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eyeless/*Pax6* controls the production of glial cells in the visual center of *Drosophila melanogaster*

Takumi Suzuki [a], Rie Takayama [a,c], Makoto Sato [a,b,c,*]

[a] Laboratory of Developmental Neurobiology, Brain/Liver Interface Medicine Research Center, Kanazawa University, 13-1 Takaramachi Kanazawa-shi, Ishikawa 920-8640, Japan
[b] Graduate School of Medical Sciences, Kanazawa University, 13-1 Takaramachi Kanazawa-shi, Ishikawa 920-8640, Japan
[c] CREST, JST, 4-1-8 Honcho Kawaguchi, Saitama 332-0012, Japan

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ABSTRACT

Pax6 is known as a neurogenic factor in the development of the central nervous system and regulates proliferation of neuronal progenitor cells and promotes neuronal differentiation. In addition to neurogenesis, Pax6 is also involved in the specification and maturation of glial cells. Here, we show that Eyeless (Ey), *Drosophila* homolog of Pax6, regulates the production of glial cells in the brain. In the developing fly visual center, the production of neurons and glial cells are controlled by the temporal transcription factors that are sequentially expressed in neuroblasts (NBs). Among them, NBs of the last temporal window produce astrocyte-like glial cells. Ey is strongly expressed in the middle aged NBs, whose temporal window is earlier compared with glia producing older NBs. Weak Ey expression is also detected in the glia producing NBs. Our results suggest that Ey expression in the middle aged NBs indirectly control gliogenesis from the oldest NBs by regulating other temporal transcription factors. Additionally, weak Ey expression in the NBs of last temporal window may directly control gliogenesis. Ey is also expressed in neurons produced from the NBs of Ey-positive temporal window. Interestingly, neuron-specific overexpression of Ey causes significant increase in glial cells suggesting that neuronal expression of Ey may also contribute to gliogenesis. Thus, Pax6-dependent regulation of astrocyte-like glial development is conserved throughout the animal kingdom.

1. Introduction

Pax6 was identified as a member of paired box transcription factor (Walther and Gruss, 1991) and is a highly conserved from vertebrates to invertebrates. *Pax6* is also well known as a causative gene of human aniridia disorder (Ton et al., 1991), mouse *Small eye* (Hill et al., 1991), and *Drosophila eyeless* (*ey*, *Drosophila* homolog of *Pax6*) mutants (Quiring et al., 1994). Although *Pax6* is broadly accepted as the master regulator of eye formation, its functions are not limited to the regulation of eye development. During the embryonic development of central nervous system in mice, Pax6 is specifically expressed in the forebrain, hindbrain, and spinal cord. In the developing forebrain, Pax6 is strongly expressed in the radial glial cells (Götz et al., 1998) and its mutation results in smaller and thinner brain (Schmahl et al., 1993), it has been suggested that Pax6 is involved in the neurogenesis of the cerebral cortex by regulating neuronal differentiation. In fact, loss- and gain-of-function analysis revealed that Pax6 positively controls the ability of radial glial cells to produce neurons (Heins et al., 2002).

Pax6 is also involved in the cell type specification in the central nervous system. The cortical progenitor cells, which produce glutamatergic neurons in wild type, generate GABAergic interneurons in *Pax6* mutant mice (Kroll and O'Leary, 2005). The stage-specific knockout of *Pax6* in the mouse embryo resulted in significantly thinner superficial cortical layers compared to those in wild type (Georgala et al., 2011). These observations implicate that Pax6 plays critical roles in the specification of the cortical neuronal types. Pax6 also shows crucial effects on the astrocyte subtype specification by providing the positional identities in the mouse spinal cord (Hochstim et al., 2008) and regulates development of astrocytes (Sakurai and Osumi, 2008). Thus Pax6 is widely known as a master gene for eye formation but it plays extraordinarily diverse roles during animal development.

Pax6 is also involved in the neuronal type specification in the visual center of fruit fly, *Drosophila melanogaster*. *Drosophila* visual center is composed of the four ganglia, lamina, medulla, lobula and lobula plate. Among them, medulla is the largest component and contains more than 100 types of 40,000 neurons (Fischbach and Dittrich, 1989). During development, the medulla primordium

* Corresponding author at: Laboratory of Developmental Neurobiology, Brain/Liver Interface Medicine Research Center, Kanazawa University, 13-1 Takaramachi Kanazawa-shi, Ishikawa 920-8641, Japan.
E-mail address: makotos@staff.kanazawa-u.ac.jp (M. Sato).

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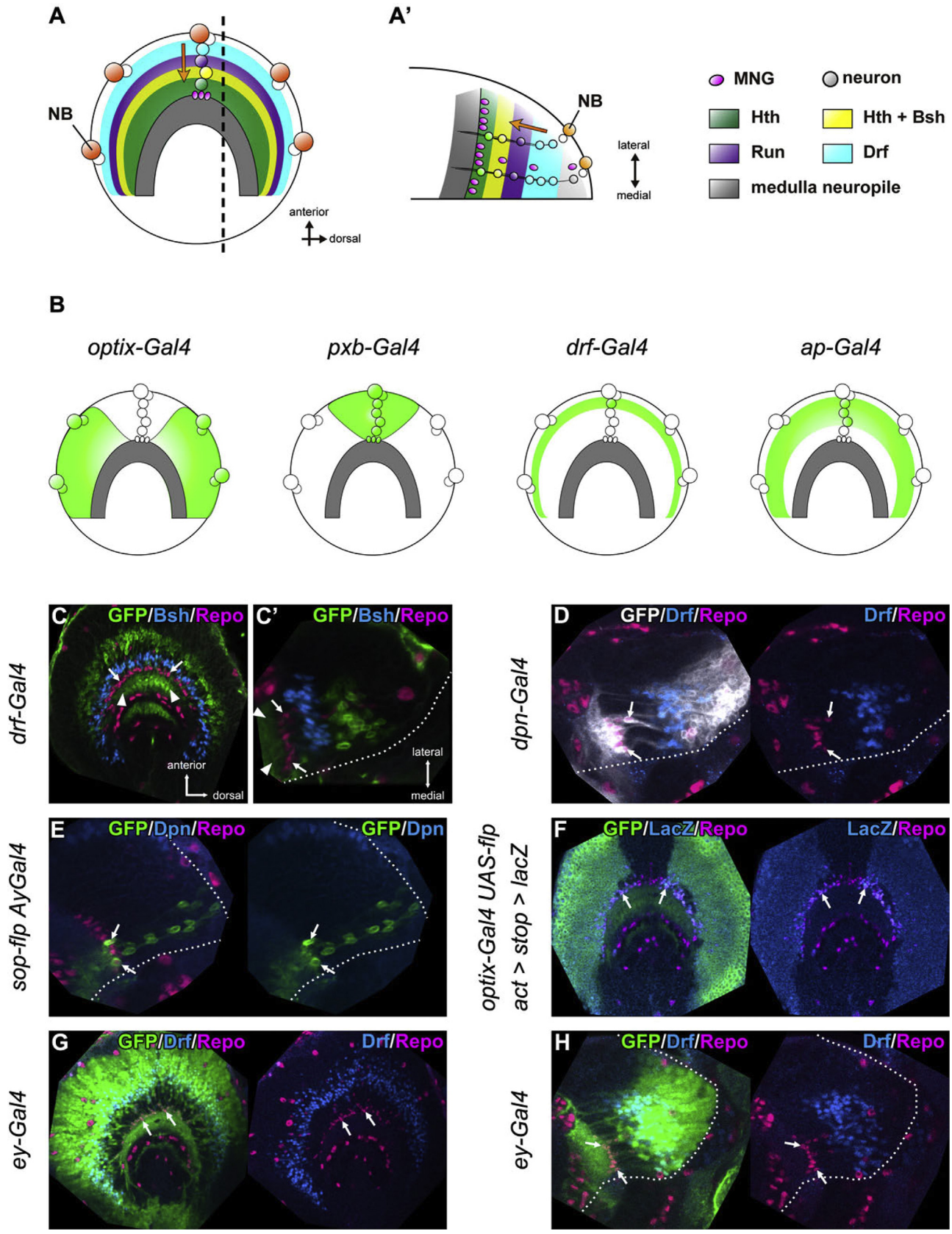

