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# Formation and specification of a *Drosophila* dopaminergic precursor cell

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## SUMMARY

Dopaminergic neurons play important roles in animal behavior, including motivation, reward and locomotion. The *Drosophila* dopaminergic H-cell interneuron is an attractive system for studying the genetics of neural development because analysis is focused on a single neuronal cell type. Here we provide a mechanistic understanding of how MP3, the precursor to the H-cell, forms and acquires its identity. We show that the *gooseberry/gooseberry-neuro* (*gsb/gsb-n*) transcription factor genes act to specify MP3 cell fate. It is proposed that *single-minded* commits neuroectodermal cells to a midline fate, followed by a series of signaling events that result in the formation of a single $gsb^{+}/gsb\text{-}n^{+}$ MP3 cell per segment. The *wingless* signaling pathway establishes a midline anterior domain by activating expression of the forkhead transcription factors *sloppy paired 1* and *sloppy paired 2*. This is followed by *hedgehog* signaling that activates *gsb/gsb-n* expression in a subgroup of anterior cells. Finally, *Notch* signaling results in the selection of a single MP3, with the remaining cells becoming midline glia. In MP3, *gsb/gsb-n* direct H-cell development, in large part by activating expression of the *lethal of scute* and *tailup* H-cell regulatory genes. Thus, a series of signaling and transcriptional events result in the specification of a unique dopaminergic precursor cell. Additional genetic experiments indicate that the molecular mechanisms that govern MP3/H-cell development might also direct the development of non-midline dopaminergic neurons.

**KEY WORDS: Cell fate, CNS midline, Dopamine, *Drosophila*, *gooseberry*, Neuron**

## INTRODUCTION

A key aspect of neurogenesis concerns how neural precursors are generated and acquire specific fates. The simplified view is that patterning proteins, consisting of intercellular signaling pathway components and transcription factors, activate downstream transcription factors that promote neural precursor formation and direct specific precursor fates (Skeath and Thor, 2003). The combined action of these proteins activates additional factors that control neuron-specific differentiation. Despite conceptual understanding of the factors involved, there are few in vivo examples in which the developmental progression of individual neurons has been comprehensively followed from the undifferentiated neuroectoderm to the differentiated neuron. Yet, studies of individual neurons and their precursors are particularly valuable for their detailed, mechanistic insights.

An attractive system for the systematic study of neuronal development is provided by *Drosophila* CNS midline cells (Fig. 1) (Wheeler et al., 2006). These cells reside between the two hemiganglia of the *Drosophila* ventral nerve cord (VNC). Initially consisting of 16 ectodermal cells per segment (referred to as 'mesectoderm'), these cells express *single-minded* (*sim*), which acts as a master regulator of midline cell development (Nambu et al., 1991). From this seemingly uniform set of precursor cells emerge an array of diverse interneurons, motoneurons, neurosecretory neurons, axon-ensheathing anterior midline glia (AMG) and non-ensheathing posterior midline glia (PMG) (Wheeler et al., 2006). Five of the 16 mesectodermal cells give rise to midline precursors (MPs; MP1, MP3, MP4, MP5 and MP6) that divide only once to generate two neurons (Wheeler et al., 2008). MP1 divides symmetrically to generate two identical MP1 peptidergic motoneurons, MP3 divides asymmetrically into the dopaminergic (DA) H-cell and glutamatergic H-cell sib interneurons, and MP4-6 each divide asymmetrically to yield a GABAergic iVUM interneuron and a glutamatergic/octopaminergic mVUM motoneuron. One additional midline neural precursor, the median neuroblast (MNB), is a neural stem cell that divides throughout embryonic and postembryonic development. Analysis of midline cell development has been greatly facilitated by large-scale identification of midline-expressed genes and the ability to identify each cell type at all stages of embryonic development (Kearney et al., 2004; Wheeler et al., 2006; Wheeler et al., 2008; Wheeler et al., 2009).

Recent work has focused on the development of the midline DA H-cell neuron (Stagg et al., 2011; Wheeler et al., 2008). The MP3 precursor to the H-cell emerges during stage 11 from the mesectodermal cells (Fig. 1), rotates its spindle perpendicular to the longitudinal axis and divides asymmetrically into a basal H-cell and an apical H-cell sib. *Notch* signaling is required for H-cell sib fate, but the asymmetric localization of Numb in the H-cell blocks *Notch* signaling, leading to its divergent fate (Wheeler et al., 2008). Both the Lethal of scute [L(1)sc] and Tailup (Tup) transcription factors are present in the H-cell (Stagg et al., 2011; Thor and Thomas, 1997). Embryos mutant for *l(1)sc* fail to express any of the genes required for H-cell-specific differentiation, whereas *tup* mutants lack expression of a subset of H-cell differentiation genes, including *pale* (*ple*; tyrosine hydroxylase), *Dopamine transporter* (*DAT*) and *Dopa decarboxylase* (*Ddc*). *l(1)sc* is also required for the formation of MP4-6 and controls mVUM differentiation (Stagg et al., 2011). Since *l(1)sc* function leads in one case (H-cell) to a DA fate and in the other case (mVUM) to a motoneuron fate, this suggests that the distinct identities and properties of their precursors (MP3 and MP4-6) lead to alternative neuronal fates.

