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A temporal mechanism that produces neuronal diversity in the *Drosophila* visual center

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ABSTRACT

The brain consists of various types of neurons that are generated from neural stem cells; however, the mechanisms underlying neuronal diversity remain uncertain. A recent study demonstrated that the medulla, the largest component of the *Drosophila* optic lobe, is a suitable model system for brain development because it shares structural features with the mammalian brain and consists of a moderate number and various types of neurons. The concentric zones in the medulla primordium that are characterized by the expression of four transcription factors, including Homothorax (Hth), Brain-specific homeobox (Bsh), Runt (Run) and Drifter (Drf), correspond to types of medulla neurons. Here, we examine the mechanisms that temporally determine the neuronal types in the medulla primordium. For this purpose, we searched for transcription factors that are transiently expressed in a subset of medulla neuroblasts (NBs, neuronal stem cell-like neural precursor cells) and identified five candidates (Hth, Klumpfuss (Klu), Eyeless (Ey), Sloppy paired (Slp) and Dichaete (D)). The results of genetic experiments at least explain the temporal transition of the transcription factor expression in NBs in the order of Ey, Slp and D. Our results also suggest that expression of Hth, Klu and Ey in NBs trigger the production of Hth/Bsh-, Run- and Drf-positive neurons, respectively. These results suggest that medulla neuron types are specified in a birth order-dependent manner by the action of temporal transcription factors that are sequentially expressed in NBs.

Introduction

For successful functional brain development, a large number of various types of neurons must be generated at the optimal time and location. The molecular mechanisms underlying neuronal diversity and the spatio-temporal regulation of neurogenesis are largely unknown because the brain is too complex to elucidate its entire developmental mechanisms. Our previous study revealed that the medulla, the largest component of the *Drosophila* optic lobe, is a suitable model system for brain development (Hasegawa et al., 2011). The medulla has similar structural features to the mammalian brain, such as layer and columnar structures and contains at least 60 types of 40,000 neurons (Fischbach and Dittrich, 1989; Hofbauer and Campos-Ortega, 1990). Thus, the medulla is genetically tractable and sufficiently complex to be considered as a model of brain development. The developing medulla is subdivided into concentric zones that are characterized by the expression of the genes encoding conserved transcription factors *homothorax* (*hth*), *brain-specific homeobox* (*bsh*), *runt* (*run*) and *drifter* (*drf*), which are collectively called concentric genes (Hasegawa et al., 2011). This type of subdivision also exists in the developing mammalian spinal cord (Jessell, 2000), telencephalon and eye (Lupo et al., 2006). Thus, the developmental mechanism of the medulla could be highly similar to that of the mammalian central nervous system.

During the development of the central nervous system, neural stem cells generate a variety of neuronal cells depending on spatial and temporal information. In mammals, neural stem cells generate neurons and then glia in a stereotypical order that is determined by the temporal restriction of the precursor cell fate. The transition from neurogenesis to gliogenesis is controlled by extrinsic and intrinsic factors (Cepko, 1999). The cell-intrinsic mechanism that restricts the competence of stem cells has been well investigated in the developing embryonic central nervous system of *Drosophila*. Neuroblasts (NBs), stem cell-like multipotent precursors, divide asymmetrically to produce a ganglion mother cell (GMC) and

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a NB in a self-renewal fashion (Bossing et al., 1996). The GMC further divides into two post-mitotic neurons that are specified upon the birth of their mother cells (Doe and Goodman, 1985). The GMC birth-order identity is determined by the expression of heterochronic transcription factors, including Hunchback (Hb), Krüppel (Kr), Pdm1/Pdm2 (Pdm), Castor (Cas) (Isshiki et al., 2001; Kambadur et al., 1998) and Granyhead (Grh) (Almeida and Bray, 2005; Chen et al., 2012). These transcription factors are expressed sequentially in each NB and are maintained in their daughter GMC to contribute to specifying the neuronal identity of their progeny (Isshiki et al., 2001). Although the sequential expression of various transcription factors in NBs elicits temporal neuronal specification in the embryonic central nervous system, the mechanisms underlying neuronal diversity during adult brain development are poorly understood.

Medulla NBs are located on the cortical surface and generate neurons in both a linear and radial orientation. Thus, a single NB generates many medulla neurons of various identities that are characterized by the expression of each concentric gene (Hasegawa et al., 2011). The expression of these genes correlates with the birth order of the medulla neurons, suggesting that the medulla neurons are also specified in a birth order-dependent manner as observed in the embryonic central nervous system. Additionally, the wave of differentiation called 'proneural wave' progresses in medial-to-lateral orientation and induces the transition of neuroepithelia (NE) into medulla NBs (Fig. 1A). As a result, early-born NBs are situated medially while later-born NBs are situated laterally on the surface of the larval medulla primordium. An advantage of using the medulla as a model could be that NBs of different ages can be observed and compared at the same time (Yasugi et al., 2008). A group of genes that are transiently and sequentially expressed in the medulla NBs may be involved in temporal neuronal specification in the medulla.

In this study, we examine the molecular mechanism that produces neuronal diversity in the developing medulla. Among four concentric transcription factors that are expressed in medulla neurons, Hth expression is also detected in NEs and newly differentiated NBs, suggesting that Hth expression is inherited from NB to neurons (Hasegawa et al., 2011; Reddy et al., 2010). Hth might also be one of the heterochronic transcription factors expressed in the medulla NBs. Additionally, we searched for transcription factors that are transiently expressed in medulla

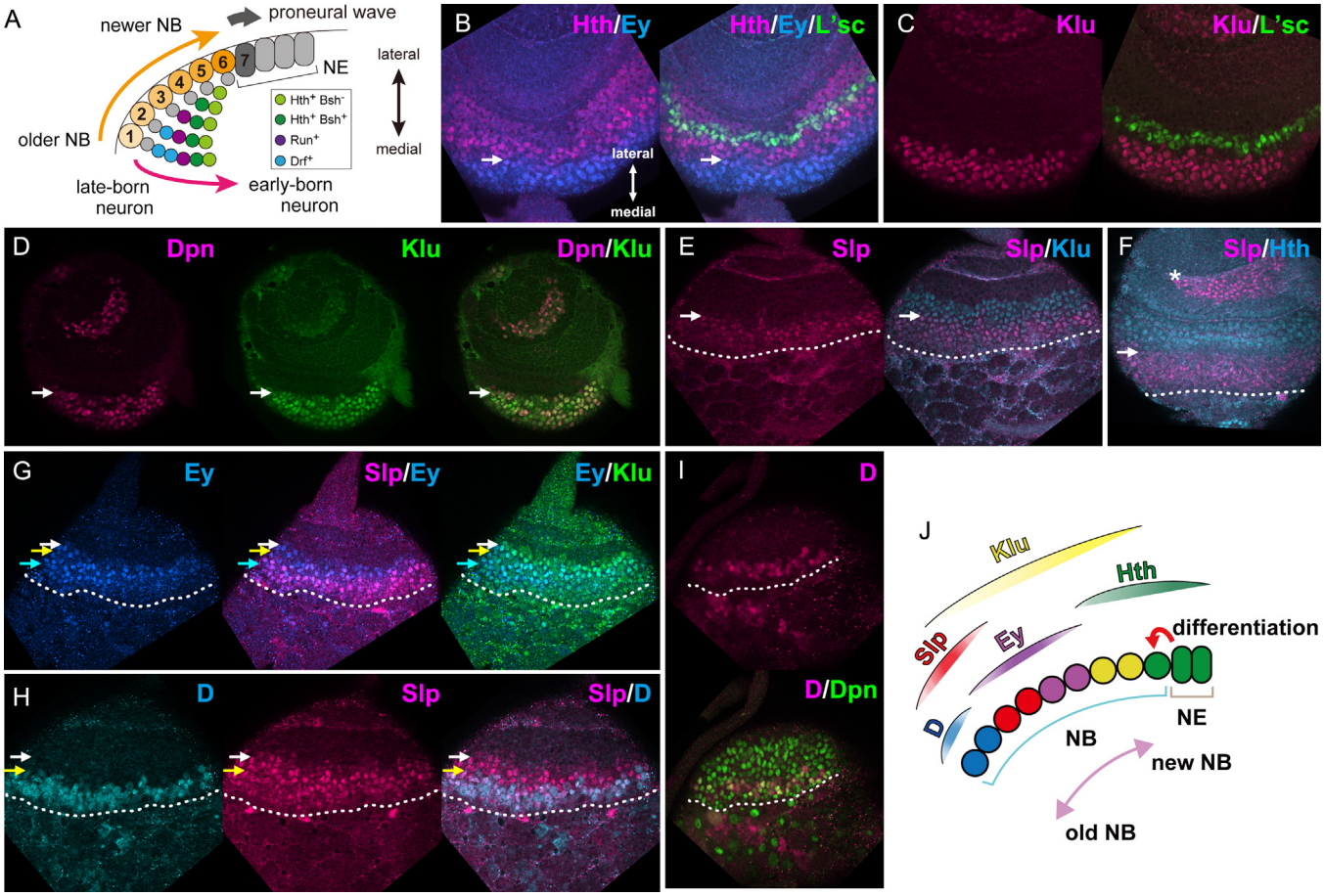

Fig. 1. Hth, Klu, Ey, Slp and D are temporally expressed in the medulla NBs. (A) Schematic model of NB production in the medulla primordium. NBs are numbered in a numerical order: the NB1 is firstly differentiated from NE and the NB6 is the last. NE7 is the NE cell that is just differentiating to NB. NBs sequentially produce medulla neurons in the order of cells labeled in light green (Hth+Bsh−), dark green (Hth+Bsh+), magenta (Run+) and blue (Drf+). (B–I) The cortical surface of medulla primordium in frontal views at wandering late third larval instar. Lateral to the top, medial to the bottom as indicated in (B). (E–I) White dots indicate the borders between the medulla and central brain. (B) Hth expression (magenta) is observed in L'sc expressing NEs (green) and lateral NBs. Ey (blue) expression does not overlap Hth. Arrows indicate the border between Hth and Ey domains. (C) Klu expression (magenta) is observed in NBs adjacent to L'sc-positive NEs (green). (D) Klu (green) is strongly expressed in the lateral-most NBs (Dpn; magenta, arrow). (E) Slp (magenta) is expressed in medial NBs compared to Klu-positive NBs (blue). Slp is weakly expressed in Klu-positive NBs. Arrows indicate the lateral borders of Slp domain. (F) Hth (blue) is not expressed in Slp-positive NBs (magenta). Slp expression in the lamina is indicated by asterisk. Arrow indicates the border between Hth and Slp domains. (G) Ey (blue) is expressed in Klu-positive NBs (green) except for the most lateral NBs. Ey and Slp (magenta) expression partially overlaps. White, yellow, and blue arrows indicate the lateral borders of the Klu, Ey and Slp domains respectively. (H) Slp (magenta) and D (blue) are expressed in intermediate and medial NBs, respectively. Slp and D expression partially overlaps. White and yellow arrows indicate the lateral borders of Slp and D domains, respectively. (I) D (magenta) is expressed in medial NBs (Dpn; green). (J) Schematic model illustrating the expression domains of Hth, Klu, Ey, Slp and D in medulla NBs.