Fig. 1. Origins of the medulla MNGs. (A, A′) Schematics of the larval medulla primordium in lateral (A) and horizontal views (A′; a horizontal section along the dotted line in A). NBs produce medulla neurons in a linear and radial orientation toward the center of the larval medulla primordium (orange arrows). (B) Schematics of the expression patterns of Gal4 drivers used in this study (lateral views as shown in A). The Gal4 expressing regions are colored in green in each panel. Lateral (C, F, G; anterior to the top and dorsal to the right, same orientation as in A) and horizontal views (C′, D, E, H; lateral to the top and anterior to the right, same orientation as in A′) of the medulla primordium at wandering late third instar larva. The boundaries between the optic lobe and other region of the brain are indicated by dotted white lines. (C, C′) The MNGs visualized with the expression of Repo (magenta; arrows) are located adjacent to the medulla neuopile (arrowheads) and are also observed in the cortex of medulla primordium. (D) Expression of CD8GFP (white) driven by *dpn-Gal4* is strongly detected in the MNGs (magenta; arrows) as well as in Drf+ neurons (blue). (E) Lineages of NBs in OPC are visualized with CD8GFP expression under the control of *AyGal4* (green). Progenies of the NBs contain MNGs (magenta; arrows). (F) Lineages of NBs in OPC are also visualized with LacZ expression (blue) under the control of *optix-Gal4* (green). MNGs are labeled with LacZ (arrows). (G, H) GFP expression driven by *ey-Gal4* is observed in MNGs visualized with Repo expression (magenta; arrows) as well as in neurons located at the outer region of medulla cortex containing Drf+ neurons (blue).

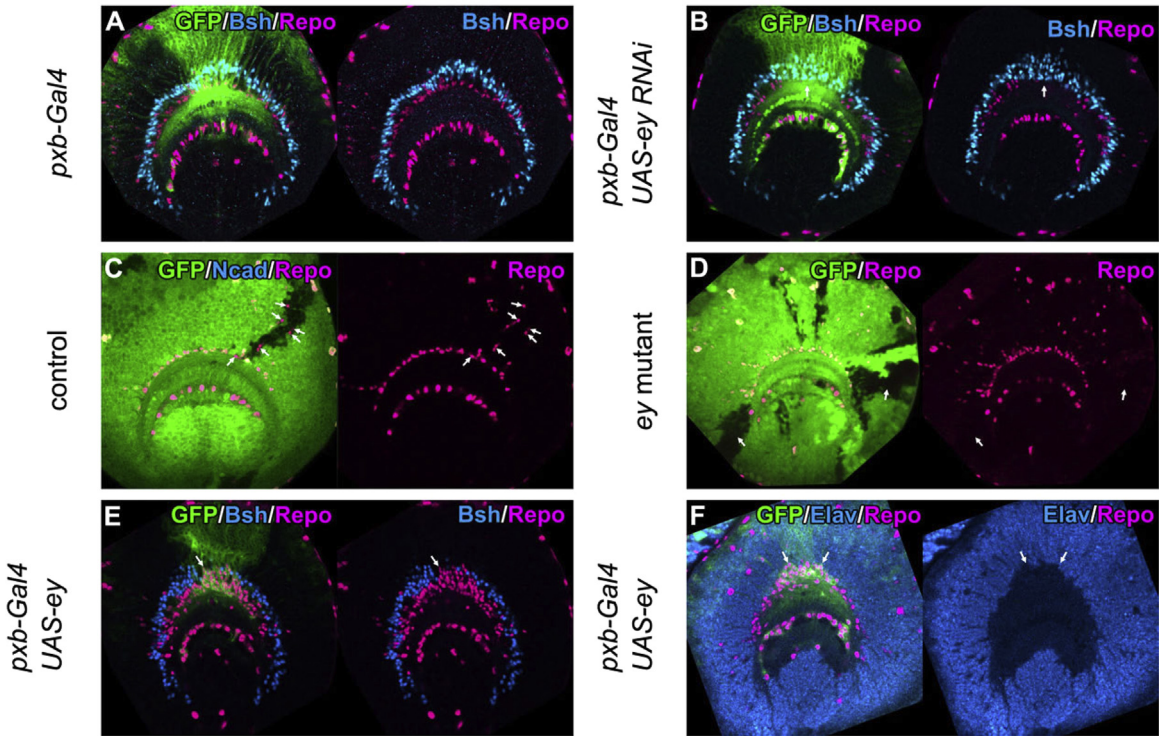

Fig. 2. *ey* regulates the production of MNGs. Lateral views (anterior to the top and dorsal to the right, same orientation as in Fig. 1A) of the medulla primordium at wandering late third instar larva. MNGs are visualized with Repo expression (magenta). (A) *pxb-Gal4* visualized with CD8GFP (green) is expressed specifically in the anterior region. (B) MNGs are almost completely lost in the region expressing *ey RNAi* ($ey^{KK107100}$) under the control of *pxb-Gal4* (green) as indicated by arrows. (C) MNGs are normally observed in the control clones as indicated by arrows. (D) MNGs are never found in the *ey* mutant clones as indicated by arrows. (E) Dramatic increase in MNGs is observed in the region expressing *ey* under the control of *pxb-Gal4* as indicated by arrows. (F) Instead of the increase in MNGs, neurons as visualized with Elav (blue) are lost in the region adjacent to the medulla neuropile as indicated by arrows.

is subdivided into concentric zones that are characterized by the expression of conserved transcription factors and these medulla neurons are produced from neuroblasts (NB), neural stem cell-like cells, with linear and radial orientation toward the center of the medulla primordium (Hasegawa et al., 2011). Several types of neurons are contained in each lineage of NBs and form a line that intersects such concentric zones. The medulla neurons born earlier are located in the inner region, while those born later in development are located in the outer region. The concentric expression of each transcription factors is correlated with the birth order of the medulla neurons and in fact their neuronal types are specified in a birth order-dependent manner (Li et al., 2013b; Suzuki and Sato, 2014). Specification of the medulla neuron types is controlled by the temporal transcription factors that are sequentially expressed in NBs (temporal factors): Homothorax (Hth), Klumpfuss (Klu), Ey, Sloppy paired (Slp), Dichaete (D) and Tailless (Tll) (Li et al., 2013a; Suzuki et al., 2013).

In the present study, we examined the roles of Ey, *Drosophila* homolog of Pax6, in the brain development and demonstrate that Ey/Pax6 regulates gliogenesis. Pax6/Ey is also involved in the cell type specification in *Drosophila* visual center as a member of the temporal factors (Li et al., 2013a, 2013b; Suzuki et al., 2013; Suzuki and Sato, 2014). Among the temporal factors, Ey is strongly expressed in the middle aged NBs to produce Ey+ neurons. Tll+ medial NBs of the last temporal window located at the border between optic lobe and central brain produce the medulla neuroplie glial cells (MNGs), which differentiate to astrocyte-like glial cells (Edwards et al., 2012). We also found that Ey is weakly expressed in these oldest glia producing NBs in addition to strong Ey expression in the middle aged NBs (Li et al., 2013a; Suzuki et al., 2013). The present results implies that Ey expression in the middle aged NBs may indirectly control gliogenesis from the oldest NBs by regulating other temporal factors. Additionally, weak Ey expression in the oldest NBs may also control gliogenesis. Ey is also expressed in the subset of medulla neurons that are produced from Ey+ NBs (Suzuki et al., 2013). Surprisingly neuron-specific induction of Ey resulted in significant increase of MNGs, suggesting that neuronal Ey may indirectly contribute to the regulation of MNG production through unknown mechanism. Thus the Ey/Pax6 action in the control of gliogenesis may be conserved throughout the animal kingdom.

