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Brain-specific-homeobox is required for the specification of neuronal types in the *Drosophila* optic lobe

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ABSTRACT

The *Drosophila* optic lobe comprises a wide variety of neurons forming laminar and columnar structures similar to the mammalian brain. The *Drosophila* optic lobe may provide an excellent model to investigate various processes of brain development. However, it is poorly understood how neuronal specification is regulated in the optic lobe to form a complicated structure. Here we show that the Brain-specific-homeobox (Bsh) protein, which is expressed in the lamina and medulla ganglia, is involved in specifying neuronal identity. Bsh is expressed in L4 and L5 lamina neurons and in Mi1 medulla neurons. Analyses of loss-of-function and gain-of-function clones suggest that Bsh is required and largely sufficient for Mi1 specification in the medulla and L4 specification in the lamina. Additionally, Bsh is at least required for L5 specification. In the absence of Bsh, L5 is transformed into glial cells.

Introduction

The establishment of precise neuronal circuits is essential for correct brain function. Complex neuronal circuits contain various types of neurons that are connected intricately with one another. Processes that result in the formation of the correct circuits include the specification of neuronal types, the extension of axons to the appropriate places, and the formation of synapses with their correct partners. As a first step to make complete neuronal circuits, the specification of neuronal types is an important process.

The *Drosophila* visual system may serve as a powerful model for neuronal circuit formation because it has only a limited number of neurons but forms sufficiently complex neuronal circuits that can be analyzed comprehensively (Sanes and Zipursky, 2010). In addition, neurogenetic tools that are available in *Drosophila* allow artificial manipulation of neuronal activity in temporally and spatially restricted manner (Kitamoto, 2001; Pulver et al., 2009). However, the full picture of development of the *Drosophila* optic lobe awaits further investigation.

The *Drosophila* retina is composed of 750–800 ommatidia that contain eight types of photoreceptor neurons, denoted as R1–R8. The visual information received in the retina is transmitted to the optic lobe, which is composed of four ganglia; the lamina, medulla, lobula and lobula plate. The complex neuronal circuits in the visual center process various types of visual information, such as motion, color and shape (Gao et al., 2008; Katsov and Clandinin, 2008; Morante and Desplan, 2008; Rister et al., 2007). In this paper, we focus on the development of the lamina and medulla.

The development of the lamina has been studied in some detail. During the third instar larva, photoreceptor neurons extend retinal axons (R axons) to the optic lobe and deliver the inductive signal Hedgehog (Hh) to the lamina precursor cells (LPCs) (Huang and Kunes, 1996). LPCs divide and become lamina neurons and activate the expression of Dachshund (Dac) and EGF receptor. Spitz, an EGF ligand, is delivered by R axons, received by EGFR and promotes further differentiation of lamina neurons, including the expression of Elav and the formation of lamina columns. Finally, the five types of lamina neurons, L1–L5, become closely associated with R1–R8 axons, forming a lamina cartridge (Huang et al., 1998). Although the differentiation of lamina neurons is understood to some extent, how the distinction among L1–L5 neurons is regulated remains unclear.

The second visual ganglion, the medulla, contains 40,000 neurons forming tangentially oriented stratifications, which are defined as 10 layers (Fig. 1G; Fischbach and Dittrich, 1989). Medulla neurons are classified by their pattern of arborizations. Some neurons arborize only in the medulla (medulla intrinsic neurons, Mi-neurons), and some send projections back to the lamina (lamina wide-field neurons, Lawf-neurons). Other neurons

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arborize in both the medulla and the lobula (transmedullary neurons, Tm-neurons) and in the lobula complex (transmedullary Y neurons, TmY neurons)(Fischbach and Dittrich, 1989). The medulla is the largest part of the optic lobe and is thought to process both color and motion. Although the medulla is considered to play an important role in visual processing, the developmental mechanisms of the medulla remain elusive. During the third instar larva, neuroblasts (NBs) located in the outer proliferation center divide to make ganglion mother cells (GMCs), which divide to produce differentiated neurons (Egger et al., 2007; Hofbauer and Campos-Ortega, 1990; Toriya et al., 2006; Yasugi et al., 2008). Expression of specific transcription factors in a subset of medulla neurons was examined (Hasegawa et al., 2011; Morante and Desplan, 2008). We reported previously that neurons produced from NBs express different types of transcription factors according to their birth order to form a concentric expression pattern (Hasegawa et al., 2011). However, how the differentiation of the medulla neurons is controlled is still unclear.

