Drosophila hedgehog signaling and *engrailed-runt* mutual repression direct midline glia to alternative ensheathing and non-ensheathing fates

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SUMMARY

The *Drosophila* CNS contains a variety of glia, including highly specialized glia that reside at the CNS midline and functionally resemble the midline floor plate glia of the vertebrate spinal cord. Both insect and vertebrate midline glia play important roles in ensheathing axons that cross the midline and secreting signals that control a variety of developmental processes. The *Drosophila* midline glia consist of two spatially and functionally distinct populations. The anterior midline glia (AMG) are ensheathing glia that migrate, surround and send processes into the axon commissures. By contrast, the posterior midline glia (PMG) are non-ensheathing glia. Together, the *Notch* and *hedgehog* signaling pathways generate AMG and PMG from midline neural precursors. *Notch* signaling is required for midline glial formation and for transcription of a core set of midline glial-expressed genes. The Hedgehog morphogen is secreted from ectodermal cells adjacent to the CNS midline and directs a subset of midline glia to become PMG. Two transcription factor genes, *runt* and *engrailed*, play important roles in AMG and PMG development. The *runt* gene is expressed in AMG, represses *engrailed* and maintains AMG gene expression. The *engrailed* gene is expressed in PMG, represses *runt* and maintains PMG gene expression. In addition, *engrailed* can direct midline glia to a PMG-like non-ensheathing fate. Thus, two signaling pathways and *runt-engrailed* mutual repression initiate and maintain two distinct populations of midline glia that differ functionally in gene expression, glial migration, axon ensheathment, process extension and patterns of apoptosis.

KEY WORDS: CNS midline, *Drosophila*, Engrailed, Glia, Hedgehog, Runt

INTRODUCTION

Diverse glial cell types populate the CNS. Different kinds of glia can ensheath axons, support neuronal function, act as phagocytes and constitute a blood-brain barrier. Even closely related glial cell types have different functions. For example, vertebrate Schwann cells can develop into two distinct populations: those that myelinate axons and those that are non-myelinating (Mirsky et al., 2008). *Drosophila* possess a set of glia that reside at the midline of the ventral nerve cord. These midline glia (MG) play multiple roles in development, including: (1) ensheathing commissural axons, (2) directing axon guidance and muscle cell migration, and (3) controlling formation of several embryonic cell types via cell signaling pathways (Crews, 2003). Similarly, the vertebrate spinal cord and brain have a specialized set of glial-like midline cells, the floor plate, that also ensheath commissural axons and use cell signaling proteins to direct axon guidance and pattern the spinal cord (Campbell and Peterson, 1993; Yoshioka and Tanaka, 1989). Although relatively little is known regarding how floor plate cells ensheath and interact with crossing axons, the *Drosophila* MG have been well-characterized, both at the cellular and molecular levels. In *Drosophila*, the MG also exist as two functionally distinct

populations, anterior MG (AMG) and posterior MG (PMG), which have different gene expression, migratory and ensheathment properties (Dong and Jacobs, 1997; Kearney et al., 2004). Given the increasing awareness of the significance of glia to CNS function (Allen and Barres, 2009), it is important to understand how diverse glial cell types are generated. In this paper, we describe the regulatory mechanisms that establish AMG and PMG cell fates, how those differences are maintained and how they impact MG function.

The *Drosophila* CNS midline cells are an outstanding system to study how glia and neurons acquire their identities. The midline cells, although small in number (22 cells per segment at the end of embryogenesis), nevertheless comprise motorneurons, interneurons, neurosecretory cells and two types of MG (AMG and PMG) (Wheeler et al., 2006). Midline neurons are derived from the median neuroblast (MNB) and five midline precursors (MPs) named MP1 and MP3-6. During stage 10, the 16 midline cells are arranged in three equivalence groups consisting of four to six MPs each: the MP1, MP3 and MP4 groups (the MP4 group gives rise to MP4-6 and the MNB). From these equivalence groups, *Notch* signaling directs the formation of MG (Menne and Klämbt, 1994; Wheeler et al., 2008). After MG formation, the differences in AMG and PMG become apparent; these include position, function, cell survival and differential gene expression (Fig. 1A). AMG arise in the anterior part of the segment and express high levels of *wrapper*, which encodes an immunoglobulin domain-containing GPI-linked membrane protein involved in MG-axonal adhesion (Noordermeer et al., 1998). During stage 12, AMG move internally (Fig. 1A,B) to contact commissural axons and those that are not in close proximity to the commissures undergo apoptosis (Bergmann et al., 2002). The surviving AMG then migrate posteriorly to ensheath the

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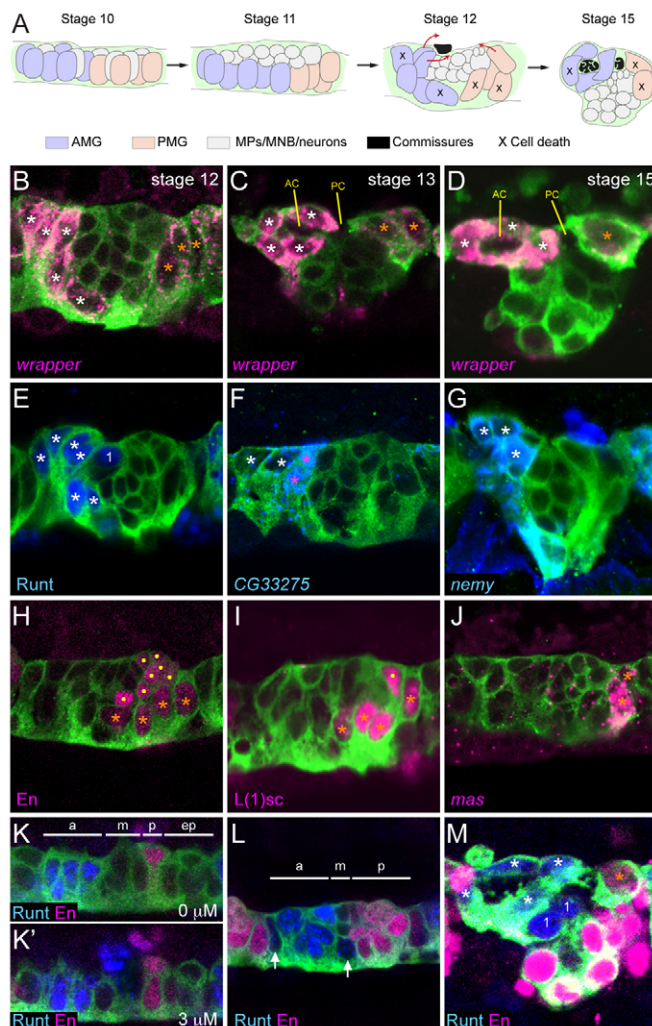
Fig. 1. Anterior midline glia (AMG) and posterior midline glia (PMG) differ in gene expression and origins. (**A**) Schematic summary of midline glia (MG) positioning and migration in sagittal views. The schematic depicts an idealized view; actual segments vary in MG number and position. Colored objects represent nuclei, and pathways of AMG and PMG migration are indicated at stage 12 by red arrows. MNB, median neuroblast; MP, midline precursor. (**B-M**) Fluorescent confocal images of single segments in sagittal view from *sim-Gal4 UAS-tau-GFP* embryos. Anterior is to the left and dorsal (internal) is up. White asterisks indicate AMG; orange asterisks, PMG; dots, midline neurons; '1', MP1 neurons. RNA (italicized) and antibody (non-italicized) stains and corresponding colors are indicated in the lower left corner of each panel except for anti-GFP staining (green) that is present in all images. (B) During stage 12, AMG (high levels of *wrapper* RNA) and PMG (low levels of *wrapper* RNA) are elongating and moving to the dorsal (internal-most) surface of the CNS. Midline neurons are the centrally located *wrapper⁻* cells flanked by *wrapper⁺* MG. (C) During stage 13, AMG migrate posteriorly above and below the anterior commissure (AC). PMG abut the posterior commissure (PC). (D) By stage 15, AMG completely surround the AC and are poised to ensheath the PC. Most PMG have undergone apoptosis; the remainder stay in contact with the PC. (E-G) Runt is present in all AMG and the MP1 neurons, whereas *CG33275* and *nemy* expression is restricted to AMG only. The AMG closest to the midline neurons and developing commissure (pink asterisks) have high levels of *CG33275*, compared with those that are more distant (white asterisks). (H-J) En and L(1)sc are present in all PMG and in a subset of midline neurons whereas *mas* is expressed in only one or two PMG in each segment. (K,K') Two focal planes (separated by 3 µm) of a stage 10 segment; Runt is present in four to five cells in the anterior cells (a), Runt and En are absent from the middle (m) and extreme posterior cells (ep), and En is present in two cells in the posterior (p). (L) During stages 10-11, Runt expands to additional MG (arrows) and the number of En⁺ cells increases (stage 11 is shown). The segment is divided into anterior (a) *runt⁺ en⁻*, middle (m) *runt⁻ en⁻* and posterior (p) *runt⁻ en⁺* domains. (M) In stage 15 MG, Runt and En are present in AMG and PMG, respectively.

