Development 134, 2783-2793 (2007) doi:10.1242/dev.02870

# Helt determines GABAergic over glutamatergic neuronal fate by repressing Ngn genes in the developing mesencephalon

**Tomoya Nakatani*, Yasuko Minaki, Minoru Kumai and Yuichi Ono*,†**

The mechanism underlying the determination of neurotransmitter phenotype in the developing mesencephalon, particularly GABAergic versus glutamatergic fate, remains largely unknown. Here, we show in mice that the basic helix-loop-helix transcriptional repressor gene Helt (also known as Megane and Heslike) functions as a selector gene that determines GABAergic over glutamatergic fate in the mesencephalon. Helt was coincidently expressed in all the progenitor domains for mesencephalic GABAergic neurons. In the mesencephalon of Helt-deficient embryos, GABAergic neurons were mostly absent and glutamatergic neurons emerged instead. Conversely, ectopically expressed Helt suppressed glutamatergic formation and induced GABAergic neurogenesis. However, the Helt mutants showed normal progenitor domain formation. In consequence, postmitotic expression of the homeodomain factor Nkx2.2, which was specifically expressed by GABAergic populations in wild-type embryos, was maintained despite the transmitter phenotype conversion from GABAergic to glutamatergic in the Helt mutants, suggesting that Helt is not involved in neuronal identity specification. Furthermore, we identified proneural genes Ngn1 and Ngn2, which were selectively expressed in glutamatergic progenitors in the developing mesencephalon and had the ability to confer the glutamatergic fate, as downstream target genes of Helt. These results suggest that Helt determines GABAergic over glutamatergic fate, at least in part, by repressing Ngn (Neurog) genes and that basic helix-loop-helix transcription factor networks involving Helt and Ngns are commonly used in the mesencephalon for determination of the GABAergic versus glutamatergic transmitter phenotype.

**KEY WORDS: Helt, Neurogenin, Basic-helix-loop-helix, Transcriptional repressor, GABAergic neurons, Glutamatergic neurons, Mesencephalon, Neuronal identity, Transmitter phenotype determination**

## INTRODUCTION

During development, the neuronal subtype is diversified by extrinsic signals secreted from the organizer and by downstream intrinsic pathways regulated by transcription factors (Helms and Johnson, 2003; Jessell, 2000; Schuurmans and Guillemot, 2002). The subtype identity, which is determined by the expression of selective sets of transcription factors, defines the neurotransmitter usage, migration and axonal innervation pattern.

GABAergic and glutamatergic neurons are the principal inhibitory and excitatory neurons in the brain, respectively. One of the best-characterized regions involved in GABAergic versus glutamatergic transmitter decision control is the developing dorsal spinal cord. In this region, homeodomain and basic helix-loop-helix (bHLH) transcription factors play pivotal roles in the specification of the progenitors and derived neurons (Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005; Gross et al., 2002; Mizuguchi et al., 2006; Muller et al., 2002). Among these postmitotically expressed transcription factors, *Ptf1a* and *Tlx1/3* function as selector genes that switch alternative transmitter fates (Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005). By contrast, in the telencephalon, the proneural bHLH factors Mash1 and Ngn2 (also known as Ascl1 and Neurog2, respectively – Mouse Genome Informatics) are selectively expressed in progenitors for GABAergic and glutamatergic neurons, respectively, and are involved in the determination of transmitter phenotype (Fode et al., 2000; Parras et al., 2002), suggesting that the transmitter is chosen at the progenitor state. Proneural factors are also involved in the specification of dorsal spinal interneurons (Gowan et al., 2001; Helms et al., 2005; Muller et al., 2005; Nakada et al., 2004). However, the concept that transmitter selection is simply controlled by proneural genes at the progenitor state, as in the telencephalon, is unlikely in the dorsal spinal cord (Mizuguchi et al., 2006; Wildner et al., 2006). Thus, acquisition of the GABAergic or glutamatergic phenotype is controlled by distinct pathways in different brain areas. The mechanisms of transmitter selection have not yet been elucidated in other brain regions, such as the mesencephalon.

Previous studies have described arcuate expression patterns of transcription factors that might specify the neuronal subtypes in the mesencephalon (Agarwala and Ragsdale, 2002; Sanders et al., 2002). However, the transmitter patterns have not been accorded with the neuronal subtypes defined by transcription factor codes. Thus, the neuronal identity, location of neuron emergence and control of GABAergic and glutamatergic neuron development in the mesencephalon remain largely unknown.

The bHLH-Orange (bHLH-O) family consists of members related to Hes proteins that show conserved primary structures and transcriptional repressor functions (Davis and Turner, 2001). In neuronal development, Hes genes act as downstream effectors for the Notch pathway and inhibit neuronal differentiation by repressing proneural bHLH factor expression (Bertrand et al., 2002; Kageyama, 1999; Ross et al., 2003). This inhibitory function of Hes genes is required for lateral inhibition of neurogenesis, which controls progenitor maintenance and the timing of neuronal birth, and local organizer development in the neural tube (Baek et al., 2006;

KAN Research Institute Inc., KobeMI R&D Center, 6-7-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan.

*These authors contributed equally to this work
†Author for correspondence (e-mail: y-ono@kan.eisai.co.jp)

**Accepted 22 May 2007**

Bertrand et al., 2002; Kageyama, 1999; Ross et al., 2003). However, whether bHLH-O factors are involved in neuronal subtype specification remains uncertain.

Recently, we and others identified a novel bHLH-O family member, *Helt* (also known as *Heslike* and *Megane*), which is selectively expressed in neural progenitors in the developing mesencephalon and diencephalon (Guimera et al., 2006a; Miyoshi et al., 2004; Nakatani et al., 2004). Gain- and loss-of-function studies revealed that *Helt* has a potent activity for promoting mesencephalic GABAergic neuron development (Guimera et al., 2006b; Miyoshi et al., 2004). However, it has not been addressed whether *Helt* is only involved in the promotion of GABAergic neuron differentiation or whether it also plays a role in transmitter phenotype selection. Furthermore, downstream target genes of Helt have not yet been identified, and thus the mechanism of action of *Helt* remains to be clarified.

In the present study, we found that *Helt* functions as a selector gene that determines the GABAergic over glutamatergic transmitter phenotype in the mesencephalon. Furthermore, we identified Ngn (Neurog) genes, which are selectively expressed in glutamatergic progenitors in the mesencephalon and show activity for promoting glutamatergic differentiation, as downstream target genes of Helt. By using a mesencephalic domain map delineated by transcription factor expression, we further found that the *Helt* and Ngn pathways are commonly used in mesencephalic GABAergic and glutamatergic differentiation and that transmitter choice in mesencephalic neurons is not completely coupled with the specification of neuronal subtype, which might be regulated by homeodomain transcription factors. Taken together, our results illustrate the strategy of the transmitter phenotype decision in mesencephalic neuron development.

## MATERIALS AND METHODS

### Mice

A *Helt* targeting vector was assembled using ploxP-GFP-neo-DT-A, which contains GFP cDNA and loxP-flanked HSV TK-neomycin gene cassettes in pBluescript SK+ (Stratagene). Genomic sequences encompassing the mouse *Helt* gene were isolated from a 129SV genomic phage library. A 3 kb 5′-arm-containing genomic fragment just upstream of the initiation codon of *Helt*, and a 3.7 kb 3′-arm-containing fragment, were amplified by PCR and separately cloned into the *Sma*I and *Eco*RV sites of ploxP-GFP-neo-DT-A to generate the *Helt* targeting vector. *Helt*-null mice were generated by homologous recombination in the 129SVEV embryonic stem cell line according to standard procedures, and germline transmission of the mutation was confirmed by Southern blotting and PCR. *Helt*$^{-/-}$ mice were generated by crossing heterozygous mutant mice on a 129SV×C57Bl/6 background and genotyped by PCR.

pNE was constructed by ligating the SV40 poly(A) signal and genomic fragments for the intronic enhancer and promoter of the nestin gene (Miyoshi et al., 2004) into pSP73 (Promega). pNE-Helt was constructed by ligating a *Helt* cDNA (Nakatani et al., 2004) into the *Mlu*I/*Not*I sites of pNE. pNE-Ngn1 was constructed by ligating an *Ngn1* cDNA into the *Kpn*I/*Not*I sites of pNE. The primer sequences used for amplification of these fragments are available upon request. Linearized pNE constructs were injected into fertilized eggs and founder embryos were collected at E12.5. The embryos were genotyped by PCR.

### Immunohistochemistry and in situ hybridization

Immunohistochemistry was performed as described previously (Nakatani et al., 2004). The polyclonal rabbit anti-Helt antibody was also described previously (Nakatani et al., 2004). A rat anti-Nkx6.1 monoclonal antibody was raised against GST-Nkx6.1 (aa 60-122). The other primary antibodies used were: anti-Lim1/2, anti-Nkx2.2 and anti-Pax7 (Developmental Studies Hybridoma Bank); anti-Mash1 (BD Pharmingen); anti-Lim1, anti-Ngn1, anti-Ngn2 and anti-Hnf3β (Santa Cruz Biotechnology); and anti-Brn3a (Chemicon).

In situ hybridization was performed as described previously (Nakatani et al., 2004). The primer sequences used for amplification of probe cDNAs (*Gad1*, *Vglut2* and *GFP*) are available upon request.