The Bsx family transcription factors are widely conserved homeodomain proteins that are involved in various neuronal processes. For example, mouse Bsx regulates hyperphagia, locomotory behavior (Sakkou et al., 2007), growth, and nursing (McArthur and Ohtoshi, 2007). *Xenopus* Bsx links daily cell cycle rhythms to pineal photoreceptors (D'Autilia et al., 2010). The *Drosophila* Bsx protein, Brain-specific homeobox (Bsh), is expressed in the embryonic brain (Jones and McGinnis, 1993), and in the lamina and medulla neurons of larvae and adults (Choe et al., 2006; Chu et al., 2006; Hasegawa et al., 2011; Huang and Kunes, 1998; Huang et al., 1998; Poeck et al., 2001; Zhu et al., 2009). However, the molecular function of *Drosophila bsh* has not been studied.

In our previous paper, we showed that Bsh is expressed in the medulla and that Bsh-positive neurons differentiate into a single type of medulla intrinsic neuron, Mi1. Moreover, Hth, which is expressed in Mi1 together with Bsh, is essential for Mi1 neuron identity (Hasegawa et al., 2011). Here, we show that Bsh is also required for Mi1 neuron specification in the medulla. *bsh* mutant neurons were transformed to Tm-type neurons, and overexpression of Bsh induced Mi1-like neurons. Moreover, Bsh expression was required for L4 neuronal specification in the L4 neurons of the lamina, and overexpression of Bsh in the lamina induced L4-like neurons. Therefore, Bsh may have roles in neuronal type specification in both the lamina and the medulla. Relatively weak Bsh expression found in L5 lamina neurons may be required for neuronal differentiation of L5. In the absence of *bsh*, L5 cells were transformed into glial cells.

Materials and methods

Fly strains

Fly strains were reared on standard *Drosophila* medium at 25 °C. Following fly strains were used: *elav-Gal4*, *hs-flp*, *FRT19A*, *FRT40A*, *tub-Gal80*, *UAS-CD8GFP* (Lee and Luo, 1999), *AyGal4* (Ito et al., 1997), dac^3 (Mardon et al., 1994), *UAS > CD2 > CD8GFP* (Wong et al., 2002), *bsh-Gal4*, $drfGal4^{G1}$ (Hasegawa et al., 2011),$UAS\text{-}hth^{12}$ (Pai et al., 1998), *ap-lacZ* (Cohen et al., 1992), *dac-flp* (Millard et al., 2007), *NP6013-Gal4* (Hasegawa et al., 2011), *L2-Gal4* (Rister et al., 2007), *L4-Gal4* (*Rdl-Gal4*; (Kolodziejczyk et al., 2008)), *L3-Gal4, L5-Gal4* (Nern et al., 2008), *ptub > Gal80 >* (Gordon and Scott, 2009), *UAS-bshIR* (VDRC106634), $UAS\text{-}bshPA^{1M}$, $UAS\text{-}bshPB^{10F}$ and $UAS\text{-}bshPB^{8M}$. *UAS-bshPA* and *UAS-bshPB* were generated by inserting *bsh-PA* and *bsh-PB* cDNA into pUAST, respectively. $UAS\text{-}bshPA^{1M}$ and $UAS\text{-}bshPB^{10F}$ show a similar expression level of Bsh (not shown).

Generation of bsh mutant alleles

Delta2–3 transposase (Fig. 1A). The deletion mutants produced by the imprecise excision of the P element were screened using PCR. Primer sets used were P1 and P4, or P1 and P7 (P1: AACCATCACCGCGATCACTC; P4: CAACGGCAACTGATAGCACG; P7: TTCTGGAACCAGGTCTTCAC). Sequence analysis indicated that the bsh^1 allele carries a 3062-bp deletion, which corresponds to the region from 156 bp upstream of the first start codon to 336 bp downstream of the stop codon. The bsh^2 allele carries a 498-bp deletion, which corresponds to the region from 144 bp upstream of the first start codon to 72 bp upstream of the second start codon in the first exon, in addition to a 176-bp insertion of an unknown origin.