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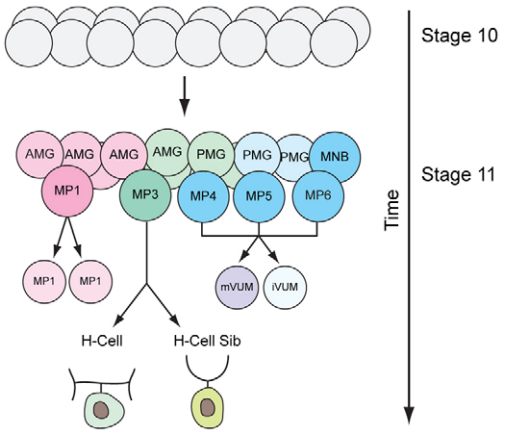


**Fig. 1. Summary of *Drosophila* midline neurogenesis.** During stages 10 to 11, 16 mesectodermal cells (gray) develop into six midline neural precursors [MP1, MP3, MP4, MP5, MP6 and the median neuroblast (MNB)] and two groups of midline glia [anterior midline glia (AMG) and posterior midline glia (PMG)]. Each MP divides once to generate two neurons.

This proposition raises several related issues: (1) what is the molecular basis of MP3 formation and MP3 identity specification; (2) how do these genes regulate *l(1)sc* and *tup* to ultimately control H-cell differentiation; (3) are midline cells pre-patterned into domains permissive and non-permissive for MP3 specification; and (4) are the genes that specify MP3 development also used to control development of other *Drosophila* DA neuronal lineages? Here, we address the genetic mechanisms involved in MP3 formation and specification.

## MATERIALS AND METHODS

### *Drosophila* strains and genetic analysis

*Drosophila* mutant strains included: *Df(2R)gsb* (Nusslein-Volhard et al., 1984), *Df(2R)Kr10* (Gutjahr et al., 1993), $hh^{AC}$ (Lee et al., 1992), $ptc^{7}$ $wg^{1-12}$ (Nusslein-Volhard et al., 1984), $ptc^{9}$ (Nusslein-Volhard et al., 1984), $slp^{\Delta 34B}$ (Grossniklaus et al., 1992), $slp1^{2}$ (Nusslein-Volhard et al., 1984) and $wg^{1-8}$ (Nusslein-Volhard et al., 1984). Mutant strains were obtained from the Bloomington Drosophila Stock Center. *Gal4* and *UAS* lines employed were: *sim-Gal4* (Xiao et al., 1996), *prd-Gal4* (Xiao et al., 1996), *UAS-ci.VP16* (Larsen et al., 2003), *UAS-en* (Guillén et al., 1995), *UAS-gsb* (Marie et al., 2010), *UAS-gsb-n* (Colomb et al., 2008), *UAS-hh* (Porter et al., 1996), *UAS-slp1* (Sato and Tomlinson, 2007) and *UAS-tau-GFP* (Brand, 1995). The $ptc^{7}$ $wg^{1-12}$ strain was grown at 29°C to reduce *wg* function.

Wild-type, mutant and misexpression embryos contained *sim-Gal4 UAS-tau-GFP* in the background to assist in visualizing midline cells. Homozygous mutant embryos were identified by either: (1) staining for the absence of marked balancer *TM3 P[ftz-lacZ]* expression; (2) staining by in situ hybridization for lack of gene expression in deletion mutants; or (3) assaying for characteristic alterations in gene expression.

### In situ hybridization, immunostaining and microscopy

Embryo collection, in situ hybridization and immunostaining were performed as previously described (Kearney et al., 2004). Embryos were commonly hybridized to two RNA probes, one labeled with digoxygenin and another with biotin, along with immunostaining with two antibodies (see http://midline.bio.unc.edu/MDB_Home.aspx, under Information>protocols). RNA probes for in situ hybridization were generated from the *Drosophila* Gene Collection (Open Biosystems) (*en*, *Gad1*, *gsb-n*, *odd*, *ple*, *slp1*, *slp2* and *VGlut*) or PCR amplified from genomic DNA (*gsb*, *hh*, *tup*). Primary antibodies used were: rat anti-Elav (1:3; Developmental Studies Hybridoma Bank), mouse anti-En (1:25) (Patel et al., 1989), rabbit anti-GFP (1:100; Abcam), rabbit anti-Hb (1:100) (Tran and Doe, 2008), guinea pig and rat anti-L(1)sc (1:250 with TSA) (Stagg et al., 2011), guinea pig anti-Lim3 (1:250) (Broihier and Skeath, 2002), guinea pig anti-Runt (1:400 with TSA) (Kosman et al., 1998), mouse anti-Tau (1:100; Tau-2, Sigma) and guinea pig anti-Zfh1 (1:250) (Vogler and Urban, 2008). Stained embryos were imaged on Zeiss LSM-PASCAL, LSM-510 and LSM-710 confocal microscopes.

## RESULTS

MP3 gives rise to both the H-cell and H-cell sib, and genes were sought that affected the development of MP3 and its progeny. Since MPs arise in defined positions along the anterior-posterior axis (Bate and Grunewald, 1981; Wheeler et al., 2008), we hypothesized that anterior-posterior patterning genes [*hedgehog* (*hh*), *patched* (*ptc*), *wingless* (*wg*), *slp1*, *slp2*, *gsb*, *gsb-n*, *engrailed* (*en*)] might play a role in directing MP cell fate. Eight of these genes were assayed for effects on MP3 lineage development using mutant and misexpression/overexpression approaches.

### *gsb and gsb-n* transcription factor genes specify MP3 fate

The *gsb* and *gsb-n* genes are related in sequence, reside within 9.7 kb, have similar patterns of embryonic expression and are partially redundant (Duman-Scheel et al., 1997). At stages 10-11, both *gsb* and *gsb-n* are expressed in neuroectodermal stripes that are collinear with, and include, MP3 (Fig. 2A,C; supplementary material Fig. S1A,E) (Bossing and Brand, 2006). MP3 is the only MP in which either gene is expressed. Both genes are also expressed in the H-cell and H-cell sib at stage 11 (Fig. 2B,D), but are absent from all other midline neurons and MPs. Expression of both genes is absent in midline cells by late stage 12.

