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Ascl1 and *Helt* act combinatorially to specify thalamic neuronal identity by repressing *Dlxs* activation

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ABSTRACT

The mammalian thalamus is an essential diencephalic derivative that plays unique roles in processing and relaying sensory and motor information to and from the cerebral cortex. The profile of transcription factors and lineage tracing experiments revealed a spatiotemporal relationship between diencephalic progenitor domains and discrete differentiated neurons contributing to thalamic nuclei. However, the precise molecular mechanisms by which heterogeneous thalamic neurons become specified and assemble into distinct thalamic nuclei are still poorly understood. Here, we show that a combinatorial interaction between the bHLH transcription factors *Ascl1* and *Helt* is required for acquiring thalamic progenitor identity. Surprisingly, in the combined absence of *Ascl1* and *Helt*, rostral thalamic progenitors (TH-R) adopt a molecular profile of a more rostral diencephalic derivative, the prethalamus. Furthermore, we show that the prethalamic factors *Dlxs* upregulated by *Ascl1*/*Helt* deficiency play unique roles in regulating thalamic progenitor specification, and that derepression of *Dlx2* and *Dlx5* suppress generation of TH-R neurons. Taken together, our results suggest a model whereby the combined activity of two distinct bHLH factors plays a key role in the development of discrete classes of thalamic interneurons.

Introduction

Spatial coordination of thalamic nuclei assembly is essential for performing its principal roles in processing and relaying afferent sensory inputs to the cerebral cortex, as well as regulating consciousness, sleep and awareness (Jones, 2007). The profile of transcription factors expressed by the developing thalamus (previously called dorsal thalamus) has enabled precise defining of thalamic progenitor cell populations and has led to a better understanding of lineage relationships between each progenitor domain and distinct postmitotic interneurons populating thalamic nuclei (Bulfone et al., 1993; Kitamura et al., 1997; Nakagawa and O'Leary, 2001; Vue et al., 2007; Chen et al., 2009; Suzuki-Hirano et al., 2011; Jeong et al., 2011; Nakagawa and Shimogori, 2012). The neurons that populate thalamic nuclei are derived from at least two distinct progenitor domains of diencephalic alar plate. The progenitors of a large caudal region of the thalamus (TH-C) give rise to glutamatergic neurons, and contribute to all thalamic nuclei that project to the neocortex via thalamocortical axons. The rostral population of thalamic progenitors (TH-R), positioned between TH-C and the zli, generate GABAergic neurons that contribute to two nuclei, the ventrolateral geniculate nucleus (vLG) and the intergeniculate leaflet (IGL).

While significant advances have been made in deciphering the roles for distinct extracellular signals such as Shh, Wnt, and Fgf8 in the rostro-caudal (TH-R *vs* TH-C) regionalization (Kiecker and Lumsden, 2004; Vieira et al., 2005; Kataoka and Shimogori, 2008; Vue et al., 2009; Szabó et al., 2009; Jeong et al., 2011; Bluske et al., 2012), relatively little is known about the requirement of transcription factors regulated by these signals for specifying heterogeneous clusters of thalamic neurons (reviewed in Hagemann and Scholpp (2012)). Loss- and/or gain-of-function analyses have elucidated the roles for *Otx2*, *Pax6*, and *Neurog1/2* in regulating TH-C/glutamatergic lineage fate (Fode et al., 2000; Puelles et al., 2006; Wang et al., 2011; Bluske et al., 2012; Robertshaw et al., 2013). By contrast, *Her6* and *Gata2* have been shown to promote TH-R/GABAergic fate (Scholpp et al., 2009; Virolainen et al., 2012). The requirement of *Ascl1* for GABAergic lineage neurogenesis in various CNS regions has also been the subject of much investigation (Fode et al., 2000; Bertrand et al., 2002; Peltopuro et al., 2010; Virolainen et al., 2012). However, the identity of TH-R progenitors does not appear to depend on *Ascl1*, as loss of *Ascl1* had no consequences on rostral thalamic

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specification (Virolainen et al., 2012). A bHLH-Orange factor *Helt* expressed in a pattern similar to *Ascl1* has also been shown to play crucial roles in GABAergic neurogenesis in the midbrain and pretectum (Miyoshi et al., 2004; Guimera et al., 2006; Nakatani et al., 2007; Delogu et al., 2012). While a recent gain-of-function study showed that ectopic *Helt* is capable of inducing TH-R cell types in TH-C (Sellers et al., 2014), loss of *Helt* did not affect TH-R neuronal identity (Guimera et al., 2006; Delogu et al., 2012).

While inactivation of single transcription factors can lead to dysregulated lineage decisions, it is also possible that specific combinations of transcription factors regulate correct lineage decisions. Given the similar expression pattern and shared activities between *Ascl1* and *Helt*, we considered the possibility that a developmental program that functionally couples their activities might be required for thalamic differentiation. Surprisingly, our study shows that the identity of rostral thalamic progenitors (TH-R) is established by a cooperative interaction between *Ascl1* and *Helt*. Combined loss of *Ascl1* and *Helt* abrogated TH-R specification and instead led to a fate switch to a more rostral diencephalic derivative, the prethalamus (previously called ventral thalamus). Moreover, the prethalamic factors *Dlx2, Dlx5,* and *Arx* that are induced by *Ascl1*/*Helt* deficiency play distinct roles in thalamic specification: *Dlx2* and *Dlx5* suppress TH-R fate while *Arx* represses TH-C fate. Our results propose a new model whereby heterogeneous thalamic progenitors become specified and assemble into distinct thalamic nuclei.

Materials and methods

Mouse lines

All animal procedures were carried out in accordance with the guidelines and protocols approved by the Kyung Hee University Institutional Animal Care and Use Committee. Generation of *Ascl1* and *Helt* mice was described previously (Leung et al., 2007; Guimera et al., 2006). *Ascl1*$^{+/-}$ mice (The Jackson Laboratory, Bar Harbor, ME) have green fluorescent protein (GFP) knocked into the *Ascl1* locus, disrupting *Ascl1* coding sequences. *Helt*$^{+/-}$ (Mgn$^{tz/+}$) mice were generated to replace exons 2 and 3 (the bHLH-Orange domain) with tau-*lacZ*. *Ascl1*$^{+/-}$ mice were bred to *Helt*$^{+/-}$ mice to generate double heterozygous F_1 progeny. These were maintained on an outbred ICR background and intercrossed to generate *Ascl1*$^{-/-}$; *Helt*$^{-/-}$ embryos. Embryos were staged from the time point of vaginal plug detection, which was designated as embryonic day (E) 0.5.

Plasmids

All regulatory sequences assayed were cloned into the *NotI* restriction site of a reporter vector comprising the β-globin minimal promoter, *lacZ* cDNA, SV40 large T antigen poly(A) site. The specific DNA sequences (ECR1–ECR5) and vertebrate homologs of Gbe1 were generated by PCR amplification. To test the requirement of each E box binding site in the Gbe1, mutations designed to disrupt DNA binding at the recognition sequences were constructed by a three-component assembly ligation of two PCR product and reporter vector. Each primer sequence is shown in Supplementary material text S1.

For misexpression constructs, mouse full length *Dlx1*, *Dlx2*, *Dlx5* (J. L. Rubenstein, UCSF), *Dlx6* (E. N. Olson, University of Texas Southwestern Medical Center), and *Arx* (Origene, Rockville, MD, USA) cDNAs were cloned into the *SnaBI* site of an expression vector consisting of the *Nestin* enhancer, β-globin minimal promoter, SV40 large T antigen poly(A) site. Fused cassettes *VP16-Dlx2* and *EnR-Dlx2* (D. Eisenstat, U. of Alberta, Canada; Le et al., 2007), as well as *VP16-Dlx5* and *EnR-Dlx5* (A. J. Bendall, U. of Guelph, Canada; Hsu et al., 2006), were also cloned into the *SnaBI* site of the same expression vector.