NBs and identified Klumpfuss (Klu) (Yang et al., 1997), Eyeless (Ey) (Quiring et al., 1994; Morante et al., 2011), Sloppy paired (Slp) (Grossniklaus et al., 1992) and Dichaete (D) (Russell et al., 1996) as candidates for the heterochronic factors that temporally determine neuronal types. The results of our genetic experiments at least explain the temporal transition of transcription factor expression in NBs in the order of Ey, Slp and D. The temporal windows that are specified by the expression of Hth, Klu and Ey in NBs approximately correspond to the production of Hth/Bsh-, Run- and Drf-positive neurons from these NBs, respectively. Indeed, our results suggest that expression of Hth, Klu and Ey in NBs trigger the production of Hth/Bsh-, Run- and Drf-positive neurons, respectively. These observations reveal that medulla neurons are also specified in a birth order-dependent manner by the sequential expression of heterochronic transcription factors; however, the genes involved in the specification of medulla neurons differ from that required for the embryonic central nervous system.

Materials and methods

Fly strains

All fly strains were reared on standard *Drosophila* medium at 25 °C unless otherwise noted. The fly strains used were *hs-flp*, *FRT40A*, *FRT2A*, *FRT82B*, *tub-Gal80*, *actin-Gal4*, *tub-Gal4*, *UAS-CD8GFP* (Lee and Luo, 1999), *tub-Gal80^{ts}* (McGuire et al., 2003), *UAS-GFP*, *ubi-GFP*, *actin > yellow > Gal4* (Ito et al., 1997), *UAS-hth^{12}*, *hth^{P2}* (Hasegawa et al., 2011), *klu^{R51}*, *UAS-klu* (Yang et al., 1997), *UAS-ey RNAi* (*ey^{KK107100}* and *ey^{HMS00489}*), *UAS-ey*, *slp^{S37A}*, *UAS-slp1^{IF}*

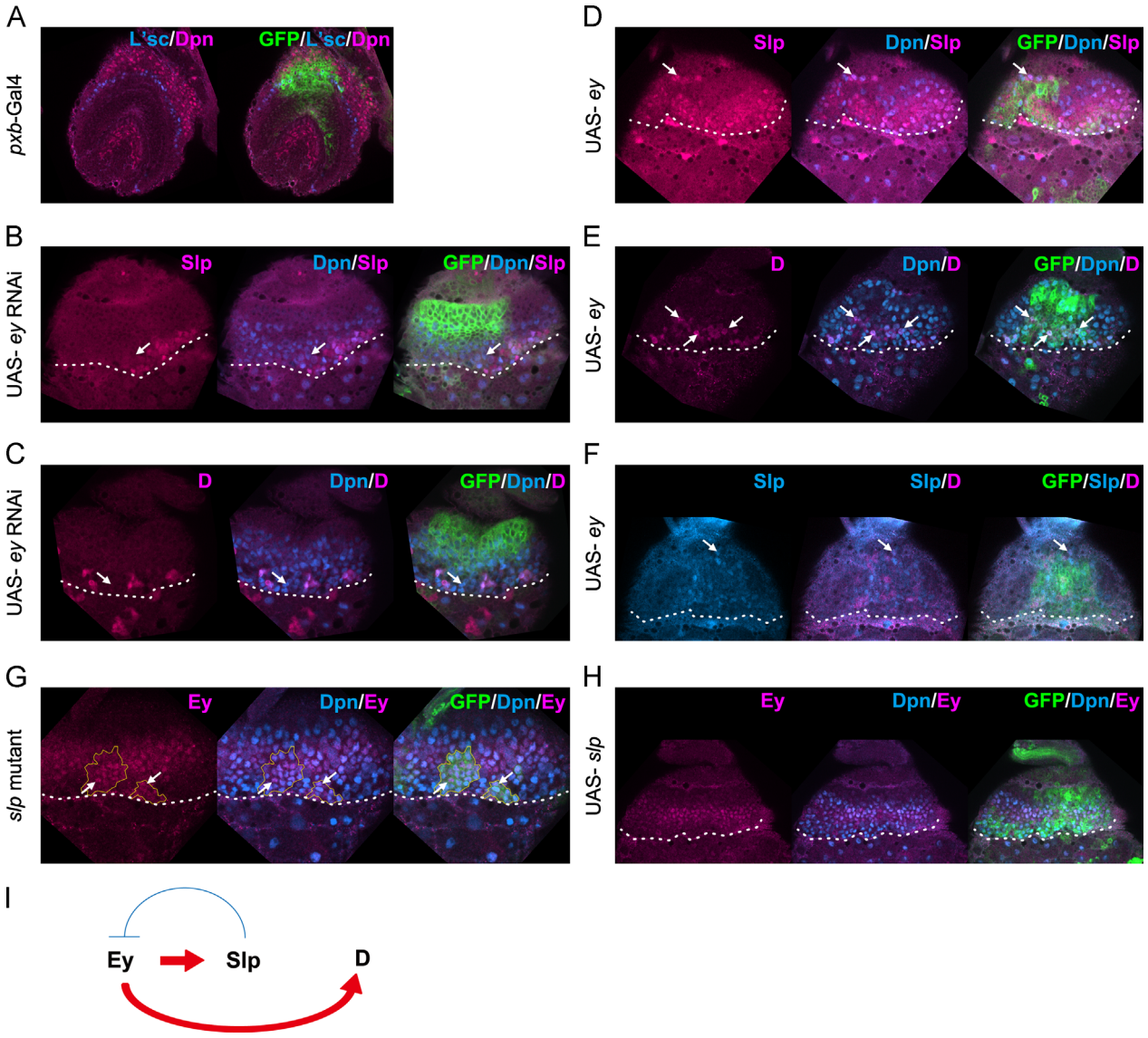

Fig. 2. Mutual regulation between Ey and Slp in the NBs. Lateral views (A; anterior to the top) and frontal surface views ((B–H); lateral to the top) of the medulla primordium at wandering late third instar. (A) *pxb-Gal4* UAS-*CD8GFP* (green) is expressed in the anterior region including NEs and NBs as visualized by L'sc (blue) and Dpn (magenta) expression. (B, C) Both Slp (magenta in B) and D expression (magenta in C) in NBs are disappeared without affecting NB formation (Dpn; blue in B and C) in the region expressing *ey RNAi* under the control of *pxb-Gal4* (GFP; green). (D, E) Ectopic expression of Ey under the control of *pxb-Gal4* (visualized by GFP; green) induces precocious expression of both Slp (magenta in D) and D (magenta in E) in lateral NBs without affecting NB formation (Dpn; blue in D and E). (F) Ectopic Ey expression occasionally induces D expression (magenta) without affecting Slp expression (blue). (G) Ey expression (magenta) is derepressed in medial NBs in *slp* mutant clones (GFP; green) without affecting NB formation (Dpn; blue). (H) Ey expression (magenta) in NBs (Dpn; blue) is normal in the region expressing Slp under the control of *pxb-Gal4* (GFP; green). (I) Schematic model of the genetic interaction between Ey, Slp and D in medulla NBs. Ey positively regulates the expression of Slp and D, conversely Slp negatively regulates that of Ey. The thin blue line is supported by the results of loss-of-function experiments, while the red bold lines are supported by the results of loss-of-function and gain-of-function experiments.

(Sato and Tomlinson, 2007), *UAS-D* (Soriano and Russell, 1998), *P(XP)D02427*, *I(3)rN346* (*pxb-lacZ*), *drf-Gal4* (Hasegawa et al., 2011) and *OK107* (*ey-Gal4*) (Connolly et al., 1996). D^{d23} and *pxb-Gal4* were generated as described below.

Generation of the D mutant allele

The P element insertion line *P(XP)D02427* (*Drosophila* Genome P Element Disruption Project) was mobilized by crossing to a strain that expresses Delta 2–3 transposase. The deletion mutant produced by the imprecise excision of the P element was screened by PCR. Primer sequences were designed according to the nucleotide sequence of *D* (5′-CTCCAAATCAAAGCGAAGCG-3′ and 5′-CTGCTGACCCTGATTGTTGA-3′). Sequence analysis indicated that the D^{d23} allele possesses a 1116 bp deletion that includes the translational start site and two-thirds of the open reading frame of *D*, suggesting that D^{d23} is a null allele.

Generation of the pxb-Gal4 strain

To convert the P element insertion line *P(PZ)l(3)rN346* (*pxb-lacZ*) into a Gal4 driver line, we used the gene conversion technique (Sepp and Auld, 1999) and substituted *Gal4* for the *lacZ* encoding region, generating *pxb-Gal4* line. The expression pattern of *pxb-Gal4* as visualized by *UAS-GFP* was almost the same as that of LacZ in *P(PZ)l(3)rN346* (not shown).