two axon commissures in a stepwise fashion: first the anterior commissure (AC) at stages 13-15 (Fig. 1A,C,D) and then the posterior commissure (PC) at stages 16-17 [for a more detailed discussion, see Wheeler et al. (Wheeler et al., 2009)]. One aspect of ensheathment is the further subdivision of the commissures by glial projections (Stollewerk and Klämbt, 1997; Stork et al., 2009; Wheeler et al., 2009). These MG projections might enhance neuronal survival by increasing MG secretion of neurotrophins onto commissural axons (Zhu et al., 2008), and might also enhance MG survival by increased exposure to the axonal-derived *spitz* survival signal (Bergmann et al., 2002). In contrast to AMG, PMG arise in the posterior of the segment and express low levels of *wrapper* (Wheeler et al., 2006; Wheeler et al., 2008). PMG move internally (Fig. 1A,B; stage 12) and from stages 13-17 they abut the posterior commissure, but do not ensheath or extend projections (Fig. 1C,D). The function of PMG is unknown and all PMG undergo apoptosis by mid-stage 17 (Dong and Jacobs, 1997; Sonnenfeld and Jacobs, 1995).

Mechanistic insight into MG migration and ensheathment emerged from studies showing that the Wrapper protein is a heterophilic cell adhesion molecule present on MG that interacts with the Neurexin IV (Nrx-IV) transmembrane protein present on the surface of neuronal axons and cell bodies (Noordermeer et al., 1998; Stork et al., 2009; Wheeler et al., 2009). Genetic studies demonstrated that MG migration and ensheathment require both

Nrx-IV and *wrapper*. Thus, the ability of AMG, and the corresponding inability of PMG, to migrate along and ensheath axons might be due, in part, to the levels of Wrapper present on their surfaces. Given the importance of differences between AMG and PMG, we sought to understand the developmental and genetic basis of their divergent characteristics.

In this paper, we explore the differentiation of AMG and PMG. We demonstrate that Hedgehog (Hh), secreted from cells adjacent to the midline, directs MG in the posterior of the segment to become PMG. By contrast, MG in the anterior of the segment do not respond to Hh and become AMG. Hh functions both by repressing AMG gene expression and activating PMG gene expression. One of the targets of Hh in PMG is the *engrailed* (*en*) gene, which encodes a homeodomain protein. AMG gene expression is repressed by *en* in PMG, and *en* also directs MG, in part, to function as PMG. The RUNX-family transcription factor Runt is present in AMG, but not PMG. *runt* is required for AMG gene expression, functions by repressing *en* in AMG, and is required for the expression of at least one AMG-specific gene. Similarly, *en* represses *runt* in PMG. Thus, two transcriptional repressors, En and Runt, are partitioned into different populations of MG, and are key regulators in directing cell type-specific gene expression and function. This includes regulating levels of *wrapper* in MG that might lead to functional differences in MG migration and axon ensheathment.

MATERIALS AND METHODS

Drosophila strains

Drosophila mutant strains used included: *Df(2R)en^E^* (Gustavson et al., 1996), *Df(2R)en-A* (Gubb, 1985), *hh^{AC}^* (Lee et al., 1992), *hh^2^* (Nusslein-Volhard and Wieschaus, 1980), *runt^3^* (Gergen and Wieschaus, 1986), *runt^{29}^* (Wieschaus et al., 1984) and *Df(1)sc-B57* (Jimenez and Campos-Ortega, 1990). *Gal4* and *UAS* lines employed were: *sim-Gal4* (Xiao et al., 1996), *UAS-ci.VP16* (Larsen et al., 2003), *UAS-en* (Guillen et al., 1995), *UAS-VP16En* (Alexandre and Vincent, 2003), *UAS-hh* (Porter et al., 1996), *UAS-runt^{U15}^* (Tracey et al., 2000) and *UAS-tau-GFP* (Brand, 1995).

In situ hybridization, immunostaining and microscopy

Embryo collection, in situ hybridization and immunostaining were performed as previously described (Kearney et al., 2004). Digoxygenin-labeled antisense RNA probes for in situ hybridization were generated from either: (1) cDNA clones from the *Drosophila* Gene Collection (Open Biosystems, AL, USA) (*CG33275*: GM01778; *Fhos*: LD24110; *mas*: LP06006; and *wrapper*: GH03113) or (2) genomic DNA PCR-amplified using gene specific primers (*hh*, *nemy* and *ptc*). Primary antibodies used were: mouse MAb BP102 [Developmental Studies Hybridoma Bank (DSHB)], mouse and rat MAb anti-Elav (DSHB), mouse MAb anti-En (Patel et al., 1989), rabbit anti-GFP (Ab290, Abcam), guinea pig and rat anti-L(1)sc (S.B.S., unpublished), rabbit anti-Nrx-IV (Baumgartner et al., 1996), guinea pig anti-Runt (Kosman et al., 1998), mouse anti-Tau (Tau-2, Sigma) and mouse anti-Wrapper MAb 10D3 (DSHB) (Noordermeer et al., 1998). Secondary antibodies used were conjugated with AlexaFluor 488, 543, 568 and 647 (Invitrogen). Stained embryos were imaged on Zeiss LSM-PASCAL, LSM-510 and LSM-710 confocal microscopes. All images are from abdominal segments and are single optical planes derived from *z*-series stacks or single cuts taken from stacks that were rotated using the Zen 2008 software (Zeiss).

RESULTS

Differential gene expression of AMG and PMG

Insights into the functional differences between AMG and PMG can arise from identifying genes differentially expressed in AMG and PMG. In a previous in situ hybridization screen, we identified 54 genes expressed in MG (Kearney et al., 2004; Wheeler et al., 2006). Using fluorescence in situ hybridization and confocal microscopy, we analyzed the expression patterns of eight MG-expressed genes in the *sim-Gal4 UAS-tau-GFP* genetic background, in which the position and morphology of midline cells can be visualized. Each gene was differentially expressed in AMG and PMG during stage 12, a time when AMG-PMG differences are readily observed (Table 1). *wrapper* was expressed at high levels in AMG and at low levels in PMG (Fig. 1B-D). The genes *runt*, *CG33275*, *no extended memory* (*nemy*) and *Fhos* (Fig. 1E-G; not shown) were expressed in AMG, and *en*, *lethal of scute* [*l(1)sc*] and

masquerade (*mas*) (Fig. 1H-J) were expressed in PMG. Whereas *runt*, *nemy* and *Fhos* were expressed at comparable levels in all AMG, *CG33275* was expressed at high levels in a subset of AMG (Fig. 1F). Similarly, *en* and *l(1)sc* were expressed at comparable levels in all PMG, whereas *mas* was expressed in only one to two PMG (Fig. 1J). The high level of expression of *CG33275* in a subset of AMG, and expression of *mas* in a subset of PMG indicate that both AMG and PMG might also possess functionally distinct subtypes.