Transgenic mouse embryos

Plasmid transgenes were prepared and linearized with *SacI or SacII* for microinjection as previously described (Jeong and Epstein, 2003). Transient transgenic embryos or mouse lines were generated by pronuclear injection into fertilized eggs derived from FVBN strain mice.

β-galactosidase staining and in situ hybridization

The activity of β-galactosidase was assessed by histochemical staining with X-gal (Roche) as substrate (Jeong and Epstein, 2003). Whole-mount *in situ* hybridization was performed using digoxygenin-UTP-labeled riboprobes essentially as described (Jeong and Epstein, 2003). For genes expression studies at E12.5 and E13.5, heads were bisected sagitally along the midline prior to initiating the whole-mount *in situ* hybridization protocol. After staining, left and right medial surfaces of bisected brains were photographed. For section *in situ* hybridization (Lee et al., 2012), embryos were fixed in 4% formaldehyde overnight, sunk in 30% sucrose, embedded in OCT, and cryosectioned at 25 μm.

Results

Acquisition of rostral thalamic progenitor identity is dependent on the combined functions of Ascl1 and Helt

While *Ascl1* and *Helt* are each known to affect the development of several GABAergic lineages, neither *Ascl1* nor *Helt* mutants display obvious defects in the thalamic specification (Miyoshi et al., 2004; Guimera et al., 2006; Nakatani et al., 2007; Peltopuro et al., 2010; Virolainen et al., 2012). Furthermore, single mutants of either *Ascl1* or *Helt* continue to express *Helt* and *Ascl1*, respectively, in the thalamus. Therefore, we investigated whether these two factors cooperate to regulate thalamic progenitor specification. To address this question, we systematically studied the expression profiles of a panel of regional markers in the developing thalamus of wild-type, *Ascl1*$^{-/-}$, *Helt*$^{-/-}$, and *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants at E12.5. The TH-R domain comprises a narrow band, sandwiched between TH-C and the zli, and expresses several transcription factors including *Tal2*, *Tal1*, *Gata2*, *Gata3*, *Six3* and *Sox14* (Fig. 1A1–F1; Supplementary material Fig. S1A1–F1). Loss of *Ascl1* function led to severe downregulation or complete loss of expression of these transcription factors in the pretectum; however, their expression was maintained in TH-R (Fig. 1A2–F2; Supplementary material Fig. S1A2–F2). Similarly, in *Helt*$^{-/-}$ embryos, reduced expression of these markers was observed in the pretectum, but not in TH-R (Fig. 1A3–F3; Supplementary material Fig. S1A3–F3). In contrast to single knockout mutants, combined inactivation of *Ascl1* and *Helt* led to a dramatic loss of TH-R marker expression (Fig. 1A4–F4; Supplementary material Fig. S1A4–F4). This failure of rostral thalamic specification in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants was not likely due to a defect in early regional patterning, as *Shh*, *Nkx2.2* and *Gsx1* expression was normally detected (Supplementary material Fig. S2). Concurrent with the loss of TH-R progenitor identity, the neurotransmitter profile of TH-R neurons was also compromised in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants. In *Ascl1* or *Helt* single mutants, *Gad1* expression was largely unaffected in TH-R despite an almost complete lack or downregulation in pretectal neurons (Fig. 1G2 and G3; Supplementary material Fig. S1G2 and G3). By contrast, in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants, there was a significant loss of *Gad1* expression in the TH-R domain (Fig. 1G4;

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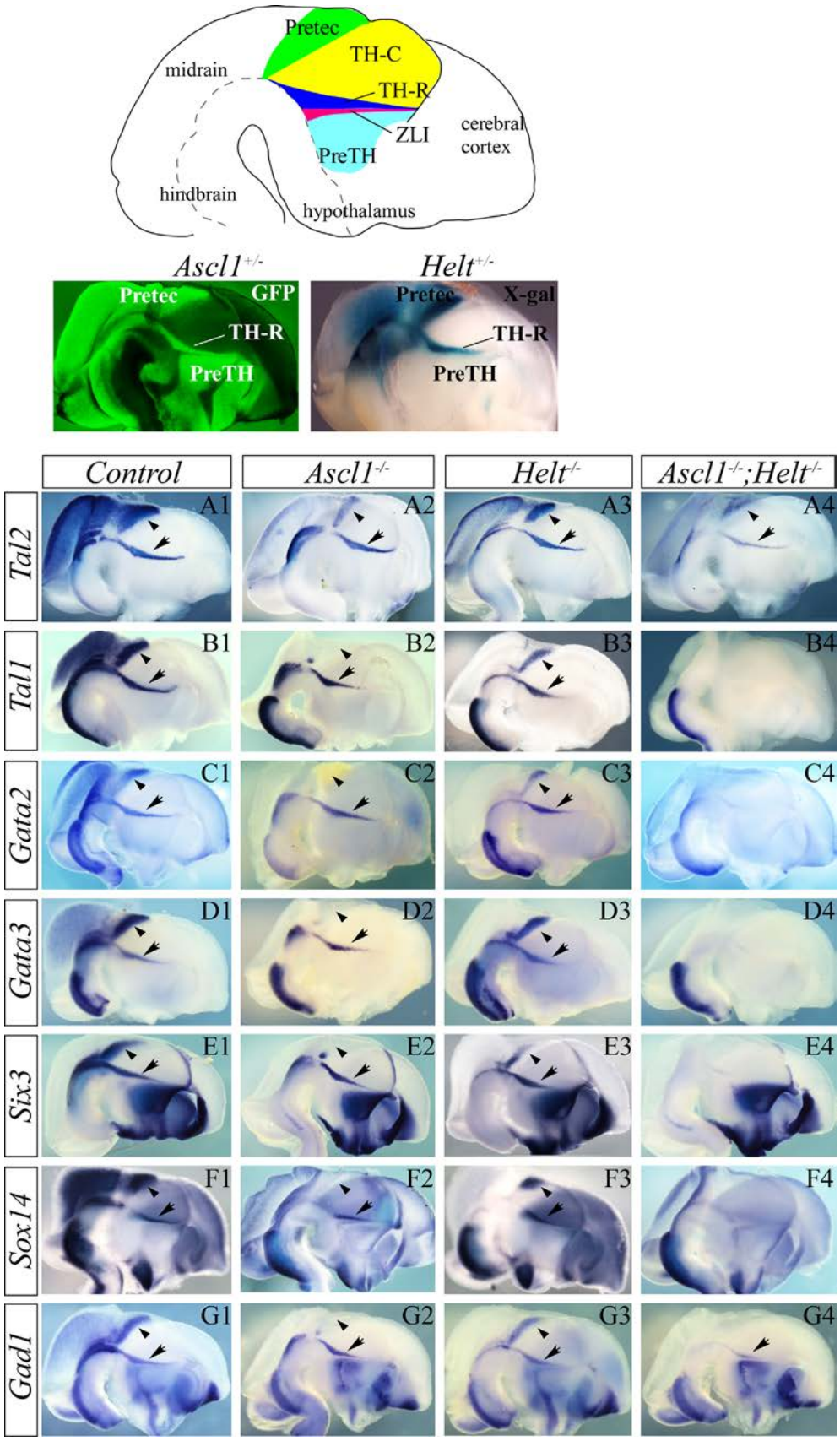

Fig. 1. Combinatorial activity of *Ascl1* and *Helt* is required for specifying TH-R progenitor identity. (Top) Schematic representation of a sagittal view through the mouse diencephalon, color coded as follows: green, pretectum; yellow, TH-C; blue, TH-R; red, zli; aqua, prethalamus. Below are shown GFP fluorescence of $Ascl^{+/-}$ embryo and X-gal staining of $Helt^{+/-}$ embryo at E12.5. (A1–G4) Whole-mount *in situ* hybridization of genes expressed in the pretectum (arrowhead) and TH-R (arrow) of wild-type, $Ascl1^{-/-}$, $Helt^{-/-}$, and $Ascl1^{-/-}$;$Helt^{-/-}$ compound mutant embryos at E12.5. The expression of *Tal2, Tal1, Gata2, Gata3, Six3, Sox14* and *Gad1* is greatly downregulated or absent in the midbrain and pretectum of $Ascl1^{-/-}$ (A2–G2) or $Helt^{-/-}$ embryos (A3–G3), while the TH-R expression was largely unaffected in *Ascl1*- or *Helt*-deficient embryos. Remarkably, in $Ascl1^{-/-}$;$Helt^{-/-}$ compound mutant embryos, the TH-R expression was severely reduced (*Tal2, Gad1*) or completely missing (*Tal1, Gata2, Gata3, Six3, Sox14*). Pretec, pretectum; PreTH, prethalamus; TH-C, caudal thalamus; TH-R, rostral thalamus.