The highly specific expression of *gsb* and *gsb-n* in MP3 and its progeny suggested that one or both of these genes play important roles in MP3 cell fate. This was initially tested using a *gsb gsb-n* double-mutant strain [*Df(2R)gsb*]. In these experiments and throughout, midline cell identity was based on position, morphology and the use of cell-specific markers in multiply stained embryos (for examples, see Fig. 2I-N, Fig. 5B,B′,E,I,J, Fig. 6H,N,P). Cell fate changes were assessed by analyzing embryos at stages 14-16 for the following midline neuronal differentiation markers: H-cell (*ple*, *tup*), H-cell sib (high levels of *VGlut*), MP1 (Lim3, *odd*, Runt), iVUMs (En, *Gad1*) and mVUMs (Zfh1, low *VGlut*) (Fig. 2E-H) (Stagg et al., 2011; Wheeler et al., 2006; Wheeler et al., 2008); these data are quantitated in supplementary material Figs S2-S5.

The *Df(2R)gsb* embryos showed an absence of cells expressing *ple* (H-cell) or high *VGlut* (H-cell sib) and a corresponding doubling of $Lim3^{+}$ MP1 neurons was often observed (29% of segments scored) (Fig. 2I-K). $Zfh1^{+}$ mVUM numbers also increased in 50% of *Df(2R)gsb* segments, most often from three to four cells (Fig. 2L). The interpretation of these findings is that *gsb*/*gsb-n* are required for MP3 cell fate, and in their absence MP3 is transformed into either an MP1 or MP4-6. Since in *Df(2R)gsb* embryos MP3 fate is transformed rather than fails to appear, this indicates that *gsb*/*gsb-n* are not required for MP3 formation or cell division. However, we note that the timing of division, as revealed by the appearance of some Tau-dense dividing cells adjacent to MP1 neurons, is delayed compared with the normal timing of the MP3 division (Fig. 2W).

The respective roles of *gsb* and *gsb-n* were analyzed by examining embryos homozygous mutant for only *gsb*. Since single-gene mutants for *gsb* and *gsb-n* are not available, we analyzed