### FACS

Ventral mesencephalons were dissected from E12.5 homozygous and heterozygous *Helt* mutant embryos and dissociated using Accumax (Chemicon). Cell sorting was performed using a FACS Aria (BD Biosciences). Total RNA was isolated from ~$5\times10^4$ sorted GFP$^+$ cells. cDNA amplicons were prepared as described (Osada et al., 2005) and the expression of homeobox and proneural genes was analyzed by RT-PCR (primer sequences available upon request).

## RESULTS

### Helt is commonly expressed in all GABAergic progenitor domains in the mesencephalon

In order to understand the mechanism of mesencephalic neuron specification and the role of *Helt* in transmitter phenotype determination, we first compared the expression pattern of Helt with those of transmitter markers and transcription factors potentially involved in the specification of subtype identity of the progenitors and derived neurons. By precisely analyzing the patterns of transcription factors expressed in the ventricular zone (VZ), such as Nkx2.2, Nkx6.1, Hnf3β (also known as Nkx2-2, Nkx6-1, Foxa2, respectively – Mouse Genome Informatics), Sim1 and Helt, we mapped seven progenitor domains in the anterior mesencephalon (Fig. 1A,B; summarized in Fig. 2). The ventral-most m7 domain generated dopaminergic neurons marked by Lmx1a expression (Fig. 1B) (Andersson et al., 2006), whereas the adjacent m6 domain produced glutamatergic red nucleus (RN) neurons (Fig. 1B) (Fedtsova and Turner, 2001). Helt was selectively expressed in the m2 to m5 domains at E11.5 and *Gad1*$^+$ GABAergic neurons emerged from these domains as previously reported (Fig. 1A,B) (Guimera et al., 2006a; Miyoshi et al., 2004; Nakatani et al., 2004). The neurons emerging from the m3 to m5 domains appeared to have distinct identities despite their common GABAergic phenotype and Helt expression in their progenitors, as they expressed different sets of postmitotically expressed transcription factors, such as Nkx2.2 and Hnf3β, in addition to the distinct transcription factor codes in their progenitors (Fig. 1A). The m2 domain, marked by Nkx2.2 expression in the mantle layer (ML), generated both GABAergic and glutamatergic neurons in a mosaic fashion (Fig. 1B). Nkx2.2 appeared to selectively mark GABAergic neurons in the m2 domain (Fig. 1B). The dorsal-most m1 domain marked by Pax7 expression only generated glutamatergic neurons at E11.5 (Fig. 1A,B). However, at later stages (E12.5-13.5), both GABAergic and glutamatergic neurons were generated in a salt-and-pepper pattern (Fig. 1A). From E11.5 onward, Helt started to be expressed in this domain as a ventral-to-dorsal wave, and at E12.5 Helt was expressed in all areas of the domain (Fig. 1A). Taken together, these observations indicate that GABAergic neurons are generated from five of the seven mesencephalic progenitor domains, and that Helt is coincidently and commonly expressed in all the GABAergic progenitor domains, but is excluded from glutamatergic domains, in the developing mesencephalon (summarized in Fig. 2).

### Helt is required for selection of GABAergic over glutamatergic neuronal fate in the mesencephalon

To examine the role of *Helt* in GABAergic neuron development, we generated *Helt*-null mutant mice by a targeted disruption approach, and confirmed the null phenotype by the complete loss of Helt expression in homozygous mutant (*Helt*$^{-/-}$) embryos (Fig. 3A). At

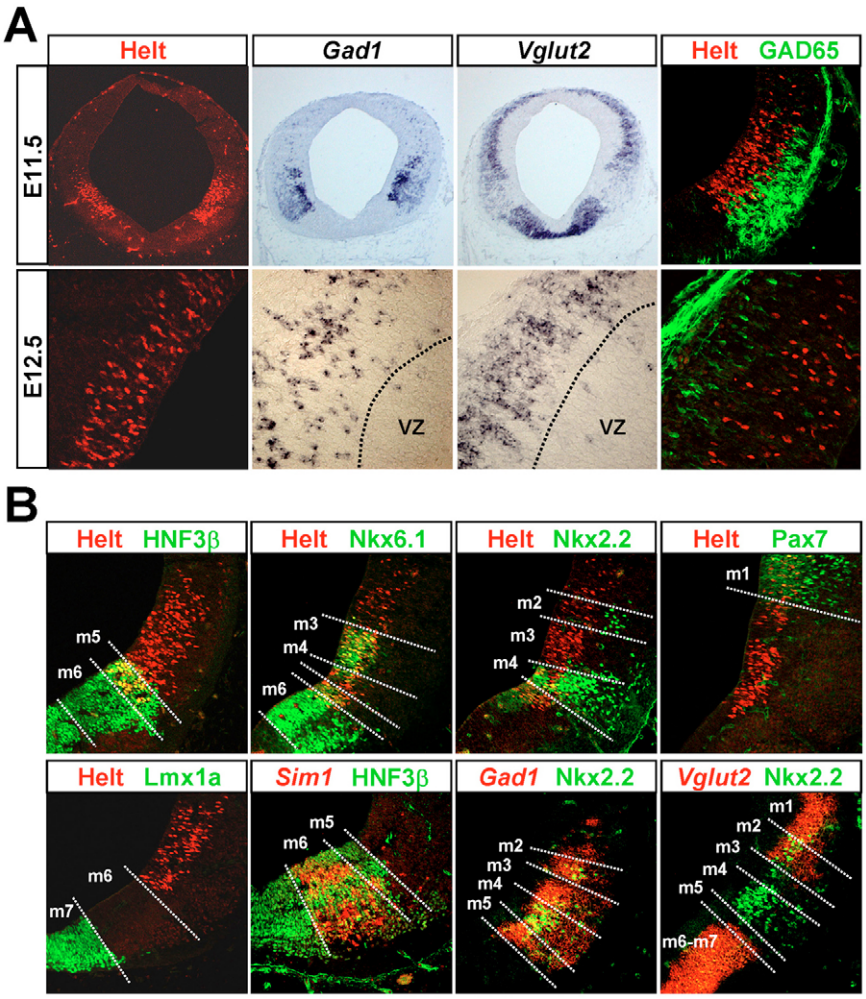


**Fig. 1. Helt is selectively expressed in GABAergic progenitors in the developing mouse mesencephalon.** (**A**) Comparison of the expression pattern of Helt with those of transmitter markers in the mesencephalon at E11.5 and E12.5. The lower panels show the dorsal mesencephalon region at E12.5. Helt is selectively expressed in GABAergic domains and excluded from glutamatergic domains. In the dorsal m1 domain, only glutamatergic neurons are generated and Helt is not expressed in the VZ at E11.5. At later stages, both GABAergic and glutamatergic neurons are generated, and Helt is expressed by subpopulations of the dorsal progenitors. (**B**) Domain structure of the developing mesencephalon. The expression patterns of transcription factors in the E11.5 mesencephalon are shown. Each domain is indicated as m1 to m7 (summarized in Fig. 2). Helt is expressed in the ventricular zone (VZ) of the m2 to m7 domains. Note that some Helt$^+$ cells are observed in the ventral-most region of the m1 domain at E11.5. These Helt$^+$ cells in the m1 domain are not obvious in embryos at a slightly earlier stage (data not shown).

E11.5, *Gad1* expression was mostly absent from *Helt*$^{-/-}$ mesencephalons, with the exception of some *Gad1*$^+$ neurons that emerged from the m5 domain (Fig. 3B). Until at least E13.5, some *Gad1*$^+$ neurons were generated in the m3 and m5 domains (see also Fig. 4B), but the total number of *Gad1*$^+$ neurons in the ventral mesencephalon region of the mutant embryos did not increase to the level found in wild-type control embryos (Fig. 3B; data not shown), indicating that a delay in GABAergic neuron generation or *Gad1* induction in postmitotic neurons does not cause this phenotype. In the dorsal m1 domain, GABAergic neurons were completely lost in the mutants at E12.5 and E13.5 (Fig. 3B; data not shown). These results are consistent with recently published observations that superior colliculus GABAergic neurons derived from the dorsal mesencephalon are absent in another *Helt*-knockout mouse strain (Guimera et al., 2006b). In addition to this dorsal phenotype, our precise analysis (see also the results described below) revealed that *Helt* is required for GABAergic differentiation in the ventral mesencephalon.

Owing to the structural similarities between Helt and the Hes and Hey family proteins that control neurogenesis in many brain regions, it is possible that neurons fated to be GABAergic are not generated owing to an effect of *Helt* loss on the neurogenesis of GABAergic progenitors. However, neural progenitor cells were maintained normally and neurons were similarly generated in all regions of *Helt*$^{-/-}$ mutant mesencephalons, as compared with their wild-type littermates (data not shown; see also Fig. 4A). In addition, cell death was not increased in any of the mesencephalic regions of the mutants, as revealed by the lack of activated caspase-3 staining (data not shown). These results indicate that *Helt* is not involved in the control of neurogenesis, in contrast to other bHLH-O factors and despite their structural conservation, and that neurons generated from GABAergic domains cannot acquire the GABAergic phenotype without *Helt* activity.

To determine whether the phenotype was caused by the inability of GABAergic precursors to mature into *Gad1*$^+$ neurons despite maintaining their correct identity, or by conversion of their fate into other neuronal subtypes, we examined the expression of other transmitter markers, including *Vglut2* (also known as *Slc17a6* – Mouse Genome Informatics) (Fig. 3B; data not shown). At E11.5, *Vglut2* was ectopically expressed at the expense of *Gad1* expression in the m3 to m5 domains of the mutant mesencephalons (Fig. 3B). At E12.5, *Vglut2* expression in the m1 domain was upregulated in the absence of *Helt* (Fig. 3B). Thus, *Helt* activity appears to be required for suppression of glutamatergic neuron generation as well as for induction of GABAergic neurons.