Genetic crosses

The genetic crosses and heat shock conditions used in this study are as follows. Figs. 2D–F, H, 5B–C, 6C–E: *tub-Gal80*^{ts}; *pxb-Gal4 UAS-CD8GFP* was crossed to either *UAS-klu*, *UAS-ey* or *UAS-slp1*^{IF} and raised at 17 °C to suppress lethality. The larvae were then raised at 30 °C for 24 h prior to dissection to allow transgene expression. Figs. 2B–C, 6A–B: *ey* RNAi (*ey*^{KK107100} or *ey*^{HMS00489}) was expressed under the control of *pxb-Gal4* at 30 °C to knock down *ey*. Figs. 2G, 3A–B and 7A–C: *hs-flp*; *slp*^{S37A} *FRT40A* was crossed to *hs-flp*; *tub-Gal80 FRT40A*; *tub-Gal4 UAS-CD8GFP* (37 °C for 60 min at late embryo or first instar). Figs. 3C and 7D–G: *hs-flp*; *UAS-slp1*^{IF} was crossed to *hs-flp*; *actin > yellow > Gal4* (*AyGal4*) *UAS-GFP* (34 °C for 30 min at late embryo or first instar). Figs. 3D and 8A–D: *UAS-D* was crossed to *hs-flp*; *AyGal4 UAS-GFP* (34 °C for 60 min at late embryo or first instar). Figs. 3E–F and 8E: D^{d23} *FRT2A* was crossed to *hs-flp*; *act-Gal4 UAS-GFP*; *tub-Gal80 FRT2A* (37 °C for 90 min at late embryo or first instar). Fig. 4C–F: *hs-flp UAS-CD8GFP* was crossed to *AyGal4* (32 °C for 15 min at second or early third instar). Fig. S2: *klu*^{R51} *FRT2A* was crossed to *hs-flp*; *act-Gal4 UAS-GFP*; *tub-Gal80 FRT2A* (37 °C for 60 min at late embryo or first instar).

Immunohistochemistry

Immunohistochemistry was performed as previously described (Hasegawa et al., 2011). The following primary antibodies were used: guinea pig anti-Dpn (1:500; James Skeath, Washington University, St Louis, MO, USA), rabbit anti-Dpn (1:500; Bier et al., 1992), mouse anti-Mira (1:20; Ohshiro et al., 2000), rabbit anti-Hth (1:1000; Kurant et al., 1998), rabbit anti-Klu (1:1000; Yang et al., 1997), rabbit anti-D (1:1000; Soriano and Russell, 1998), mouse anti-SoxN (1:400; Buescher et al., 2002), guinea pig anti-Slp (1:300; Asian Distribution Center for Segmentation Antibodies), guinea pig anti-Bsh (1:800; Hasegawa et al., 2011), guinea pig anti-Run (1:1000; NIG), and rat anti-Drf (1:3000; Hasegawa et al., 2011). The rat anti-Elav (1:100) and mouse anti-Ey (1:20) antibodies were obtained from the Developmental Studies Hybridoma Bank. A custom-made antibody against L'sc was raised as previously described (Martin-Bermudo et al., 1991). The secondary antibodies used were anti-mouse Cy3, anti-mouse Cy5, anti-mouse FITC, anti-rat Cy3, anti-guinea pig Cy5, anti-guinea pig Cy3, and anti-rabbit FITC (Jackson ImmunoResearch Laboratories, West Grove, PA); and anti-rat Ax647, anti-rat Ax488, and anti-rabbit Ax546 (Invitrogen, Carlsbad, CA). The images were processed using the Zeiss LSM image browser and Adobe Photoshop.

Results

Hth, Klu, Ey, Slp and D are sequentially and transiently expressed in medulla NBs

The differentiation of NEs into medulla NBs progresses in a medial-to-lateral orientation, and this progress can be clearly monitored by the expression of Lethal of scute (L'sc or L(1)sc), encoded by a member of the bHLH proneural gene family (Yasugi et al., 2008). Given that L'sc is expressed transiently in 1–2 rows of cells within the NEs that are situated at the border between NEs and NBs, it can be used as a molecular marker of differentiation from NEs to NBs. During third larval instar, the lateral NBs situated closer to L'sc-positive NEs are differentiated more recently (newer NBs), whereas medial NBs situated far from such NEs are differentiated at earlier stages (older NBs), indicating that the distance between each NB and the L'sc-positive NE reflects the age of the NB (Fig. 1A). The NBs that are situated between the lateral and medial NBs are designated as intermediate NBs. We examined the expression of conserved transcription factors with L'sc to identify genes that are transiently expressed in NBs at late third larval instar.

It has been reported that Hth is expressed in NEs and NBs (Hasegawa et al., 2011; Reddy et al., 2010). Therefore, we examined the expression pattern of Hth in NBs and discovered that Hth is expressed in L'sc-positive NEs and in lateral NBs but not in intermediate and medial NBs (Fig. 1B, F). Similar expression pattern was observed at earlier stages in third larval instar (Fig. S1A–C). This result suggests that Hth expression is maintained during differentiation from NEs to NBs during third instar.

In addition to Hth, we found that four transcription factors, Klu, Ey, Slp and D are transiently and sequentially expressed in NBs. Klu was strongly expressed in NBs located in the lateral region of the medulla cortical surface adjacent to L'sc-positive NEs and gradually weakened in NBs located in more medial region (Fig. 1C–E). Ey expression was detected in the Klu-expressing NBs except for the most lateral cells (Fig. 1G) (Morante et al., 2011). Slp expression was detected in intermediate NBs in which Klu were weakly expressed (Fig. 1E, G). Ey and Slp expression overlaps, but Slp domain was found medial to the Ey domain (Fig. 1G). As observed for the expression pattern of Klu, both the Ey and Slp expression levels gradually decreased from lateral to medial NBs. These observations suggest that Hth and Klu are strongly expressed in newer NBs; however, Ey and Slp are expressed in middle-aged NBs. In contrast to these four genes, D expression was detected in NBs located in the medial region, indicating that D is expressed in older NBs (Fig. 1H, I). A subset of these D-positive NBs was also Slp-positive (Fig. 1H), implying that D is expressed subsequently to Slp and that they regulate each other in NBs.

The expression pattern of these five transcription factors suggests that the expression of Hth, Klu, Ey, Slp and D is sequentially initiated and decreased according to the ages of the medulla NBs (Fig. 1J). Essentially the same relative expression pattern of each gene was also observed at earlier stages during third larval instar, suggesting that each one of NB goes through a temporal cascade of transient transcription factor expression (Fig. S1). The overlapping expression pattern of the transcription

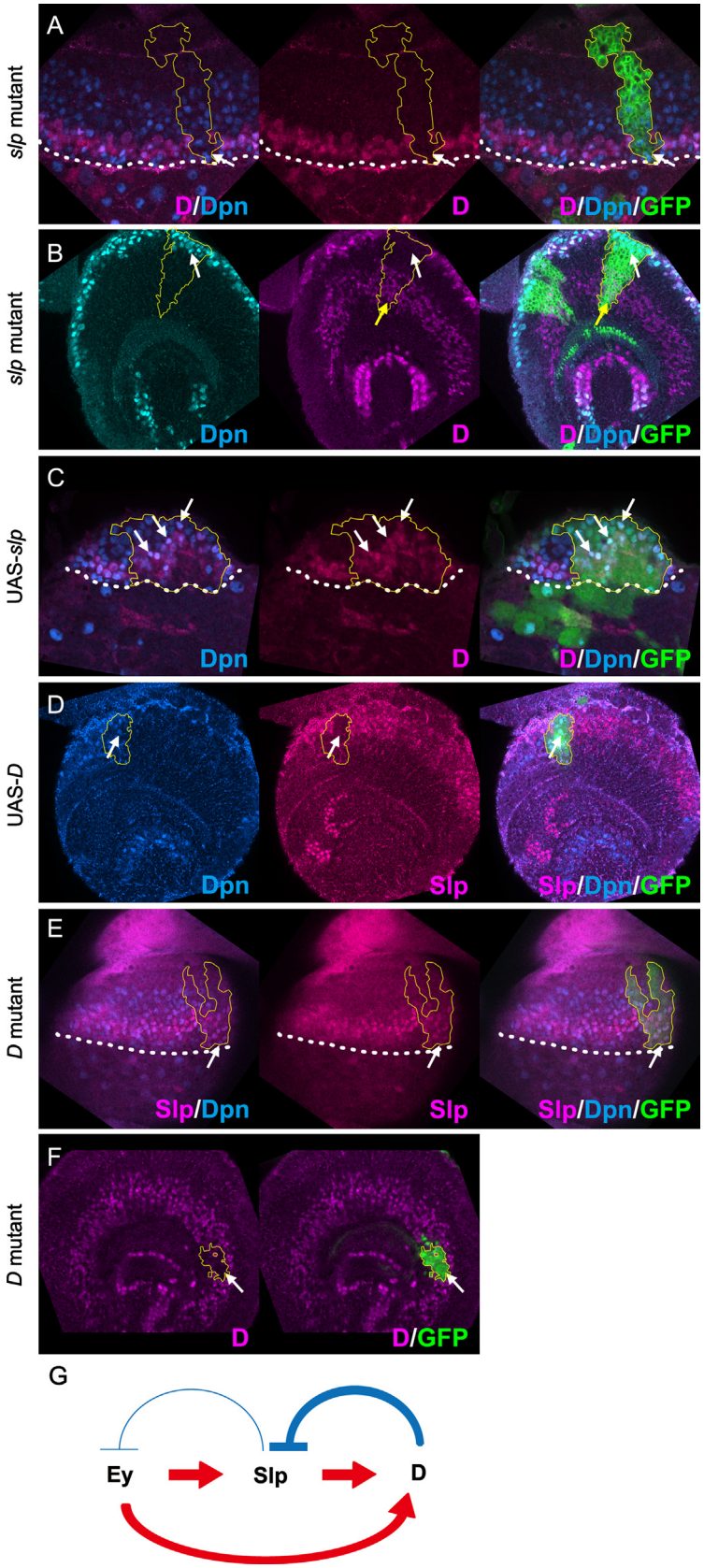

Fig. 3. Mutual regulation between Slp and D in the NBs. Frontal surface views (A, C, E) and lateral views (B, D, F) of the medulla primordium at wandering late third instar. (A, B) D expression (magenta) in NBs is disappeared (white arrows) without affecting NB formation (Dpn; blue) in *slp* mutant clones (GFP; green). D expression in neurons is not influenced (yellow arrow in B). (C) In clones expressing Slp (green), ectopic D expression (magenta) is observed in NBs (Dpn; blue, white arrows). (D) Slp expression (magenta) in NBs is lost in clones expressing D (green) without affecting NB formation (Dpn; blue, arrow). (E) In *D* mutant clones (green), Slp (magenta) is expressed even in medial NBs (Dpn; blue, arrow). (F) D expression is completely lost in *D* mutant clone. (G) Schematic model of the genetic interaction between Ey, Slp and D in medulla NBs. Ey positively regulates the expression of Slp and D, conversely Slp negatively regulates that of Ey. Slp positively regulates D expression while D suppresses Slp expression in the medulla NBs. The thin blue line is supported by the results of loss-of-function experiments, while the blue and red bold lines are supported by the results of loss-of-function and gain-of-function experiments.