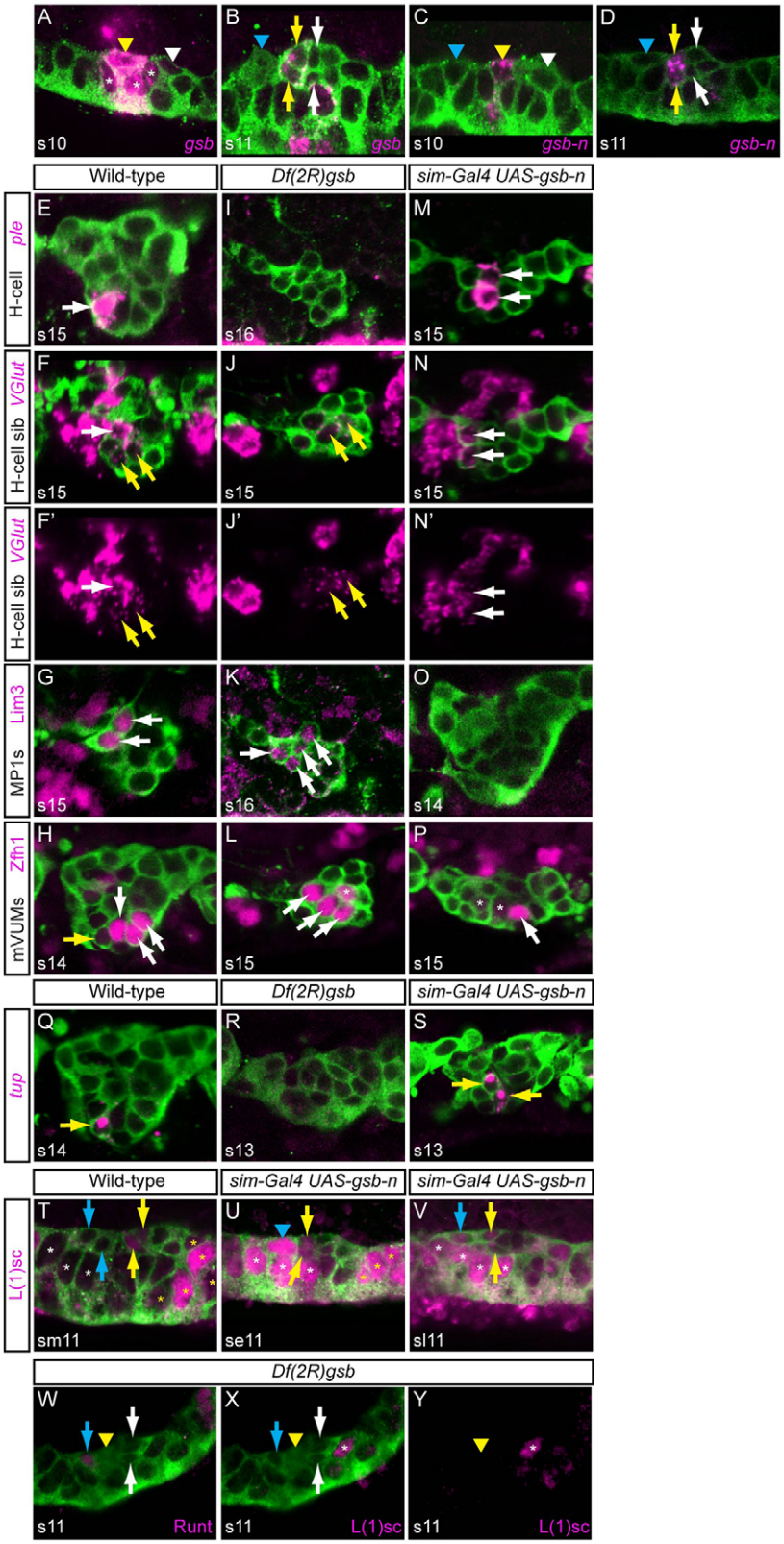


**Fig. 2. *gsb* and *gsb-n* direct MP3 cell fate.** All panels are sagittal views of single segments; anterior is to the left and internal up. Embryos carry *sim-Gal4 UAS-tau-GFP* and are stained with anti-Tau to illustrate midline cells (green), with in situ hybridization or immunostaining as indicated. (**A**) At stage 10, *gsb* RNA is present in MP3 (yellow arrowhead) and surrounding MG (asterisks), but not in MP4 (white arrowhead). (**B**) At stage 11, *gsb* is present in the H-cell and H-cell sib (yellow arrows) but is absent in MP1 (blue arrowhead) and mVUM4 and iVUM4 (white arrows). (**C**) At stage 10, *gsb-n* is expressed only in MP3 (yellow arrowhead), not MP1 (blue arrowhead) and MP4 (white arrowhead). (**D**) At stage 11, *gsb-n* is expressed in the H-cell and H-cell sib (yellow arrows), but is absent from MP1 (blue arrowhead) and mVUM4 and iVUM4 (white arrows). (**E-P**) Stage 14-16 embryos. (F′,J′,N′) Only *VGlut* expression is shown (compare with F,J,N). (E-H) Wild-type expression of (E) *ple* in H-cell (arrow), (F,F′) high levels of *VGlut* in H-cell sib (white arrow) and low levels in mVUMs (yellow arrows), (G) Lim3 in two MP1 neurons (arrows) and (H) Zfh1, which is strong in the three mVUMs (white arrows) and weak in the *tup*$^{+}$ H-cell (yellow arrow). (I-L) *Df(2R)gsb* embryos. (I) *ple* expression is absent. (J,J′) High *VGlut* expression is absent; yellow arrows indicate low *VGlut* mVUMs. (K) Four Lim3$^{+}$ MP1 neurons are present (arrows). (L) Three Zfh1$^{+}$ mVUMs are present (arrows); the additional Zfh1$^{+}$ cell (asterisk) might be a fourth mVUM. (M-P) *sim-Gal4 UAS-gsb-n* embryos. Two *ple*$^{+}$ cells (M) and two high *VGlut* cells (N,N′) are present (arrows). (O) Lim3 protein is absent. (P) Only one strong Zfh1$^{+}$ mVUM (arrow) is present. There are two weak Zfh1$^{+}$ cells (asterisks) that are *ple*$^{+}$ H-cells. (**Q-V**) Embryos at (Q-S) stages 13-14, (T) mid stage 11 (sm11), (U) early stage 11 (se11) and (V) late stage 11 (sl11). (Q) Wild-type expression of *tup* in H-cell (arrow). (R) Expression of *tup* is absent in *Df(2R)gsb* embryos. (S) Misexpression of *gsb-n* results in two *tup*$^{+}$ cells (arrows). (T) Wild-type embryo shows L(1)sc protein in H-cell and H-cell sib (yellow arrows), but its absence in MP1 neurons (blue arrows) and AMG (white asterisks). L(1)sc is also present in MP5, MP6, MNB and PMG (yellow asterisks). (U) Misexpression of *gsb-n* results in activation of L(1)sc in MP1 (blue arrowhead) and AMG (white asterisks). H-cell and H-cell sib are indicated by yellow arrows and PMG by yellow asterisks. (V) In older *gsb-n* misexpression embryos, L(1)sc was present in MP1 neurons (blue arrow; only one neuron shown), AMG (white asterisks) and H-cell and H-cell sib (yellow arrows). (**W-Y**) Stage 11 *Df(2R)gsb* embryo stained for (W) Runt and (X,Y) L(1)sc. Shown is a Runt$^{+}$ MP1 neuron (blue arrow), dividing MP3 (yellow arrowhead) and mVUM4 and iVUM4 (white arrows). L(1)sc is present in posterior cells but not in MP3/H-cell [compare with wild-type H-cell and H-cell sib L(1)sc staining in T; yellow arrows]. Note that the timing of the dividing MP3 is delayed compared with wild type (Wheeler et al., 2008).

transheterozygous embryos that were heterozygous for *gsb-n* but lacked both copies of *gsb* [*Df(2R)Kr10/Df(2R)gsb*]. Expression of *gsb-n* was significantly reduced in the lateral CNS (supplementary material Fig. S1G, compare with S1A), as observed previously (Gutjahr et al., 1993). In the midline, *gsb-n* was present in some segments, but absent in others (supplementary material Fig. S1G). Examination of embryos for *ple* and high *VGlut* expression indicated an absence of *ple* expression in 85% of segments (supplementary material Fig. S1H, Fig. S2) and an absence of high *VGlut* in 54% of segments (supplementary material Fig. S1I, Fig. S3). Thus, mutant analysis indicates that *gsb* plays a role in activating *gsb-n* expression in MP3 and is important for MP3

lineage development. However, as *Df(2R)Kr10/Df(2R)gsb* embryos do not show as severe a phenotype as *Df(2R)gsb* embryos (supplementary material Figs S2, S3), both *gsb* and *gsb-n* are required for MP3 development.