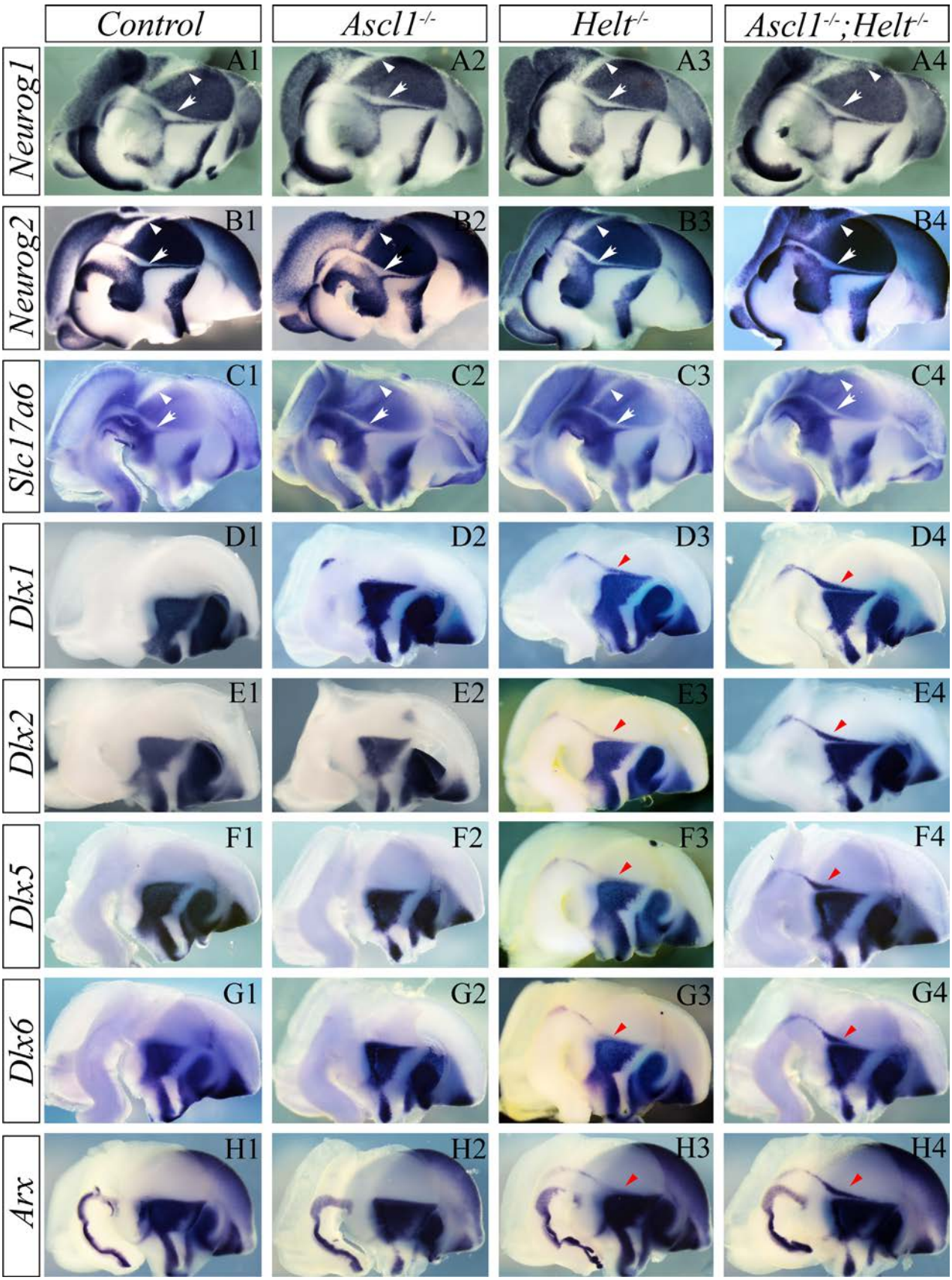

Fig. 2. TH-R progenitors adopt prethalamic fate in the combined absence of *Ascl1* and *Helt*. (A1–H4) Whole-mount *in situ* hybridization of TH-C (*Neurog1, Neurog2,* and *Slc17a6*) and prethalamic markers (*Dlx1, Dlx2, Dlx5, Dlx6,* and *Arx*) in wild-type, *Ascl1*$^{-/-}$, *Helt*$^{-/-}$, and *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutant embryos at E12.5. (A1–C4) The expression of TH-C markers was ectopically upregulated in the pretectum (white arrowhead) of *Ascl1*$^{-/-}$, *Helt*$^{-/-}$, and *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutant embryos, while the white gap of these genes expression between TH-C and zli, corresponding to TH-R (white arrow) was still maintained. (D1–H4) Weak upregulation of prethalamic genes was detected in TH-R (red arrowhead, D3, E3, F3, G3, and H3) of *Helt*$^{-/-}$ embryos, while in the combined absence of *Ascl1* and *Helt*, robust expression of these prethalamic genes was induced in TH-R of *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutant embryos (D4, E4, F4, G4, and H4).