To examine whether *Helt* is indeed capable of suppressing the glutamatergic phenotype as well as inducing the GABAergic phenotype, we generated transgenic mice expressing *Helt* under the control of the nestin enhancer. As previously reported using similar transgenic mice (Miyoshi et al., 2004), ectopically expressed *Helt* induced *Gad1*$^+$ neurons (Fig. 3C). Importantly, ectopic *Helt* expression suppressed the generation of *Vglut2*$^+$ neurons (Fig. 3C).

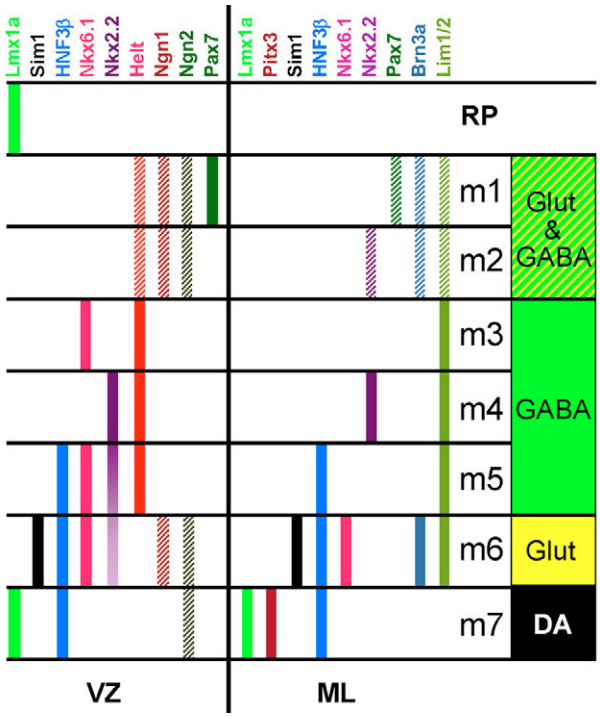


**Fig. 2. Gene expression map of the mouse anterior mesencephalon at E12.5.** Summary of the gene expression pattern and transmitter phenotype of each domain (m1 to m7), obtained from the results shown in Figs 1, 5 and 7. In the m1 and m2 domains, both GABAergic and glutamatergic neurons emerge. The hatched lines indicate expression in subpopulations within each domain (i.e. expression of Helt and Ngns, Lim1 and Brn3a, and of *Gad1* and *Vglut2*, is mutually exclusive). Note that the motoneurons of the oculomotor complex are not shown in this map as their generation is completed at an earlier stage and the motoneuron progenitor domain changes to the RN domain at E11.5 (data not shown). RP, roof plate; VZ, ventricular zone; ML, mantle layer; Glut, glutamatergic; DA, dopaminergic.

Taken together, the results obtained from these loss- and gain-of-function experiments suggest that *Helt* plays key roles in the selection of the GABAergic over glutamatergic transmitter phenotype at the progenitor state in the mesencephalon.

## Helt is not involved in progenitor domain formation and neuronal subtype identity specification

Since *Helt* is selectively expressed in the GABAergic progenitor domains and the onset of its expression precedes neurogenesis in these domains (Miyoshi et al., 2004), *Helt* might be involved in the regional patterning of the neural tube. To better understand the cause of the *Helt* mutant phenotype and the mechanism of action of *Helt* in controlling the transmitter phenotype decision, we searched for genes up- or downregulated by the loss of *Helt* activity. For this purpose, we compared the gene expression patterns in presumptive Helt$^+$ progenitors sorted from *Helt*$^{+/-}$ and *Helt*$^{-/-}$ mesencephalons. As expected from the targeting strategy of replacing the *Helt* coding sequence with a *GFP* cDNA, *GFP* was transcribed with an essentially identical pattern to that of *Helt*, and GFP$^+$ cells could be sorted by FACS (see Fig. S1A,B in the supplementary material). Among approximately 200 homeodomain factors screened by RT-PCR, none of the factors selectively expressed in VZ progenitors in the mesencephalon was identified as showing altered expression after loss of *Helt* (data not shown). Consistently, the homeodomain factors expressed within the Helt$^+$ domain, such as Nkx2.2 and Nkx6.1, were normally expressed in the VZ of the mutants, and the expression of the VZ transcription factors Hnf3β and Sim1 was also unaffected (Fig. 4A; data not shown). In addition, as expected from these observations, the expression patterns of these factors were not affected by ectopic *Helt* expression in the transgenic embryos (see Fig. S2 in the supplementary material). These results suggest that *Helt* is not involved in progenitor domain formation. Importantly, postmitotic expression of Nkx2.2, which was selectively expressed by *Gad1*$^+$ populations among postmitotic neurons in the mesencephalon, was not affected in the m4 domain, although Nkx2.2 expression in the postmitotic neurons in the m2 domain was lost (Fig. 4A). In addition, although some Nkx2.2$^+$ neurons retained *Gad1* expression in the *Helt*$^{-/-}$ mutants at E12.5, most Nkx2.2$^+$ neurons expressed *Vglut2*, suggesting that the fate of Nkx2.2$^+$ neurons was converted from GABAergic to glutamatergic in the absence of *Helt*, even though their neuronal identity was maintained, at least in part (Fig. 4B). Thus, Helt appears to select the transmitter phenotype without completely changing the neuronal subtype identity defined by the homeodomain factor codes.

## Helt controls pan-GABAergic and pan-glutamatergic pathways in postmitotic neurons

We reasoned that the expression of postmitotic transcription factors that control *Gad1* or *Vglut2* expression might be changed by the loss of *Helt* activity. Owing to the long half-life of GFP proteins, the sorted GFP$^+$ cells contained postmitotic precursors that originated from the Helt$^+$ domain (see Fig. S1C in the supplementary material). Thus, by the above-described RT-PCR screening for homeodomain factors, we were able to examine the postmitotic events affected by the *Helt* mutation. We found that some postmitotically expressed factors were differentially expressed between *Helt*$^{+/-}$ and *Helt*$^{-/-}$ GFP$^+$ cell populations (data not shown). Among these, we focused on Lim1 and Brn3a (also known as Lhx1 and Pou4f1, respectively – Mouse Genome Informatics).

We first examined the normal expression patterns of these factors. At E11.5, Lim1 was selectively expressed in the m2 to m6 domains (Fig. 5A; data not shown). At later stages, Lim1 expression was initiated in some populations in the m1 domain along with *Gad1* (Fig. 5A,B). Brn3a expression was detected in the ventral-most region of the basal plate (m6) and the dorsal m1 and m2 domains (Fig. 5A). Double staining revealed coexpression of these factors in the m6 domain, which probably consists of RN neurons (Agarwala and Ragsdale, 2002; Fedtsova and Turner, 2001). In all the other regions, Lim1 and Brn3a showed mutually exclusive expression patterns. By comparison with *Gad1* and *Vglut2* expression, we found that Lim1$^+$ domains, except for the RN domain, were well matched with *Gad1* expression, and confirmed the coexpression of Lim1 and *Gad1* at the single cell level (Fig. 5B). By contrast, Brn3a expression was specific for *Vglut2*$^+$ cells, although *Vglut2* expression was also detected in dopaminergic neurons in the m7 domain. In summary, Brn3a specifically marks glutamatergic neurons, whereas Lim1$^+$ Brn3a$^-$ populations define GABAergic neurons, in the developing mesencephalon (summarized in Fig. 2).

Next, we examined whether these homeodomain factors were affected by the loss or gain of *Helt* activity. In the *Helt*-null mutant at E11.5, Lim1 expression was mostly absent from the m2 to m4 domains, although Lim1$^+$ neurons were observed within the m5 domain, where *Gad1* expression was also partially retained (Fig.