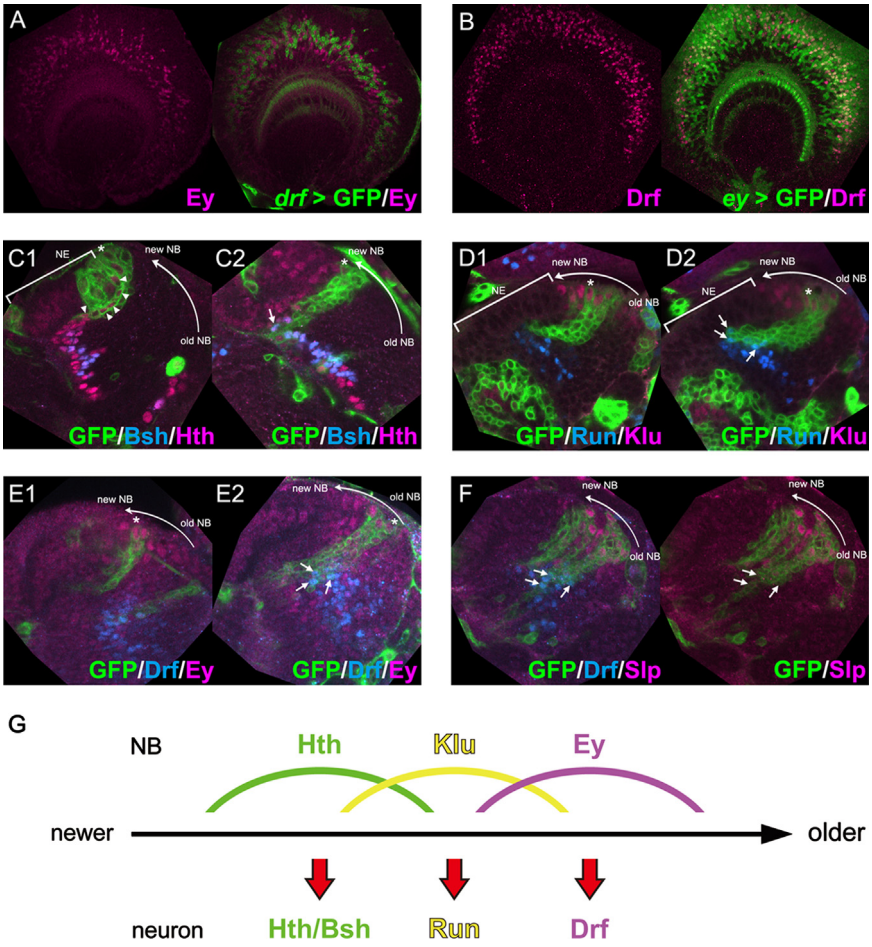

Fig. 4. Temporal windows of NBs that express Hth, Klu, Ey and Slp. The cortex of medulla primordium in lateral (A, B) and horizontal views (C–F) at wandering late third instar. (A) Ey (magenta) is not expressed in the Drf positive neurons as visualized by *drf-Gal4 UAS-CD8GFP* (green). (B) Drf (magenta) is expressed in neurons labeled with GFP driven by *ey-Gal4* (green). (C–F) Lineages of the NBs expressing Hth, Klu, Ey or Slp are visualized with CD8GFP under the control of *AyGal4* (green). Lateral to the top, medial to the bottom. (C1 and C2) Hth-positive NBs (magenta) and Bsh-positive neurons (blue). Progenies of Hth-positive NBs adjacent to NEs (asterisk and arrowheads) do not contain Bsh-positive neurons (C1), while those of more medial NBs contain a Bsh-positive neuron (C2; arrow). (D1 and D2) Klu-positive NBs (magenta) and Run-positive neurons (blue). Progenies of Klu-positive NBs (asterisk) do not contain Run-positive neurons (D1), while those of more medial NBs contain Run-positive neurons (D2; arrows). (E1 and E2) Ey-positive NBs (magenta) and Drf-positive neurons (blue). Progenies of Ey-positive NBs (asterisk) do not contain Drf-positive neurons (E1), while those of more medial NBs contain Drf-positive neurons (E2; arrows). (F) Slp-positive NBs (magenta) have larger number of progenies which contain Drf-positive neurons (blue, arrows). (G) Schematic model that shows the temporal windows of NBs that express Hth, Klu and Ey, which produce Hth/Bsh-, Run- and Drf-positive neurons, respectively.

factors shown above suggests these factors could act partially redundantly and explains the partial penetrance of the following genetic experiments.

A temporal cascade that produces the sequential and temporal expression of Hth, Klu, Ey, Slp and D in the medulla NBs

To examine if the temporal transcription factors shown above regulate each other to establish their sequential expression in the NBs, we performed a set of genetic experiments as shown below. We initially focused on the relationships between Ey, Slp and D. Ey and Slp are co-expressed in medial NBs, and their expression levels are conversely related (Fig. 1G). Slp is weakly expressed in NBs that strongly express Ey but is strongly expressed in those that weakly express Ey. This observation implies a mutual regulation between Ey and Slp. Similarly, expression levels of Slp and D are conversely related (Fig. 1H), suggesting a mutual regulation between Slp and D.

To test these possibilities, we examined Slp and D expression in the absence of Ey function. As *ey* is located on the 4th chromosome, we were not able to generate *ey* homozygous mutant clones. We instead knocked down *ey* in the anterior NBs by expressing *ey RNAi* under the control of *pxb-Gal4*, which is expressed in anterior NEs and NBs (Fig. 2A). Note that two RNAi strains designed to target different portions of the *ey* transcript were used and essentially the same results were obtained in the following experiments. Ey expression was eliminated by *ey* RNAi under the control of *pxb-Gal4* (not shown). Slp and D were downregulated in NBs without affecting NB formation as visualized by Dpn expression (Fig. 2B and C; $n=10/14$ and 6/10, respectively). In contrast, ectopic expression of Ey under the control of *pxb-Gal4* induced precocious expression of Slp and D in lateral NBs without affecting Dpn expression (Fig. 2D and E; $n=6/6$ and 6/8, respectively). These results suggest that Ey positively regulates the expression of Slp and D in the medulla NBs. Since Slp positively regulates D expression as shown below, Ey may regulate D expression by upregulating Slp expression. However, ectopic Ey expression occasionally induced D expression without affecting Slp expression (Fig. 2F), suggesting that Ey can regulate D expression independently from Slp expression. In clones mutant for *slp*, we observed derepression of Ey expression in medial NBs without affecting Dpn expression (Fig. 2G; $n=19/28$). Since a double mutant allele for *slp1* and *slp2* were used in this study, the results cannot specify if *slp1* and/or *slp2* function is required. Although ectopic Slp expression under the control of *pxb-Gal4* did not affect Ey expression, the results at least suggest Slp is necessary to repress Ey expression in medial NBs (Fig. 2H).

We next examined D expression in *slp* mutant clones. In *slp* mutant clones, D expression in NBs was abolished without affecting NB formation, as visualized by Dpn expression (Fig. 3A and B; $n=15/17$), indicating that Slp function is necessary for D expression in NBs. In addition to the expression in the NBs, D is also expressed in medulla neurons (Fig. 3B). The neuronal expression of D was not affected in *slp* mutant clones despite the loss of D expression in the NBs, also suggesting that *slp* regulates D expression specifically in NBs but not in neurons. Ectopic D expression was observed in clones expressing Slp (Fig. 3C; $n=11/25$). In contrast, Slp expression was abolished in clones expressing D without affecting NB formation, as visualized by Dpn expression (Fig. 3D; $n=4/6$). To analyze Slp expression in the loss-of-function clones of *D*, we generated a null mutant allele D^{d23}. The expression of D was completely lost in clones homozygous for D^{d23} (Fig. 3F). In *D* mutant clones, Slp expression was derepressed in the medial NBs (Fig. 3E; $n=8/11$). These results suggest that Slp is necessary and sufficient for inducing D expression, while D is necessary and sufficient for suppressing Slp expression in NBs. These regulatory relationships may be responsible for the temporal transition of transcription factor expression in the order of Ey, Slp and D in medulla NBs (Fig. 3G).

Unfortunately, we did not find the regulatory relationships between Hth, Klu and the other temporal transcription factors. Our loss-of-function and gain-of-function experiments at least suggest that Hth and Klu do not regulate each other and that Klu and Ey do not regulate each other (data not shown). Although ectopic Ey expression under the control of *pxb-Gal4* repressed Hth expression in NBs, *ey* RNAi did not affect Hth expression. Ey expression was not affected in either loss-of- or gain-of-function clones for *hth* (not shown). Thus, the regulatory mechanisms that guarantee the temporal transition through Hth, Klu and Ey expression in NBs remain elusive.

The temporal windows in NBs that express Hth, Klu, Ey, Slp and D

Medulla NBs located on the cortical surface generate many medulla neurons of various identities that are characterized by the expression of each concentric transcription factor Hth, Bsh, Run and Drf, which correlates with the birth order of the medulla neurons (Hasegawa et al., 2011). The organization of the concentric zones containing Hth/Bsh-, Run- and Drf-positive cells from the inner to outer domains in the larval medulla suggests that NBs produce medulla neurons in the order of Hth/Bsh-, Run- and Drf-positive cells. Hth/Bsh-positive neurons, situated in the inner concentric zone, mature into only Mi1 neurons. Co-expression of Hth and Bsh specifies the neuronal identify of Mi1 (Hasegawa et al., 2011, 2013). In contrast, Drf-positive neurons, situated in the outer concentric zone, mature into nine types of neurons, including various types of Tm and TmY neurons (Hasegawa et al., 2011). Although Run expression persists in adult, the neuronal type of Run-positive cells has not been identified. However, it is very likely that the neurons in each concentric zone mature into unique types of neurons and that the concentric transcription factors can be used as molecular markers for each neuronal type in the medulla.