In a complementary experiment, *sim-Gal4 UAS-gsb-n* embryos, in which *gsb-n* is expressed in all midline cells at stages 10-11, showed an increase in *ple*$^{+}$ H-cells (Fig. 2M) and high *VGlut* H-cell sibs (Fig. 2N,N′) from one to two cells per segment. Accordingly, Lim3 expression was absent (Fig. 2O), suggesting that MP1 was transformed to MP3. There was also a general decrease in VUM neurons from six to two cells, as assayed by *zfh1* expression (Fig. 2P). However, these cells were not transformed to additional H-cells and H-cell sibs, so their fate is unclear. In contrast to *UAS-gsb-n*, misexpression of *gsb* (*sim-Gal4 UAS-gsb*) did not show an obvious effect on midline neuron cell fate (not shown). In summary, the *gsb*/*gsb-n* mutant and misexpression data are consistent with *gsb-n* and *gsb* driving MP3 cell fate, but not its formation. Mechanistically, *gsb*/*gsb-n* normally repress MP1 and MP4 fate in MP3, while also promoting MP3 fate.

The *l(1)sc* and *tup* transcription factor genes are both expressed in the H-cell and control H-cell differentiation and gene expression (Fig. 2Q,T) (Stagg et al., 2011). We addressed whether their expression was controlled by *gsb*/*gsb-n*. H-cell expression of *tup* was absent in *Df(2R)gsb* (Fig. 2R) and misexpression of *gsb-n* most often led to the appearance of two or more *tup*$^{+}$ H-cells in 28/38 segments scored (Fig. 2S). Similarly, *l(1)sc* expression was absent from the H-cell in *Df(2R)gsb* mutants (Fig. 2W-Y), and *gsb-n* misexpression resulted in a strong increase in *l(1)sc* expression in MP1 neurons and AMG (Fig. 2U,V). These results indicate that *gsb*/*gsb-n* (directly or indirectly) regulate the expression of *tup* and *l(1)sc*.

## *slp1/2* establish a permissive anterior midline domain for MP3 and MP1 cell fates

Having established that *gsb*/*gsb-n* control MP3 cell fate, the next issue concerns how *gsb*/*gsb-n* expression is activated in the midline cells. The first developmental event involves establishing an anterior midline domain, compatible with the formation of MP3. This hypothesis is based on the observation that ectopic *gsb-n* most often generates a single additional MP3 at the expense of the more anterior MP1 or adjacent MP4; it does not generally convert more posterior MPs (MP5 and MP6) and the MNB to MP3. We analyzed the role of *slp1* and *slp2*, which are closely related in sequence, reside within 9.7 kb, have similar expression patterns and genetically have largely redundant segmentation phenotypes (Cadigan et al., 1994). At stage 9, both genes are expressed in mesectodermal rows D-F, which are adjacent to, but do not overlap with, the *en* expression domain (rows G,H) (Wheeler et al., 2006). Rows D-F are likely to give rise to MP3. At stage 10, both genes are expressed in anterior midline cells, including MP1, MP3 and