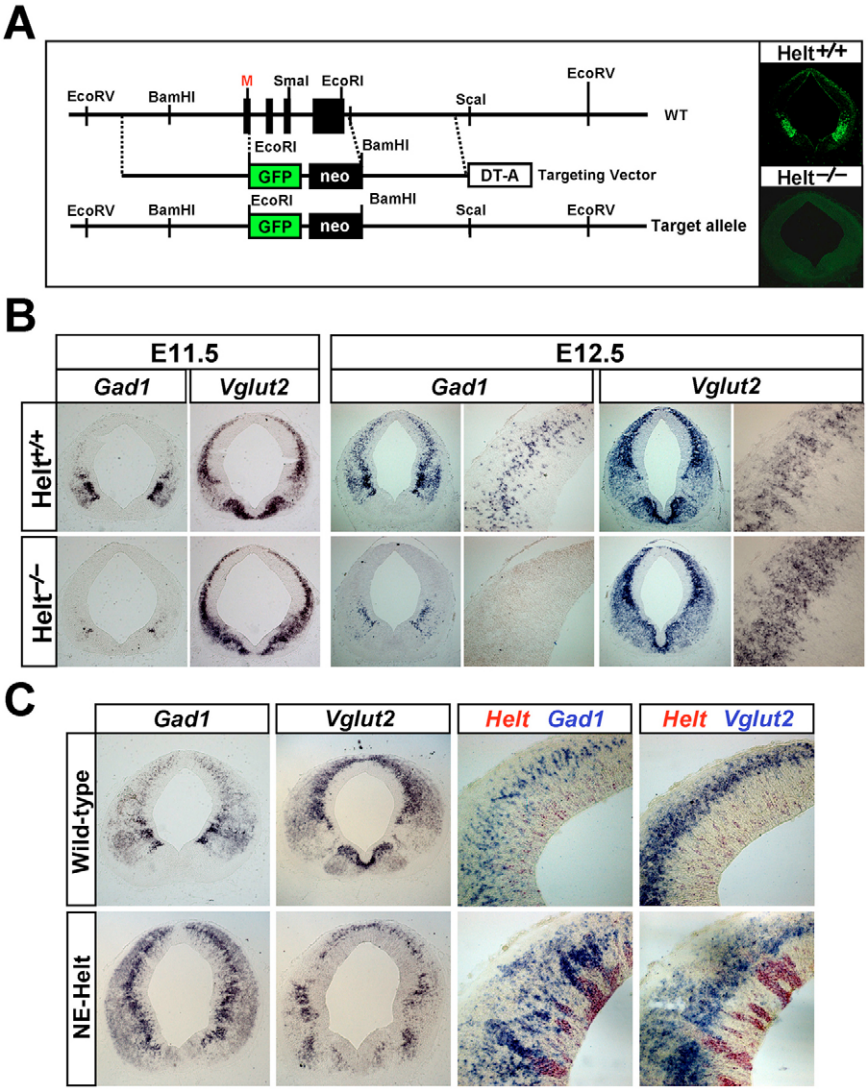


**Fig. 3. Helt selects the GABAergic versus glutamatergic phenotype in the mesencephalon.** (**A**) Gene targeting strategy. All exons encoding the *Helt* ORF were replaced with a *GFP* cDNA, and recombination was confirmed by Southern blotting (data not shown). The null phenotype in homozygous mutant mouse embryos was confirmed by complete loss of Helt expression (right-hand panels). (**B**) *Helt* is required for induction of GABAergic neurons and suppression of glutamatergic differentiation. In the *Helt*$^{-/-}$ mutant mesencephalon, *Gad1*$^{+}$ neurons are only detected in the ventral-most m5 region of the presumptive GABAergic domains, whereas *Vglut2*$^{+}$ neurons are generated in essentially all domains of the mesencephalon at E11.5. At E12.5, although *Gad1*$^{+}$ neurons start to emerge in a broad area of the ventral GABAergic domains (m3 to m5), the frequency is still lower than that in wild-type control embryos and ectopic *Vglut2*$^{+}$ neurogenesis continues. Dorsal *Gad1*$^{+}$ neurons are completely absent in the mutants. (**C**) Helt suppresses glutamatergic neurogenesis and induces GABAergic neurons. In the transgenic embryos expressing *Helt* under the control of the nestin enhancer, the number of *Vglut2*$^{+}$ cells is decreased and the number of *Gad1*$^{+}$ cells is increased instead. Note that GABAergic neurons are efficiently generated and glutamatergic neurons are suppressed in the ML just outside of the VZ positive for exogenous Helt, suggesting a cell-autonomous effect of *Helt*.

6A). At later stages, Lim1 expression in the m5 domain was maintained and some Lim1$^{+}$ neurons emerged from the m3 and m4 domains (data not shown), consistent with the above observation that some Nkx2.2$^{+}$ Gad1$^{+}$ neurons were detected in the mutants. In the dorsal m1 domain, Lim1 expression was completely lost at E12.5 and E13.5 (Fig. 6A; data not shown). By contrast, ectopic Brn3a expression was observed in the m2 to m4 domains of the mutants, where Lim1 expression was lost, and virtually all the neurons emerging from the m1 domain were Brn3a$^{+}$ (Fig. 6A). GFP$^{+}$ neurons were generated and migrated toward the ML in the null mutants and, importantly, 95.82±1.09% of GFP$^{+}$ neurons in the m1 domain expressed Brn3a (Fig. 6A, $n$=5), indicating that neurogenesis by presumptive Helt$^{+}$ progenitors was unaffected, but the derived neurons were transfated to become Brn3a$^{+}$ glutamatergic neurons following the loss of *Helt* activity. Consistent with these mutant phenotypes, ectopic expression of *Helt* induced Lim1$^{+}$ neurons that coexpressed Gad65 (also known as Gad2 – Mouse Genome Informatics) and inhibited Brn3a$^{+}$ neurogenesis instead (Fig. 6B). Taken together, these results suggest that *Helt* functions as a selector gene that promotes the pan-GABAergic pathway involving Lim1 by suppressing the pan-glutamatergic pathway involving Brn3a.

## Helt represses Ngn1 and Ngn2 in GABAergic progenitors

Since Helt is a transcriptional repressor that is selectively expressed in the VZ (Nakatani et al., 2004), we reasoned that factors expressed in the VZ and controlling glutamatergic fate might be downstream targets of Helt. The fact that bHLH proneural factors play important roles in neuronal subtype specification and transmitter selection as well as neurogenesis control in other brain regions (Fode et al., 2000; Gowan et al., 2001; Helms et al., 2005; Muller et al., 2005; Nakada et al., 2004; Parras et al., 2002), and that bHLH-O factors frequently regulate bHLH genes (Davis and Turner, 2001), led us to examine the possibility that Helt might regulate bHLH gene expression to determine the transmitter phenotype. We screened for bHLH factors expressed in the VZ that were differentially expressed in GFP$^{+}$ cells sorted from *Helt*$^{+/-}$ and *Helt*$^{-/-}$ mesencephalons, and identified the Ngn genes (data not shown).

First, we examined the expression patterns of these proneural factors in the mesencephalon of wild-type embryos. Ngn1 (also known as Neurog1 – Mouse Genome Informatics) was selectively expressed in the VZ of the Brn3a$^{+}$ glutamatergic m1, m2 and m6 domains (Fig. 7A,B). Ngn2 was selectively expressed in the RN domain (m6) and ventral midline dopaminergic domain (m7) at high levels and in the dorsal m1 domain at a relatively low level and

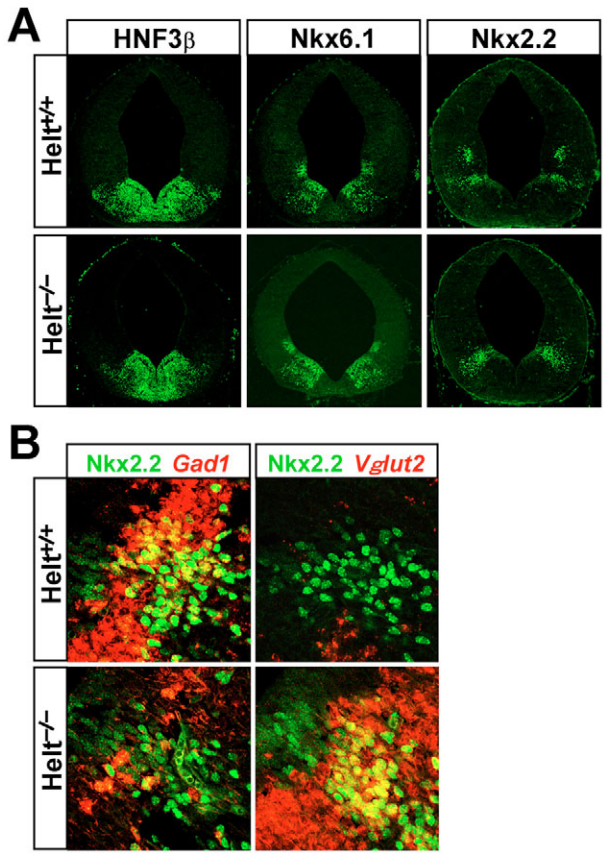


**Fig. 4. *Helt* determines the transmitter phenotype without affecting neuronal subtype identity specification.** (**A**) Progenitor domain formation is not affected by the *Helt*-null mutation. Note that Nkx2.2 expression in the VZ is not changed by the *Helt* mutation, although postmitotic expression of this factor in the m2 domain is lost in the mutants. By contrast, expression of Nkx2.2 in m4 neurons is maintained in the mutants. (**B**) Conversion of the transmitter phenotype in Nkx2.2$^+$ m4 neurons by the *Helt*-null mutation. In wild-type mouse embryos, most Nkx2.2$^+$ neurons in the anterior mesencephalon express *Gad1* but not *Vglut2*. By contrast, in the *Helt* mutants, most Nkx2.2$^+$ neurons express *Vglut2*, although some populations maintain *Gad1* expression.

frequency at E11.5 (Fig. 7A,B) (Fedtsova and Turner, 2001; Kele et al., 2006). At later stages, Ngn2 expression was increased in the m1 domain (Fig. 7A). Importantly, Helt and the Ngns showed mutually exclusive patterns in all domains at all stages (Fig. 7A). By contrast, Mash1 was expressed in all progenitor domains of the developing mesencephalon and coexpressed with Helt (Fig. 7A) (Miyoshi et al., 2004).

These expression patterns suggest that Helt might repress the Ngn genes. To directly examine this, we analyzed Ngn expression in *Helt*-transgenic embryos. The numbers of Ngn1$^+$ and Ngn2$^+$ cells in both the dorsal and ventral glutamatergic domains were significantly reduced and Ngn expression was completely excluded from Helt$^+$ cells in these regions (Fig. 7C), indicating that Helt has the potency to repress Ngns by itself. As expected from the coexpression of Mash1 with Helt (Fig. 7A), Mash1 expression remained unaffected by ectopic *Helt* expression.