2. Materials and methods

2.1. 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*, *FRT80B*, *FRT2A*, *FRT82B*, *tub-Gal4*, *elav-Gal4*, *UAS-GFP*, *UAS-CD8GFP* (Lee and Luo, 1999), *UAS-CD2RFP*, *UAS-MirGFP*, *UAS-MirCD2* (Yu et al., 2009), *UAS-dicer2*, *ubi-GFP*, *tub-Gal80^{ts}* (McGuire et al., 2003), *UAS-flp, actin > stop > lacZ*, *actin > yellow > Gal4* (*Ay-Gal4*) (Ito et al., 1997), *dpn-Gal4*, *SOP-flp* (Shimono et al., 2014), *UAS-ey RNAi* ($ey^{KK107100}$ and $ey^{HMS00489}$), *UAS-ey*, *UAS-ey^{DN}*, *ey-GFP*, *UAS-gcm RNAi* ($gcm^{JF01075}$ and $gcm^{HM05124}$), *UAS-gcm1*, *UAS-slp1^{IF}*, *slp^{S37A}* (Sato and Tomlinson, 2007), *UAS-D*, *D^{d23}* (Suzuki et al., 2013), *UAS-tll*, *tll^{149}*, *NP2631* (*optix-Gal4*), *pxb-Gal4* (Suzuki et al., 2013), *drf-Gal4* (Hasegawa et al., 2011), *ap-Gal4*, *gcm-lacZ*, *gcm-Gal4*, *Df(2L)132*, *ey$^{+tCH321-01A12}$* (Li et al., 2013a), *ey$^{l5.71}$*, *OK107* (*ey-Gal4*) (Connolly et al., 1996).

2.2. Genetic crosses

The genetic crosses and heat shock conditions used in this

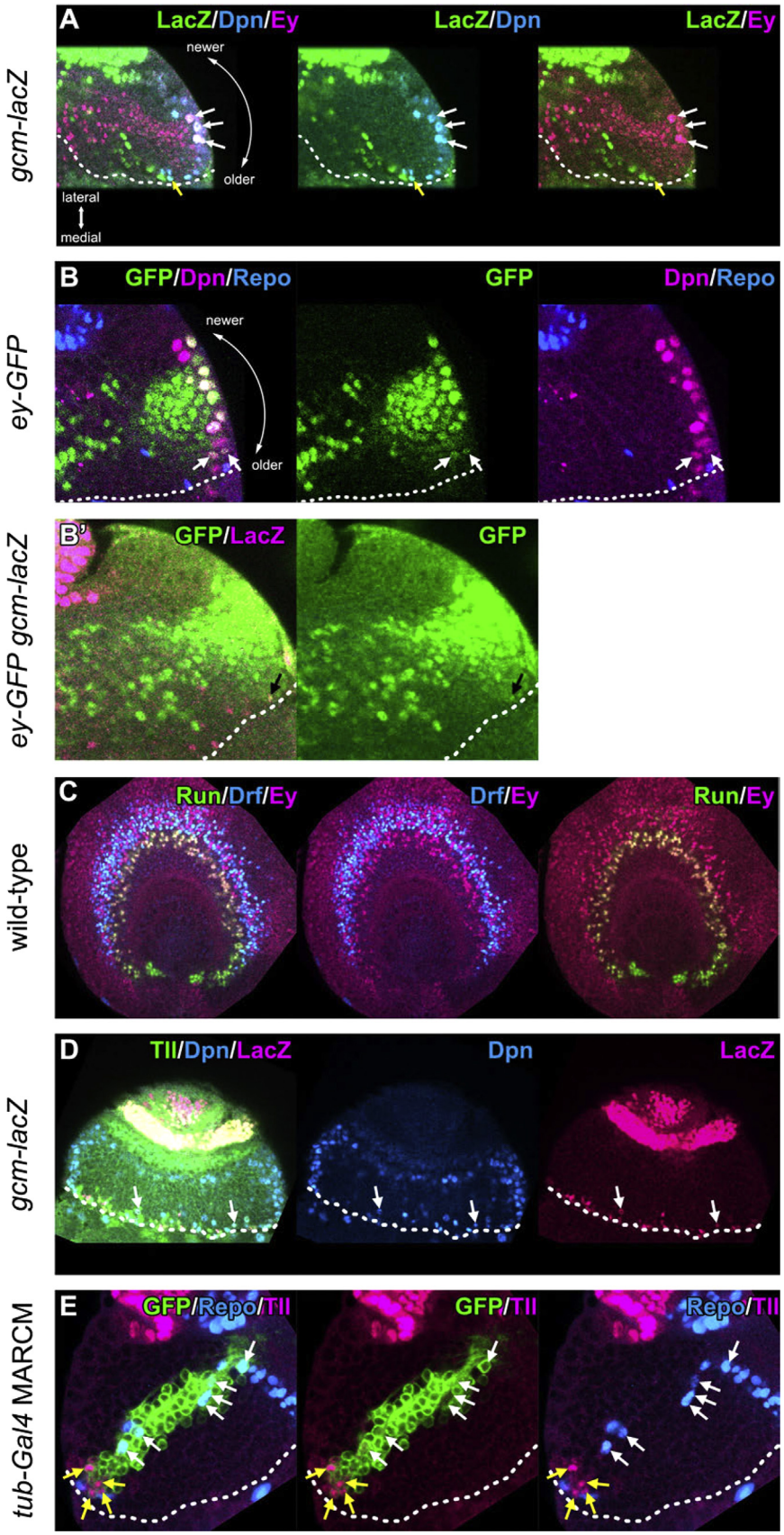

Fig. 3. Expression pattern of *ey* in NBs and neurons. Horizontal (A, B, B′, D, E; lateral to the top and anterior to the right, same orientation as in Fig. 1A′) and lateral views (C; anterior to the top and dorsal to the right, same orientation as in Fig. 1A). (A) *gcm* expression is visualized with LacZ (green) in *gcm-lacZ* larvae. LacZ expression is observed in small NBs (visualized with Dpn; blue) adjacent to the border between the optic lobe and central brain as indicated by yellow arrows. Ey expression (magenta) is observed in lateral NBs as indicated by white arrows. (B) *ey* expression is visualized with GFP (green) in *ey-GFP* larvae. GFP expression is weakly observed in the NBs (visualized with Dpn; magenta) located near the medial edge of the medulla primordium (arrows). (B′) *gcm* and *ey* expression are visualized with LacZ (magenta) and GFP (green) in *gcm-lacZ*; *ey-GFP* larvae. A few LacZ+/GFP+ cells are observed as indicated by black arrows. (C) Concentric expression of Ey is observed in the medulla cortex. Its expression is detected in Run+ neurons (green) and neurons adjacent to the Drf+ cells (blue). (D) *gcm* expression is visualized with LacZ (magenta) in *gcm-lacZ* larvae. LacZ expression is observed in a subset of Tll+ (green) NBs (visualized with Dpn; blue) as indicated by arrows. (E) A lineage of Tll+ NBs (magenta) visualized with CD8GFP (green) by MARCM method using *tub-Gal4* contains MNGs (visualized with Repo; blue) as indicated by arrows.

study are as follows.

Fig. 1E: *sop-flp* was crossed to *AyGal4 UAS-CD8GFP*. Fig. 1F: *UAS-flp*; *actin > stop > lacZ* was crossed to *optix-Gal4 UAS-CD8GFP*. Fig. S1A: *UAS-flp*; *actin > stop > lacZ* was crossed to *dpn-Gal4 UAS-CD8GFP*. Fig. S1B and C: *UAS-flp*; *actin > stop > lacZ* was crossed to *UAS-GFP*; *ey-Gal4*. Figs. 2B and S2A: *ey* RNAi (*ey*^{KK107100} or