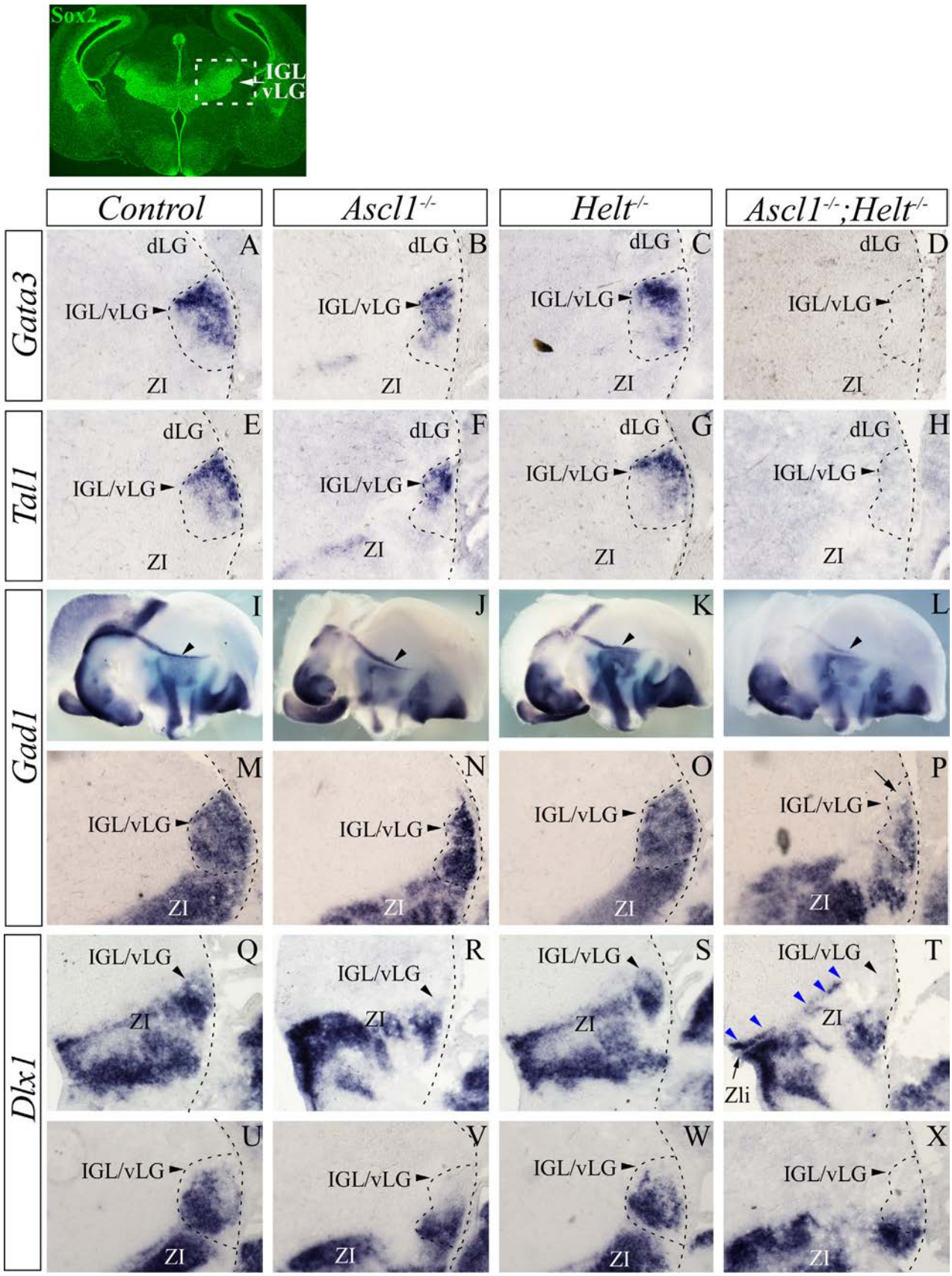

Fig. 3. IGL/vLG neurons are compromised in the combined absence of *Ascl1* and *Helt*. (Top) Sox2 immunostaining shows a sharp boundary between the dorsal lateral geniculate nucleus and IGL/vLG (white arrow) in a wild-type embryo at E16.5. (A–H) Section *in situ* hybridization of *Gata3* (A–D) and *Tal1* (E–H) in IGL/vLG of wild-type, *Ascl1*$^{-/-}$, *Helt*$^{-/-}$, and *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutant embryos at E16.5. These TH-R progenitor markers are still detected in the IGL/vLG in wild-type, *Ascl1*$^{-/-}$, and *Helt*$^{-/-}$ mutant embryos, but are missing in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutant embryos. (I–P) Whole-mount (I–L) and section *in situ* hybridization (M–P) of *Gad1* at E13.5 and E16.5, respectively. *Gad1* expression is normally present throughout IGL/vLG (M), but is lacking in the IGL and part of the vLG of *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutant embryos (P, arrow). (Q–X) Section *in situ* hybridization of *Dlx1* at E14.5 (Q–T) and E16.5 (U–X). *Dlx1* expression is principally detected in the medial region of the vLG. In contrast with wild-type, *Ascl1*$^{-/-}$, and *Helt*$^{-/-}$ embryos, a stream of *Dlx1*$^{+}$ cells migrating from the TH-R ventricular zone was detected in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutant embryos (T, blue arrowheads). This staining is separated from *Dlx1* expression in the prethalamus by a thin gap, corresponding to the zli. Staining with Sox2 and *Gadl* on adjacent sections was used to position the area of IGL/vLG.

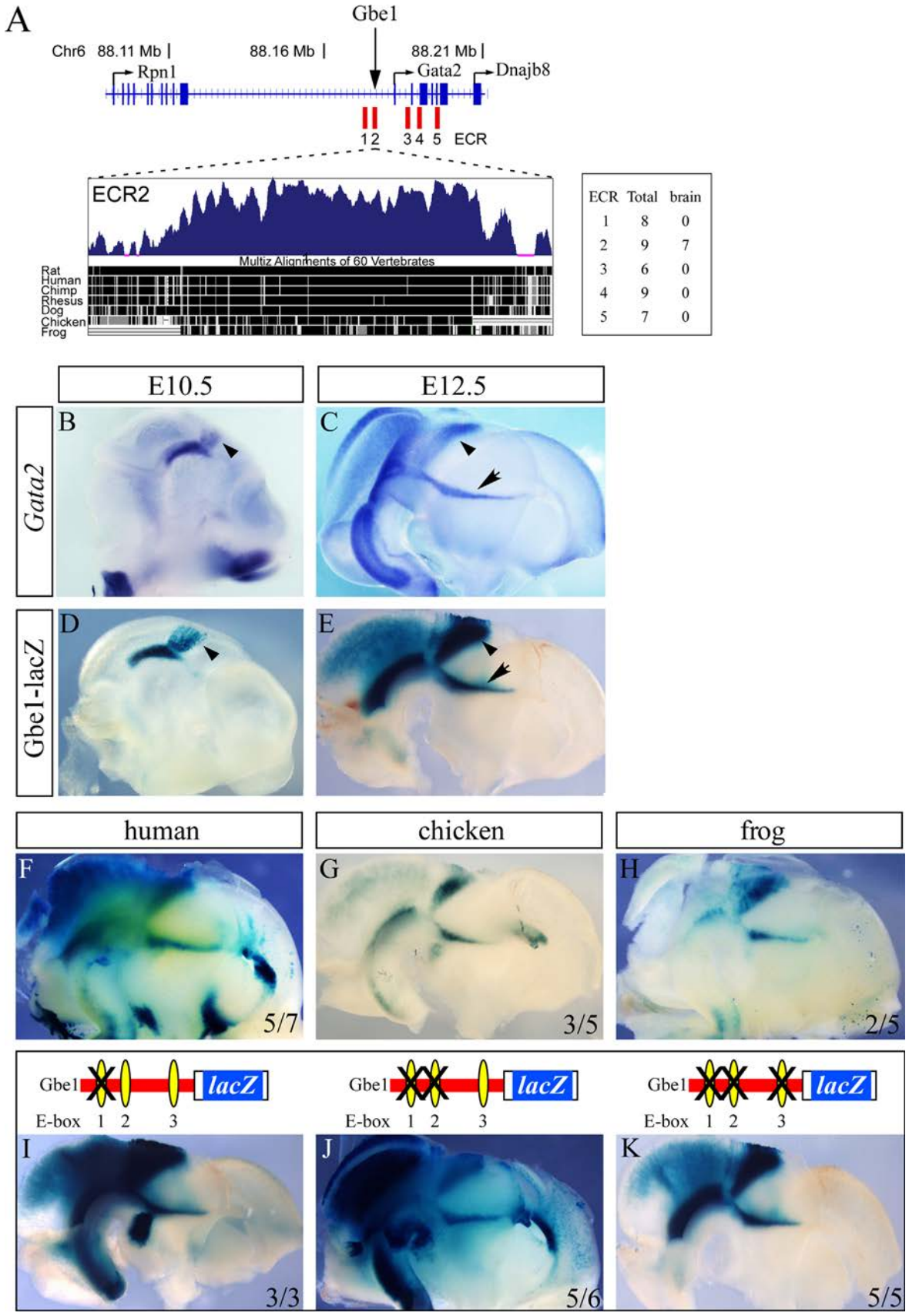

Fig. 4. Conserved function of a *Gata2* forebrain enhancer is not dependent on bHLH factor binding sites. (A) Genomic location of Gbe1 enhancer and Multiz alignment comparing Gbe1 sequences of vertebrate species (UCSC genome browser, http://genome.ucsc.edu). The table to the right indicates the total number of transgenic embryos generated for each constructs (ECR1-5, red bars) and the number of embryos showing *Gata2*-like reporter expression. (B–E) Time course of *Gata2* expression (B and C) and Gbe1 enhancer activity (D and E) in the pretectum (arrowhead) and TH-R (arrow). (F–H) Gbe1 reporter activity derived from human, chicken, and frog. The ratio of embryos exhibiting *Gata2*-like reporter activity over the total number of transgenic embryos is indicated below. (I–K) X-gal staining of transgenic embryos at E12.5 carrying reporter constructs with mutant E box sites. Mutation of one core sequence (I) or generating two (J) or three combined mutations (K) did not result in alteration of transgene expression in TH-R.