To examine whether Helt indeed represses Ngn gene expression in Helt$^+$ GABAergic progenitors, we analyzed the Ngn expression patterns in *Helt*-deficient embryos. At E11.5, ectopic expression of both factors was observed in the ventral GFP$^+$ m2 to m4 domains, where Brn3a was ectopically induced and Lim1 expression was lost in the mutants (Fig. 7D). At E12.5, Ngn expression was upregulated in the m1 domain of the mutant embryos. More importantly, Ngns were expressed in the majority of the GFP$^+$ presumptive *Helt*-expressing progenitors in the VZ (Fig. 7D; Ngn1, 57.0±4.3%; Ngn2, 73.6±1.5%). Thus, Ngn expression was derepressed in the Helt$^+$ progenitors after the loss of *Helt* activity. By contrast, Mash1 expression was not affected by the loss of *Helt* function, indicating that Helt selectively regulates the Ngn genes and that the role of Helt in promoting GABAergic differentiation does not involve maintenance of Mash1 expression, which is essential for GABAergic differentiation in the ventral mesencephalon (Miyoshi et al., 2004). Taken together, these observations demonstrate that Ngns are downstream target genes of Helt in the developing mesencephalon.

## Ngn1 can promote glutamatergic differentiation

The selective Ngn expression in glutamatergic progenitors and its repression by Helt suggest that the repression of Ngn genes might be a key function of *Helt* for suppression of the glutamatergic fate. To examine whether derepression of Ngns by loss of *Helt* is a primary cause of transmitter phenotype switching, we first compared the increase in the number of Ngn1-expressing progenitors in the VZ at E12.5 with the increase in glutamatergic neurogenesis, as indicated by the rate of accumulated glutamatergic neurons in the ML at E13.5, in the m1 domain of *Helt* mutants as compared with wild-type controls. In the VZ at E12.5, an approximately 1.51-fold increase in the percentage of Ngn1$^+$ cells was observed in the m1 domain of the mutant embryos (wild type, 13.86±2.34%; mutant, 20.89±2.05%; $n$=5). In the ML at E13.5, the percentage of Brn3a$^+$ glutamatergic neurons in the m1 domain of the mutant embryos increased from 58.96±2.06% to 93.83±2.07% ($n$=5), which is consistent with the reduction in Lim1$^+$ GABAergic neurons from 37.73±1.64% to 0% ($n$=5). Thus, glutamatergic generation was concomitantly increased ~1.59-fold by the loss of *Helt*. These results support the idea that derepression of Ngn1 expression by loss of *Helt* leads to acquisition of the glutamatergic fate, at least in the m1 domain.

To examine whether *Ngn1* is indeed capable of determining the glutamatergic fate, we generated transgenic mice expressing *Ngn1* under the control of the nestin enhancer. In the ventral Helt$^+$ domains of Ngn1-transgenic embryos at E11.5, ectopic Brn3a$^+$ neurons were observed, despite the fact that Helt expression did not appear to be affected (Fig. 8A). We next examined whether Ngn1 could promote glutamatergic differentiation in the m1 domain, where a strong correlation between *Ngn1* derepression and increased glutamatergic generation was observed in the *Helt*-null mutants. However, possibly owing to premature differentiation induced by the proneural activity of *Ngn1*, transgene-expressing progenitors were rare in the transgenic embryos at E12.5. To mark the neurons derived from exogenous Ngn1-expressing progenitors, we generated transgenic mice expressing Ngn1 and IRES-controlled GFP under the control of the nestin enhancer (NE-Ngn1-GFP). As expected from the long duration of GFP protein expression, GFP$^+$ postmitotic neurons could be detected in the ML of the transgenic embryos (Fig. 8B). Importantly, more than 96% of the GFP$^+$ neurons expressed Brn3a (193/201, $n$=2), whereas none of them expressed Lim1 (0/201, $n$=2), indicating that exogenous *Ngn1*-expressing progenitors only generated glutamatergic neurons. Taken together, these results demonstrate that *Ngn1* has the potency to promote glutamatergic differentiation and that repression of Ngns by Helt is a prerequisite for GABAergic differentiation in the mesencephalon.

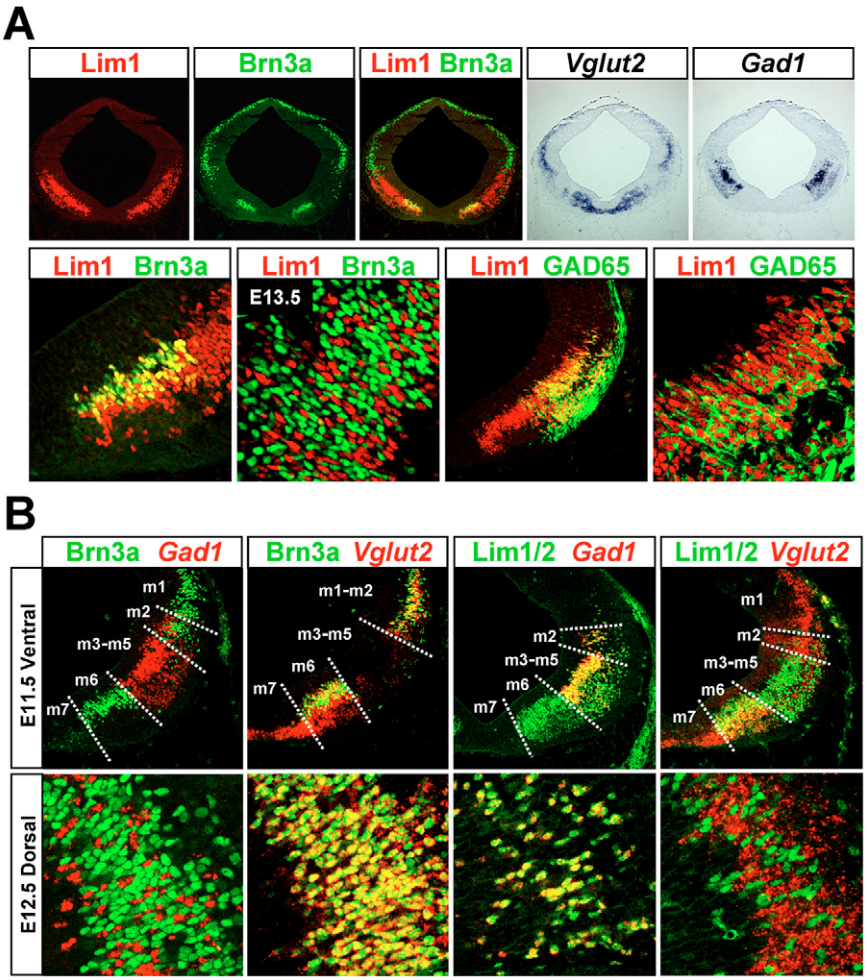


**Fig. 5. Identification of postmitotic transcription factors selectively expressed in GABAergic and glutamatergic neurons in the mesencephalon.** (**A**,**B**) Selective expression of Lim1 and Brn3a by GABAergic and glutamatergic neurons in the developing mouse mesencephalon. (A) Lim1 and Brn3a are mutually exclusively expressed, with the exception of the RN (m6) domain. (B) Brn3a is coincidently expressed by *Vglut2*$^+$ neurons, although *Vglut2* is also expressed by ventral midline dopaminergic neurons and not by *Gad1*$^+$ neurons. By contrast, Lim1 is selectively expressed by *Gad1*$^+$ neurons, with the exception of coexpression with *Vglut2* and Brn3a in the RN (m6) domain.

## DISCUSSION

### Mechanism of transmitter phenotype determination in the mesencephalon

Previous studies have shown that the developing mesencephalon is subdivided into arc structures defined by patterns of transcription factor expression (Agarwala and Ragsdale, 2002; Sanders et al., 2002). However, the characteristics of each neuronal subtype regarding transmitter usage and function remain largely elusive. In the present study, we have delineated the transcription factor codes of the progenitor domains in the mesencephalon and defined the transmitter phenotype of the derived neurons in each domain. Importantly, Helt defines all the GABAergic progenitors, whereas the Ngns, which are repressed by Helt, mark glutamatergic progenitors. Furthermore, we identified pan-GABAergic and pan-glutamatergic postmitotically expressed transcription factors (Lim1 and Brn3a, respectively). Definition of the neuronal subtypes and their progenitor domains will be useful for understanding the developmental mechanism of the mesencephalon.

Previous studies revealed that *Helt* is required for GABAergic differentiation (Guimera et al., 2006b; Miyoshi et al., 2004). Our present knockout approach revealed a pivotal role for *Helt* in the selection of the GABAergic over glutamatergic transmitter phenotype fate. The phenotype of embryos lacking *Helt* in the mesencephalon was similar to that of mutants of another bHLH factor, *Ptf1a*, in the cerebellum and dorsal spinal cord, in which GABAergic neurons were lost and glutamatergic neurons were generated instead (Glasgow et al., 2005; Hoshino et al., 2005). Thus, specification of GABAergic neurons over glutamatergic neurons appears to be achieved using similar mechanisms with distinct bHLH factors, Helt and Ptf1a, in each brain region. However, the mechanisms of transcriptional control by these factors might be different, as Helt is a bHLH-O type transcriptional repressor and Ptf1a is a bHLH factor that heterodimerizes with E proteins and has a transcriptional activator function (Beres et al., 2006; Miyoshi et al., 2004; Nakatani et al., 2004). Thus, the downstream genes regulated by these factors are likely to differ. Consistent with this notion, the phenotypes of null mutants for *Helt* and *Ptf1a* show important differences. In the *Ptf1a* mutant, GABAergic neurons originating from Ptf1a$^+$ progenitors were completely transfated to become other neuronal populations with the glutamatergic phenotype in the dorsal spinal cord (i.e. dI4 to dI5, dILA to dILB) (Glasgow et al., 2005). By contrast, in the *Helt* mutant, despite the almost complete conversion of the transmitter phenotype and the expression of pan-GABA and pan-glutamatergic transcription factors, the expression of Nkx2.2, which was specifically expressed in subpopulations of GABAergic neurons in the mesencephalon, was maintained. These observations suggest that Helt, and also possibly the Ngns, might select the transmitter phenotype without directly affecting the expression of transcription factors that determine the neuronal subtype identity in the mesencephalon. Thus, it is suggested that in the ventral mesencephalic neurons, the pathways that determine neurotransmitter identity are not completely linked to the pathways regulating neuronal subtype identity.

