mash1 rather than mis-specified. In this case, non-GABAergic neurons could be differentiated from distinct precursors, which depend on other proneural genes such as Ngn1 (Ma et al., 1997). Whatever the case, combination of Heslike and Mash1 is important for GABAergic neurogenesis, because misexpression of Heslike does not induce GABAergic neurons in the regions that do not express Mash1.

The results shown above suggest that the caudal diencephalon and the mesencephalon may use different strategies from the telencephalon to generate neuronal subtype diversity. In the telencephalon, GABAergic neurons are generated ventrally and migrate tangentially to the dorsal telencephalon, indicating that neuronal migration contributes to the neuronal diversity of the dorsal telencephalon. In contrast, in the caudal diencephalon and the mesencephalon, the ventricular cells change their expression profile of bHLH factors over time and gain competency to produce GABAergic neurons, thereby increasing neuronal diversity.

Co-expression of Heslike and Mash1 may be involved in GABAergic neurogenesis in other regions

Although GABAergic neurons are virtually missing in the region between the ZLI and the isthmus of Mash1-null mice, they are generated in other regions (rostral to the ZLI and caudal to the isthmus), suggesting that GABAergic neurogenesis depends on different transcription factors in such regions. In the ventral telencephalon, homeodomain factors such as Nkx2.1 and Dlx1/2 are involved in GABAergic neurogenesis (Anderson et al., 1997a,b; Casarosa et al., 1999; Sussel et al., 1999; Marin et al., 2000). These homeodomain factors are not expressed in other regions. In the region caudal to the isthmus, Heslike is not expressed, and other homeodomain factors are essential for GABAergic neurogenesis (Jessell, 2000; Caspary and Anderson, 2003). These results suggest that the three regions rostral to the ZLI, between the ZLI and the isthmus, and caudal to the isthmus use different transcription factor sets for GABAergic neurogenesis.

Although Heslike may not be an essential factor for GABAergic neurogenesis in the region rostral to the ZLI, it is always coexpressed with Mash1 in this region by the ventricular cells that give rise to GABAergic neurons. The number of those Heslike+/Mash1+ cells is much smaller compared with the extensive number of GABAergic neurons in this area. Thus, although Heslike is not required for generation of the majority of GABAergic neurons, it could be involved in differentiation of subsets of GABAergic neurons in the region rostral to the ZLI. Consistent with this notion, we found that coexpression of Heslike and Mash1 in neural precursor cells of the telencephalon promotes generation of GABAergic neurons.

Figure 8. Lack of GABAergic neurons in the mesencephalon of Mash1-null mice. A–F, A’–F’. The wild-type (A, A’,C,C, E, E’) and Mash1-null (B, B’, D, D’, F, F’) mice were analyzed at E11.5 by immunohistochemistry. In Mash1-null embryos, Heslike+ ventricular cells are increased in number (compare A’ and C with B’ and D’). Although neurons (TuJ1+) are generated (F, F’), virtually no GABAergic neurons (GABA-/GAD65+) are formed in Mash1-null mesencephalon (D, D’, F, F’), whereas many GABAergic neurons are generated in the mantle layer located outside the Heslike+ Mash1+ region of the wild type (C,C, E,E’). Scale bars, 200 μm.

Combinations of distinct transcription factors increase the repertoire of neuronal subtypes

It has been shown that combinations of bHLH and homeodomain factors specify neuronal subtypes. For example, in the retina, a combination of the bHLH factor Math3 and the homeodomain factor Chx10 generates bipolar neurons (Hatakeyama et al., 2001), whereas a combination of Math3 and the homeodomain factor Pax6 generates amacrine and horizontal neurons (Inoue et al., 2002). Thus, Math3 promotes specification of distinct neuronal subtypes depending on the combinatorial partners. The precise mechanism for this combinatorial action between bHLH and homeodomain factors is not known, but it was reported that some bHLH and homeodomain factors physically interact with each other. The bHLH factor Pan1 and the homeodomain factor Pitx1 form a complex through the bHLH domain and the homeodomain and synergistically induce gene expression (Poulin et al., 2000). It was also reported that functional coupling of bHLH and homeodomain factors is mediated by an adaptor protein (Lee and Pfaff, 2003).

The mechanism for combinatorial actions of Heslike and Mash1 also remains to be determined. One most likely mechanism is that Heslike and Mash1 may form a heterodimer complex through the bHLH domain and bind to a DNA sequence distinct from those recognized by their homodimers or heterodimers with the ubiquitous bHLH cofactor E47. Our results suggest that combinations of distinct bHLH factors promote formation of distinct neuronal subtypes. Similarly, coexpression of Ngn2 and Olig2 promotes somatic motor neuron formation, whereas each factor alone induces distinct cell types. Thus, a combinatorial action of distinct bHLH factors seems to be a general mechanism to increase the cell type diversity.
Similarities and differences between Heslike and Hes1

Although Heslike has a high sequence homology in the bHLH domain to Hes1, there are some structural differences between the two. The proline residue in the middle of the basic region conserved among all Hes factors is not present in Heslike. Furthermore, the carboxy-terminal WRPW sequence conserved among all Hes factors is not present in Heslike. Hence, the number of ectopic GABAergic neurons induced by misexpression of Heslike is relatively small. We thus speculate that, although Heslike endows ventricular cells with the GABAergic fate, downregulation of Heslike expression is required for maturation of GABAergic neurons. Additional analysis of Heslike will reveal the mechanism for specification of the GABAergic neuronal fate and the combinatorial actions of bHLH factors.

References