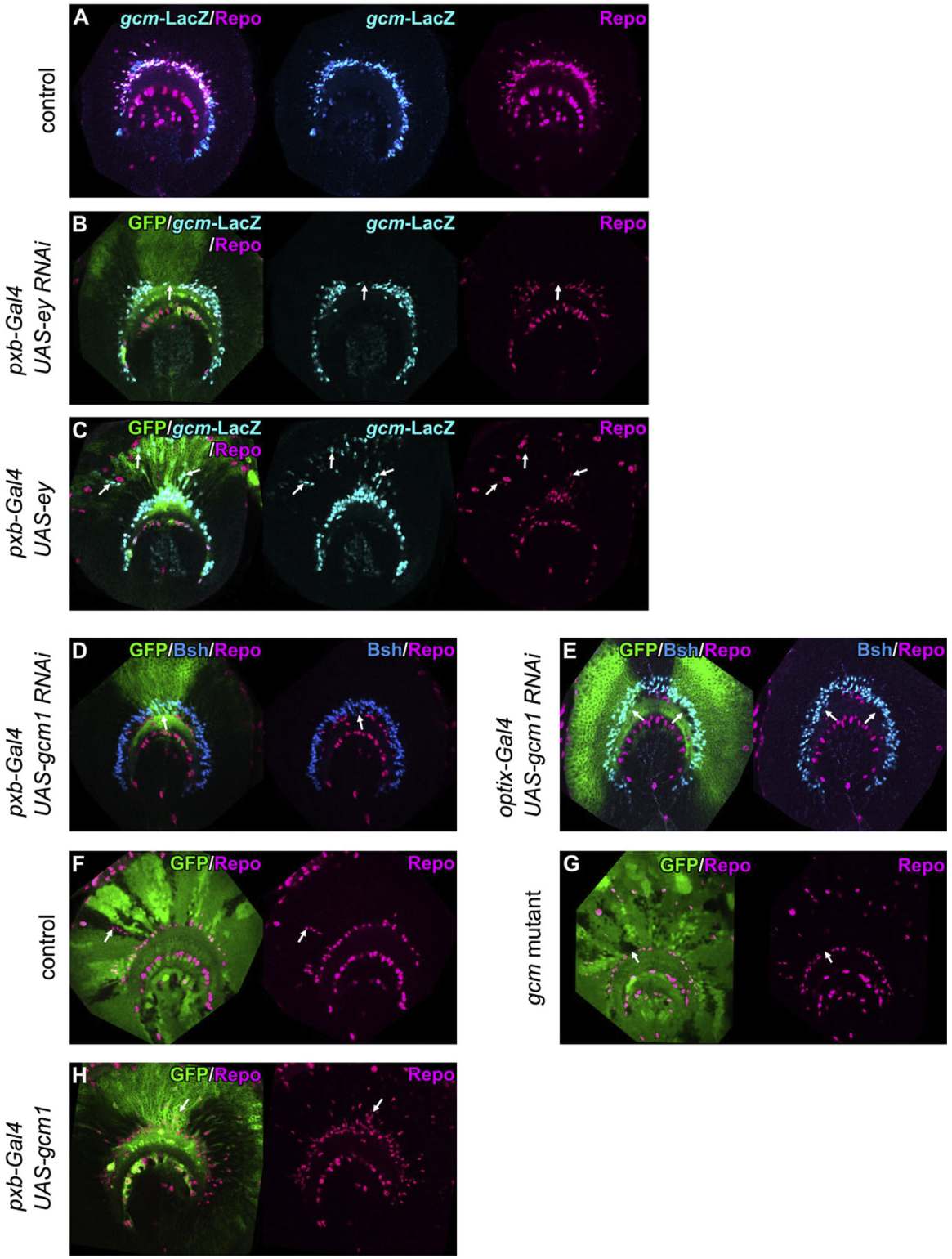

Fig. 4. *ey* regulates MNG production by regulating gcm expression. Lateral views (anterior to the top and dorsal to the right, same orientation as in Fig. 1A). (A–C) *gcm* expression is visualized with LacZ (blue) in *gcm-lacZ* larvae. (A) LacZ expression is observed in MNGs visualized with Repo (magenta). (B) LacZ expression is almost completely lost in the region expressing *ey RNAi* (*ey*^{HMS00489}) under the control of *pxb-Gal4* (green) as indicated by arrows (n=10/11). (C) By contrast, LacZ expression is strongly induced in the region expressing *ey* under the control of *pxb-Gal4* (green) as indicated by arrows (n=8/8). (D) MNGs are almost completely lost in the region expressing *gcm RNAi* (*gcm*^{lF01075}) under the control of *pxb-Gal4* (green) as indicated by arrows. (E) MNGs are lost in the region expressing *gcm RNAi* (*gcm*^{HM05124}) under the control of *optix-Gal4* (green) as indicated by arrows. (F) MNGs are normally observed in control clones as indicated by arrows. (G) MNGs are never found in *gcm* mutant clones as indicated by arrows. (H) An increase in MNGs is observed in the region expressing *gcm1* under the control of *pxb-Gal4* as indicated by arrows.

ey^{HMS00489}) was expressed under the control of *pxb-Gal4* to knock down *ey*. Fig. S2C and D: *ey* RNAi (*ey*^{KK107100} or *ey*^{HMS00489}) was expressed under the control of *optix-Gal4*. Fig. 2C: *FRT80B* was crossed to *hs-flp*; *FRT80B ubi-GFP* (37 °C for 60 min at first or second instar larvae). Fig. 2D: *ey*$^{+tCH321\text{-}01A12}$ *FRT80B*; *ey*$^{J5.71}$ was crossed to *hs-flp*; *FRT80B ubi-GFP*; *ey*$^{J5.71}$ (37 °C for 60 min at first or second instar larvae). Figs. 2E, 2F, 4H, S3B, S3D, and S3F: *tub-Gal80*^{ts}; *pxb-Gal4 UAS-CD8GFP* was crossed to either *UAS-ey*, *UAS-gcm1; UAS-gcm1*, *UAS-slp1*^{IF}, *UAS-D*, or *UAS-tll* and raised at 17 °C to suppress lethality. The larvae were then raised at 30 °C for 24 or 40 h prior to dissection to allow transgene expression. Fig. 3F, *hs-flp*; *UAS-CD8GFP UAS-MirRFP FRT40A* was crossed to *UAS-RFP UAS-MirGFP*; *tub-Gal4* (34 °C, 30 min at first or second instar larva). Fig. S3A: *hs-flp*; *slp*^{S37A} *FRT40A* was crossed to *hs-flp*; *ubi-GFP FRT40A* (37 °C for 60 min at late embryo or first instar). Fig. S3C: *hs-flp*; *D*^{d23} *FRT2A* was crossed to *hs-flp*; *ubi-GFP FRT2A* (37 °C for 60 min at late embryo or first instar). Fig. S3E: *hs-flp*; *tll*^{149} *FRT82B* was crossed to *hs-flp*; *ubi-GFP FRT82B* (37 °C for 60 min at late embryo or first instar). Fig. 4B and S4A–C: *tubGal80*^{ts}; *gcm-lacZ*; *pxb-Gal4 UAS-CD8GFP* was crossed *UAS-ey* RNAi lines (*ey*^{KK107100} or *ey*^{HMS00489}) at 30 °C. Figs. 4C and S8A: *tubGal80*^{ts}; *gcm-lacZ*; *pxb-Gal4 UAS-CD8GFP* was crossed to *UAS-ey* and raised at 17 °C to suppress lethality. The larvae were then raised at 30 °C for 24 or 40 h prior to dissection to allow transgene expression. Fig. 4D, 4E and S4F and G: *UAS-gcm* RNAi lines (*gcm*^{JF01075} and *gcm*^{HM05124}) were crossed to either *UAS-dicer2*; *pxb-Gal4 CD8GFP* or *optix-Gal4*