runt and *en* expression defines distinct midline domains

Prior to stage 10, both *runt* and *en* are expressed in pair-rule and segment polarity patterns in the ectoderm, including the corresponding midline cells (Bossing and Brand, 2006; Wheeler et al., 2006). During stages 10 and 11, the expression patterns of both *runt* and *en* change, becoming more expansive. At stage 10, *runt* was present in four to five midline cells and *en* in two midline cells (Fig. 1K,K′). These patterns are referred to as 'early *runt*' and 'early *en*' and they subdivide the midline into four regions: anterior (*runt^+^ en^−^*), middle (*runt^− en^−^*), posterior (*runt^− en^+^*) and extreme posterior (*runt^− en^−^*) (Fig. 1K,K′). Owing to *Notch* signaling, the MP1 and approximately four AMG arise from the *runt^+^ en^−^* region, and the MP3 and additional MG arise from the middle *runt^− en^−^* region (Wheeler et al., 2008). At stage 11, an additional, late phase of *runt* expression was initiated in two to three additional cells flanking the *runt^+^ en^−^* region (Fig. 1L). This includes the MG from the middle *runt^− en^−^* region, thus generating about six *runt^+^* AMG in the anterior of each segment. A second, late phase of *en* expression was activated in the extreme posterior *runt^− en^−^* cells that, in combination with the early *en^+^* cells, generated a single posterior *en^+^* region containing around eight cells (Fig. 1L). These *en^+^* cells give rise to PMG, MP4-6 and the MNB (Wheeler et al., 2008). Thus, by the end of stage 11, *runt* expression was present in all AMG and *en* expression was present in all PMG; this pattern of MG gene expression persisted throughout embryonic development with no overlap in expression (Fig. 1M).

hh signaling is a key regulator of midline glial cell fate

AMG and PMG reside at different positions along the anterior/posterior (A/P) axis, suggesting that segmentation genes direct their formation. Previous work indicated that *hh* function is required for late *en* expression in the posterior midline cells (Bossing and Brand, 2006), and we addressed whether *hh* is controlling aspects of PMG development. The *patched* (*ptc*) gene

Table 1. Genes differentially expressed in anterior midline glia (AMG) and posterior midline glia (PMG)

Gene	Function
High expression in AMG, low expression in PMG	
wrapper	Immunoglobulin domain membrane protein, Nrx-IV binding
Expressed in AMG only	
*CG33275**	Guanine nucleotide exchange factor
*Fhos**	Actin-binding protein
nemy	Carbon monoxide oxygenase
runt	Runt domain transcription factor
Expressed in PMG only	
en	Homeodomain transcription factor
l(1)sc	basic helix-loop-helix (bHLH) transcription factor
mas	Serine-type endopeptidase

*During stage 13, expression of *CG33275* and *Fhos* is at low levels in PMG.
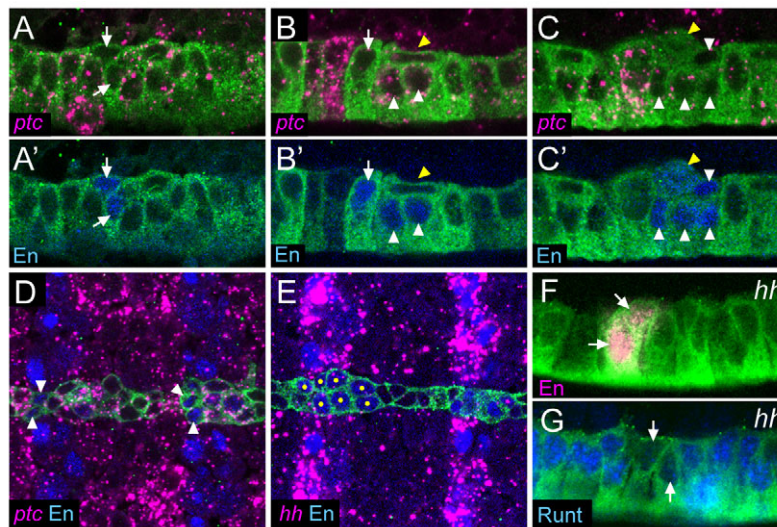
Fig. 2. *hh* activates *en* and represses *runt* expression. (A-G) Sagittal (A-C′,F,G) or horizontal (D,E) views of stage 10 *sim-Gal4 UAS-tau-GFP* embryos. **(A,A′)** Initially, En is present in two cells in each segment (arrows; referred to as early *en*) that do not express *ptc*. Expression of *ptc* is present in all other midline cells. **(B,B′)** Later, the En+ *ptc*– cells (arrow) elongate and En appears in cells just to the posterior (white arrowheads). The flattened En– MP4 (yellow arrowhead) has delaminated and resides internally and immediately posterior to the early *en*+ cells. **(C,C′)** At the end of stage 10, about eight cells are En+ (white arrowheads) and *ptc* is decreasing in these cells. Not all cells are shown in this focal plane. Expression of *en* is activated in the MP4 prior (yellow arrowheads) to its division. The tau-dense appearance of MP4 is indicative of cell division (Wheeler et al., 2008). **(D)** *ptc* is expressed in the midline cells flanking the early En+ cells (arrowheads). **(E)** During stage 10, *hh* and En colocalize in stripes in the non-midline ectoderm; *hh* is nearly absent in the midline cells. Yellow dots indicate En+ midline cells. **(F)** In *hh* mutant embryos, two En+ cells are present (arrows), but En is absent from posterior cells. **(G)** Runt is expanded to all but two cells (arrows) in each segment in stage 10 *hh* mutants.

encodes a receptor for Hh, and the presence of *ptc* expression is an indication that a cell can respond to Hh signaling (Chen and Struhl, 1996). In situ hybridization with a *ptc* probe revealed *ptc* expression at stage 10 in most midline cells, with the early *en*+ cells being an exception (Fig. 2A,B,D). As late *en* levels increased in posterior cells, *ptc* expression was reduced (Fig. 2B,C). This is likely to be due to the ability of *en* to repress *ptc* expression (Hidalgo and Ingham, 1990). At this time, *hh* was expressed in ectodermal stripes, but only at very low levels in midline cells (Fig. 2E). Thus, most midline cells were initially *ptc*+ and able to respond to *hh* signaling [as also noted by Bossing and Brand (Bossing and Brand, 2006)], and any *hh* signaling affecting the midline cells at stage 10 probably originates from cells adjacent to the midline.

The effects of *hh* signaling on *en* and *runt* MG expression were assessed in both *hh*^AC^ homozygous null and *hh*^AC^/*hh*^2^ mutant embryos and the phenotypes were the same. At late stage 10-early stage 11, only two *en*+ cells were present (Fig. 2F), instead of eight *en*+ cells in wild type (Fig. 1L, Fig. 2C′). These are likely to be the early *en*+ cells: they lined up with the *en* CNS stripe in both wild-type and *hh* mutant embryos, and in wild-type embryos, the early *en*+ cells were *ptc*– and thus unable to respond to *hh* signaling. By contrast, *runt* was expanded in all midline cells, except for two cells (Fig. 2G). The presence of *en* and *runt* was further examined in stage 12 *hh*^AC^ mutant embryos: *en* was absent from all MG, as identified by *wrapper* staining (Fig. 3A,B), and *runt* was present in all MG (Fig. 3D,E). These results indicate a requirement for *hh* in activating *en* and repressing *runt* expression in PMG.