Supplementary material Fig. S1G4). These data suggest that the combined function of *Ascl1* and *Helt* is required for correct specification of the TH-R thalamic domain.

Loss of Ascl1 and Helt mis-specify TH-R into prethalamic progenitors

We then asked whether down-regulation of TH-R markers by deficiency of *Ascl1* and *Helt* might reflect their respecification to a more caudal thalamic progenitor TH-C identity, as *Ascl1* and *Helt* repress *Neurog1* and *Neurog2* expression in the pretectum and ventral midbrain, respectively (Nakatani et al., 2007; Virolainen et al., 2012; Fig. 2A2, A3, B2, and B3). Normally, expression of *Neurog1/2* in the zli and TH-C is separated by a gap, corresponding to TH-R (Fig. 2A1 and B1). This gap in *Neurog1/2* expression was still present in the compound mutants despite strong upregulation of *Neurog1* and *Neurog2* expression in the pretectum (Fig. 2A4 and B4). Consistently, an expression gap of *Slc17a6*, a glutamatergic marker, was also maintained (Fig. 2C1–C4), indicating no apparent identity switch into TH-C.

Recently, in *Gata2*-deficient embryos, the prethalamic progenitor markers *Dlx1* and *Arx* were shown to be ectopically induced in the TH-R intermediate zone (Virolainen et al., 2012). Our results pointing towards a failure of TH-R specification upon *Ascl1* and *Helt* deficiency, therefore, prompted us to investigate whether rostral thalamic progenitors adopt a molecular profile of prethalamic progenitors. At E12.5, four *Dlx* homeodomain factors *Dlx1*, *Dlx2*, *Dlx5*, *Dlx6* and *Arx* are normally expressed in the forebrain anterior to the zli (Fig. 2D1, E1, F1, G1, and H1). Remarkably, in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants, we observed strong induction of these transcription factors in TH-R (Fig. 2D4, E4, F4, G4, and H4). Upon sectioning, in addition to ectopic staining in the intermediate zone, robust expression of prethalamic genes was also observed in the ventricular zone (Supplementary material Fig. S3D, H, L, P, and T). These results demonstrate that in the absence of *Ascl1* and *Helt*, the TH-R domain acquire a more rostral diencephalic identity. Surprisingly, we also observed weak activation of the four *Dlxs* and *Arx* in *Helt*-deficient TH-R (Fig. 2D3, E3, F3, G3, and H3; Supplementary material Fig. S3C, G, K, O, and S), suggesting that a combinatorial interaction between *Ascl1* and *Helt* is critical for proper suppression of a prethalamic cell phenotype in the thalamus.

Previous studies have shown that the vLG nucleus is composed of a heterogeneous group of neurons originating from both TH-R and prethalamus (Vue et al., 2007; Jeong et al., 2011; Bluske et al., 2012). TH-R progenitors contribute to the IGL nucleus and lateral part of the vLG nucleus, while prethalamic progenitors give rise principally to neurons that are medially positioned within the vLG. To determine the consequence of aberrant specification in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants, we examined vLG/IGL nuclei at later stages of embryogenesis. At E16.5, *Gata3* and *Tal1* were normally expressed in the IGL and laterally in the vLG (Fig. 3A and E). Consistently, their expression was still detectable in *Ascl1*$^{-/-}$ or *Helt*$^{-/-}$ single mutants, (Fig. 3B, C, F, and G), but was absent in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants (Fig. 3D and H). In the absence of *Ascl1*/*Helt*, reduced *Gad1* expression was also persistent at E13.5–E16.5 (Fig. 3L and P). Instead, a stream of *Dlx1*$^{+}$ neurons appeared to migrate radially from the TH-R ventricular zone at E14.5 in *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants (Fig. 3T). However, these neurons eventually seemed to silence *Dlx1* expression (Fig. 3T and X), indicating that ectopic upregualtion of prethalamic factors in *Ascl1*/*Helt*-deficient TH-R is transient.

Combined activity of Ascl1 and Helt indirectly regulates Gata2 expression

We next asked whether the combined inactivation of *Ascl1* and *Helt* is directly responsible for loss of TH-R progenitor identity. As *Gata2* is a downstream target regulated by the combined activity of *Ascl1* and *Helt*, and *Gata2*-deficient thalamus displays overlapping phenotypes with that of *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants (Virolainen et al., 2012), the functional role for *Helt* and

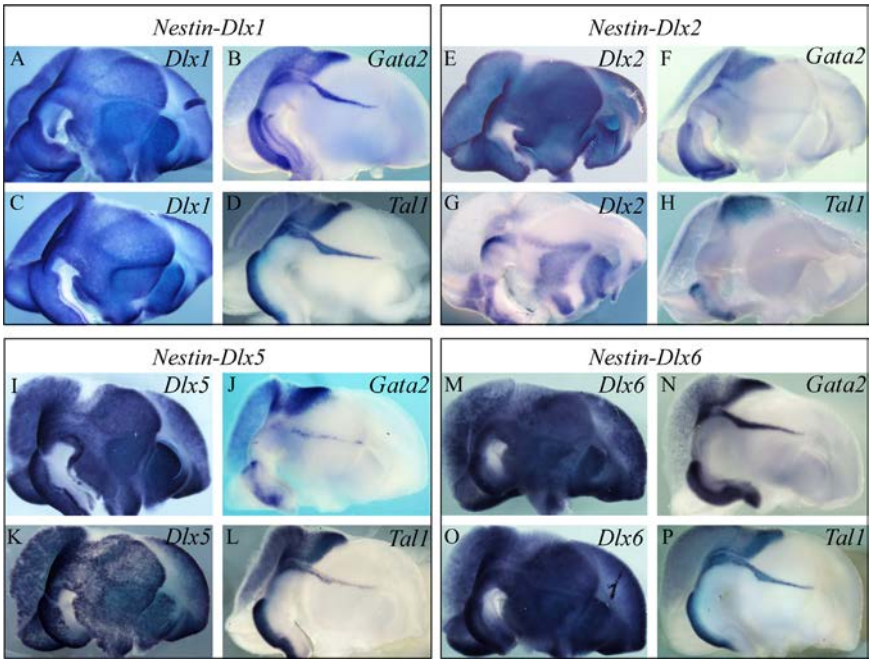

Fig. 5. *Dlx2* and *Dlx5* repress TH-R specification. Analysis of *Gata2* and *Tal1* expression in transgenic embryos carrying misexpression constructs for *Dlx1* (A–D), *Dlx2* (E–H), *Dlx5* (I–L), and *Dlx6* (M–P). For each transgenic embryo, one brain half from bisected head was used to estimate the pattern of transgene expression (*Dlx1, Dlx2, Dlx5,* and *Dlx6*) and the other half stained for *Gata2* and *Tal1*. Images from one half of each brain pair are flipped horizontally. Misexpression of *Dlx1* (A and C) did not alter *Gata2* (B; $n=0/4$) and *Tal1* expression (D; $n=0/5$). By contrast, the expression of *Gata2* (F; $n=4/4$) and *Tal1* (H; $n=4/5$) was completely lacking or severely reduced in TH-R in transgenic embryos misexpressinng *Dlx2* (E and G). Forced expression of *Dlx6* (M and O) did not affect *Gata2* (N; $n=0/3$) and *Tal1* expression (P; $n=0/5$), while *Dlx5* misexpression (I and K) resulted in marked reduction of *Gata2* (J; $n=3/4$) and *Tal1* expression (L; $n=3/3$) in TH-R.