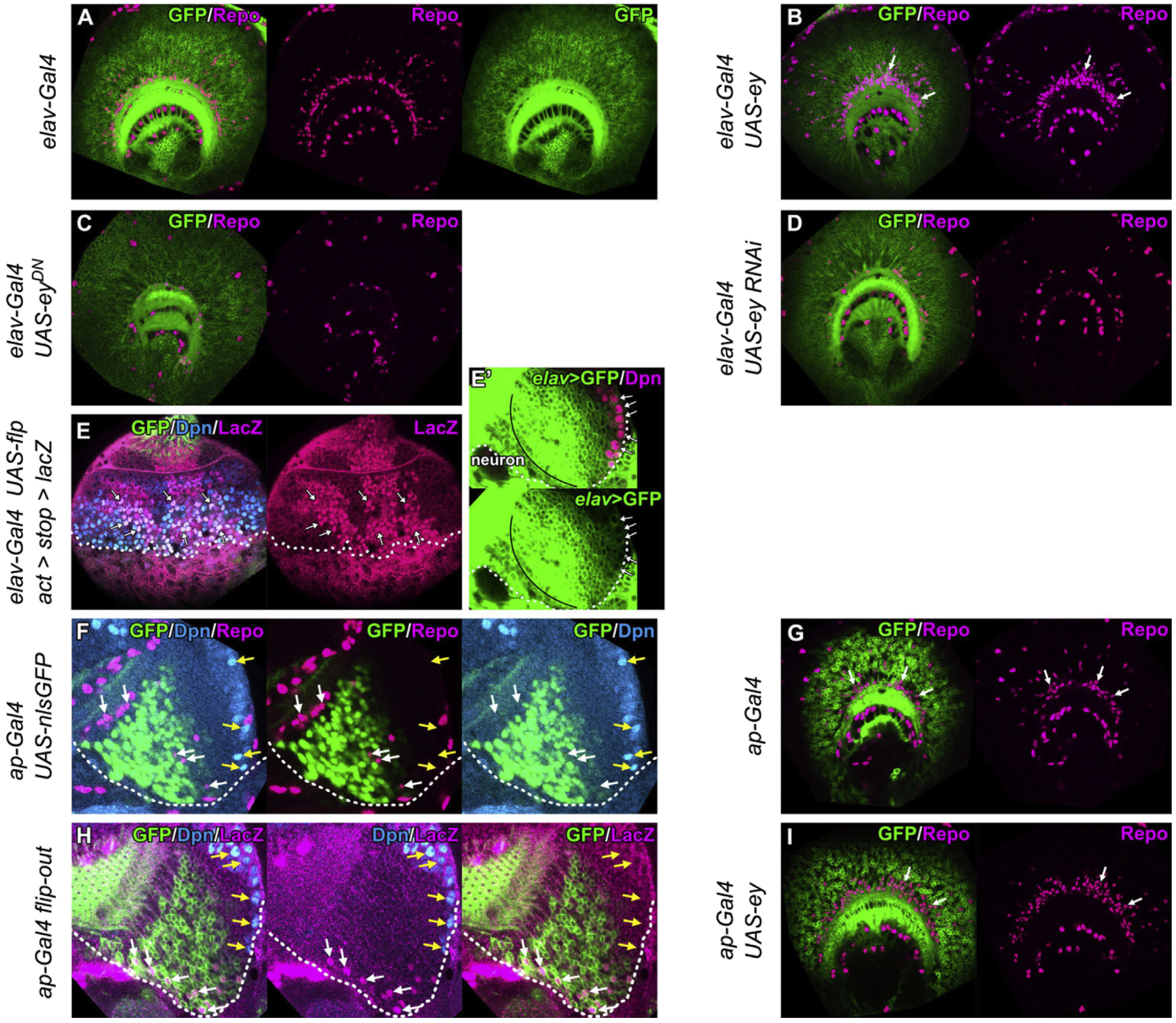

Fig. 5. Neuronal expression of *ey* is involved in the production of MNGs. Lateral (A–D, G, I; anterior to the top and dorsal to the right, same orientation as in Fig. 1A) and horizontal views (E-F, H; lateral to the top and anterior to the right, same orientation as in Fig. 1A′). (A) *elav-Gal4* expression visualized with CD8GFP (green) is found in the medulla neurons. (B) A dramatic increase in MNGs (magenta) is observed in the innermost area of medulla primordium by over-expression of *ey* under the control of *elav-Gal4* (green) as indicated by arrows. (C) MNGs (magenta) are significantly decreased by over-expression of *ey* dominant negative form under the control of *elav-Gal4* (green). (D) MNGs (magenta) are significantly decreased by expression of *ey* RNAi under the control of *elav-Gal4* (green). (E) Lineages of *elav-Gal4*+ (green) cells are labeled with LacZ (magenta). NBs (visualized with Dpn; blue) are labeled with LacZ (arrows). (E′) *elav-Gal4* is expressed in medulla neurons and NBs (visualized with Dpn; magenta). The intensity of GFP signal in NBs (white arrows) is much weaker than that in the neurons (black lines). (F-G) *ap-Gal4* visualized with nlsGFP (F) or CD8GFP (G) is specifically expressed in medulla neurons, but not in MNGs (magenta, white arrows) or NBs (visualized with Dpn; blue, yellow arrows in F). (H) Lineages of *ap-Gal4*+ (green) cells are labeled with LacZ (magenta). Although neurons are labeled with LacZ (magenta, white arrows), none of NBs (visualized with Dpn; blue, yellow arrows) is labeled. (H) The number of MNGs (magenta) is dramatically increased by overexpression of *ey* under the control of *ap-Gal4* as indicated by arrows.

UAS-CD8GFP; *UAS-dicer2*. Fig. 4F and G: *FRT40A* or *Df(2)132 FRT40A* was crossed to *hs-flp*; *FRT40A ubi-GFP* (37 °C for 60 min at first or second instar larvae). Fig. S4D-E: *UAS-ey* RNAi lines ($ey^{KK107100}$ or $ey^{HMS00489}$) were crossed to *UAS-dicer2*; *gcm-Gal4 CD8GFP*. Fig. 5B-C and S5C-D: *elav-Gal4 UAS-CD8GFP*; $tub\text{-}Gal80^{ts}$ was crossed to either *UAS-ey* or $UAS\text{-}ey^{DN}$ and raised at 17 °C to suppress lethality. The larvae were then raised at 30 °C for 24 or 40 h prior to dissection to allow transgene expression. Figs. 5D, S5E and S5F: *elav-Gal4 UAS-CD8GFP*; *UAS-dicer2* was crossed to *UAS-ey* RNAi lines ($ey^{KK107100}$ or $ey^{HMS00489}$) at 30 °C to knock down *ey*. Figs. 5I, S5G, S6B, S6D, S6F and S8B: $tubGal80^{ts}$; *ap-Gal4 UAS-CD8GFP* was crossed to *UAS-ey* and raised at 17 °C to suppress lethality. The larvae were then raised at 30 °C for 40 h prior to dissection to allow transgene expression.

2.3. Immunohistochemistry

Immunohistochemistry was performed as previously described (Hasegawa et al., 2011). The following primary antibodies were used: guinea pig anti-Dll (1:2000; Richard Mann), guinea pig anti-Dpn (1:1000; James Skeath), rabbit anti-Dpn (1:1000, Bier et al., 1992), guinea pig anti-Bsh (1:1600, Hasegawa et al., 2011), rabbit anti-D (1:1000, Soriano and Russell, 1998), rat anti-Drf (1:4000, Hasegawa et al., 2011), and chicken anti-lacZ (1:2000; abcam). Rabbit anti-Tll (1:200), guinea pig anti-Slp (1:200), and guinea pig anti-Run (1:1000) were obtained from the Asian Distribution Center for Segmentation Antibodies. Rabbit anti-PH3 (1:1000). Rabbit anti-cleaved Caspase3 (1:100). Mouse anti-BrdU (1:100). Rat anti-Elav (1:100), rat anti-Ncad (1:20), mouse anti-Repo (1:10), and mouse anti-Ey (1:10) antibodies were obtained from the Developmental Studies Hybridoma Bank. The secondary antibodies used were anti-mouse Cy3, anti-mouse Cy5, anti-mouse FITC, anti-rat Cy3, anti-rat Dylight 649, anti-guinea pig Cy5, anti-guinea pig Dylight 649, anti-guinea pig Cy3, anti-chicken Alexa Fluor 647, and anti-rabbit FITC (Jackson ImmunoResearch Laboratories); and anti-rat Ax647, anti-rat Ax488, and anti-rabbit Ax546 (Invitrogen). The images were processed using the Zeiss LSM image browser and Adobe Photoshop.