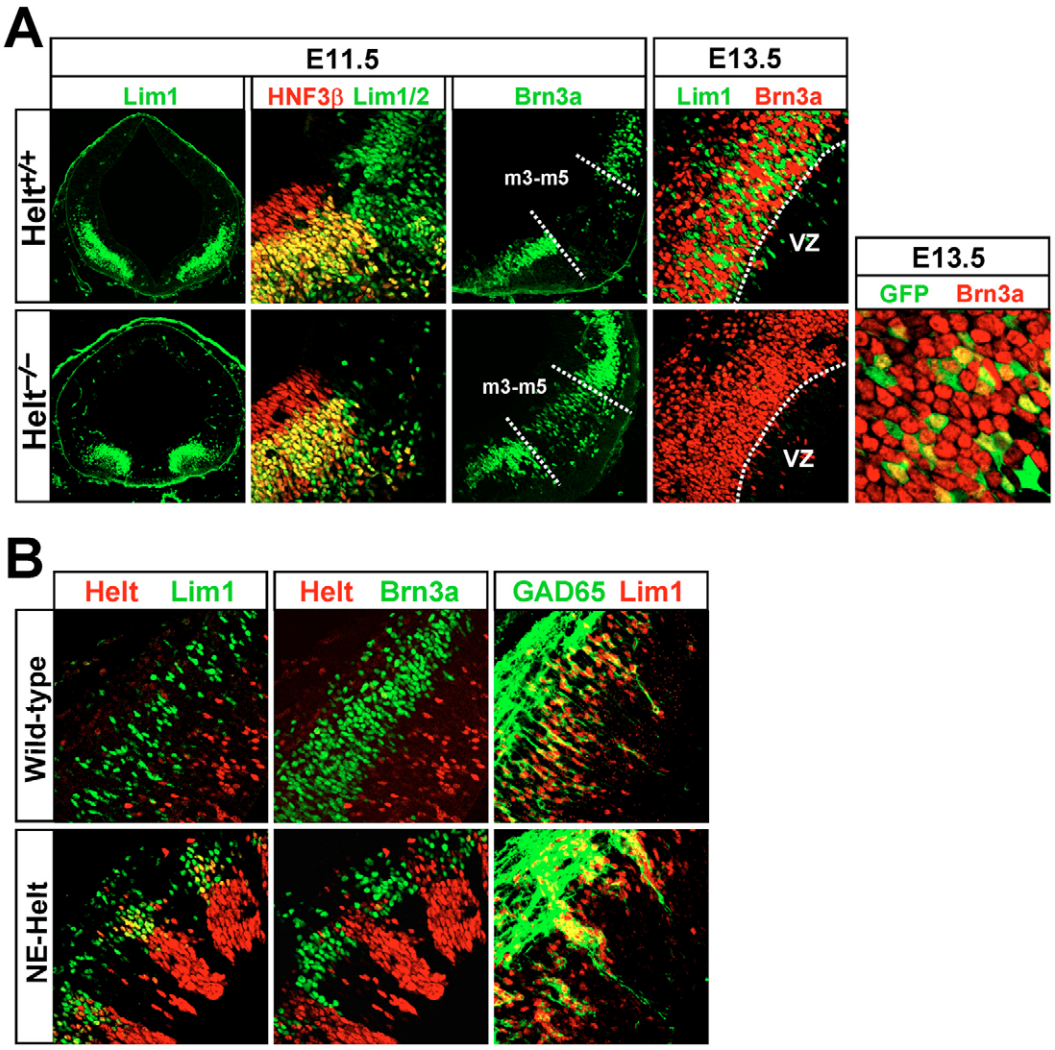


**Fig. 6. *Helt* induces Lim1$^+$ neurons and suppresses Brn3a$^+$ neurogenesis.** (**A**) Effects of the *Helt*-null mutation on Lim1 and Brn3a expression. In the *Helt*$^{-/-}$ mutant at E11.5, Lim1 expression is lost in the m2 to m4 domains, whereas expression in the m5 and m6 domains is unaffected. At E13.5, Lim1 expression in the dorsal m1 domain is completely lost. By contrast, Brn3a is ectopically expressed in the m2 to m4 domains of the mutants at E11.5. In the m1 domain, virtually all the emerging neurons are positive for Brn3a at E13.5 and coexpression of GFP and Brn3a is observed. (**B**) Exogenous *Helt* suppresses Brn3a$^+$ neurogenesis and induces Lim1$^+$ neurons. In the area efficiently expressing exogenous Helt, many Lim1$^+$ neurons are generated, but fewer Brn3a$^+$ neurons emerge compared with wild-type control mouse embryos. Exogenous *Helt*-induced Lim1$^+$ neurons express Gad65.

Although we cannot exclude the possibility that *Helt* is also involved in subtype identity specification, as other identity markers have not yet been identified, the maintenance of Nkx2.2 expression in m4 neurons of *Helt* mutants suggests that determination of the neuronal transmitter phenotype is not a downstream event of subtype identity determination, at least in m4 neurons. This idea is supported by the observation that pathways common to each transmitter fate (i.e. Helt-Lim1-Gad1 and Ngn-Brn3a-Vglut2) appear to be used in the differentiation of all mesencephalic GABAergic and glutamatergic neurons. The question then arises as to whether these types of determination pathway are commonly used for transmitter choice in all brain regions. In the dorsal spinal cord, all the known factors with transmitter selector functions, such as Ptf1a, Lbx1 and Tlx1/3, are also involved in the control of neuronal identity – the pattern of expression of transcription factors that define neuronal identity is almost completely changed to one that directs another fate in the absence of these selector factors (Cheng et al., 2004; Cheng et al., 2005; Glasgow et al., 2005; Gross et al., 2002; Muller et al., 2002). By contrast, loss of *Pax2*, which is commonly expressed by GABAergic neurons and required for acquisition of the GABAergic phenotype in the spinal cord, does not result in conversion to another transmitter phenotype (Cheng et al., 2004). Thus, *Pax2* is not a selector for the transmitter phenotype, but instead only appears to be required for the promotion of GABAergic differentiation. Taken together, at least in the dorsal spinal cord, transmitter phenotype and neuronal subtype identity appear to be tightly coupled with each other, or the transmitter phenotype might be determined as a downstream event of the specification of neuronal subtype identity, in contrast to the situation in the mesencephalon. In the present study, we did not examine whether the GABAergic phenotype was changed to the glutamatergic phenotype following maintenance of neuronal identity in the dorsal mesencephalic regions. Future detailed analyses are required to clarify whether *Helt* only plays important roles in transmitter selection or whether, in some cases, it is also involved in the selection of neuronal identity from two alternative fates, similar to *Ptf1a* in the dorsal spinal cord.

## bHLH network in cell fate specification in the mesencephalon

In many developmental systems, bHLH-O type transcription factors such as Hes1 play important roles in inhibiting cellular differentiation by sequestrating or repressing bHLH factors. In many cases, these factors are used as downstream effectors for Notch signaling. In neuronal development, the Hes and Hey families of bHLH-O factors control the differentiation of neurons and glia by repressing proneural genes (Bertrand et al., 2002; Kageyama and Ohtsuka, 1999; Ross et al., 2003; Sakamoto et al., 2003; Satow et al., 2001). Here, we have demonstrated that Helt has a similar