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Ascl1 in regulating TH-R identity is likely to be mediated, at least in part, by *Gata2*. Therefore, we set out to identify regulatory sequences that control *Gata2* expression in the developing thalamus. A previous study showed that a *Gata2* brain enhancer activity resides within a 17- kb genomic fragment of the *Gata2* locus (Zhou et al., 2000). To more precisely locate the enhancer element, five highly evolutionary conserved regions (ECR) within this interval were assayed for their regulatory potential in transgenic mouse embryos (Fig. 4A). At E12.5, of the five ECRs (ECR1–5) tested, embryos carrying ECR2 showed X-gal staining in the midbrain, pretectum and thalamus in a manner consistent with *Gata2* (Fig. 4C and E). At earlier stages, the transgene also recapitulated the pattern of *Gata2* expression (Fig. 4B and D). To further characterize this regulatory sequence, the activity of orthologous sequences from divergent organisms was also assessed in transgenic mouse embryos. Strong *lacZ* expression in embryos carrying human, chicken, or frog sequences was detected in a manner similar to mouse ECR2 (Fig. 4F–H). Therefore, these data indicate that this conserved sequence functions as a regulatory enhancer (hereafter designated *Gata2* brain enhancer 1, Gbe1) to control *Gata2* expression in the developing brain, including TH-R.

We then searched for bHLH binding sites, E box (CANNTG) by scanning Gbe1 sequences using the rVISTA tool, as well as by comparing the consensus sites manually. This analysis recognized three E boxes that were conserved in humans and mice, but not in chickens or frogs (Fig. 4I–K). Although *Helt* has been shown to function as a transcriptional repressor, we also searched for C sites (CACG(C/A)G), or N boxes (CACNAG) (Kageyama et al., 2007). There was no C site or N box found in the Gbe1 enhancer. The *in vivo* significance of these putative E boxes was assessed by introducing point mutations in each of the recognition sequences. However, abrogating one core sequence or generating two or three combined mutations did not downregulate transgene expression in TH-R or other brain regions (Fig. 4I–K), suggesting that the Gbe1 enhancer activity is not directly dependent on bHLH factor(s).

Repression of Dlx2 and Dlx5 is a key role for Ascl1 and Helt in rostral thalamic specification

Recent gain- and loss-of-function experiments revealed important functions for *Dlx2* in specifying GABAergic cell fate. In *Dlx1/2* double knockout embryos, TH-R markers were ectopically induced in the prethalamus, while by chick *in ovo* electroporation, forced expression of *Dlx2* was shown to suppress TH-R markers (Delogu et al., 2012; Sellers et al., 2014). We therefore asked whether the subsequent upregulation of prethalamic genes, resulting from *Ascl1*/*Helt* deficiency, could lead to such a phenotypic alteration. This was tested by misexpression of such prethalamic factors under the control of a *Nestin* intronic enhancer. As members of the *Dlx* family have both unique and redundant functions (Anderson et al., 1997; Stühmer et al., 2002; Robledo et al., 2002; Panganiban and Rubenstein, 2002; Cobos et al., 2005a), we injected each of the four *Dlx* expression constructs into fertilized mouse eggs, and at E12.5, heads from transgenic embryos carrying the transgenes were bisected sagitally along the midline. One brain half was then stained for the transgene and the other half for thalamic markers. In eight out of nine transgenic embryos, misexpression of *Dlx2* led to downregulation of *Gata2* and *Tal1* expression (Fig. 5E–H); however, none of the embryos misexpressing *Dlx1* showed alteration in TH-R marker expression ($n=0/9$; Fig. 5A–D). Interestingly, while TH-R marker expression was also reduced after forced expression of *Dlx5* ($n=6/7$; Fig. 5I–L), it was unchanged after misexpression of *Dlx6* ($n=0/8$; Fig. 5M–P), revealing a unique role for *Dlx2* and *Dlx5* in thalamic differentiation.

To determine whether *Gata2* and *Tal1* downregulation by ectopic *Dlx2* or *Dlx5* might result from loss of *Helt* and *Ascl1*, we also examined *Helt* and *Ascl1* expression in transgenic embryos misexpressing *Dlx2* or *Dlx5*. While *Helt* expression was slightly reduced after strong ectopic expression of *Dlx2* or *Dlx5* (Fig. 6B and F), *Ascl1* expression was not altered (Fig. 6D and H). As neither *Ascl1* nor *Helt* single mutants exhibited discernible alterations, suppression of TH-R identity by forced expression of *Dlx2* and *Dlx5* did not result from the combined loss of *Ascl1* and *Helt*. Therefore, these data demonstrate that *Dlx2* and *Dlx5* repression by *Ascl1* and *Helt* is critical for specifying correct TH-R progenitor identity.

Activator function of Dlx2 and Dlx5 mediates TH-R repression

Since *Dlx* factors both positively and negatively regulate the expression of downstream target genes (Zhang et al., 1997; Zerucha and Ekker, 2000; Le et al., 2007), we asked if *Dlx2* or *Dlx5* function as a transcriptional repressor of TH-R genes or if instead, they act as a transcriptional activator to indirectly suppress TH-R genes. For this purpose, we fused the viral coactivator VP16 or transcriptional repression domain from Drosophila Engrailed (EnR) to N-terminal truncated *Dlx2* or *Dlx5* consisting of a nuclear localization signal, homeodomain and C-terminus (Fig. 7), and tested the ability of these chimeric proteins to affect thalamic differentiation. Eight out of nine embryos expressing the *VP16-Dlx2* transgene exhibited disruption of TH-R gene expression (Fig. 7A–D). By contrast, misexpression of *EnR-Dlx2* did not alter *Gata2*

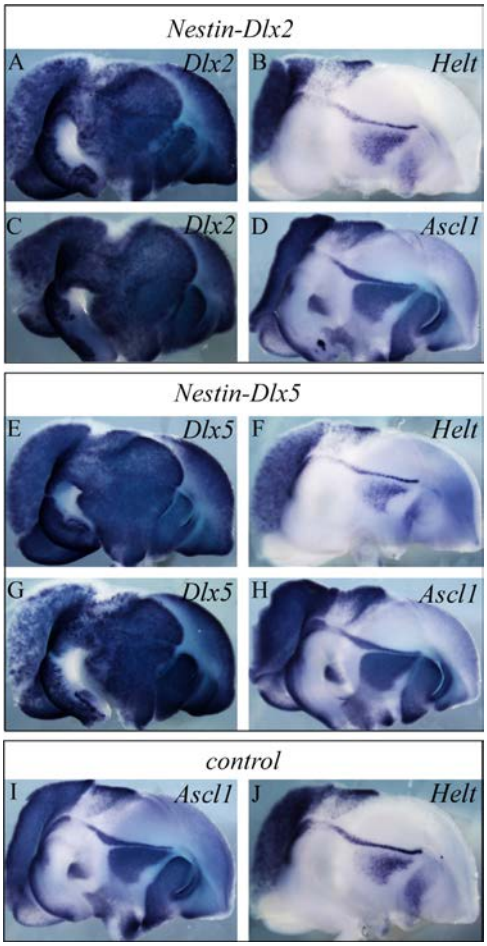

Fig. 6. TH-R fate suppression by *Dlx2* and *Dlx5* does not result from combined loss of *Ascl1* and *Helt*. (A–H) Analysis of transgenic embryos carrying *Dlx2* (A–D) or *Dlx5* (E–H) misexpression constructs. Transgenic embryos misexpressing *Dlx2* (A and C) showed a slight reduction of *Helt* in TH-R (B; $n=3/5$), and no alteration of *Ascl1* expression (D; $n=5/5$), compared to those in wild-type embryos (I and J). Similarly, misexpression of *Dlx5* led to downregulation of *Helt* (F; $n=3/4$), but did not affect *Ascl1* expression (H; $n=3/3$).

