Developmental Biology ■ (■■■■) ■■■–■■■

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Genomes and Developmental Control

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

Hobeom Song [a], Bumwhee Lee [a], Dohoon Pyun [a], Jordi Guimera [b], Youngsook Son [a], Jaeseung Yoon [a], Kwanghee Baek [a], Wolfgang Wurst [b], Yongsu Jeong [a,*]

[a] Department of Genetic Engineering, College of Life Sciences and Graduate School of Biotechnology, Kyung Hee University, Yongin-si 446-701, Republic of Korea
[b] Helmholtz Zentrum München, Institute of Developmental Genetics, D-85764 Neuherberg, Germany

ARTICLE INFO

Article history:
Received 23 October 2014
Received in revised form
2 December 2014
Accepted 4 December 2014

Keywords:
Ascl1
Dlx
Helt
Thalamus

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

* Corresponding author. Fax: +82 31 203 8127.
E-mail address: yongsu@khu.ac.kr (Y. Jeong).

http://dx.doi.org/10.1016/j.ydbio.2014.12.003
0012-1606/

Please cite this article as: Song, H., et al., *Ascl1* and *Helt* act combinatorially to specify thalamic neuronal identity by repressing *Dlxs* activation. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.12.003

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;

Please cite this article as: Song, H., et al., *Ascl1* and *Helt* act combinatorially to specify thalamic neuronal identity by repressing *Dlxs* activation. Dev. Biol. (2014), http://dx.doi.org/10.1016/j.ydbio.2014.12.003