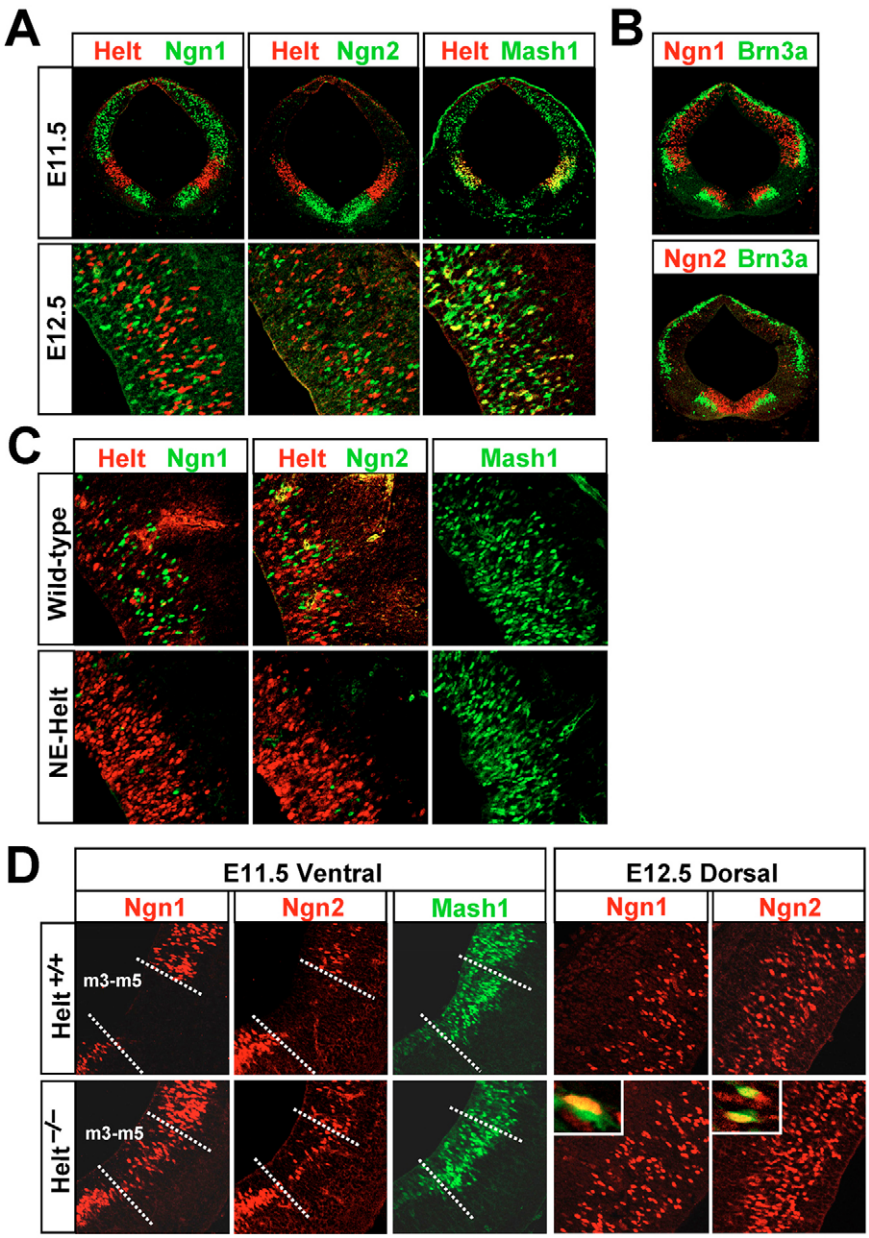


**Fig. 7. *Helt* represses Ngn genes.** (**A**) Expression patterns of proneural genes in the developing mouse mesencephalon. Expression of Ngns and Helt is mutually exclusive in all the domains. By contrast, Mash1 is expressed in essentially all domains and is coexpressed with Helt. (**B**) Ngn genes are selectively expressed in glutamatergic progenitors. Ngn1 is coincidently expressed in the VZ, where Brn3a$^+$ neurons emerge. Ngn2 is selectively expressed by glutamatergic progenitors as well as dopaminergic progenitors. (**C**) *Helt* represses Ngn expression in the mesencephalon. In transgenic embryos expressing *Helt* under the control of the nestin enhancer, expression of Ngn1 and Ngn2 is repressed. By contrast, Mash1 expression is unaffected. (**D**) Ngns are ectopically expressed in presumptive GABAergic domains in the *Helt*-null mutant. At E11.5, Ngn1 and Ngn2 are ectopically expressed in the m2 to m4 domains in the *Helt*$^{-/-}$ mutant. At E12.5, the Ngn expression in the dorsal m1 domain is upregulated and coexpression of GFP and the Ngns is observed (inset).

activity in proneural gene regulation, consistent with the structural conservation. However, there are significant differences between the activity of Helt and those of other bHLH-O members, in that Helt selectively regulates Ngn1 and Ngn2, whereas other bHLH-O factors repress all proneural genes. Thus, unlike Hes1 and Hes5, Helt is not a general regulator of neurogenesis, as loss or gain of Helt function was consistently found to have no effect on neurogenesis. Therefore, the question arises as to how Helt regulates Ngn expression. Ngn expression was affected by the gain and loss of *Helt* activity without any alterations in the expression of progenitor transcription factors, such as homeobox factors, which might be involved in the specification of progenitor identity. Thus, it is unlikely that the repression or derepression of Ngns is a secondary consequence of the change in neural progenitor identity. Consistently, Helt repressed Ngn1 promoter activity in transfection assays (our unpublished observations), suggesting the likely possibility that Helt directly represses Ngn expression.

The question also arises as to how Helt regulates the neuronal transmitter phenotype. Two important features of the developing mesencephalon are that the proneural factor Mash1 is commonly expressed by neural progenitors in all progenitor domains and that its expression is not regulated by Helt and Ngns, which is different from the situation in other brain regions such as in the telencephalon and spinal cord (Gowan et al., 2001; Helms et al., 2005). Thus, the Helt and Ngn pathways might be capable of controlling the neurotransmitter phenotype without regulating neurogenesis, which is controlled by the Mash1 pathway. Together with the established roles of Ngns in glutamatergic determination in the telencephalon (Fode et al., 2000; Parras et al., 2002), their (1) coincident expression in glutamatergic progenitor domains; (2) the strong correlation between ectopic induction or suppression of Ngn expression and glutamatergic neurogenesis in *Helt*-null mutants and *Helt*-transgenic embryos; and (3) the potency of *Ngn1* to promote glutamatergic differentiation revealed by *Ngn1* gain-of-function studies, all suggest

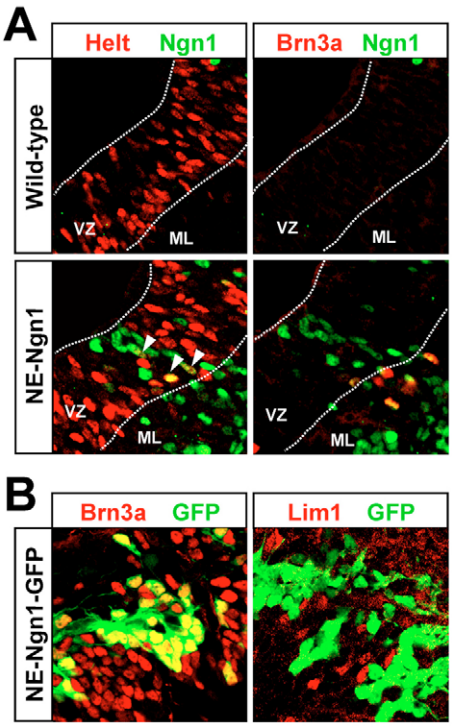


**Fig. 8. *Ngn1* promotes glutamatergic differentiation.** (**A**) Ngn1 induces ectopic Brn3a$^+$ neurons in the ventral Helt$^+$ domains. In transgenic mouse embryos expressing *Ngn1* under the control of the nestin enhancer, Brn3a$^+$ neurons are generated at E11.5 despite the fact that Helt expression is not repressed (arrowheads). Note that most of the exogenous Ngn1-expressing neurons in the ML express Brn3a. (**B**) Ngn1 determines glutamatergic fate in the m1 domain. Shown are the dorsal m1 domains of wild-type control embryos and transgenic embryos expressing *Ngn1* and IRES-controlled GFP under the control of the nestin enhancer at E12.5. Neurons generated from transgene-expressing progenitors are marked by GFP expression. Virtually all the GFP$^+$ neurons express Brn3a and do not express Lim1.

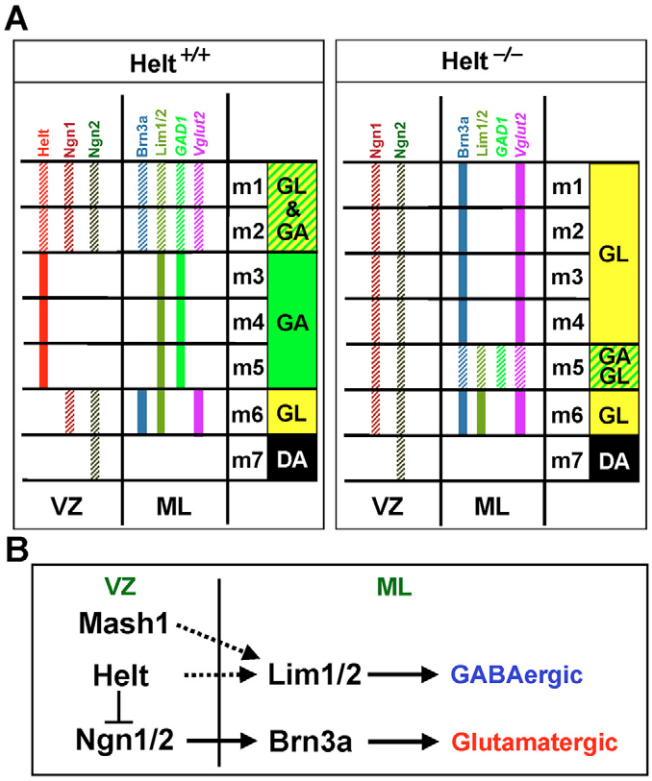


**Fig. 9. Model for transmitter phenotype determination in the developing mesencephalon.** (**A**) Summary of the phenotype of *Helt*-null mutant mouse embryos. (**B**) Model for transmitter phenotype determination by the *Helt* and Ngn genes. *Helt* determines the GABAergic phenotype by repressing the Ngn genes, which induce the glutamatergic pathway. Mash1 might also be involved in the GABAergic determination pathway (Miyoshi et al., 2004). GA, GABAergic; GL, glutamatergic; DA, dopaminergic; VZ, ventricular zone; ML, mantle layer.

that repression of the Ngn genes is likely to be a key role of Helt in transmitter selection and indicate that it is at least essential for suppressing the glutamatergic pathway.