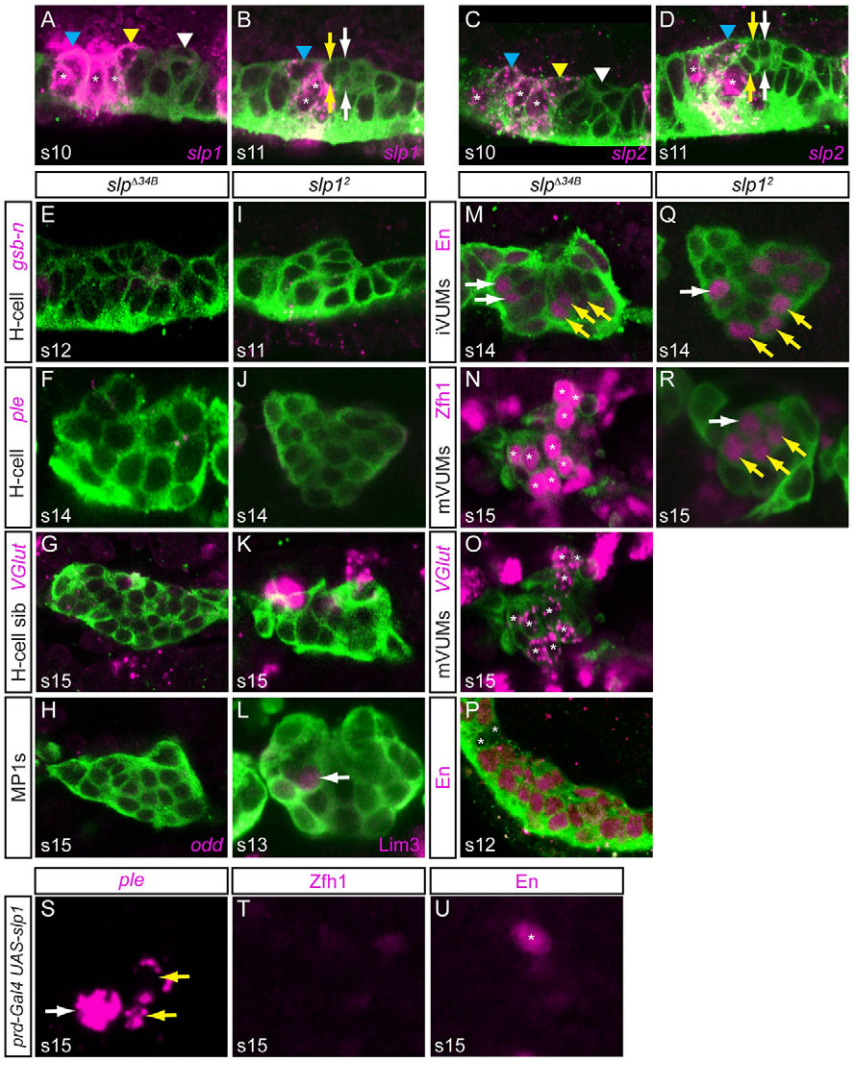


**Fig. 3. *slp1/2* establish an MP3/MP1 permissive region in anterior midline cells.** (**A**,**C**) At stage 10, *slp1* and *slp2* are expressed in MP1 (blue arrowhead), MP3 (yellow arrowhead), AMG (asterisks), but not MP4 (white arrowhead). (**B**,**D**) At stage 11, *slp1* and *slp2* expression is present in MP1 (blue arrowhead) and some AMG (asterisks), but absent in H-cell and H-cell sib (yellow arrows) and iVUM4 and mVUM4 (white arrows). (**E-H**) In *slp*$^{\Delta 34B}$ embryos, H-cell (*gsb-n*, *ple*), H-cell sib (high *VGlut*) and MP1 (*odd*) gene expression is absent. (**M**) En is present in multiple anterior midline neurons (white arrows) in addition to the three iVUMs (yellow arrows). (**N**,**O**) Additional Zfh1$^{+}$ (N, asterisks) and low *VGlut* (O, asterisks) neurons are present in *slp*$^{\Delta 34B}$ in addition to the three mVUMs (also asterisks). (**P**) At stage 12, En expands into most midline cells in *slp*$^{\Delta 34B}$ embryos. In this segment, two glia (asterisks) and four neurons (not shown) were En$^{-}$. (**I-K**) *slp1* mutant (*slp1*$^{2}$) had reductions in *gsb-n*, *ple* and *VGlut* expression. (**L**) Lim3 protein was weakly present (arrows) in *slp1*$^{2}$. (**Q**,**R**) There is an extra En$^{+}$ cell (Q, white arrow) and Zfh1$^{+}$ cell (R, white arrow) in anterior midline cells in *slp1*$^{2}$ embryos at the position of MP1 neurons. Yellow arrows mark (Q) iVUMs and (R) mVUMs. (**S-U**) Stage 15 *prd-Gal4 UAS-slp1* embryos were stained for (S) *ple*, (T) Zfh1 and (U) En. Multiple *ple*$^{+}$ cells (yellow arrows) were observed posterior to the H-cell (white arrow), whereas no Zfh1$^{+}$ mVUMs or En$^{+}$ iVUMs were observed. (U) The En$^{+}$ cell (asterisk) is either MNB progeny or PMG.

AMG (Fig. 3A,C; supplementary material Fig. S1B,F). However, after MP3 divides during stage 11, expression of both *slp1* and *slp2* is absent from the H-cell and H-cell sib (Fig. 3B,D). Thus, *slp1* and *slp2* overlap in expression in the midline cells from which MP3 will form.

The potential role of *slp1* and *slp2* in midline cell development was tested by genetic analysis using an *slp1 slp2* double-null strain ($slp^{\Delta 34B}$) and an *slp1* null mutant strain ($slp1^{2}$). In $slp^{\Delta 34B}$ embryos, MP3 and H-cell *gsb-n* expression was absent at stages 10-12 (Fig. 3E), and H-cell *ple* and *tup* expression and H-cell sib high *VGlut* expression (Fig. 3F,G; *tup* not shown) were absent at later stages. MP1 neuronal *odd* expression was also absent in $slp^{\Delta 34B}$ embryos at stages 12 and 13 (Fig. 3H). Thus, MP3 and MP1 progeny were absent. There was a corresponding increase in MP4-6 VUM progeny, as indicated by additional $En^{+}$ iVUMs and $Zfh1^{+}$ low *VGlut* mVUMs (Fig. 3M-O). Examination of earlier, stage 12 mutant embryos indicated that En expanded throughout most of the segment (Fig. 3P). This result reinforces the view that *slp1/2* repress posterior gene expression in anterior cells. Similar results were observed for the $slp1^{2}$ single mutant, except that the effects were weaker (Fig. 3I-L,Q,R), suggesting that *slp1* and *slp2* act redundantly. Together, these results indicate that *slp1/2* are required for MP1 and MP3 fates and repress MP4-6 fates in both MP3 and MP1.

The *slp1/2* mutant results suggested that misexpressing *slp1/2* in all midline cells might convert MP4-6 to MP3 or MP1. Whereas *sim-Gal4 UAS-slp1* embryos were unaffected in midline cell fates (data not shown), when we expressed *slp1* earlier using *prd-Gal4* we observed an increase in the number of $ple^{+}$ cells at the expense of $Zfh1^{+}$ mVUMs and $En^{+}$ iVUMs (Fig. 3S-U). These results reinforce the *slp1/2* mutant results and indicate that *slp1/2* influence MP3 and MP1 fate by establishing a permissive anterior midline environment at stages 9-10 for specification of MP1 and MP3 identity. *slp1/2* might accomplish this by repressing genes, including *en*, that, if expressed in these cells, would shift them toward posterior midline MP4-6 fates.

## Mutants in *wg* activate *slp1/2* expression and MP3 fate

The next issue concerns how the *slp1/2* anterior domain is established. Previous work has shown that *wg* can induce *slp1/2* expression in other cell types (Bhat et al., 2000) and *wg* can influence midline gene expression (Bossing and Brand, 2006). Consequently, we addressed whether *wg* signaling influences *slp1/2* expression and MP3 development. *wg* encodes a secreted signaling protein, and at stage 9 it is expressed in a stripe, including midline cell rows E and F, that spans the neuroectoderm (supplementary material Fig. S1C) (Wheeler et al., 2006; Xiao et al., 1996). Expression of *wg* is absent from the midline at stages 10-11, but remains in the lateral stripe adjacent to the midline.