Owing to the pleiotropy of *hh* mutants, misexpression experiments provide a more direct test than mutants of the ability of *hh* signaling to influence MG gene expression. Embryos that are *sim-Gal4 UAS-hh* express *hh* in all midline cells and were assayed for *en* and *runt* expression. The results showed a phenotype

opposite to *hh* mutants: *en* was present in all MG (Fig. 3C) and *runt* was absent (Fig. 3F). To test whether changes in midline *runt* and *en* expression are produced by a secondary effect of *hh* misexpression on non-midline cells, we used *sim-Gal4* to misexpress *UAS-ci.VP16*, a constitutively activating form of the Hh-responsive Cubitus Interruptus (Ci) transcription factor (Fig. 3M,N). This resulted in a phenotype similar to *sim-Gal4 UAS-hh* (*en* was present in all MG and *runt* was absent from MG) indicating that *hh* signaling can directly influence PMG gene expression. Analysis of additional differentially expressed genes demonstrated further that *hh* controls PMG gene expression. In wild-type embryos, *wrapper* levels were high in AMG and low in PMG (Fig. 3A′,D′), *nemy* was present in AMG and absent from PMG (Fig. 3G), and *mas* was expressed in a subset of PMG (Fig. 3J). In *hh* mutants, all MG had high levels of *wrapper* (Fig. 3B′,E′) and *nemy* (Fig. 3H), but *mas* was absent (Fig. 3K). By contrast, *sim-Gal4 UAS-hh* embryos had low-levels of *wrapper* (Fig. 3C′,F′), *nemy* was absent (Fig. 3I) and the number of *mas*+ cells increased slightly to 2.5 per segment (*n*=10 segments) (Fig. 3L) from a wild-type average of 2.1 per segment (*n*=14 segments). Unlike *en*, *mas* expression did not expand into AMG. Similar to the *wrapper* and *nemy* results, the AMG-expressed genes *CG33275* and *Fhos* showed identical results in *hh* mutant and misexpression embryos (not shown). Together, these experiments indicate that *hh* signaling is required for PMG gene expression (*en*, *mas*, low *wrapper*) and for repressing AMG gene expression (*CG33275*, *Fhos*, *nemy*, *runt*, high *wrapper*).

runt and *en* control differential midline glial gene expression

The *hh* gene partitions *runt* and *en* into different MG compartments, but do *runt* and *en* influence MG development and transcription? This was addressed in misexpression and loss-of-
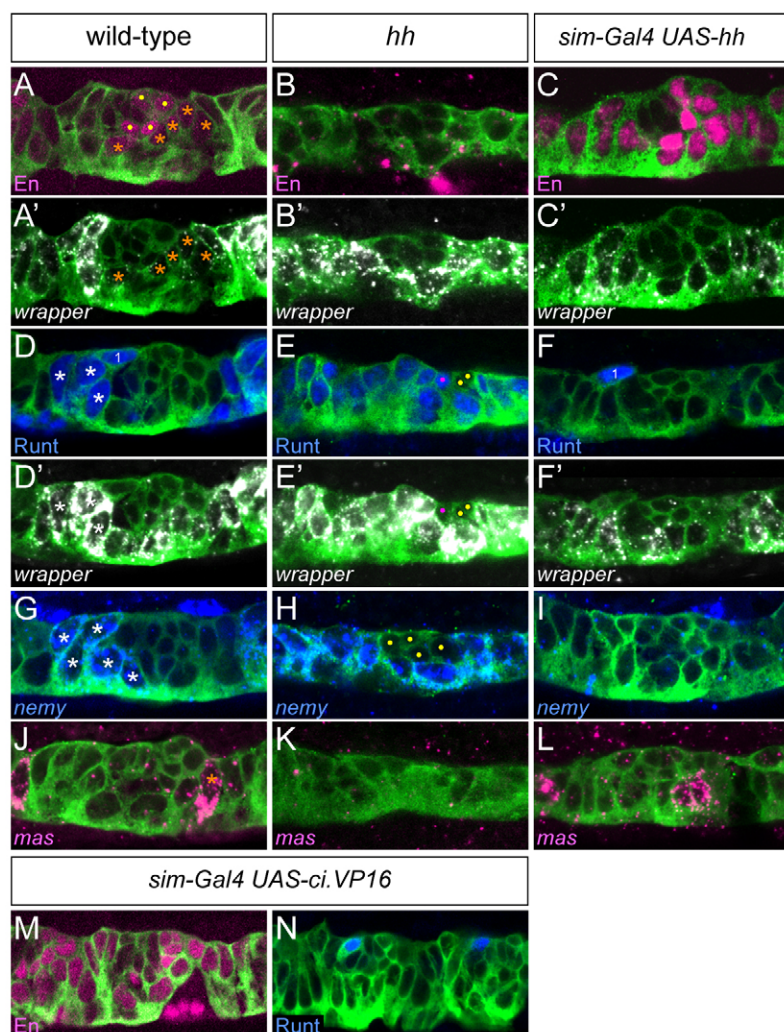
**Fig. 3. *hh* regulates anterior midline glia (AMG) and posterior midline glia (PMG) gene expression.** Sagittal views of stage 12 segments with genotypes listed above each column. (**A,A′**) In wild type, En is present in the low *wrapper*[+] PMG (orange asterisks) and a subset of *wrapper*^{−} midline neurons (dots). (**B,B′**) In the absence of *hh*, En is absent from all MG, and all MG are high *wrapper*. (**C,C′**) In *hh* misexpression embryos, all MG are En[+] and low *wrapper*. Midline neurons, which are smaller than MG, are the centrally located *wrapper*^{−} cells. (**D,D′**) In wild type, Runt is present in the high *wrapper*[+] AMG (white asterisks) and the MP1 (midline precursor 1) neurons (1); the second MP1 neuron is out of the plane of focus. (**E,E′**) In *hh* mutants, all MG are Runt[+] and high *wrapper*[+]. Four neurons are present in each segment (dots), two are Runt[+] (magenta dot; the other neuron is out of the plane of focus). (**F,F′**) *hh* misexpression results in a complete absence of Runt[+] MG, but does not affect Runt in MP1 neurons (1). (**G-I**) The AMG expression of *nemy* expands to all MG in the absence of *hh*, and is absent when *hh* is misexpressed in all midline cells. White asterisks indicate AMG and yellow dots indicate neurons. (**J-L**) *mas* is expressed in one or two PMG per segment in wild type; expression is absent in *hh* mutant embryos, and increases slightly in *hh* misexpression embryos. (**M,N**) Misexpression of *UAS-ci.VP16* using *sim-Gal4* results in MG that are En[+] and Runt[−].

function experiments with both genes. When *sim-Gal4 UAS-runt* embryos were analyzed for MG gene expression, AMG gene expression was observed in all MG, including high levels of *wrapper* (Fig. 4A,E), *nemy* (Fig. 4B,F), *CG33275* (Fig. 4C,G) and *Fhos* (not shown) expression. Concomitantly, there was a reduction in PMG gene expression, including a strong reduction in the number of *mas*-expressing cells (0.3 cells per segment; *n*=12) (Fig. 4D,H). Analysis of *runt* mutants is complicated because of the gap and pair-rule functions of *runt*, which lead to differences in ectodermal patterning along the A/P axis (Tsai and Gergen, 1994). Thus, in addition to being *runt*[−], some segments in the anterior of the embryo had expanded *hh* and *en* expression, and some posterior segments were devoid of *en* and *hh* expression (Fig. 5G,H) (Jaynes and Fujioka, 2004). In anterior segments of both *runt*[3] and *runt*[29] mutants, which were *runt*[−] *en*[+], we observed that MG gene expression was PMG-like, the opposite result to *runt* misexpression. Levels of *wrapper* were low (Fig. 4I) and expression of *nemy* and *CG33275* was absent in many segments (Fig. 4J,K). Identifying distinct segments was difficult in *runt* mutants, but *mas* expression was present in repeated clusters of cells that might correspond to individual segments (Fig. 4L). The number of *mas*^{+} cells in each cluster (5.5 cells per segment; *n*=15), was higher than in wild type (2.1 cells per segment). Thus, the *runt* mutant and misexpression data were both consistent with *runt* repressing PMG gene expression.