3. Results

3.1. Neuroblasts in outer proliferation center produce medulla neuropile glial cells

As we reported previously, the larval developing medulla is subdivided into concentric zones that are characterized by the expression of four genes encoding conserved transcription factors: *hth*, *brain-specific homeobox* (*bsh*), *runt* (*run*), and *drifter* (*drf*) (Fig. 1A and A′) (Hasegawa et al., 2011; Sato et al., 2013). The MNGs are situated in the innermost area of the developing medulla cortex adjacent to the medulla neuropile (Fig. 1A–C′) (Hasegawa et al., 2011). It has been reported that MNGs originate from the medial edge of the outer proliferation center (OPC), adjacent to the border between the optic lobe and central brain, and that they are likely to migrate radially toward the medulla neuropile (Colonques et al., 2007; Soustelle and Giangrande, 2007). Our previous observation also supports this idea through a different approach that visualizes the progeny of NBs using *dpn-Gal4*, a NB-specific Gal4 driver (Hasegawa et al., 2011). In *dpn-Gal4 UAS-GFP* flies, the progeny of NBs inherit Gal4 and GFP protein, which is then gradually lost due to degradation. Therefore, the amount of GFP protein is higher in cells that are produced later and lower in those that are produced earlier. In the previous study, we observed a high level of GFP in the MNGs (Hasegawa et al., 2011). We confirmed this observation by expressing a membrane-bound form of GFP under the control of *dpn-Gal4* (Fig. 1D). The GFP signals in the Repo+ MNGs were stronger than those found in the Drf+ cells (Drf is a marker of the later born neurons; Fig. 1A–C′), suggesting that the MNGs are produced from the NBs in OPC (OPC-NBs) after the production of the Drf+ neurons. To examine how long GFP protein remains in the progenies of each NB, we used *UAS-flp* under the control of *dpn-Gal4*, which induces FLP-FRT recombination in the progenies of each NB (Fig. S1A). The lineages of the *dpn-Gal4*+ NBs are constitutively labeled by LacZ under the control of *actin* > *stop* > *lacZ*. Among LacZ+ Repo- cells, GFP- cells were located at the inner region while GFP+ cells were located at the outer region, suggesting that GFP remains only in the later born outer neurons within the pool of lacZ+ cells derived from *dpn-Gal4*+ NBs. Similarly, LacZ+ Repo+ glial cells located in the innermost area are most likely produced at later stage.

To further confirm that MNGs derive from OPC-NBs, we performed lineage analyses of the NBs using the FLP-FRT recombination system. We first created somatic mosaic clones in the NBs using *SOP-flp*, in which *flp* expression is under the control of the binding targets of the proneural transcription factors (Shimono et al., 2014). Because the differentiation of neuroepithelial cells (NEs) into NBs is induced by the expression of proneural transcription factors, *SOP-flp* is expected to induce FLP-FRT recombination in NEs and NBs. The combination of *SOP-flp, AyGal4* (a flip-out Gal4 construct), and *UAS-CD8GFP* visualizes a subpopulation of NBs and their progeny. As expected, the mosaic clone lineages of NBs contained MNGs (Fig. 1E). As a second approach, we used *UAS-flp* under the control of *optix-Gal4* (Fig. 1B), which induces FLP-FRT recombination in the dorsal and ventral regions of the OPC (Fig. 1F). The lineages of the *optix-Gal4*+ NBs are permanently labeled by lacZ. As expected, we observed LacZ expression in the MNGs, as well as in medulla neurons and NBs (Fig. 1F). Importantly, LacZ expression was not detected in the MNGs found in the *optix-Gal4* negative region, suggesting that the MNGs produced in the dorsal or ventral sectors of the OPC do not migrate into the central part of the medulla primordium. Thus, MNGs migrate in a radial direction from the surface of the OPC to the medulla neuropile as previously suggested (Colonques et al., 2007; Soustelle and Giangrande, 2007; Hasegawa et al., 2011; Li et al., 2013a), but not in a tangential direction.

3.2. eyeless/Pax6 is necessary and sufficient for the production of MNGs

The expression pattern of *dpn-Gal4* suggests that the medulla NBs gain gliogenic competence during development. We and another group demonstrated that the competence of the NBs for medulla neuronal diversity is regulated by transcription factors that are temporally and sequentially expressed in the NBs (Li et al., 2013a; Suzuki et al., 2013). Because these factors regulate the temporal windows of the NBs to produce specific types of medulla neurons, it is possible that they also regulate the transition from neurogenesis to gliogenesis. We therefore asked whether the temporal transcription factors (Hth, Klu, Ey, Slp, and D) (Suzuki et al., 2013; Suzuki and Sato, 2014) are involved in gliogenesis and found that *ey* plays essential roles in gliogenesis in the medulla.

To examine the roles of Ey in the production of MNGs, we performed loss-of-function analyses of Ey using RNAi and mutant clones. We first knocked down *ey* by expressing an *ey* RNAi construct under the control of *pxb-Gal4*, which is expressed in the anterior half of the medulla primordium including NBs (Figs. 1B and 2A) (Suzuki et al., 2013). As described previously, Ey expression was eliminated by two independent RNAi strains (data not shown). The number of MNGs visualized with Repo expression was dramatically decreased in the region expressing *ey* RNAi (Fig. 2B). Elav expression appeared normal (data not shown),

suggesting that the loss of MNGs was not caused by non-specific defects, such as cell cycle arrest in the NBs. We obtained identical results by using two independent RNAi strains and two independent Gal4 drivers (Fig. S2A–D).

We then examined whether gliogenesis is also affected in the *ey* mutant clones. Wild type control clones frequently contained Repo+ glial cells in the medulla cortex (Fig. 2C; Repo+ cells in 50 out of 125 clones). By contrast, *ey* mutant clones never contained glial cells (Fig. 2D; Repo+ cell in 0 out of 45 clones), suggesting that *ey* function is indeed necessary for the production of MNGs.

To examine whether Ey is sufficient to induce the production of MNGs, we performed gain-of-function analysis. When Ey was ectopically expressed under the control of *pxb-Gal4*, the number of MNGs visualized with Repo expression was dramatically increased (Fig. 2E; $n=58/60$). Note that the increase in the number of glial cells is not observed in the *pxb-Gal4* negative areas, suggesting again that the MNGs do not migrate in a tangential direction. In addition, the number of medulla neurons visualized with Elav expression was decreased in the inner most area of the medulla cortex in the *pxb-Gal4*+ region (Fig. 2F; $n=12/12$), suggesting that neurogenesis in this region was converted to gliogenesis by ectopic Ey expression. By contrast, Elav expression was also observed in the innermost region at the expense of Repo expression by *ey* RNAi (Fig. S2C and D), suggesting that gliogenesis in this region was converted to neurogenesis by suppression of Ey expression. The above results strongly suggest that *ey* is both necessary and sufficient for the production of MNGs.

3.3. *Ey is expressed in the NBs of various temporal windows and controls gliogenesis*

To investigate the molecular mechanisms that regulate MNG production by Ey, we first examined the expression pattern of Ey in the medulla primordium. Strong Ey expression is detected in middle-aged NBs as reported previously (Fig. 3A) (Morante et al., 2011; Li et al., 2013a; Suzuki et al., 2013). In addition to the middle-aged NBs, Ey is weakly expressed in the older-aged NBs medially situated at the border between the optic lobe and central brain (Fig. 3B). Ey expression in NBs can be separated into two phases; a strong phase in the middle-aged NBs (strongly Ey+) and a weak phase in the older-aged NBs (weakly Ey+). We also found that weak Ey is occasionally observed in *gcm*+ differentiating glial cells (Fig. 3A and B′).

The expression pattern of *ey-Gal4* (OK107) is markedly similar to that of *dpn-Gal4* (Fig. 1D and H), and strong GFP expression driven by *ey-Gal4* is detected in both the MNGs and Drf neurons (Fig. 1G). Because Ey expression was rarely detected in the MNGs themselves (Fig. 3A–C), the GFP signals in the MNGs are thought to be due to Gal4 and/or GFP protein inherited from the Ey+ NBs. We also performed lineage analysis using *UAS-flp*, *actin > stop > lacZ*, and *ey-Gal4*. As expected, LacZ expression was detected in the MNGs (Fig. S1B and C).

However, it is unlikely that the MNGs are produced in the temporal window of strongly Ey+ NBs because MNG precursor cells visualized with *gcm*-lacZ, an early marker for differentiating glial cells, were not observed in the strongly Ey+ lineages but rather in the following temporal window situated in the medial most area of the medulla cortex such as Tailless (Tll)+ or weakly Ey+ NBs (Fig. 3A, D; Li et al., 2013a). We also found that MNGs were included in the lineages of the Tll+ NBs (Fig. 3E). These observations are consistent with the idea that the timing of gliogenesis is specified in the older aged NBs located at the medial edge of the developing medulla (Li et al., 2013a). Because Ey+/gcm+ NBs were occasionally observed in the medial edge of the medulla (Fig. 3A and B′), Ey expression in the middle aged NBs may indirectly induce the production of MNGs by regulating the expression of other temporal transcription factors. As previously reported, the temporal transcription factors (Hth, Klu, Ey, Slp, D, Tll) regulate each other to produce sequential changes in their expression in NBs, and these changes contribute to the sequential production of various types of neurons (Li et al., 2013a; Suzuki et al., 2013). For example, Ey activates Slp expression, while Slp activates D expression. Thus, if strong Ey expression in NBs is important for the production of MNGs, it is possible that Ey indirectly regulates MNG production through the induction of Slp, D and Tll.