Histochemistry

Immunohistochemistry was performed essentially as described (Kunes et al., 1993). The following primary antibodies were used: guinea pig anti-Bsh (1:1200) (Hasegawa et al., 2011), mouse anti-LacZ (1:250, promega), chicken anti-LacZ (1:2000, abcam) and rabbit anti-GFP Alexa Fluor 488 (1:1000, Invitrogen). The following monoclonal antibodies were provided by the Developmental Studies Hybridoma Bank (DSHB): mouse anti-Dac (1:20), rat anti-Elav (1:100), rat anti-Ncad (1:20) and mouse anti-Repo (1:20). The secondary antibodies used were anti-mouse Cy3, anti-mouse FITC, anti-rat Cy5, anti-guinea pig Cy3, anti-guinea pig Cy5, and anti-chicken Cy3 (Jackson ImmunoResearch Laboratories) and anti-rat Alexa Fluor 660 (Invitrogen). *in situ* hybridization was performed as described (Hasegawa et al., 2011).

Clonal analysis

Following genetic crosses and heat shock conditions were used: Fig. 1B–F: *hs-flp; tub-Gal80 FRT40A; UAS-CD8GFP* was crossed to *y+ FRT40A; bsh-Gal4 UAS-CD8GFP*, bsh^1 *FRT40A; bsh-Gal4 UAS-CD8GFP*, and bsh^2 *FRT40A; bsh-Gal4 UAS-CD8GFP. hs-flp; tub-Gal80 FRT40A; bsh-Gal4 UAS-CD8GFP* was crossed to bsh^1 *FRT40A;* $UAS\text{-}bshPA^{1M}$ *UAS CD8GFP* and bsh^1 *FRT40A;* $UAS\text{-}bshPB^{10F}$ *UAS-CD8GFP* (32 °C for 60 min at third instar); Fig. 2A–F: *tub-Gal80 hs-flp w FRT19A;* $drfGal4^{G1}$ *UAS-CD8GFP* was crossed to *FRT19A*, *FRT19A;* $UAS\text{-}bshPB^{10F}$, *FRT19A;* $UAS\text{-}bshPA^{1M}$, *FRT19A;* $UAS\text{-}hth^{12}$, *FRT19A;* $UAS\text{-}bshPB^{10F}$ $UAS\text{-}hth^{12}$, and *FRT19A;* $UASbshPA^{1M}$ $UAS\text{-}hth^{12}$ (33 °C for 20 min at third instar); Fig. 3A –I: *elav-Gal4 UAS-CD8GFP hs-flp w; tub-Gal80 FRT40A* was crossed to *y+ FRT40A;* $UAS\text{-}hth^{12}$, *y+ FRT40A;* $UAS\text{-}bshPB^{10F}$, *y+ FRT40A;* $UAS\text{-}bshPA^{1M}$, *y+ FRT40A;* $UAS\text{-}bshPB^{10F}$ $UAS\text{-}hth^{12}$, and *y+ FRT40A;* $UAS\text{-}bshPA^{1M}$ $UAS\text{-}hth^{12}$ (35 °C for 60 min at early third instar); Fig. 3J –K: *hs-flp; ubi-GFP FRT40A* was crossed to bsh^1 *FRT40A* (37 °C for 60 min at first instar); Fig. 5A –J: the same as Fig. 1; Fig. 5K –L: *hs-flp; tub-Gal80 FRT40A; UAS-CD8GFP* was crossed to *y+ FRT40A; L5-Gal4 UAS-CD8GFP* and bsh^1 *FRT40A; L5-Gal4 UAS-CD8GFP* (32–33 °C for 60 min at third instar); Fig. 6A and B: *elav-Gal4 UAS-CD8GFP hs-flp w; ptub > Gal80 >* was crossed to *dac-flp*, *dac-flp;* $UAS\text{-}bshPB^{8M}$, and *dac-flp;* $UAS\text{-}bshPB^{10F}$; Fig. 6D and E: *hs-flp; ubi-GFP FRT40A* was crossed to *y+ FRT40A* and dac^3 *FRT40A* (37 °C for 60 min at first instar); Fig. 6F: *hs-flp UAS-CD8GFP*; *UAS-bshIR* was crossed to *AyGal4 ap-lacZ* (33 °C for 30 min at second instar).

Quantification of Ncad expression level

To quantify Ncad protein expression levels, we used Photoshop CS5 (Adobe) to calculate the fluorescence intensities. In clones that show Ncad induction, a cluster of cells located at the center of the clone up-regulated Ncad. We selected a region of interest (ROI) of 15×15 pixels within the Ncad up-regulated area in clones that show Ncad induction. Or, a ROI was selected from the central part of the clone when Ncad was not up-regulated. A ROI for negative