Analysis of *sim-Gal4 UAS-en* embryos revealed a PMG-like pattern of gene expression, a phenotype similar to *hh* misexpression: *wrapper* (Fig. 4M) levels were reduced, and *nemy* (Fig. 4N), *CG33275* (Fig. 4O) and *Fhos* (not shown) expression was absent. The number of *mas*^{+} cells was increased (3.0 cells per segment; *n*=20) (Fig. 4P). The opposite results were observed in *en* null mutant embryos (*Df(2R)En*[E]/*Df(2R)En-A*); *wrapper* (Fig. 4Q), *nemy* (Fig. 4R), *CG33275* (Fig. 4S) and *Fhos* (not shown) expression was high in all MG and *mas* expression was absent (Fig. 4T). Although the MG phenotypes of *en* mutants were opposite to (and thus consistent with) the *en* misexpression phenotypes, *en* was required for *hh* expression in the ectoderm (Lee et al., 1992). Consequently, the *en* mutant phenotype cannot be unambiguously attributed solely to loss of *en* function. The more convincing evidence was produced by the misexpression experiments, because they demonstrate that midline-expressed *runt* and *en* can directly influence MG gene expression. Together, these experiments indicate that *en* represses AMG gene expression and that *runt* represses PMG gene expression.

Mutual repression between *en* and *runt*
Because *runt* represses PMG gene expression and *en* represses AMG gene expression, we addressed whether they mutually repress each other. Misexpression of *en* in *sim-Gal4 UAS-en* embryos resulted in the absence of MG *runt* expression (Fig.
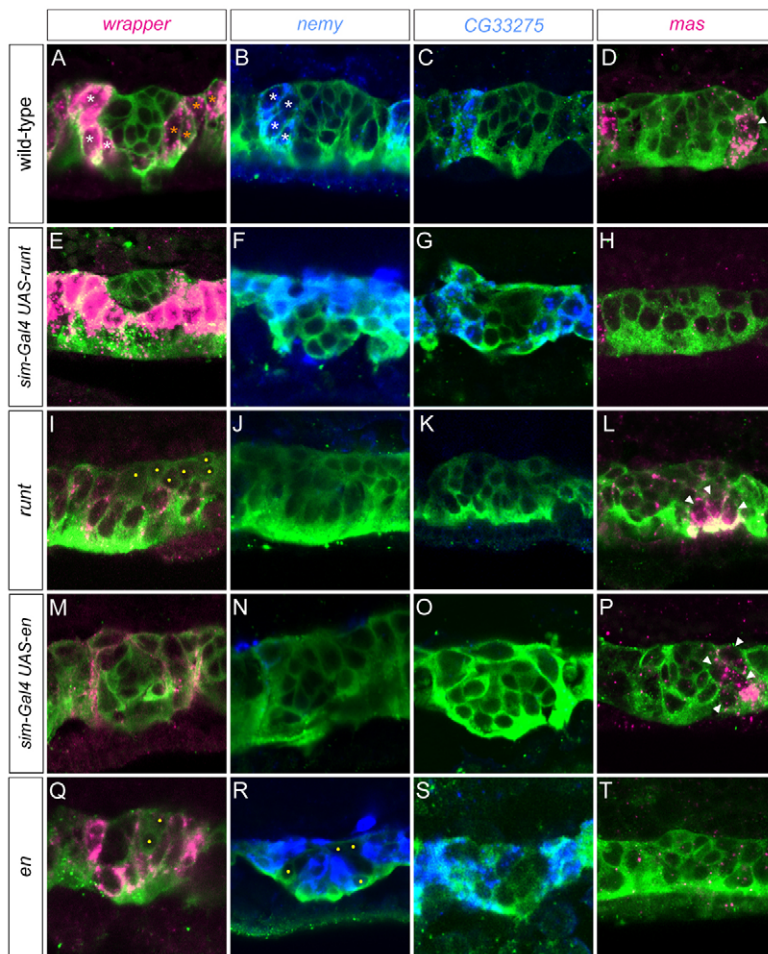
Fig. 4. *runt* and *en* control midline glia (MG) subtype-specific gene expression. Sagittal views of stage 12 segments. Dots denote *wrapper⁻* midline neurons in mutant embryos. (A-D) In wild-type embryos, *wrapper* is expressed at high levels in anterior midline glia (AMG; white asterisks) and low levels in posterior midline glia (PMG; orange asterisks); *nemy* and *CG33275* are expressed in AMG only; and *mas* (arrowhead) is expressed in PMG only. (E-H) *runt* misexpression results in MG that express high *wrapper*, *nemy* and *CG33275*, whereas *mas* expression is absent. (I-L) In anterior regions of *runt* mutant embryos (yellow bar in Fig. 5H,H' indicating *runt⁻ en⁺* cells), *wrapper* is expressed at low levels in all MG (yellow dots indicate midline neurons), *nemy* and *CG33275* expression are absent, and the number of *mas⁺* cells is increased (arrowheads). (M-P) Misexpression of *en* results in low levels of *wrapper*, absence of *nemy* and *CG33275* expression, and a slight increase in the number of *mas⁺* cells (arrowheads). (Q-T) *en* mutants have high levels of *wrapper*, *nemy* and *CG33275* in MG, whereas *mas* expression is absent. Yellow dots indicate neurons.

5A,B). Similarly, *runt* expression expanded to all MG in *en* mutant embryos (Fig. 5C). Thus, *en* represses *runt* in PMG. Misexpression of a version of *en* with an activation domain (*UAS-VP16En*) (Alexandre and Vincent, 2003) resulted in the appearance of *runt* in all MG (Fig. 5D). This indicates that *en* normally functions biochemically as a repressor in *runt* repression, rather than activating a *runt* repressor. Misexpression of *runt* in *sim-Gal4 UAS-runt* embryos resulted in an absence of *en* in MG (Fig. 5E), and *en* expanded to additional MG in anterior regions of *runt* mutant embryos (Fig. 5F). These results indicate that *runt* represses *en* in AMG. Thus, these data demonstrate *runt* and *en* mutual repression, and this repression probably ensures the maintenance of AMG and PMG patterns of gene expression.

The *runt* gene represses *en* and PMG gene expression in AMG, but is it required for AMG gene expression? This issue was addressed by examining gene expression in MG devoid of both *en* and *runt*, thus allowing the role of *runt* to be assessed in the absence of the repressive role of *en* on AMG gene expression. In the posterior regions of *runt* mutants, expanded ectodermal expression of *sloppy paired 1* (*slp1*) leads to repression of *en* (Jaynes and Fujioka, 2004) creating regions that are devoid of both *en* and *runt* (Fig. 5G,H). These *runt⁻ en⁻* regions had high levels of *wrapper* (Fig. 4I, Fig. 5I,I'), *nemy* (Fig. 4J, Fig. 5J,J') and *Fhos* (not shown). However, expression of *CG33275* was absent (Fig. 4K, Fig. 5K,K'). By contrast, the flanking *runt⁻ en⁺* regions had low *wrapper* expression (Fig. 5I,I') and no *nemy* (Fig. 5J,J'), *Fhos* (data not shown) or *CG33275* (Fig. 5K,K') expression. As AMG gene expression is present in *runt⁻ en⁻* MG, the major function of *runt* might be to

repress *en* and PMG gene expression in AMG. However, the absence of expression of at least one gene (*CG33275*) in *runt⁻ en⁻* regions indicates that *runt* can also positively influence AMG gene expression.