Note that Hth and Ey are expressed continuously through NBs to neurons (Fig. S3A and B) (Hasegawa et al., 2011; Morante et al., 2011). Thus, the expression of Hth and Ey may indicate the temporal windows of Hth- and Ey-positive NBs that produce Hth- and Ey-positive neurons, respectively. Indeed, we previously showed that Hth is continuously expressed through NBs to neurons and the Hth-positive domain contains Bsh-positive neurons. Bsh expression is lost in *hth* mutant clones and Bsh expression is induced by ectopic Hth expression in neurons under the control of *elav-Gal4*, suggesting that Hth expressing NBs produce Bsh-positive neurons through inherited Hth expression in medulla neurons (Hasegawa et al., 2011, 2013).

Similarly, Drf-positive neurons as visualized by *drf-Gal4 UAS-CD8GFP* are distributed in and around the Ey domain (Fig. 4A). However, Drf is not expressed in Ey-positive neurons. The salt-and-pepper like pattern of Ey expression in medulla neurons suggest that not all neurons produced from Ey-positive NBs become Ey-positive (Fig. 4A). For example, Ey-positive GMCs may produce Ey-positive and Ey-negative neurons. We assume that Ey-negative neurons produced from Ey-positive NBs might become Drf-positive neurons. Indeed, when the cells that are produced from Ey-positive NBs were labeled with GFP under the control of *ey-Gal4* (*OK107*; Gal4 and GFP proteins likely persist in cells even after *ey* transcription is terminated), Drf expression was found among GFP-positive cells (Fig. 4B). Taken together, it is likely that Hth/Bsh- and Drf-positive neurons are produced within the temporal windows of Hth- and Ey-positive NBs, respectively.

Although Klu, Slp and D are not continuously expressed from NBs to neurons (Fig. 5A, Fig. S3C–F), NBs expressing these transcription factors should also have their own temporal windows to produce specific types of medulla neurons. Considering that the temporal window of NBs expressing Klu is placed between those expressing Hth and Ey, and that medulla neurons are produced in the order of Hth/Bsh-, Run- and Drf-positive neurons, it is likely that the temporal window of Klu-positive NBs produce Run-positive neurons.

We next examined the correlation between temporal factor expression in NBs and neuronal types that are produced from the NBs by generating GFP-labeled clones to visualize small number of NBs and their progeny. Spatial relationships between the concentric zones expressing Bsh, Run or Drf and the neurons produced from the NBs expressing a temporal transcription factor were examined: Hth-positive NBs situated adjacent to NEs have small number of Hth-positive progenies which do not contain Bsh-positive neurons (Fig. 4C1), while those situated more medially contain Bsh-positive neurons (Fig. 4C2). Klu-positive NBs have small number of progenies which do not contain Run-positive neurons (Fig. 4D1), while progenies of more medial NBs which weakly express Klu contain Run-positive neurons (Fig. 4D2). Ey-positive NBs have larger number of progenies which do not contain Drf-positive neurons (Fig. 4E1), while progenies of more medial NBs which weakly express Ey contain Drf-positive neurons (Fig. 4E2). These results are consistent with the idea that Hth-, Klu- and Ey-positive NBs are about to produce Bsh-, Run- and Drf-positive neurons, respectively. In contrast, the clones containing Slp-positive NBs include Drf-positive neurons (Fig. 4F), suggesting that Slp-positive neurons are about to produce unidentified types of neurons that are produced after the production of Drf-positive neurons. The above idea is further tested in the following sections.

Potential roles of Klu in the production of Run-positive neurons

In contrast to Hth and Ey, Klu expression is essentially restricted to NBs and is occasionally found in GMCs, but not found in the medulla neurons (Fig. 5A). However, it is possible to speculate that Klu in NBs indirectly regulates the types of medulla neurons through an unidentified transcription factor that is continuously expressed through NBs to neurons under the control of Klu.

As the temporal window of Klu-positive NBs roughly corresponds to the production of Run-positive neurons as discussed above, we next asked if Klu regulates the production of Run-positive neurons, or not. Unfortunately, when *klu* mutant clones were induced, overproduction of NBs in the inner region of the medulla primordium was observed in addition to the disruption

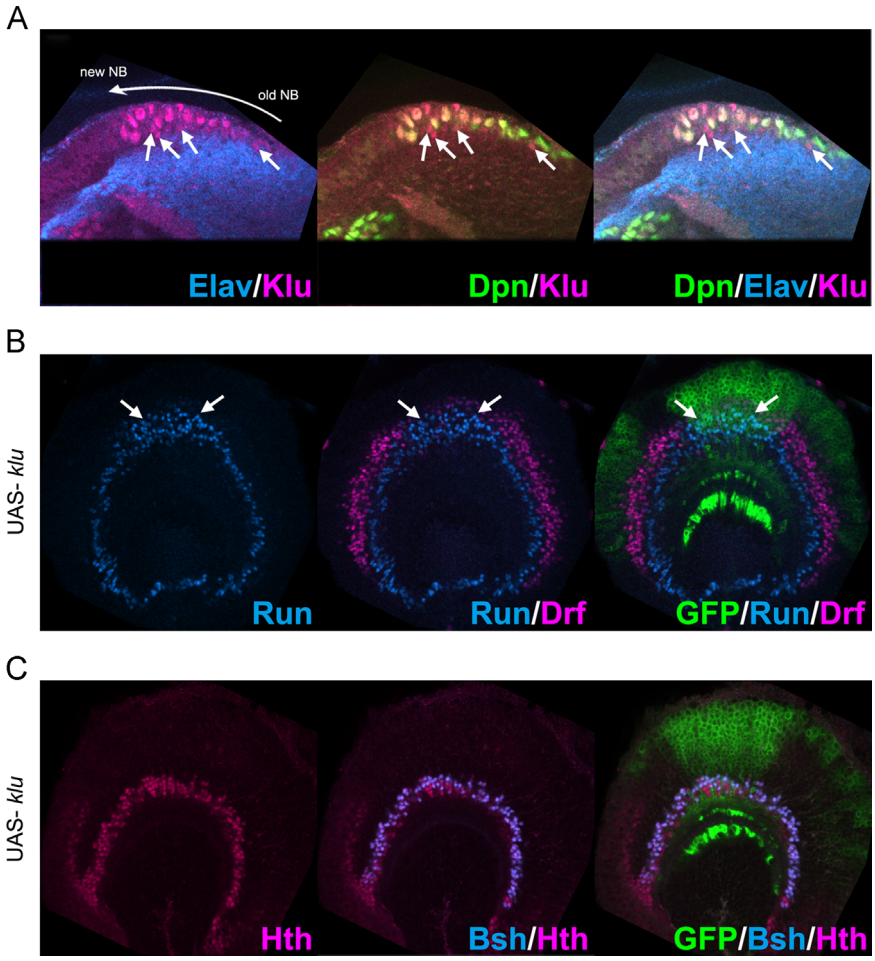

Fig. 5. Roles of Klu in specification of neuronal types. Horizontal views (A) and lateral views (B, C) of the medulla primordium at wandering late third instar. (A) Klu (magenta) is expressed in NBs (Dpn, green) and GMCs (arrow) but not in neurons (Elav, blue). (B) Klu expression under the control of *pxb-Gal4* (visualized by CD8GFP, green) induces and reduces the production of Run-(blue) and Drf-(magenta) positive neurons, respectively. (C) Hth and Bsh expression are not affected by Klu expression under the control of *pxb-Gal4*.

of concentric gene expression, suggesting that *klu* is required for normal growth of NBs and their localization to the cortical surface of the medulla primordium (Fig. S2). In such samples, it is difficult to determine the cause of the disrupted concentric gene expression (*klu* may be involved in the specification of neuronal types, or the concentric zones may be indirectly disrupted by the presence of ectopic NBs or by excessive number of neurons produced by ectopic NBs).

In contrast, ectopic Klu expression under the control of *pxb-Gal4* did not compromise the cortical organization of the medulla primordium. We observed ectopic Run expression in the outer domain while Drf expression was repressed (Fig. 5B; $n=7/12$). Expression of Hth, Bsh and Elav was not affected (Fig. 5C and not shown). These results suggest that Klu is at least sufficient to induce the production of Run-positive neurons and to repress the production of Drf-positive neurons. As Run was not induced in the inner cortical area compared to the endogenous Run domain, we assume that the NBs in the temporal window of Hth are not competent to produce Run-positive neurons even when Klu expression is induced (Fig. 5B). Hth expression in NBs was not affected by Klu expression, either (not shown).

Potential roles of Ey in the production of Drf-positive neurons

We next examined potential roles of Ey in specification of neuronal types in the medulla. If the temporal window of Ey-positive NBs corresponds to the production of Drf-positive neurons, Ey should positively regulate the production of Drf-positive neurons.

When *ey* was knocked down under the control of *pxb-Gal4*, Drf expression was abolished while Run expression was derepressed in the outer domain (Fig. 6A; $n=12/14$). Hth and Bsh expression was not significantly affected (Fig. 6B). When Ey was ectopically expressed under the control of *pxb-Gal4*, Drf expression was ectopically induced in the inner domain while Run expression was abolished (Fig. 6C; $n=21/21$). In contrast, Hth and Bsh expression was abolished (Fig. 6D and E; $n=19/19$ and $27/28$, respectively). Since Elav expression was not affected, ectopic Ey expression does not affect neuronal differentiation (Fig. 6D). These results suggest that Ey is necessary and sufficient to induce the production of Drf-positive neurons and to repress the production of Run-positive neurons, and is sufficient to repress the production of Hth- and Bsh-positive neurons. Note that Klu expression in NBs is not affected by ectopic Ey expression or by *ey* knockdown and that Ey expression in NBs is not affected by ectopic Klu expression or in *klu* mutant clones (not shown). These results suggest that Klu and Ey act in parallel to regulate the production of Run- and Drf-positive neurons. Similarly, Hth expression in NBs is not affected by *ey* knockdown (not shown). As ectopic Ey expression represses Hth expression in NBs, repression of Hth and Bsh expression in neurons may in part be explained by the loss of Hth expression in NBs (not shown).