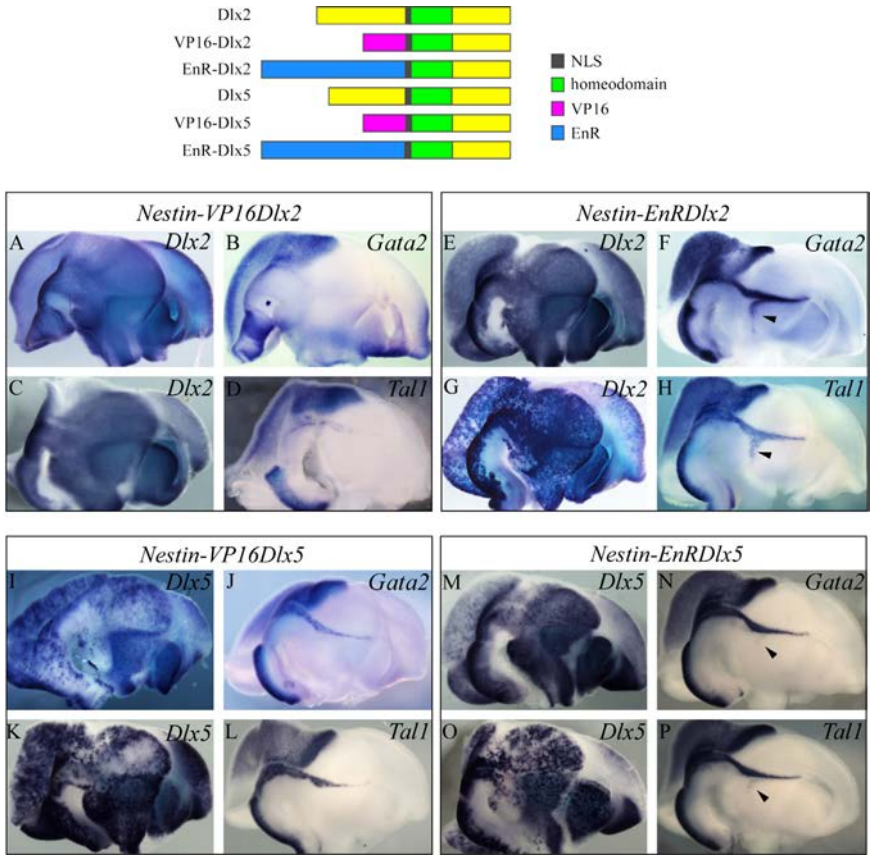

Fig. 7. *Dlx2* and *Dlx5* act as transcriptional activators to indirectly suppress TH-R identity. (Top) Schematic diagram of *Dlx2* and *Dlx5* fusion protein constructs. The N-terminal domains of wild-type *Dlx2* or *Dlx5*, leaving the nuclear localization signal (NLS, black) and homeodomain (green) intact, were replaced with the transactivation domain of herpes simplex virus VP16 or the transcriptional repression domain of *D. melanogaster* engrailed. (A–P) Analysis of *Gata2* and *Tal1* expression in transgenic embryos carrying misexpression constructs for *VP16-Dlx2* (A–D), *EnR-Dlx2* (E–H), *VP16-Dlx5* (I–L), and *EnR-Dlx5* (M–P). Ectopic expression of *VP16-Dlx2* led to downregulation of *Gata2* (B; $n=4/4$) and *Tal1* expression (D; $n=4/5$), while misexpression of *EnR-Dlx2* induced ectopic expression of TH-R markers in the prethalamus (arrowhead) without affecting *Gata2* (F; $n=0/4$) and *Tal1* expression (H; $n=0/3$) in the TH-R domain. Similarly, ectopic *VP16-Dlx5* reduced *Gata2* (J; $n=3/4$) and *Tal1* expression (L; $n=3/4$), while *EnR-Dlx5* did not affect *Gata2* (N; $n=0/5$) and *Tal1* expression (P; $n=0/3$) in TH-R.

and *Tal1* expression in the pretectum or TH-R ($n=0/7$; Fig. 7E–H). Instead, *EnR-Dlx2* appeared to interfere with endogenous *Dlx2* function in the prethalamus. Indeed, misexpression of *EnR-Dlx2* resulted in ectopic expression of TH-R markers in the prethalamus (Fig. 7E–H). This was reminiscent of *Dlx1/2* double knockout mutants in which upregulation of TH-R markers was detected in the prethalamus (Delogu et al., 2012; Sellers et al., 2014). Similar results were also obtained after misexpression of chimeric Dlx5 proteins (Fig. 7I–P). These data suggest that TH-R repression by *Dlx2* and *Dlx5* is mediated via the induction of (a) negative regulator(s).

Arx suppresses a rostral to caudal fate switch in Ascl1/Helt-deficient TH-R

Arx has been shown to be positively regulated by *Dlx* genes and thus mediate some aspects of *Dlx* functions (Colasante et al., 2008; Cobos et al., 2005b). Therefore, we asked whether *Arx* could mediate repression of TH-R fate by *Dlx2* and *Dlx5*. To answer this question, transgenic embryos forcing *Arx* expression throughout the brain were generated and analyzed for expression of thalamic marker genes. Contrary to our expectation, misexpression of *Arx* did not result in repression of *Gata2, Tal1,* or *Gad1* in the TH-R domain (Fig. 8A–F). Surprisingly, we found that the TH-R gene expression expanded caudally into the TH-C domain after forced expression of *Arx* (Fig. 8A–F). Previous experiments using *Neurog1/2* double knockout mice revealed the requirement of *Neurog1/2* to suppress GABAergic lineage markers (Fode et al., 2000; Bluske et al., 2012). We, therefore, investigated whether ectopic induction of TH-R by *Arx* in the TH-C results from alteration of *Neurog1/2*. In contrast to misexpression of *Dlx2* (Fig. 8K–N), misexpression of *Arx* indeed led to downregulation of *Neurog2* in the TH-C domain (Fig. 8G and H). Consistently, *Slc17a6* expression was also reduced (Fig. 8I and J), demonstrating that *Arx* functions independently of *Dlx* genes and instead antagonizes *Neurog1/2* functions. Notably, as described above, strong induction of *Arx* by *Ascl1/Helt*-deficiency was detected only in the TH-R domain (Fig. 2H4), thus explaining why the combined absence of *Ascl1* and *Helt* led to different fate switches in the pretectum and TH-R.

Discussion

Our results have highlighted the requirement of *Ascl1* and *Helt* function for regulating thalamic progenitor identity, and demonstrated that the single or combined activities of *Ascl1* and *Helt* play distinct and overlapping roles in discrete progenitor domains. Loss of *Ascl1* or *Helt* disrupted neuronal specification in the pretectum, causing a GABAergic to glutamatergic neuronal fate switch, and this alteration appeared to result from derepression of *Neurog2* (Virolainen et al., 2012; Delogu et al., 2012; Fig. 2B1–C4). Loss of *Ascl1/Helt* led to an almost complete abrogation of TH-R identity, but did not result in derepression of *Neurog2* in the TH-R domain. Instead, *Ascl1/Helt*-deficient TH-R acquired a fate of prethalamic progenitors. This is in contrast to Shh mutants where the TH-R markers including *Ascl1* and *Helt* were absent in the thalamus, and *Neurog1/2* expanded into the TH-R domain (Vue et al., 2009; Jeong et al., 2011; Bluske et al., 2012). Previous studies showed that