en contributes to PMG cell fate

Analysis of gene expression by *en* misexpression experiments indicates that *en* normally represses *runt* and AMG gene expression in PMG. Because *en* mutant embryos also affect *hh* signaling, it is difficult to examine the functional role of *en* from loss-of-function experiments. However, this issue could be addressed by examining commissural ensheathment, extension of MG processes into the commissures, and the neuronal accumulation of Nrx-IV in *en* misexpression embryos. In wild-type embryos, MG fully ensheathed the commissures (Fig. 6A), projected membrane projections extensively into the commissures (Fig. 6A',A") and had high levels of Nrx-IV accumulation within the commissures (Fig. 6B). By contrast, in *sim-Gal4 UAS-en* embryos, the resulting MG did not completely ensheath the axon commissures (Fig. 6C), there was an absence of membrane projections into the commissures (Fig. 6C',C") and only low levels of Nrx-IV accumulation (Fig. 6D). These results indicate that *en* is able to impart on MG properties similar to PMG. Analysis of *sim-Gal4 UAS-hh* embryos showed similar results to *sim-Gal4 UAS-en*, but the effects were even more severe in their relative lack of MG ensheathment of axons and Nrx-IV accumulation (Fig. 6E,F). These results are consistent with the view that *hh* imparts a PMG fate on MG, and that *en* is
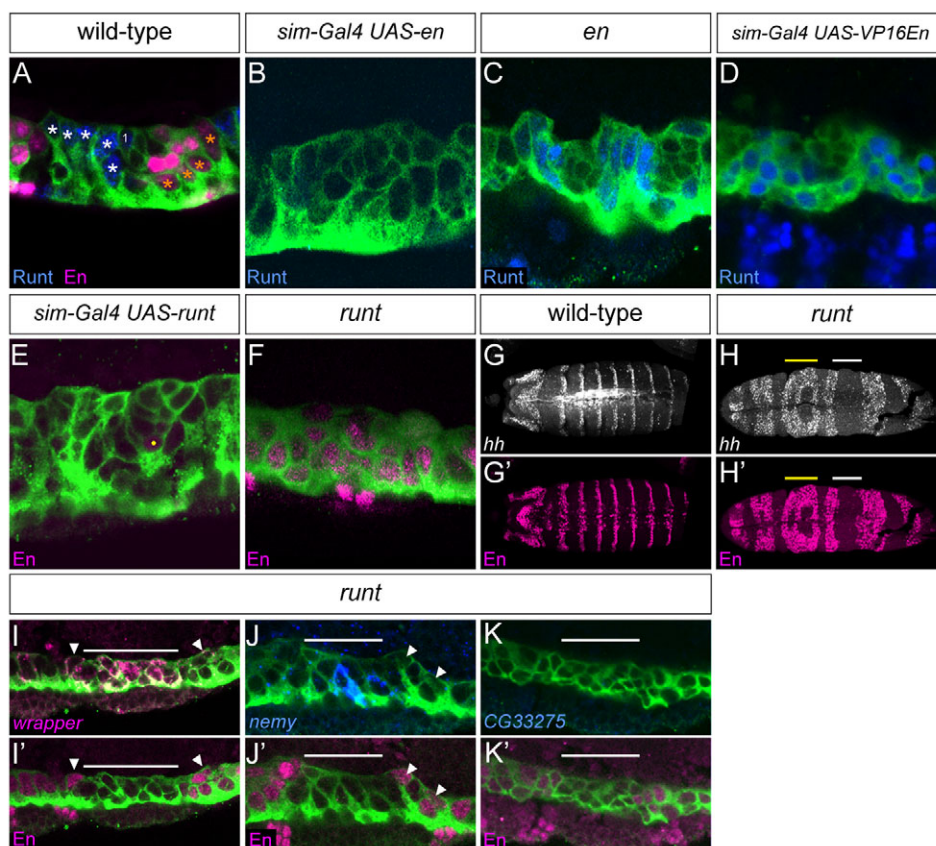
Fig. 5. *runt* and *en* mutually repress. (**A-K'**) Sagittal (A-F,I-K) and horizontal (G-H') views of stage 12 segments. (A) In wild type, Runt is present in the anterior midline glia (AMG; white asterisks) and midline precursor 1 (MP1) neurons (1), whereas En is present in posterior midline glia (PMG; orange asterisks) and a subset of midline neurons. (B) Misexpression of *en* in all midline cells results in a complete absence of Runt. (C) In *en* mutant embryos, Runt is present in all midline glia (MG). (D) Misexpression of a constitutively activating form of *en* (*UAS-VP16En*) results in the presence of Runt in all MG. (E) *runt* misexpression results in the absence of En+ MG. Yellow dot indicates an En+ neuron. (F) In anterior regions of *runt* mutant embryos (yellow bar in H,H'), En is present in all MG. (G,G') In wild-type embryos, *hh* and En are present in collinear segmentally repeated ectodermal stripes. (H,H') In *runt* mutant embryos, the regular pattern of *hh* and En stripes is disrupted, with anterior regions of expanded *hh* and En (yellow bars), and posterior regions devoid of *hh* and *en* (white bars). (I-K') MG gene expression was analyzed in posterior regions of *runt* mutant embryos that are *runt⁻ en⁻* (white bar in H,H'). (I-J') *wrapper* and *nemy* levels are high in *runt⁻ en⁻* MG (below white bar), but low or absent in adjacent *runt⁻ en⁺* regions (arrowheads). (K,K') By contrast, *CG33275* is absent in *runt⁻ en⁻* regions.

a significant *hh*-effector gene. *sim-Gal4 UAS-runt* embryos were examined and, similar to wild-type AMG, all MG were able to ensheath axons (Fig. 6G), extend cellular projections (Fig. 6G',G″) and accumulate high levels of Nrx-IV (Fig. 6H). By stage 17, all *sim-Gal4 UAS-en* and *sim-Gal4 UAS-hh* MG had undergone apoptosis (not shown). At stage 15, *sim-Gal4 UAS-runt* embryos showed increased numbers of MG compared with wild type (Fig. 6G) but, by stage 17, apoptosis had reduced the number to wild-type levels (not shown). Although the absence of MG in *en* and *hh* misexpression embryos at stage 17 is consistent with a PMG fate, the fact that apoptosis is also a common fate for AMG renders cell number a relatively weak indicator of MG cell fate.

As both *en* and *hh* have similar effects on PMG gene expression and development, is *en* required for all aspects of *hh* control of PMG fate, or does *hh* activate expression of additional PMG-expressed genes? The data reported above indicate that, similar to *hh*, *en* represses AMG gene expression, has the same limited effect on *mas* PMG expression when assayed by misexpression and can impart PMG properties with respect to axon ensheathment. To address this issue in more detail, we

analyzed the *l(1)sc* gene, which is expressed in PMG but not AMG, at stages 10-12 (Fig. 7A). The *l(1)sc* gene was also expressed in all MPs and the MNB (Fig. 7A). Expression of *l(1)sc* expanded to all MG in *hh* misexpression embryos (Fig. 7B) and was absent in *hh* mutants (Fig. 7C), indicating that its expression at stages 11-12 requires *hh*. However, misexpression of *en* failed to expand *l(1)sc* expression to additional MG (Fig. 7D), and loss of *en* in *runt* misexpression embryos also did not affect the expression of *l(1)sc* in PMG (Fig. 7E). These data indicate that *hh* controls expression of *l(1)sc* independently of *en* and, thus, *en* does not mediate all of the effects of *hh* on PMG gene expression.

The *l(1)sc* gene encodes a bHLH transcription factor that plays an important role in *Drosophila* neural precursor formation (Jimenez and Campos-Ortega, 1990; Younossi-Hartenstein et al., 1997). We investigated the potential role of *l(1)sc* in PMG gene expression and development by analyzing embryos from *Df(1)sc-B57*, which is a deletion of the *achaete-scute* complex genes, including *l(1)sc*. In mutant embryos, PMG were present and expression of *en* and *wrapper* was similar to that observed in wild type (Fig. 7F,G). At stage 14, wild-type embryos had 1.9 PMG per segment (*n*=9