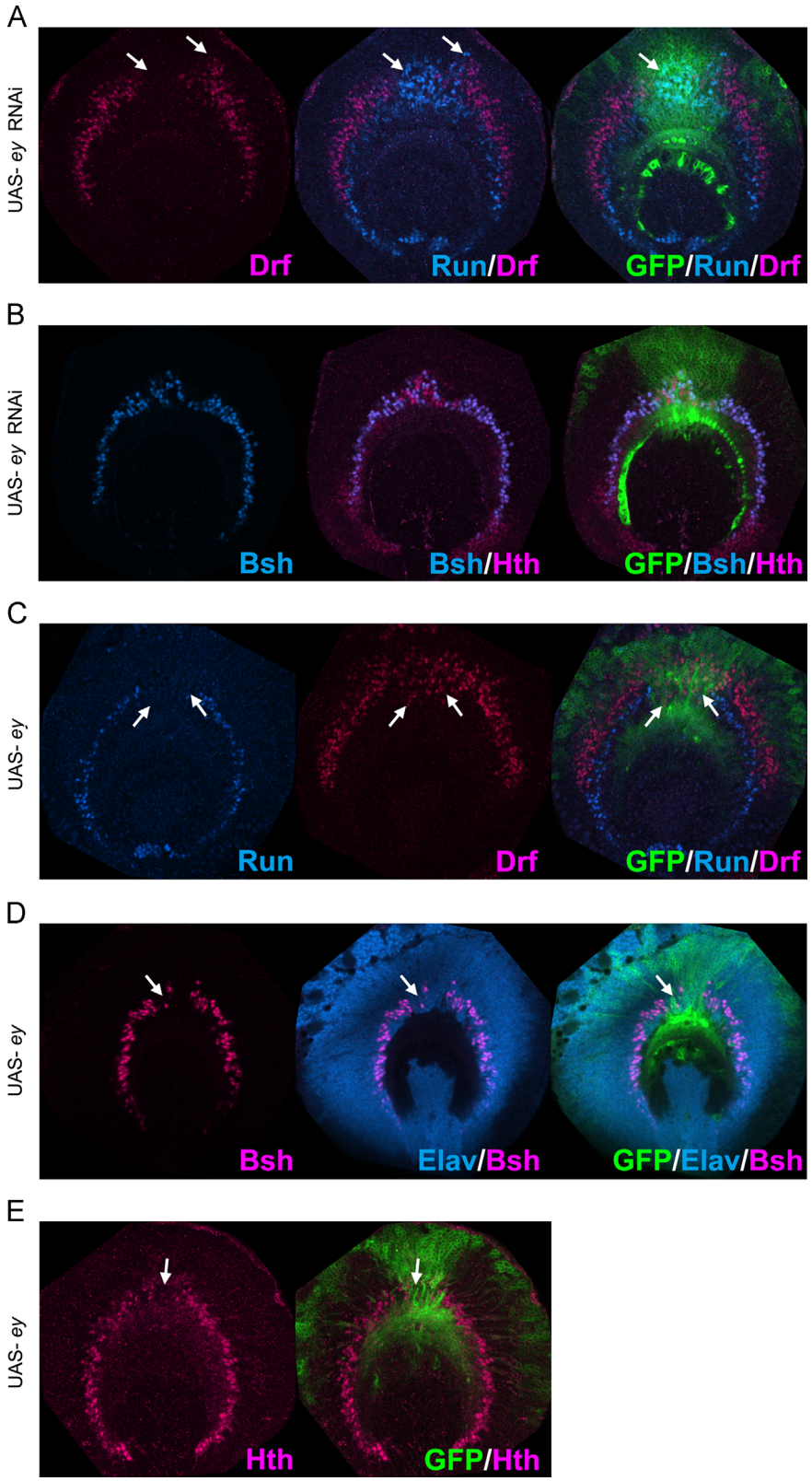

Fig. 6. Roles of Ey in specification of neuronal types. The cortex of medulla primordium in lateral view. (A) *ey* knockdown under the control of *pxb-Gal4* induces and reduces the production of Run-(blue) and Drf-(magenta) positive neurons, respectively. (B) Bsh (blue) and Hth (magenta) expression is not significantly affected by *ey* knockdown. (C–F) Ectopic expression of Ey is driven by *pxb-Gal4*. (C) Production of Drf-positive neurons (blue) is induced while that of Run-positive neurons is reduced (magenta). (D and E) Bsh-(magenta in D) and Hth-positive neurons (magenta in E) are lost while Elav expression (blue in D) is not affected.

Potential roles of Slp in specification of medulla neuron types

As discussed above, the temporal window of Slp-positive NBs appears to correspond to unidentified types of neurons that are produced after the production of Drf-positive neurons. We examined roles of *slp* in the specification of medulla neurons. In *slp* mutant clones, Run and Drf expression was derepressed in the outer domain (Fig. 7A and B; $n=22/48$ and 26/51, respectively). In contrast, Run and

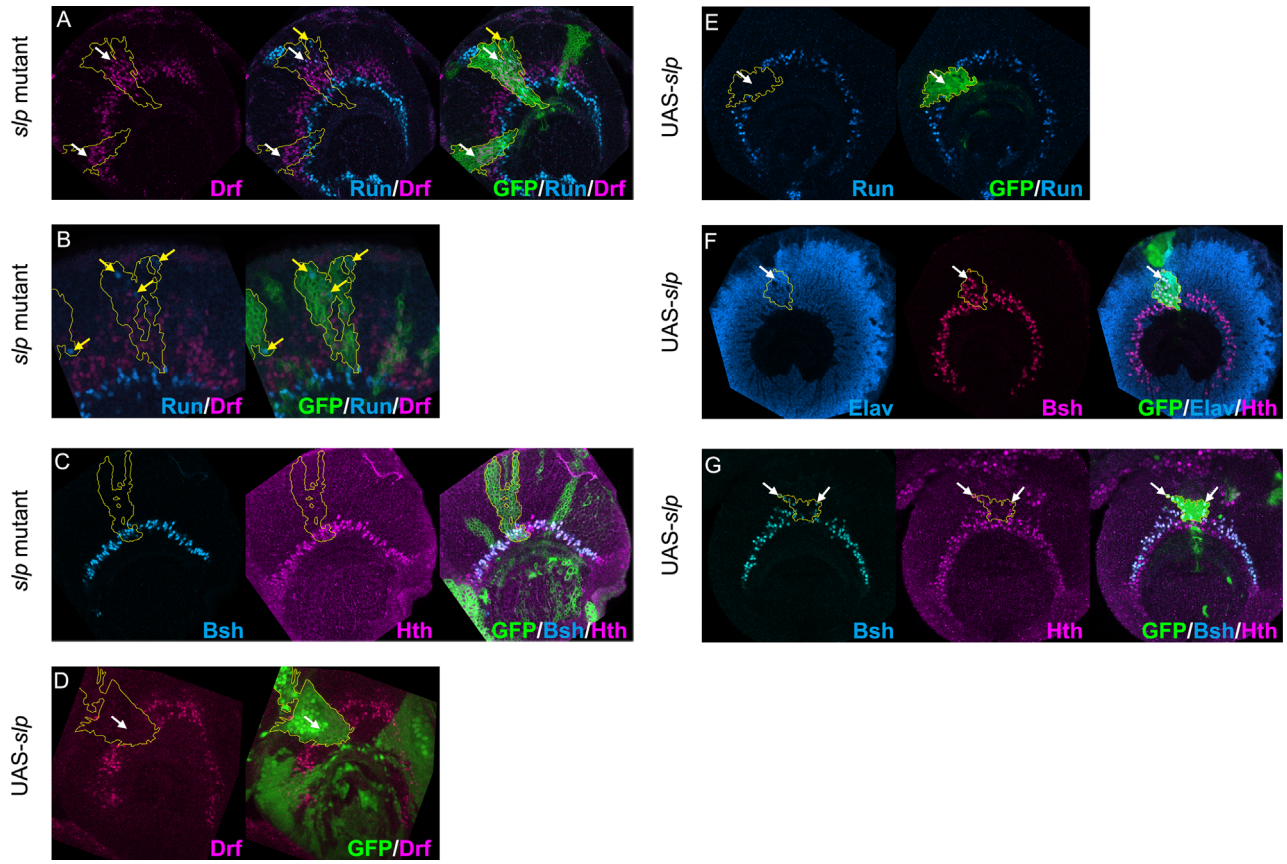

Fig. 7. Roles of Slp in specification of neuronal types. Lateral views of the medulla primordium at wandering late third instar. (A and B) In *slp* mutant clones (GFP; green), Run (blue, yellow arrows) and Drf expression (magenta, white arrows) is ectopically induced. (C) Expression of Hth (magenta) and Bsh (blue) is normal in *slp* mutant clones (green). (D) Drf (magenta) is lost in clones expressing Slp (green, arrow). (E) Run (blue) is disappeared in clones expressing Slp (green, arrow). (F) Elav (blue) expression is not affected while Bsh is ectopically expressed (magenta) in clones expressing Slp (arrow). (G) Ectopic expression of Hth (magenta) and Bsh (blue) is induced in clones expressing Slp (green, arrows).

Drf were suppressed in clones expressing Slp (Fig. 7D and E; $n=29/43$ and 27/39, respectively). Since Elav expression was not affected in Slp-expressing clones, Slp appears to regulate neuronal types without affecting neuronal differentiation (Fig. 7F). These results suggest that Slp represses the production of Run- and Drf-positive neurons in the temporal window of Slp-positive NBs.

A puzzling observation is that Bsh and Hth expression is ectopically induced in clones expressing Slp (Fig. 7F and G; $n=20/58$ and 12/43, respectively). Hth expression in NBs was not affected by ectopic Slp expression, suggesting that the induction of Hth and Bsh in neurons is not caused by Hth induction in NBs (data not shown). Bsh and Hth expression was not affected in *slp* mutant clones (Fig. 7C). As Slp is expressed in older NBs compared to Hth, and expression of Hth and Slp does not overlap in NBs (Fig. 1F), the temporal window of Slp-positive NBs is distinct from that of Hth-positive NBs. Thus, the induction of Hth- and Bsh-positive neurons by Slp expression may not reflect the normal physiological condition.