However, MNGs were normally observed in *slp* mutant clones (Fig. S3A; Repo+ cells were found in 44 out of 73 clones) and in the region ectopically expressing Slp (Fig. S3B; $n=12/12$), suggesting that *slp* is not involved in the regulation of MNG production. In contrast, MNGs were significantly lost in *D* mutant clones (Fig. S3C; Repo+ cells were found in 17 out of 142 clones), suggesting that Ey may induce the expression of D independently of Slp and consequently trigger the production of MNGs, though MNG production was not affected in the region ectopically expressing D (Fig. S3D; $n=16/16$). Note that the *D* mutant clone still contained MNGs and the phenotype was less prominent compared to *ey* mutant clones, which contained no MNG (Figs. 2D and S3C), suggesting that Ey may also control gliogenesis via additional D independent mechanisms.

MNGs were normally observed in *tll* mutant clones (Fig. S3E; Repo+ cells were found in 86 out of 151 clones), suggesting that *tll* is not involved in the *ey*-mediated regulation of MNG production. Although MNGs were significantly reduced in the region ectopically expressing Tll (Fig. S3F; $n=17/17$), this phenotype is opposite to the expected role of *tll* to induce gliogenesis. These results suggest that Ey regulates MNG production via multiple mechanisms including indirect regulation via D and direct regulation by weak Ey expression in the medial NBs. Note that no MNG was found in *ey* mutant clones (Fig. 2D). However, gliogenesis was not completely blocked in clones mutant for other temporal transcription factors. Thus, Ey is the most essential component of gliogenesis among the temporal transcription factors expressed in the medulla NBs.

3.4. *eyeless/Pax6 controls the production of MNGs via glial cells missing*

During Ey-dependent production of MNGs, Ey may activate a factor that directly regulates the production of MNGs. As a candidate for such a factor, we focused on *glial cells missing* (*gcm*), which is known as a master regulator for glial cell differentiation in the embryonic CNS (Hosoya et al., 1995). As reported previously, *gcm* expression is detected in the MNGs (Colonques et al., 2007), and its function is required for MNG production (Soustelle and Giangrande, 2007). Therefore, we examined whether Ey regulates *gcm* expression using a *gcm-lacZ* enhancer trap line. In agreement with the previous reports, *gcm* expression was observed in the MNGs (Fig. 4A). When Ey expression was suppressed by RNAi under the control of *pxb-Gal4*, *gcm* expression was diminished coincidently with the loss of MNGs (Fig. 4B; $n=8/11$, S4A–C). By contrast, *gcm* expression was induced by the overexpression of Ey under the control of *pxb-Gal4* (Fig. 4C; $n=8/8$). Note that a subset of *gcm*+ cells do not co-express Repo, which is consistent with the fact that *gcm* is the earlier marker for glial cells.

We next performed functional analyses of *gcm* in the production of MNGs. Although two *gcm* genes (*gcm* and *gcm2*) exist in the fly genome, only *gcm* is expressed in MNGs, while *gcm2* is not (Soustelle and Giangrande, 2007). Thus, we focused on the function of *gcm* in the production of MNGs. Whereas a dominant-negative form of *gcm* was used to suppress its function in the previous paper (Soustelle and Giangrande, 2007), we used null

mutant and RNAi of *gcm* to examine its function. Because it is not clear whether the dominant negative form of *gcm* specifically inactivates *gcm* functions. We examined the effects of *gcm* RNAi and *gcm* mutant clones on the number of MNGs. When *gcm* was knocked down by RNAi under the control of *pxb-Gal4*, the number of MNGs visualized with Repo expression was significantly decreased (Fig. 4D; $n=14/16$). The effect of *gcm* RNAi was also examined using another Gal4 driver, *optix-Gal4*, whose expression pattern is complementary to that of *pxb-Gal4* (Figs. 1B and S2B). Again, the number of MNGs was dramatically decreased (Fig. 4E; $n=22/23$). We performed these analyses using two independent RNAi strains designed to target distinct regions of the *gcm* transcript and obtained similar results (Fig. S4F and G). Similar results were also obtained by generating *gcm*-mutant clones. As shown in Fig. 2, control wild type clones frequently contain MNGs (Fig. 4F; Repo+ cells were found in 74 out of 143 clones). By contrast, no MNG was found in the *gcm* mutant clones (Fig. 4G; $n=56$). These results suggest that *gcm* is necessary for the production of MNGs.

To examine whether *gcm* is sufficient to induce the production of MNGs, we performed gain-of-function analysis. When *gcm* was ectopically expressed under the control of *pxb-Gal4*, the number of MNGs visualized by Repo expression was increased (Fig. 4H; $n=13/15$). However, the phenotypic severity was milder compared to those observed in Ey overexpression (Fig. 2E).

3.5. Possible roles of neuronal Ey in the gliogenesis

The above results suggest that Ey control gliogenesis by multiple pathways: indirect regulation via D by strong Ey expression in the middle aged NBs and direct regulation by weak Ey expression in the oldest NBs. In the followings, we propose a possibility of an additional mechanism.

Besides expression in NBs, Ey expression is also observed in neurons such as Run+ neurons and neurons within the concentric zones specified by Drf expression, although no overlap with Drf expression was observed (Fig. 3C) (Suzuki et al., 2013). Ey and Drf show complementary expression patterns in medulla neurons. As previously reported, neurons regulate cell fate decisions by non-autonomously affecting the stem cells via cell-extrinsic factors in mammals (Barnabé-Heider et al., 2005; Namihira et al., 2009; Seuntjens et al., 2009). To examine whether a similar strategy is also used in the medulla primordium, Ey was overexpressed under the control of *elav-Gal4*, a putative neuron-specific Gal4 driver (Fig. 5A and B). Surprisingly, a dramatic increase in MNGs was also induced via the neuronal expression of Ey (Fig. 5B; $n=12/13$). By contrast, a significant loss of MNGs was observed when a dominant-negative form of Ey was overexpressed under the control of *elav-Gal4* (Fig. 5C; $n=20/20$). A significant loss of MNGs was also caused by Ey RNAi under the control of *elav-Gal4* (Fig. 5D, $n=13/15$). Taken together, these results suggest that neuronal Ey contributes to the production of MNGs.

To validate these assays, we examined whether *elav-Gal4* is a completely neuron-specific driver, or not, by lineage analysis using *UAS-flp* and *act > stop > lacZ,* and found that NBs were labeled by LacZ (Fig. 5E). This result suggests that *elav-Gal4* is weakly but definitely expressed in NBs and we confirmed it is not completely neuron-specific in the optic lobe (Fig. 5E′) as observed in embryos (Berger et al., 2007). Therefore, we used *ap-Gal4* as another neuron-specific driver (Figs. 1B, 5F and G) to examine roles of neuronal Ey in the production of MNGs. Note that the signals of nuclear localized GFP under the control of *ap-Gal4* are not detected in glial cells and NBs (Fig. 5F). As shown by the lineage analysis, *ap-Gal4* is indeed neuron-specific in the developing medulla (Fig. 5H). The dramatic increase in MNGs was also observed upon overexpression of Ey under the control of *ap-Gal4* (Fig. 5I; $n=14/19$). Since Ey expression was not observed in *ap-Gal4*+ neurons, neuron-specific loss-of-function analysis of Ey was not possible by using *ap-Gal4*. However, above results suggest that neuronal expression of Ey contributes to the production of MNGs. Since neuronal Ey expression does not convert Ey-positive neurons to MNGs in wild type brains and the number of *ap-Gal4* positive cells was not significantly reduced by Ey overexpression, it is unlikely that *ap-Gal4* positive cells were converted to MNGs (Fig. 5I).

In the above experiments, the identification of MNGs solely relies on Repo expression, a general glial cell marker. We confirmed that MNGs are indeed affected by using Distal-less (Dll) as a marker for MNGs. In wild type animals, Dll is expressed in the MNGs located adjacent to the medulla neuropile but not in other glial cells within the medulla cortex (Fig. S5A and B). When Ey was overexpressed, the number of Repo+/Dll+ cells increased in the innermost area of the medulla cortex (Fig. S5C, G). In addition, the number of Repo+/Dll+ cells was significantly decreased by expressing a dominant negative form of Ey or *ey* RNAi (Fig. S5D–F).