segments examined) and *Df(1)sc-B57* mutant embryos had 1.9 PMG per segment (*n*=15 segments). Thus, *l(1)sc* does not have a major effect on PMG gene expression or development.
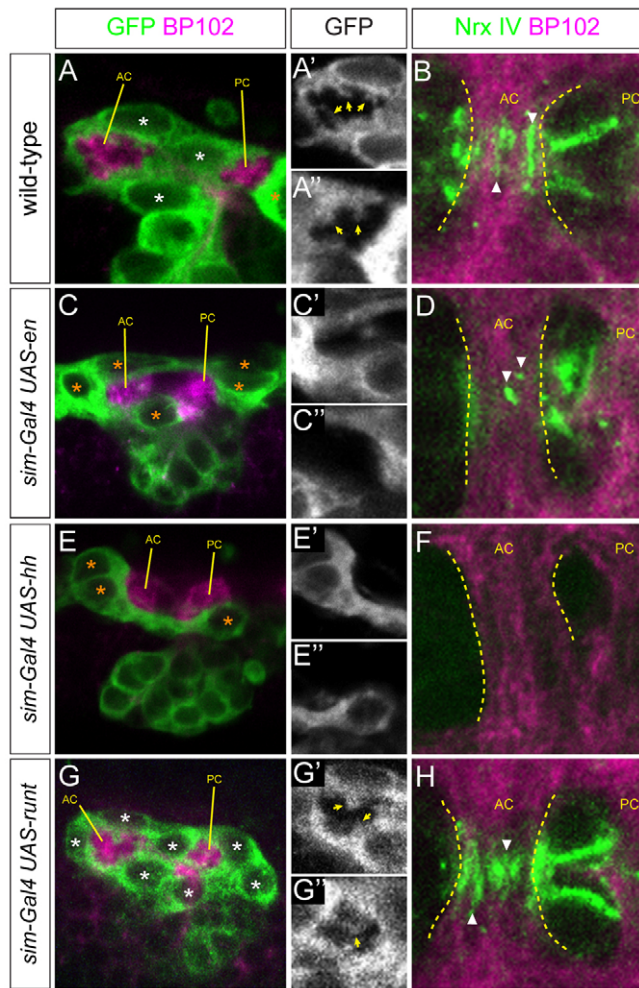
Fig. 6. *en* directs posterior midline glia (PMG) cell fate.
(**A,C,E,G**) Sagittal views of segments from stage 15 *sim-Gal4 UAS-tau-GFP* embryos stained with MAb BP102 (magenta, axon commissures) and anti-GFP (green, midline cells). (**A′,C′,E′,G′**) MG (GFP channel; white) surround the anterior commissure (AC). Arrows point to midline glia (MG) projections into the AC. (**A″,C″,E″,G″**) MG surrounding the posterior commissure (PC). (**B,D,F,H**) Horizontal views of the AC. Dotted lines indicate the anterior and posterior boundaries of the AC. The PC is not completely ensheathed at stage 15, and is not shown. Nrx-IV staining (green) within commissural axons is adjacent to MG projections. (A-A″) In wild-type embryos, three AMG ensheath the AC and extend projections into the commissures. (B) In wild-type embryos, axonal Nrx-IV accumulates at the sites of contact (arrowheads) between AMG projections and AC axons. (C-C″,E-E″) Misexpression of *en* and *hh* results in incomplete ensheathment of the commissures, an absence of MG projections and (D,F) a strong reduction in Nrx-IV accumulation at sites of MG-axonal contact. In both *sim-Gal4 UAS-en* and *sim-Gal4 UAS-hh* embryos, the normal separation of the anterior and posterior commissures is disrupted to a varying degree. This is consistent with previous work showing that genetic defects in MG number, migration and ensheathment result in commissure separation defects (Klämbt et al., 1991; Noordermeer et al., 1998; Wheeler et al., 2009). (G-G″) Misexpression of *runt* does not affect ensheathment or MG projections (arrows). There are excess MG present compared with wild type. (H) As in wild type, *runt* misexpression results in Nrx-IV accumulation at sites of MG-axonal contact.

DISCUSSION

In this paper, we describe how the Hh morphogen patterns the midline cells to generate two populations of MG with distinct functional properties (Fig. 8A). The key output of this signaling is the expression of *en* that imparts PMG cell fate, in part, by repressing *runt*. In turn, the *runt* gene maintains AMG fate by repressing *en*. Thus, morphogenetic signaling and transcriptional regulation lead to AMG and PMG with divergent molecular, morphological and functional differences.

At stage 10, the 16 midline cells per segment consist of three equivalence groups of neural precursors, four to six cells each (Fig. 8B). *Notch* signaling directs ten of these 16 cells to become MG; the remainder become MPs and the MNB (Wheeler et al., 2008). Thus, *Notch* represses neuronal development in MG and activates a core set of MG-expressed genes (e.g. *wrapper*). MG in the anterior of the segment become AMG; those in the posterior of the segment become PMG. *Notch* signaling by itself is unlikely to influence different MG fates, as expression of activated *Suppressor of Hairless* in midline cells drives all cells into a MG fate but does not affect their AMG or PMG patterns of gene expression (Wheeler et al., 2008). Thus, additional factors that can direct AMG and PMG cell fates were sought.

Previous work demonstrated that *hh* can pattern midline cells along the A/P axis (Bossing and Brand, 2006), and, indeed, we demonstrate that *hh* is required for PMG cell fate. The source of Hh is not in the midline, but in the lateral ectoderm in a stripe of cells, collinear with the pair of midline early *en*+ cells. Hh signals to midline cells posterior to the early *en*+ cells, inducing *en* in an additional six to seven cells (Fig. 8C). These late *en*+ cells plus the early *en*+ cells become about four PMG, as well as MP4-6 and the MNB (Fig. 8D). Misexpression and mutant analyses indicate that *hh* is required for all PMG gene expression and for repressing AMG expression. *hh* signaling probably has multiple target genes because *hh* is required for *en* and *l(1)sc* expression, but *en* does not regulate *l(1)sc*. Misexpression of *hh* can activate *en* expression in anterior MG, and both *hh* and *en* misexpression convert these cells functionally into non-ensheathing MG that resemble PMG, results also consistent with observations by Bossing and Brand (Bossing and Brand, 2006) that ectopic expression of *hh* and *en* in midline cells affects AMG differentiation. However, neither *hh* nor *en* can activate all PMG gene expression in anterior MG, because neither activates *mas* expression in anterior MG. The *mas* gene is expressed transiently at stage 12 in a subset of PMG, suggesting that functionally distinct classes of PMG might exist. Expression of *mas* might require other signals in addition to *hh* that are absent in anterior MG.

runt is present in AMG and represses *en* and PMG-specific gene expression. In *runt* mutant cells that are *runt*– *en*+, expression of three genes expressed in only AMG (*CG33275*, *Fhos* and *nemy*) are absent and *wrapper* is reduced. This could be due to *runt* repression of *en*, repression of other genes or activation by *runt*. In *runt* mutant cells that are *runt*– *en*–, *Fhos* and *nemy* are present, *wrapper* is at high levels, but *CG33275* expression is absent. This suggests that *runt* does not activate expression of *Fhos*, *nemy* and *wrapper* in AMG, but maintains their AMG levels by repressing *en*. By contrast, *runt* is required for expression of *CG33275*, possibly indicating a positive role for *runt* in AMG differentiation in addition to its repressive role in AMG maintenance. However, *CG33275* is most prominently expressed in a subset of AMG closest to the commissures (Fig. 1F), and this AMG expression could be dependent on additional signals, perhaps from the developing axon commissure. Thus,
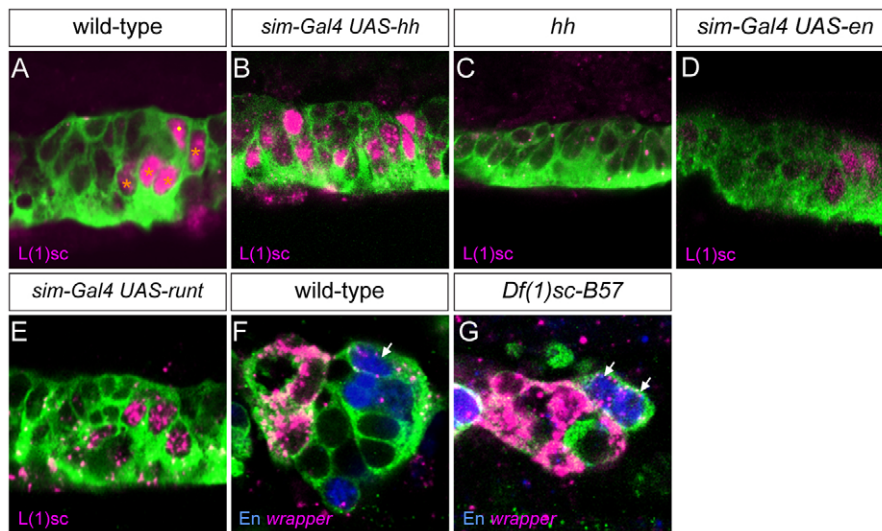
Fig. 7. L(1)sc posterior midline glia (PMG) expression is regulated by *hh* but not *en*. (**A**) In wild-type embryos, L(1)sc is present in PMG (orange asterisks) and midline neurons (dot) at stage 12. (**B**) Misexpression of *hh* results in the presence of L(1)sc in all MG, whereas (**C**) L(1)sc is absent from the midline cells in *hh* mutant embryos. (**D**) Misexpression of *en* does not result in expansion of L(1)sc. (**E**) In *runt* misexpression embryos, *l(1)sc* expression is unaffected. (**F,G**) PMG (arrows; En[+] low *wrapper*^{+} cells) are present in both wild-type (F) and *l(1)sc* mutant (*Df(1)sc-B57*) (G) embryos.

absence of *CG33275* expression in *runt* mutant embryos could alternatively be due to an effect of *runt* on developing axons or CNS development.