Analysis of *wg* null mutant ($wg^{1-8}$) embryos indicates a strong reduction of *gsb-n*, *ple*, *tup* and of high *VGlut* expression (Fig. 4A-D), consistent with a loss of MP3. There was a mild reduction in the number of MP1 neurons, with 25% of segments possessing no MP1 neurons (Fig. 4E) and 75% of segments with the wild-type number of two neurons. VUMs were relatively unaffected, although one to two additional $Zfh1^{+}$ cells were observed in 61% of segments (Fig. 4F). Of particular note is that *slp1* expression was absent from the midline in *wg* mutant embryos (Fig. 4G). In summary, these data suggest a model in which *wg* activates *slp1/2* in an anterior midline domain, thus allowing MP1 and MP3 to form.

## Ectopic *en* alters MP3 and MP1 fates

One potential role of *slp1/2* is to repress posterior gene expression in the anterior midline cells. A strong candidate is *en*, the posterior expression of which is adjacent to, but does not overlap with, *slp1/2* expression (supplementary material Fig. S1B,F). At stage 9, *en* is expressed in a stripe that includes midline rows G and H and is collinear with the lateral ectodermal stripe (Kearney et al., 2004). Expression of *en* at stage 10 is present in two MG that lie between the MP3 and MP4 neural precursors (Fig. 5A) (Watson et al., 2011). At stage 11, *en* expands into MP4, MP5, MP6, MNB and all PMG (Fig. 5B) (Wheeler et al., 2006). Most importantly, *en* is not expressed in MP1, MP3, H-cell or H-cell sib (Fig. 5B′). Later, at stage 15, En is prominently expressed in the three iVUM neurons (Fig. 5C).

Analysis of *slp1/2* mutant and *slp1* misexpression embryos indicates that they repress *en* (Fig. 3M,P,Q,U), and possibly other genes, in anterior midline cells. This suggests that if *en* is present in MP3 its fate might be altered. This was addressed by misexpressing *en* in MP3 and MP1 in *sim-Gal4 UAS-en* embryos. The expression of *gsb-n* in MP3 was not significantly affected (Fig. 5D), indicating that the presence of *en* did not block activation of *gsb-n*. However, ectopic *en* resulted in a decrease of *ple*, *tup* (both H-cell), high *VGlut* (H-cell sib) and Lim3 (MP1) expression (Fig. 5E-H). There was a small increase in $Zfh1^{+}$ mVUMs (3.4±0.5; Fig. 5I) and $Gad1^{+}$ iVUMs (3.6±0.6; Fig. 5J) compared with wild type (3.0 cells). The additional VUM cells were usually the anteriormost neurons at the position of MP1 neurons. By contrast, neurons at the position of H-cell and H-cell sib (Fig. 5I,J, asterisks) generally did not express any midline neuron marker, although occasionally low levels of *ple* were present. This suggests that the presence of *en* in the H-cell and H-cell sib alters their neuronal identity, but does not

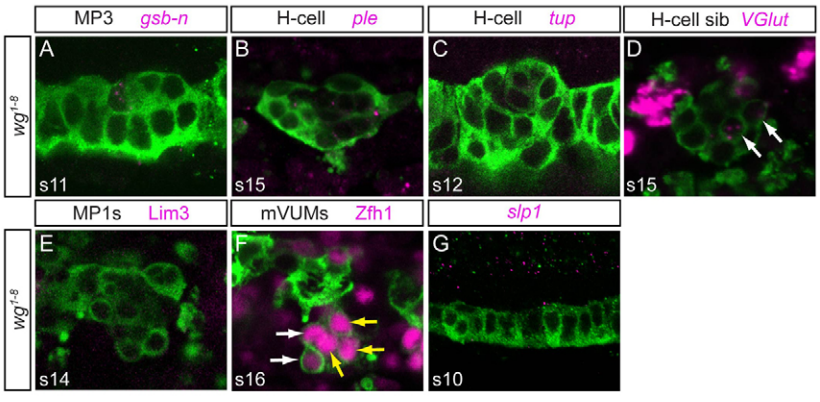


**Fig. 4. *wg* signaling influences *slp1/2* expression.** (**A-E**) In $wg^{1-8}$ embryos, expression of *gsb-n*, *ple*, *tup*, high *VGlut* and Lim3 was strongly reduced. The weak midline *VGlut* staining (D, arrows) is from mVUMs. (**F**) Additional $Zfh1^{+}$ cells (white arrows) were often observed in $wg^{1-8}$ embryos (mVUMs, yellow arrows). (**G**) Expression of *slp1* was absent in $wg^{1-8}$.