Potential roles D in specification of medulla neuron types

As D is expressed in medial NBs, which is older than Slp-positive NBs, the temporal window of D-positive NBs should correspond to the neuronal types that are produced later than the unidentified type of neurons produced from Slp-positive NBs. To examine roles of D in the specification of medulla neurons, we induced gain- and loss-of-function clones of *D*. The expression of Hth, Bsh, and Drf was lost in clones expressing D (Fig. 8A–C; $n=4/6$, 17/22, and 9/12, respectively). Neuronal differentiation as visualized by Elav was not affected (Fig. 8A), suggesting that D blocks the production of Hth-, Bsh- and Drf-positive neurons without affecting neuronal differentiation. However, expression of Drf and Hth/Bsh was not affected in *D* mutant clones (Fig. 8E and not shown). These results suggest that D is at least sufficient to repress the production of neuronal types that are produced earlier than the temporal window of D-positive NBs.

Another puzzling observation is that Run is ectopically induced in clones expressing D (Fig. 8B and C; $n=18/27$). Klu expression in NBs was not affected by ectopic D expression, suggesting that the induction of Run in neurons is not indirectly caused by Klu expression in NBs (data not shown). In contrast, Run expression was not affected in *D* mutant clones (Fig. 8E). As D is expressed in older NBs compared to Klu (Fig. 1), the temporal window of D-positive NBs appears distinct from that of Klu-positive NBs. Thus, the induction of Run-positive neurons by D expression may not reflect the normal physiological condition.

The above results suggest that the Ey expressing clones induce the production of Drf-positive neurons while activate Slp and D expression in NBs (Figs. 2E–F and 6A). However, the clones expressing Slp or D inhibit the production of Drf-positive neurons (Figs. 7D and 8C). When Ey is expressed in the NBs, production of Drf-positive neurons may be activated by a Slp/D-independent pathway and inactivated by Slp/D-dependent pathways. However, Ey expression overlaps Slp/D expression only partially in wild type NBs (Fig. 1G and H). Slp and D are expressed in medial NBs that weakly express Ey, suggesting that the activation of Slp and D expression by Ey does not take place instantaneously but is somewhat delayed. Thus, Slp/D-independent production of Drf-positive neurons under the control of Ey may be dominant over the Slp/D-dependent pathways.

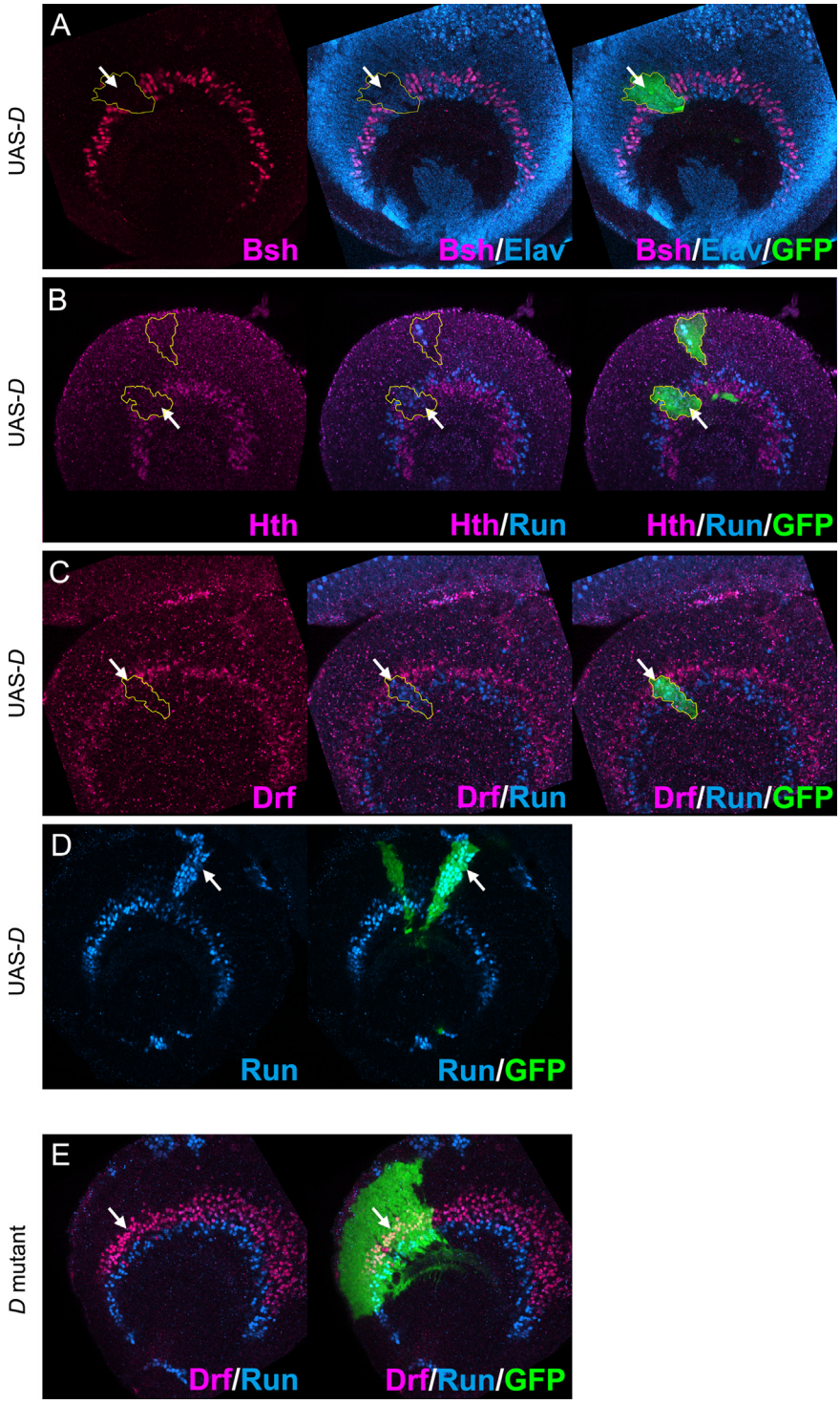

Fig. 8. Roles of D in specification of neuronal types. The cortex of medulla primordium in lateral views at wandering late third instar. (A) Bsh (magenta) is lost in clones expressing D (green, arrow) without affecting neuronal differentiation (Elav; blue). (B) Hth (magenta) is lost and Run (blue) is ectopically induced in clones expressing D (green, arrow). (C, D) Drf (magenta) is disappeared and Run (blue) is ectopically induced in clones expressing D (green, arrow). (E) Run (blue) and Drf (magenta) expression is not affected in *D* mutant clone (arrow).

Discussion

A temporal cascade of heterochronic transcription factors in the medulla NBs

In the embryonic central nervous system, the heterochronic transcription factors such as Hb, Kr, Pdm, Cas and Grh are expressed in NBs to regulate the temporal specification of neuronal identity (Isshiki et al., 2001). They regulate each other to achieve sequential changes in their expression in NBs (Baumgardt et al., 2009; Isshiki et al., 2001) without cell-extrinsic factors (Grosskortenhaus et al., 2005). However, expression of the embryonic heterochronic genes was not detected in the medulla NBs (not shown). We instead found that Hth, Klu, Ey, Slp and D are transiently and sequentially expressed in medulla NBs. The expression of Hth and Klu was observed in lateral NBs, while that

of Ey/Slp and D was observed in intermediate and medial NBs, respectively (Fig. 1). These observations suggest that the expression of heterochronic transcription factors changes sequentially as each NB ages, as observed in the development of the embryonic central nervous system.

In this study, we demonstrated that at least three of the temporal factors Ey, Slp and D regulate each other to form a genetic cascade that ensure the transition from Ey expression to D expression in the medulla NBs (Figs. 2 and 3). Ey expression in NBs activates Slp, while Slp inactivates Ey expression. Similarly, Slp expression in NBs activates D expression, while D inactivates Slp expression. In fact, the expression of Slp is not strong in newer NBs in which Ey is strongly expressed, but is upregulated in older NBs in which Ey is weakly expressed in the wild type medulla. A similar relationship is found between Slp and D, supporting the idea that Ey, Slp and D regulate each other's expression to control the transition from Ey-expression to D-expression (Fig. 1). In the embryonic central nervous system, similar interaction is mainly observed between adjacent genes of the cascade *hb-Kr-pdm-cas-grh* (Baumgardt et al., 2009), and this concept may also be applied to the medulla primordium. The expression pattern and function of Ey, Slp and D suggest that they are adjacent to each other in the cascade of transcription factor expression in medulla NBs.

However, we found no such relationship between Hth, Klu and the other temporal factors. The sequential expression of Hth and Klu could be regulated by an unidentified mechanism that is totally different from the genetic cascade that controls the transition through Ey–Slp–D. Or, there might be unidentified temporal factors that are expressed in lateral NBs which act upstream of Hth and Klu to regulate their expression. We need to identify additional transcription factors that are transiently expressed in medulla NBs.

Temporal windows of NBs that produce specific types of medulla neurons

The expression of concentric transcription factors in the medulla neurons correlates with the temporal sequence of neuron production from the medulla NBs (Hasegawa et al., 2011). In the larval medulla primordium, the neurons are located in the order of Hth/Bsh-, Run- and Drf-positive cells from inside to outside, and these domains are adjacent to each other (Hasegawa et al., 2011). Given that NBs generate neurons toward the center of the developing medulla, Hth/Bsh-positive neurons are produced at first, and then Run-positive and Drf-positive neurons. We thus used Hth/Bsh, Run and Drf as markers to examine roles of Hth, Klu, Ey, Slp and D expressed in NBs in specifying types of medulla neurons.

The continuous expression of Hth and Ey from NBs to neurons and the results of clonal analyses that visualize the progeny of NBs expressing each one of the temporal transcription factors suggest that the temporal windows of NBs expressing Hth, Klu and Ey approximately correspond to the production of Hth/Bsh-, Run- and Drf-positive neurons, respectively (Fig. 4). Indeed, the results of our previous paper and our genetic study suggest that Hth and Ey are necessary and sufficient to induce the production of Hth/Bsh- and Drf-positive neurons, respectively (Hasegawa et al., 2011, 2013) (Fig. 6). Ectopic Klu expression at least induces the production of Run-positive neurons (Fig. 5).