As most AMG gene expression is not dependent on *runt*, we propose that *Notch* signaling initially induces an AMG pattern of gene expression in all glia (Fig. 8A,B) and, either simultaneously or soon after, Hh signaling in the posterior of the segment generates PMG (Fig. 8C). One important downstream target of *Notch* signaling is likely to be the *sim* gene, which encodes a bHLH-PAS protein that functions as a DNA-binding heterodimer with the Tango (Tgo) bHLH-PAS protein (Nambu et al., 1991; Sonnenfeld et al., 1997). During early development, *sim* is
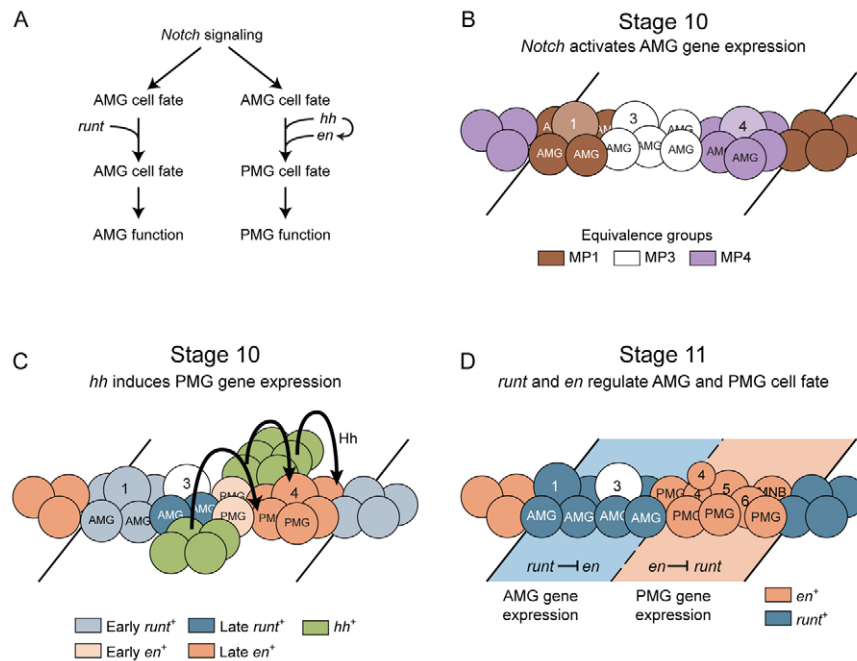
Fig. 8. Model of *hh*, *en* and *runt* regulation of midline glia (MG) cell fate. (**A**) Flow diagram describing the genetic regulation of anterior midline glia (AMG) and posterior midline glia (PMG) development. (**B,C**) Schematics of stage 10 segments. Anterior is to the left and lines indicate segmental boundaries. (B) *Notch* signaling results in the activation of MG gene expression and the formation of MP1 (1), MP3 (3) and MP4 (4) from three equivalence groups. (C) Hh, originating in cells lateral to the midline, signals (arrows) to the midline cells that are posterior to the early *en*^{+} cells, activating late *en* expression. Late *runt* is initiated in cells flanking the early *runt*^{+} cells. MP1 is shown arising from early *runt*^{+} cells, as it is *runt*[+] and forms during stage 10 prior to the initiation of late *runt* expression. The early *en*^{+} cells are shown as PMG and not AMG, MP1, MP3-6, or the median neuroblast (MNB), because: (1) AMG, MP1 and MP3 arise from *en*^{−} cells (Wheeler et al., 2006); (2) MP4 arises from *en*^{−} cells just posterior to the early *en*^{+} cells (Fig. 2B'); and (2) MP5, MP6 and the MNB all arise posterior to MP4 (Wheeler et al., 2006). This leaves only PMG as the descendants of the early *en*^{+} cells. The other two PMG arise from the late *en*^{+} cells, along with MP4-6 and the MNB. (**D**) Schematic of early stage 11, in which the MP4 has divided into two ventral unpaired median 4 (VUM4) neurons (4). Other MPs are designated 1, 3, 5 and 6, along with the MNB. Dashed line indicates the boundary between *runt*[+] and *en*[+] domains. In the *runt*[+] domain, *runt* represses *en* expression, and in the *en*[+] domain, *en* represses *runt* expression. This cross-repression maintains distinct AMG and PMG cell fates. MP, midline precursor.

expressed in all midline primordia and is required for midline cell development (Nambu et al., 1990). However, later in development, *sim* is restricted to MG and a subset of midline neurons (Crews et al., 1988; Wheeler et al., 2008). Genetically, *sim* expression is absent in embryos mutant for *Notch* signaling (Wheeler et al., 2008). The *sim* gene is likely to be an important aspect of MG transcription, because mutation of Sim-Tgo binding sites in the *slit* and *wrapper* MG enhancers results in loss of MG expression (Estes et al., 2008; Wharton et al., 1994), and Sim-Tgo binding sites are present in other identified MG enhancers (Fulkerson and Estes, 2010). The Hh morphogen only transforms posterior MG into PMG. It is unknown why *hh* does not affect anterior MG, but it is likely to be owing to the presence of unknown factors in these cells that inhibit *hh* signaling. As *Notch* signaling, rather than *runt*, is primarily required for AMG gene expression, the key role of *runt* is probably to maintain AMG gene expression by repressing *en*. Similarly, *en* functions to maintain PMG gene expression by repressing *runt*, but also contributes positively to PMG cell fate, as *en* misexpression confers PMG-like function to AMG.

The most striking features of AMG are their ability to migrate around the commissures, ensheath them and extend processes into the axons. The function of PMG is unknown, but they are unable to ensheath the commissures, even though they are in close proximity. One of the major factors influencing AMG-axon interactions is Nrx-IV-Wrapper adhesion (Stork et al., 2009; Wheeler et al., 2009). Levels of *wrapper* expression in AMG are higher than in PMG, and this is likely to be a key determinant of why AMG ensheath commissures, and PMG do not, because loss of *wrapper* expression results in incomplete migration and ensheathment (Noordermeer et al., 1998; Stork et al., 2009; Wheeler et al., 2009). Recent work has demonstrated that *sim* directly regulates *wrapper* expression, and *spitz* signaling from axons might also form a positive feedback loop to control *wrapper* levels and strengthen Nrx-IV-Wrapper interactions (Crews, 2009; Estes et al., 2008). As *en* genetically reduces *wrapper* levels in PMG, it will be interesting to determine if this regulation is direct or indirect. Although the control of *wrapper* levels is likely to be a major factor in AMG-PMG differences and the ability of glia to ensheath axons, other genes whose levels differ between AMG and PMG might also contribute. This illustrates why it will be important to identify target genes and understand better the roles that *Notch*//*Suppressor of Hairless*, *sim*, *hh*, *Ci*, *en*, *runt* and other MG transcription factors play in regulating MG gene expression and function.

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