It is possible to speculate that Ey expressed in neurons may non-autonomously affect the expression of temporal transcription factors in the NBs to indirectly regulate gliogenesis. However, the expression of Slp, D and Tll in NBs was not affected by expressing Ey under the control of *ap-Gal4* (Fig. S6). These results suggest that neuronal Ey does not regulate MNG production by regulating the expression of temporal factors such as Slp, D and Tll in NBs.

Ey expression in neurons may non-autonomously regulate the cell cycle of glia-producing NBs to indirectly control gliogenesis, because a subset of gcm+ NBs are mitotically active (Fig. S7A). We examined this idea by visualizing cell proliferation in NBs by using BrdU and PH3 antibody in loss-of and gain-of-function conditions of *ey*. However, we could not find any change in NB proliferation in OPC. The BrdU+ or PH3+ cells were normally observed in the region expressing *ey* or *ey RNAi* under the control of *pxb-Gal4* (data not shown). In addition, we also examined the possibility that Ey regulates cell death of the MNGs by the induction of p35 expression, because caspase-3 is activated in a subset of *gcm*+ cells in wild type OPC (Fig. S7B). However, cell death inhibition did not affect the number of MNGs (data not shown).

4. Discussion

The present results support the hypothesis that the OPC, in addition to the glia precursor cells located in the posterior region of the medulla primordium, provides glial cells (Dearborn and Kunes, 2004; Colonques et al., 2007; Soustelle and Giangrande, 2007). Our results clearly demonstrate the relative birth-order of MNGs and medulla neurons. When GFP expression was driven by *dpn-Gal4*, a stronger signal was detected in the MNGs than in the most of medulla neurons (Fig. 1D and S1A). This result suggests that the MNGs are produced at a later stage than the medulla neurons are. A previous study predicted that the MNGs are produced from the oldest Tll+ OPC-NBs because these NBs are located adjacent to the *gcm*+ putative glial precursor cells (Li et al., 2013a). This is consistent with our present lineage analysis because MNGs are contained in the lineages of Tll+ NBs (Fig. 3E). We also observed *gcm* expression in the Tll+ NB lineages located at the border between the optic lobe and the central brain (Fig. 3D), implying that the gcm+ NBs are the glioblasts. Taken together, our results support the previous prediction that medulla NBs become gliogenic over time to produce the MNGs following neurogenesis (Li et al., 2013a).

Pax6 is well known as a neurogenic transcription factor that regulates neuronal specification, neuronal migration, and the projection patterns of axons in mammals (Simpson and Price, 2002). Previously, we and other groups revealed that Ey also plays these neurogenic functions in the *Drosophila* central nervous

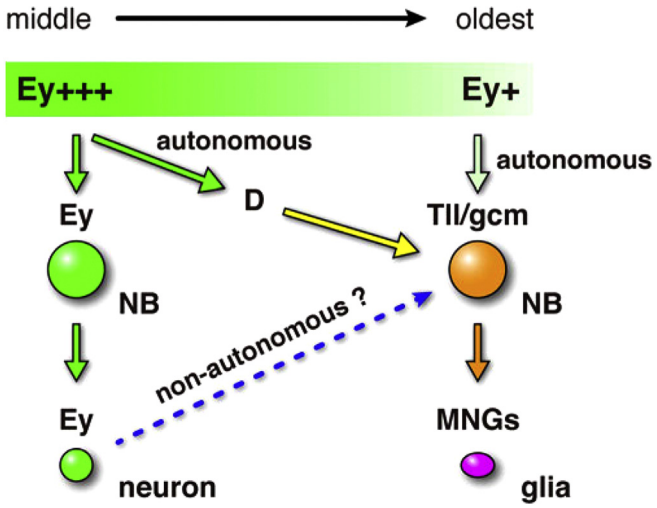

Fig. 6. Multiple pathways by which Ey regulates gliogenesis. Ey Expression in NBs is separated into two phases; a strong phase (Ey+++) in the middle-aged NBs and a weak phase (Ey+) in the older NBs. In the temporal window of Ey+++ phase, Ey neurons (green) are produced by the NBs. On the other hand, in the window of Ey+ phase (also Tll+) NBs begin to produce MNGs (magenta) that migrate toward the center of the medulla. Ey cell-autonomously regulates the expression of other temporal factor(s) such as D in NBs, in turn D may activate the expression of Tll (and also that of Ey weakly) to induce the initiation of gliogenesis. The neuronal expression of Ey would also contribute to the gliongenesis by indirect ways.

system (Morante et al., 2011; Bayraktar and Doe, 2013; Li et al., 2013a; Suzuki et al., 2013). In the present study, we revealed that *ey* is also involved in gliogenesis in the medulla primordium. Although the medulla contains several types of glial cells: medulla glia, MNGs, perineurial glia, and subperineurial glia (Edwards et al., 2012), MNGs were specifically affected by the manipulation of *ey* expression (Fig. 2 and S5). It is known that *Pax6* regulates the proliferation, maturation, and migration of astrocytes in mammals (Sakurai and Osumi, 2008). Because MNGs seem to differentiate into astrocyte-like glial cells (Edwards et al., 2012), it is possible that the *ey*/*Pax6*-dependent regulation of astrocyte development is evolutionarily conserved. However, the molecular mechanisms that underlie this regulation in mammals and *Drosophila* appear different. *Pax6* is expressed in the astrocytes and their progenitors to regulate their development as a cell-intrinsic factor in mammals, whereas *ey* expression was not observed in the MNGs in *Drosophila* (Fig. 3A–C). Because *ey* RNAi under the control of *gcm-Gal4* did not affect the production of MNGs (Fig. S4D-E), *ey* is unlikely to regulate MNG development in a cell-intrinsic manner in differentiating glial cells.

The above observation does not conflict with the loss-of- and gain-of-function analyses of *ey* in subdomains of the medulla, which suggest that *ey* regulates gliogenesis in a cell-autonomous manner (Fig. 2B–E, S2C and D). Under the control of *pxb-Gal4* and *optix-Gal4*, *ey* function is manipulated in NBs. Thus, *ey* expression in NBs, but not in differentiating glial cells, autonomously controls the production of glial cells.

Ey is expressed strongly in the middle-aged NBs (Ey+++) and weakly in the older-aged NBs (Fig. 6). Strong Ey expression in NBs induces the temporal window transition of Ey-Slp-D (Li et al., 2013a; Suzuki et al., 2013). Although *slp* mutant clones did not show defects in gliogenesis, number of MNGs was reduced in *D* mutant clones (Fig S3C). Thus, strong Ey expression in NBs may control the gliogenesis by regulating D, but not Slp, in NBs. However, MNGs were not completely lost in *D* mutant clones in contrast to *ey* mutant clones (Figs. 2D and S3C), suggesting that Ey may also regulate gliogenesis via distinct D independent mechanisms. Since weakly Ey+ NBs are located near *gcm*+ cells at the medial edge of the developing medulla (Fig. 3B), such weak expression of Ey may play direct roles in the initiation of MNG production. However, Ey expression in these medial NBs is very weak and is only occasionally detected in gcm+ NBs. Moreover, *ey* RNAi under the control of *gcm-Gal4* did not affect the production of MNGs (Fig. S4D-E). These results imply the presence of additional mechanisms by which Ey regulates MNG production.

Surprisingly, the neuronal expression of *ey* was sufficient for the production of MNGs (Fig. 5I). Ectopic glial cells were found adjacent to Gal4 expressing cells (Fig. S8). Recent studies indicate that neurons positively regulate cell fate decisions in the neuronal stem cells via cell-extrinsic signalings, such as JAK-STAT, Notch, NTF, and FGF, in mammals (Barnabé-Heider et al., 2005; Namihira et al., 2009; Seuntjens et al., 2009). Thus, Ey+ neurons may also contribute to the gliogenesis via unidentified secreted factors or membrane-bound ligands as observed in mammals.

In conclusion, we propose that Ey/Pax6 contributes to the control of gliogenesis through the induction of expression of *gcm* and other genes. It will be interesting to determine the extent to which the *ey*/*Pax6*-dependent regulation of astrocyte-like glial cell development is conserved between insects and mammals.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.12.004.

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