Slp and D expression in NBs may correspond to the temporal windows that produce medulla neurons in the outer domains of the concentric zones, which are most likely produced after the production of Drf-positive neurons (Fig. 4). Our results at least suggest that Slp is necessary and sufficient and D is sufficient to repress the production of Drf-positive neurons (Figs. 7 and 8). Identification of additional markers that are expressed in the outer concentric zones compared to the Drf-positive domain would be needed to elucidate the roles of Slp and D in specification of medulla neuron types.

D mutant clones did not produce any significant phenotype except for derepression of Slp expression in NBs (Fig. 3E). Drf expression in neurons was not affected either (Fig. 8E). Since D is a Sox family transcription factor, SoxN, another Sox family transcription factor, is a potential candidate molecule that acts together with D in the medulla NBs (Buescher et al., 2002). However, its expression was found in NEs and lateral NBs that overlap with Hth-positive cells but not with D-positive cells (Fig. S4).

All the potential heterochronic transcription factors examined in this study are expressed in three to five cell rows of NBs (Fig. 1). Nevertheless, one NB has been observed to produce one Bsh-positive and one Run-positive neurons (Hasegawa et al., 2011). Therefore, the expression pattern of the heterochronic transcription factors is not sufficient to explain the stable production of one Bsh-positive and one Run-positive neurons from a single NB. The combinatorial action of multiple temporal factors expressed in NBs may play important roles in the specification of Bsh- and Run-positive neurons.

Another possible mechanism that guarantees the production of limited number of the same neuronal type from multiple rows of NBs expressing a temporal transcription factor could be a mutual repression between concentric transcription factors expressed in medulla neurons. For example, Hth/Bsh, Run and Drf may repress each other to restrict the number of neurons that express either of these transcription factors. However, expression of Run and Drf was not essentially affected in *hth* mutant clones and in clones expressing Hth (Hasegawa et al., 2011). Similarly, expression of Hth and Drf was not essentially affected in clones expressing *run* RNAi under the control of *AyGal4*, in which Run expression is eliminated (Yusuke Kitada and M. S., unpublished observations). Hth and Run expression was not affected in *drf* mutant clones (Hasegawa et al., 2011). These results suggest that Hth/Bsh, Run and Drf do not essentially regulate each other during the formation of concentric zones in the medulla.

Inheritance of temporal transcription factor expression from NBs to neurons

During the embryonic development, the heterochronic genes that are expressed in NBs (*hb-Kr-pdm-cas-grh*) are maintained and act in GMCs to specify neuronal type (Isshiki et al., 2001; Baumgardt et al., 2009). Similarly, Hth and Ey are continuously expressed from NBs to neurons, suggesting that their expression may also be inherited through GMCs (Hasegawa et al., 2011). However, this type of regulatory mechanism may be somewhat modified in the case of Klu, Slp and D.

Klu is expressed in NBs and GMCs, but not in neurons (Fig. 5A). Slp and D are predominantly detected in NBs and neurons visualized by Dpn and Elav, respectively (Fig. S3C and D). Occasionally, however, expression of D was found in putative GMCs, which are situated between NBs and neurons (Fig. S3C). Additionally, both D-positive and D-negative cells were found among Miranda-positive GMCs (Fig. S3E). Slp expression was not found in Miranda-positive GMCs (Fig. S3F). Finally, D is expressed in medulla neurons forming a concentric zone in addition to its expression in medial NBs. However, D expression was abolished in *slp* mutant NBs but remained in the mutant neurons (Fig. 3B), suggesting that D expression in medulla neurons is not inherited from the NBs. These results suggest that Slp and D expression are not maintained from NBs to neurons and that not all the temporal transcription factors expressed in NBs are inherited through GMCs. However, it is possible to speculate that Klu, Slp and D regulate expression of unidentified transcription factors in NBs that are inherited from NBs to neurons through GMCs.

Conclusions

This study suggests that the types of medulla neurons are specified by transcription factors that are transiently and sequentially expressed in the medulla NBs. Although neuronal identity determinants differ from those of the embryonic central nervous system, the identities of the medulla neurons are determined in a temporal manner. Our study provides a basis for an alternative model system for studying molecular mechanisms that govern birth-order dependent production of neuronal diversity.

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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.2013.05.002.

References

Almeida, M.S., Bray, S.J., 2005. Regulation of post-embryonic neuroblasts by Drosophila Grainyhead. Mech. Dev. 122, 1282–1293.
Baumgardt, M., Karlsson, D., Terriente, J., Diaz-Benjumea, F.J., Thor, S., 2009. Neuronal subtype specification within a lineage by opposing temporal feed-forward loops. Cell 139, 969–982.
Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L.Y., Jan, Y.N., 1992. deadpan, an essential pan-neural gene in Drosophila, encodes a helix-loop-helix protein similar to the hairy gene product. Genes Dev. 6, 2137–2151.
Bossing, T., Udolph, G., Doe, C.Q., Technau, G.M., 1996. The embryonic central nervous system lineages of *Drosophila melanogaster*. I. Neuroblast lineages derived from the ventral half of the neuroectoderm. Dev. Biol. 179, 41–64.
Buescher, M., Hing, F.S., Chia, W., 2002. Formation of neuroblasts in the embryonic central nervous system of *Drosophila melanogaster* is controlled by SoxNeuro. Development 129, 4193–4203.
Cepko, C.L., 1999. The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr. Opinion Neurobiol. 9, 37–46.
Chen, Z., Li, X., Desplan, C., 2012. Deterministic or stochastic choices in retinal neuron specification. Neuron 75, 739–742.
Connolly, J.B., Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T., O'Kane, C.J., 1996. Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. Science 274, 2104–2107.
Doe, C.Q., Goodman, C.S., 1985. Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. Dev. Biol. 111, 206–219.
Fischbach, K.F., Dittrich, A.P.M., 1989. The optic lobe of *Drosophila melanogaster*. I. A Golgi analysis of wild-type structure. Cell Tissue Res. 258, 441–475.
Grosskortenhaus, R., Pearson, B.J., Marusich, A., Doe, C.Q., 2005. Regulation of temporal identity transitions in Drosophila neuroblasts. Dev. Cell 8, 193–202.
Grossniklaus, U., Pearson, R.K., Gehring, W.J., 1992. The Drosophila sloppy paired locus encodes two proteins involved in segmentation that show homology to mammalian transcription factors. Genes Dev. 6, 1030–1051.
Hasegawa, E., Kitada, Y., Kaido, M., Takayama, R., Awasaki, T., Tabata, T., Sato, M., 2011. Concentric zones, cell migration and neuronal circuits in the Drosophila visual center. Development 138, 983–993.
Hasegawa, E., Kaido, M., Takayama, R., Sato, M., 2013. Brain-specific-homeobox is required for the specification of neuronal types in the Drosophila optic lobe. Dev. Biol..
Hofbauer, A., Campos-Ortega, J.A., 1990. Proliferation pattern and early differentiation of the optic lobes in *Drosophila melanogaster*. Rouxs Arch. Dev. Biol. 198, 264–274.
Isshiki, T., Pearson, B., Holbrook, S., Doe, C.Q., 2001. Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. Cell 106, 511–521.
Ito, K., Awano, W., Suzuki, K., Hiromi, Y., Yamamoto, D., 1997. The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. Development 124, 761–771.
Jessell, T.M., 2000. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat. Rev. Genet. 1, 20–29.
Kambadur, R., Koizumi, K., Stivers, C., Nagle, J., Poole, S.J., Odenwald, W.F., 1998. Regulation of POU genes by castor and hunchback establishes layered compartments in the Drosophila CNS. Genes Dev. 12, 246–260.
Kurant, E., Pai, C.Y., Sharf, R., Halachmi, N., Sun, Y.H., Salzberg, A., 1998. Dorsotonals/homothorax, the Drosophila homologue of meis1, interacts with extradenticle in patterning of the embryonic PNS. Development 125, 1037–1048.
Lee, T., Luo, L., 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451–461.
Lupo, G., Harris, W.A., Lewis, K.E., 2006. Mechanisms of ventral patterning in the vertebrate nervous system. Nat. Rev. Neurosci. 7, 103–114.
Martin-Bermudo, M.D., Martinez, C., Rodriguez, A., Jimenez, F., 1991. Distribution and function of the lethal of scute gene product during early neurogenesis in Drosophila. Development 113, 445–454.
McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., Davis, R.L., 2003. Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302, 1765–1768.
Morante, J., Erclik, T., Desplan, C., 2011. Cell migration in Drosophila optic lobe neurons is controlled by eyeless/Pax6. Development 138, 687–693.
Ohshiro, T., Yagami, T., Zhang, C., Matsuzaki, F., 2000. Role of cortical tumour-suppressor proteins in asymmetric division of Drosophila neuroblast. Nature 408, 593–596.
Quiring, R., Walldorf, U., Kloter, U., Gehring, W.J., 1994. Homology of the eyeless gene of Drosophila to the small eye gene in mice and Aniridia in humans. Science 265, 785–789.
Reddy, B.V., Rauskolb, C., Irvine, K.D., 2010. Influence of fat-hippo and notch signaling on the proliferation and differentiation of Drosophila optic neuroepithelia. Development 137, 2397–2408.
Russell, S.R., Sanchez-Soriano, N., Wright, C.R., Ashburner, M., 1996. The Dichaete gene of *Drosophila melanogaster* encodes a SOX-domain protein required for embryonic segmentation. Development 122, 3669–3676.
Sato, A., Tomlinson, A., 2007. Dorsal-ventral midline signaling in the developing Drosophila eye. Development 134, 659–667.
Sepp, K.J., Auld, V.J., 1999. Conversion of lacZ enhancer trap lines to GAL4 lines using targeted transposition in *Drosophila melanogaster*. Genetics 151, 1093–1101.
Soriano, N.S., Russell, S., 1998. The Drosophila SOX-domain protein Dichaete is required for the development of the central nervous system midline. Development 125, 3989–3996.
Yang, X., Bahri, S., Klein, T., Chia, W., 1997. Klumpfuss, a putative Drosophila zinc finger transcription factor, acts to differentiate between the identities of two secondary precursor cells within one neuroblast lineage. Genes Dev. 11, 1396–1408.
Yasugi, T., Umetsu, D., Murakami, S., Sato, M., Tabata, T., 2008. Drosophila optic lobe neuroblasts triggered by a wave of proneural gene expression that is negatively regulated by JAK/STAT. Development 135, 1471–1480.