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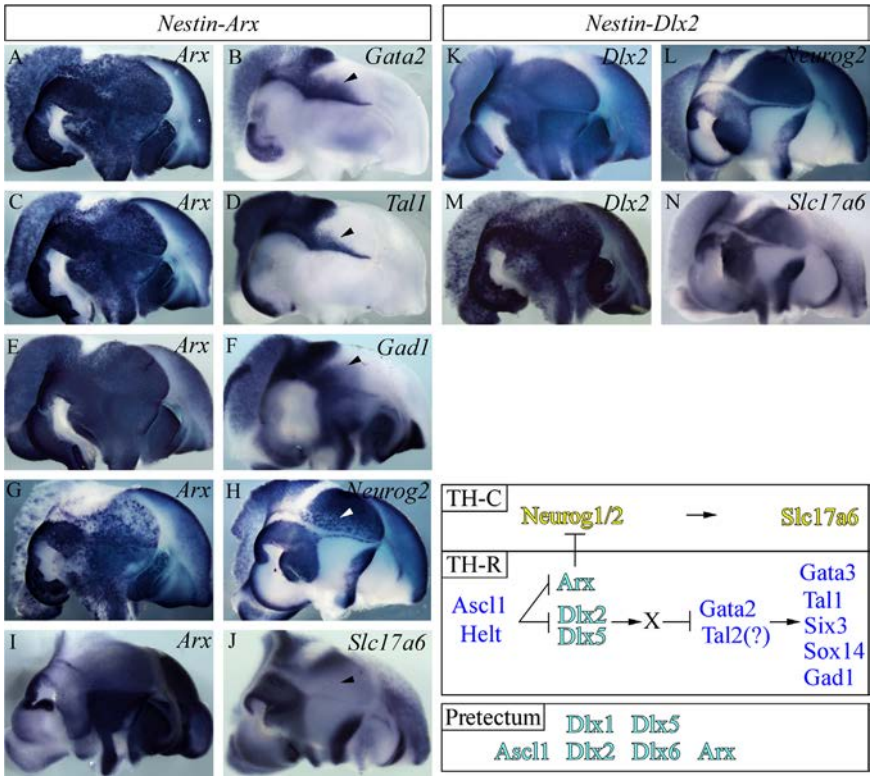

Fig. 8. *Arx* suppresses TH-C identity. (A–N) Analysis of thalamic specification in transgenic embryos carrying *Arx* (A–J), and *Dlx2* misexpression constructs (K–N). Misexpression of *Arx* resulted in ectopic induction of *Gata2* (B; $n=5/5$), *Tal1* (D; $n=3/3$) and *Gad1* (F; $n=3/3$) in TH-C, but did not affect their expression in TH-R. Concurrent with the elevation of TH-R markers, the expression of *Neurog2* (H; $n=4/5$) and *Slc17a6* (J; $n=3/4$) was repressed in TH-C (arrowhead; H and J). Misexpression of *Dlx2* did not alter *Neurog2* (L; $n=0/3$) or *Slc17a6* expression (N; $n=0/3$). A schematic model for the regulation of thalamic specification is shown to the right bottom corner. Color coded as follows: yellow, TH-C; blue, TH-R; aqua, prethalamus. The identity of X to repress TH-R genes remains to be determined.

prethalamic patterning is also dependent on Shh signaling (Kiecker and Lumsden, 2004; Vieira et al., 2005; Scholpp et al., 2005). Enhancing or attenuating Shh signaling in chicken or zebrafish embryos resulted in the enlargement or reduction of prethalamic gene expression. Interestingly, our results suggested that combined function of *Ascl1* and *Helt* regulates TH-R specification by repressing *Dlx2*, *Dlx5* and *Arx*. Ectopic activation of *Dlx2* and *Dlx5* resulting from *Ascl1*/*Helt* deficiency repress TH-R genes, while derepressed *Arx* has a differential ability to suppress *Neurog2*, thereby blocking a rostral to caudal fate switch in *Ascl1*/*Helt*-deficient TH-R. Therefore, this may, in part, reflect differences in the dependency of TH-R on extrinsic Shh signal and its downstream intrinsic factors. Taken together, our data revealed previously unrecognized epistatic relationships between these transcription factors in thalamic development (Fig. 8).

Previous analyses of conditional *Gata2* mutants showed that *Gata2* functions as a selector gene for GABAergic neurons in the midbrain and diencephalon (Kala et al., 2009; Virolainen et al., 2012). In the absence of *Gata2*, TH-R domain adopted a fate of prethalamic progenitors (Virolainen et al., 2012). These alterations implicate a role for *Gata2* as a downstream effector of combined *Ascl1*/*Helt* activity. Interestingly, the lack of *Gad1* expression in *Gata2*-deficient TH-R is transient, which is different from that of combined *Ascl1*/*Helt* mutants that show persistent loss of *Gad1* expression throughout the later embryonic stages. In a recent study, loss of *Tal2* resulted in downregulation of *Gata3*, *Tal1*, *Six3* and *Gad1* expression and in upregulation of *Slc17a6* expression in the midbrain without affecting *Gata2* expression (Achim et al., 2013). As *Tal2* expression was also shown to be independent of *Gata2*, these two factors must act in parallel to regulate GABAergic neurogenesis. Notably, as *Tal2* expression was severely downregulated in the combined absence of *Ascl1* and *Helt*, both *Gata2* and *Tal2* appear to be required as downstream mediators of *Ascl1* and *Helt* function for normal GABAergic neurogenesis in TH-R, although *Tal2* function in the thalamus remains to be determined.

Recent results from lineage tracing experiments suggest that neurons derived from TH-R and prethalamic progenitors are intermingled within the vLG nucleus (Vue et al., 2007; Jeong et al., 2011; Bluske et al., 2012). Loss of *Gata3*, *Tal1*, and *Gad1* expression in IGL/vLG neurons of *Ascl1*$^{-/-}$;*Helt*$^{-/-}$ compound mutants raises several questions about the fate of mis-specified TH-R neurons. Analysis of *Ascl1*/*Helt*-deficient embryos harvested at E14.5 showed a pattern of *Dlx1* expression in a stream of cells extending from the TH-R ventricular zone (Fig. 3T), implicating that the mis-specified TH-R neurons are likely to migrate radially to a lateral position close to the IGL/vLG. Nevertheless, we did not observe *Dlx*$^{+}$ neurons in the IGL and lateral part of the vLG of *Ascl1*/*Helt* compound mutants at E14.6-16.5. Therefore, it remains unclear whether the mis-specified TH-R neurons still reach their final destinations in the IGL/vLG, or fail to survive.

Our data suggest that proper acquisition of thalamic progenitor identity is established by sequential repression/derepression of transcription factors. Full suppression of prethalamic genetic programs by *Ascl1* and *Helt* is a prerequisite to turn on TH-R gene expressions. Previous studies have suggested that *Ascl1* acts as an upstream regulator of *Dlx* members in the developing forebrain (Casarosa et al., 1999; Fode et al., 2000). Forced activation of *Ascl1* in the dorsal telencephalon resulted in ectopic induction of *Dlx1* (Fode et al., 2000). Moreover, a detailed analysis of a *Dlx1/2* forebrain enhancer, I12b, implicated a direct role for *Ascl1* in regulating *Dlx1/2* expression (Ghanem et al., 2003; Poitras et al., 2007). Therefore, *Ascl1* appears to play opposite regulatory roles in the thalamus and more rostral forebrain regions. *Helt*, which is closely related to *Hes* members, functions as a transcriptional

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repressor of target gene expression (Miyoshi et al., 2004; Nakatani et al., 2007), and loss of *Helt* led to weak activation of prethalamic genes in the TH-R domain. As I12b enhancer does not contain any class C sites or N boxes (Kageyama et al., 2007), it remains to be elucidated how the distinct factors *Ascl1* and *Helt* cooperate to repress the prethalamic gene expression. Adding an additional layer of complexity to the genetic hierarchy that regulates TH-R specification is the existence of a third factor that may act as a transcriptional repressor downstream of *Dlx2* and *Dlx5* transcriptional activators. As our data ruled out Arx as a candidate repressor, the downstream target of *Dlx2* and *Dlx5* is presently unknown and awaits further investigation.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.12.003.

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