The question remains as to whether Helt constructively promotes GABAergic differentiation or whether the default phenotype in mesencephalic neurons is GABAergic when the glutamatergic pathway is suppressed. The observation that exogenous Ngn1 could not repress Helt expression in the transgenic mice, and that derepressed Ngns did not repress GFP expression in the *Helt*-null mutants, suggest that Ngns cannot regulate Helt expression. Nevertheless, ectopic expression of Ngn1 in the Helt$^+$ domains could suppress GABAergic differentiation and promote the glutamatergic pathway. Based on these observations, together with the fact that Helt appears to have a selector function by suppressing the alternative fate, an instructive role of Helt in GABAergic promotion seems unlikely. Instead, the GABAergic phenotype is likely to be determined by Mash1, which is essential for GABAergic neuron formation in the ventral mesencephalon (Miyoshi et al., 2004). Thus, the default status of mesencephalic progenitors might be GABAergic as conferred by the ubiquitous expression of Mash1, and Helt might promote GABAergic differentiation by repressing the Ngns that promote the glutamatergic pathway (Fig. 9). This idea is supported by the observation that some GABAergic neurons were generated in the ventral domains even in the absence of *Helt* (Fig. 3B), and the fact that the increased GABAergic production in these regions with development correlated well with Ngn downregulation (data not shown), which is regulated by an unknown mechanism independent of *Helt*. Loss-of-function studies for the Ngn genes will be needed to clarify this point and to gain further understanding of the overall picture of transmitter phenotype determination in the mesencephalon.

We are grateful to Dr Yoshimi Takai (Department of Molecular Biology and Biochemistry, Osaka University Graduate School of Medicine/Faculty of Medicine, Japan) for constant support and fruitful discussions. We thank Dr T. Imai (KAN Research Institute Inc.) for helpful comments and encouragement, E. Mizuhara for the FACS technique and Y. Sakamoto and T. Inoue for critical reading of the manuscript. The monoclonal anti-Nkx2.2, anti-Lim1/2 and anti-Pax7 antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained at the Department of Biological Sciences, The University of Iowa, Iowa City, IA 52242.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/15/2783/DC1

**References**

**Agarwala, S. and Ragsdale, C. W.** (2002). A role for midbrain arcs in nucleogenesis. *Development* **129**, 5779-5788.

**Andersson, E., Tryggvason, U., Deng, Q., Friling, S., Alekseenko, Z., Robert, B., Perlmann, T. and Ericson, J.** (2006). Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* **124**, 393-405.

**Baek, J. H., Hatakeyama, J., Sakamoto, S., Ohtsuka, T. and Kageyama, R.** (2006). Persistent and high levels of Hes1 expression regulate boundary formation in the developing central nervous system. *Development* **133**, 2467-2476.

**Beres, T. M., Masui, T., Swift, G. H., Shi, L., Henke, R. M. and MacDonald, R. J.** (2006). PTF1 is an organ-specific and Notch-independent basic helix-loop-helix complex containing the mammalian Suppressor of Hairless (RBP-J) or its paralogue, RBP-L. *Mol. Cell. Biol.* **26**, 117-130.

**Bertrand, N., Castro, D. S. and Guillemot, F.** (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.

**Cheng, L., Arata, A., Mizuguchi, R., Qian, Y., Karunaratne, A., Gray, P. A., Arata, S., Shirasawa, S., Bouchard, M., Luo, P. et al.** (2004). Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. *Nat. Neurosci.* **7**, 510-517.

**Cheng, L., Samad, O. A., Xu, Y., Mizuguchi, R., Luo, P., Shirasawa, S., Goulding, M. and Ma, Q.** (2005). Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. *Nat. Neurosci.* **8**, 1510-1515.

**Davis, R. L. and Turner, D. L.** (2001). Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. *Oncogene* **20**, 8342-8357.

**Fedtsova, N. and Turner, E. E.** (2001). Signals from the ventral midline and isthmus regulate the development of Brn3.0-expressing neurons in the midbrain. *Mech. Dev.* **105**, 129-144.

**Fode, C., Ma, Q., Casarosa, S., Ang, S. L., Anderson, D. J. and Guillemot, F.** (2000). A role for neural determination genes in specifying the dorsoventral identity of telencephalic neurons. *Genes Dev.* **14**, 67-80.

**Glasgow, S. M., Henke, R. M., Macdonald, R. J., Wright, C. V. and Johnson, J. E.** (2005). Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. *Development* **132**, 5461-5469.

**Gowan, K., Helms, A. W., Hunsaker, T. L., Collisson, T., Ebert, P. J., Odom, R. and Johnson, J. E.** (2001). Crossinhibitory activities of Ngn1 and Math1 allow specification of distinct dorsal interneurons. *Neuron* **31**, 219-232.

**Gross, M. K., Dottori, M. and Goulding, M.** (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* **34**, 535-549.

**Guimera, J., Vogt Weisenhorn, D., Echevarria, D., Martinez, S. and Wurst, W.** (2006a). Molecular characterization, structure and developmental expression of Megane bHLH factor. *Gene* **377**, 65-76.

**Guimera, J., Vogt Weisenhorn, D. and Wurst, W.** (2006b). Megane/Heslike is required for normal GABAergic differentiation in the mouse superior colliculus. *Development* **133**, 3847-3857.

**Helms, A. W. and Johnson, J. E.** (2003). Specification of dorsal spinal cord interneurons. *Curr. Opin. Neurobiol.* **13**, 42-49.

**Helms, A. W., Battiste, J., Henke, R. M., Nakada, Y., Simplicio, N., Guillemot, F. and Johnson, J. E.** (2005). Sequential roles for Mash1 and Ngn2 in the generation of dorsal spinal cord interneurons. *Development* **132**, 2709-2719.

**Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y. V., Fukuda, A., Fuse, T., Matsuo, N., Sone, M. et al.** (2005). Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. *Neuron* **47**, 201-213.

**Jessell, T. M.** (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1**, 20-29.

**Kageyama, R. and Ohtsuka, T.** (1999). The Notch-Hes pathway in mammalian neural development. *Cell Res.* **9**, 179-188.

**Kele, J., Simplicio, N., Ferri, A. L., Mira, H., Guillemot, F., Arenas, E. and Ang, S. L.** (2006). Neurogenin 2 is required for the development of ventral midbrain dopaminergic neurons. *Development* **133**, 495-505.

**Miyoshi, G., Bessho, Y., Yamada, S. and Kageyama, R.** (2004). Identification of a novel basic helix-loop-helix gene, Heslike, and its role in GABAergic neurogenesis. *J. Neurosci.* **24**, 3672-3682.

**Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q. and Goulding, M.** (2006). Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. *Nat. Neurosci.* **9**, 770-778.

**Muller, T., Brohmann, H., Pierani, A., Heppenstall, P. A., Lewin, G. R., Jessell, T. M. and Birchmeier, C.** (2002). The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* **34**, 551-562.

**Muller, T., Anlag, K., Wildner, H., Britsch, S., Treier, M. and Birchmeier, C.** (2005). The bHLH factor Olig3 coordinates the specification of dorsal neurons in the spinal cord. *Genes Dev.* **19**, 733-743.

**Nakada, Y., Hunsaker, T. L., Henke, R. M. and Johnson, J. E.** (2004). Distinct domains within Mash1 and Math1 are required for function in neuronal differentiation versus neuronal cell-type specification. *Development* **131**, 1319-1330.

**Nakatani, T., Mizuhara, E., Minaki, Y., Sakamoto, Y. and Ono, Y.** (2004). Helt, a novel basic-helix-loop-helix transcriptional repressor expressed in the developing central nervous system. *J. Biol. Chem.* **279**, 16356-16367.

**Osada, A., Kiyozumi, D., Tsutsui, K., Ono, Y., Weber, C. N., Sugimoto, N., Imai, T., Okada, A. and Sekiguchi, K.** (2005). Expression of MAEG, a novel basement membrane protein, in mouse hair follicle morphogenesis. *Exp. Cell Res.* **303**, 148-159.

**Parras, C. M., Schuumans, C., Scardigli, R., Kim, J., Anderson, D. J. and Guillemot, F.** (2002). Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. *Genes Dev.* **16**, 324-338.

**Ross, S. E., Greenberg, M. E. and Stiles, C. D.** (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39**, 13-25.

**Sakamoto, M., Hirata, H., Ohtsuka, T., Bessho, Y. and Kageyama, R.** (2003). The basic helix-loop-helix genes Hesr1/Hey1 and Hesr2/Hey2 regulate maintenance of neural precursor cells in the brain. *J. Biol. Chem.* **278**, 44808-44815.

**Sanders, T. A., Lumsden, A. and Ragsdale, C. W.** (2002). Arcuate plan of chick midbrain development. *J. Neurosci.* **22**, 10742-10750.

**Satow, T., Bae, S. K., Inoue, T., Inoue, C., Miyoshi, G., Tomita, K., Bessho, Y., Hashimoto, N. and Kageyama, R.** (2001). The basic helix-loop-helix gene hesr2 promotes gliogenesis in mouse retina. *J. Neurosci.* **21**, 1265-1273.

**Schuurmans, C. and Guillemot, F.** (2002). Molecular mechanisms underlying cell fate specification in the developing telencephalon. *Curr. Opin. Neurobiol.* **12**, 26-34.

**Wildner, H., Muller, T., Cho, S. H., Brohl, D., Cepko, C. L., Guillemot, F. and Birchmeier, C.** (2006). dILA neurons in the dorsal spinal cord are the product of terminal and non-terminal asymmetric progenitor cell divisions, and require Mash1 for their development. *Development* **133**, 2105-2113.