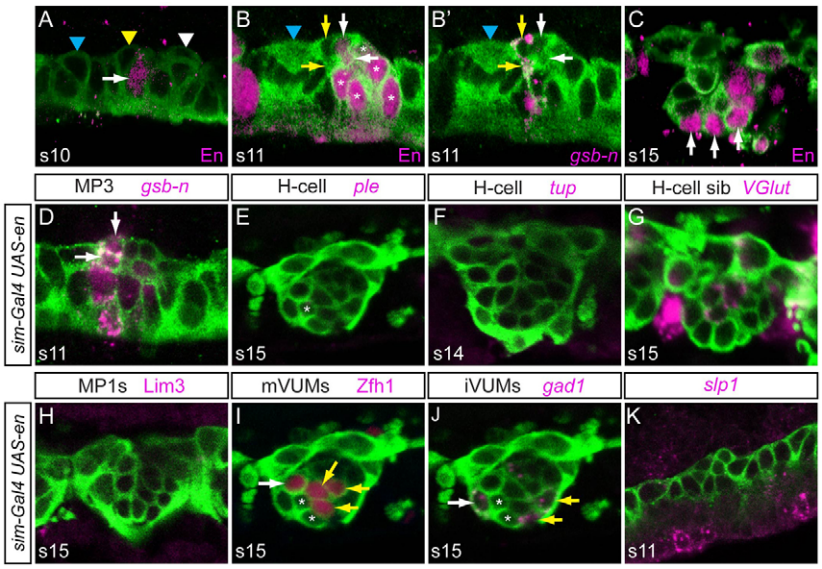


**Fig. 5. Ectopic *en* alters MP1 and MP3 cell fate.** (**A**) At stage 10, En is present in only two MG cells (arrow; only one cell is shown). En is absent from all neural precursors, including MP1 (blue arrowhead), MP3 (yellow arrowhead) and MP4 (white arrowhead). (**B**,**B'**) At stage 11, En defines the posterior midline cells. The same segment is shown stained for (B) En and (B′) *gsb-n*. (B) En is present in VUM4 progeny (white arrows) and MP5, MP6, MNB and PMG (asterisks). En is absent from H-cell and H-cell sib (yellow arrows), which are both *gsb-n*$^{+}$ (B′), and En is also absent in MP1 (blue arrowheads). (**C**) At stage 15, En is present in the three iVUM4-6 neurons (arrows). (**D-H**) In stage 11 *sim-Gal4 UAS-en* embryos, *gsb-n* expression in H-cell and H-cell sib (arrows) was unaffected (D) and expression of *ple*, *tup*, high *VGlut* and *lim3* was greatly reduced (E-H). Low levels of *ple* could be detected in a single cell (asterisk), indicating that it is likely to be an H-cell. (**I**,**J**) Additional Zfh1$^{+}$ and *Gad1*$^{+}$ cells are present in the position of MP1 neurons (white arrows) in *sim-Gal4 UAS-en* embryos. Yellow arrows indicate (I) mVUMs and (J) iVUMs, and probable H-cell and H-cell sib are indicated by asterisks (see E). (**K**) Absence of *slp1* midline expression in a stage 11 *sim-Gal4 UAS-en* embryo.

transform these cells into VUMs; MP1s might be more permissive to VUM transformation by *en*.

Ectopic *en* also resulted in an absence of *slp1* from midline cells (Fig. 5K). Since *slp1/2* repress *en*, these results indicate that *en* and *slp1/2* mutually repress each other. If *en* is expressed in MP1 and MP3 it can alter their fates and thus it is important to restrict its expression, which is a function of *slp1/2*. These results also indicate that misexpressed *en* has the ability to drive MP1 cells into an MP4-6 fate or that *en* interferes with the establishment of MP1 fate leading to a default state resembling MP4-6.

## MP3 fate is dependent on *hh* signaling

*slp1/2*, activated by *wg*, establish an anterior domain that is necessary for the expression of *gsb-n* in MP3. However, we propose that *wg* and *slp1/2* commit anterior midline cells to an MP1 fate and that an additional factor is required to commit a group of those cells to an MP3 fate. An attractive candidate for this factor is Hh, a prominent secreted signaling protein that directs alternative MG fates (Watson et al., 2011). At stages 10-11, *hh* is largely absent from the midline, but is expressed as a stripe in the lateral neuroectoderm (supplementary material Fig. S1D). We addressed whether *hh* signaling plays a role in MP3 cell fate.

Initially, *elav* expression was examined at stages 12-14 in *hh* mutants to assess whether *hh* affects midline neuronal cell number (Robinow and White, 1991). Segments contained 4.2±1.1 Elav$^{+}$ cells, whereas wild-type embryos contained 12.0 neurons at these stages (Fig. 6A,B; supplementary material Fig. S6A). However, 72% of embryos contained either four (46%) or six (26%) neurons. These data indicate that *hh* is required for the production of many midline neurons. Staining with neuron-specific markers indicated that when four or six neurons were present in *hh* null mutant (*hh*$^{AC}$) embryos, they comprised two MP1 neurons (the progeny of a single MP1 precursor), one to two mVUMs and one to two iVUMs (Fig. 6G,I,K; supplementary material Fig. S6B-D; stages 12-14). By contrast, H-cell (Fig. 6M), H-cell sib (Fig. 6O), one to two iVUMs, one to two mVUMs, and the MNB and its progeny were absent. Consistent with the absence of H-cell and H-cell sib gene expression, *gsb-n* expression was absent at stages 10-11 (Fig. 6Q) in the midline of *hh*$^{AC}$ embryos. As expected, *tup* was also absent (Fig. 6S) at stage 11 and later, and L(1)sc staining was absent in the midline of *hh*$^{AC}$ stage 11 embryos (Fig. 6U). The presence of only four to six neurons in *hh*$^{AC}$ embryos indicates that, generally, one or two of three MP4-6 cells and the MNB require *hh* signaling for formation. Surprisingly, the *slp1* expression domain is increased in some segments in *hh* mutant embryos at stages 10-11, although levels are reduced compared with wild type (Fig. 6C-F). This suggests that *hh* signaling helps in maintaining a sharp boundary of *slp1/2* expression. Despite this expansion of *slp1/2*, *hh* mutants fail to generate an MP3 or additional MP1s. Thus, we hypothesize that *hh* signaling plays three roles in midline neuronal development: (1) a proneural role for MPs and the MNB; (2) a role in specifying MP3 cell fate; and (3) a role in restricting *slp1/2* expression to anterior midline cells.

Consistent with the *hh* MP3 cell fate mutant phenotype, overactivation of *hh* signaling in all midline cells resulted in an increase in MP3s. *ptc* encodes an Hh receptor that, in the absence of *hh* signaling, inhibits the ability of Smoothened (Smo) to activate the *hh* signaling pathway (Ingham and McMahon, 2001). When activated by Hh, Smo is released from Ptc inhibition. Thus, *ptc* expression is present in cells responding to *hh* signaling, and *ptc* mutants act as constitutive activators of *hh* signaling. At stage 10, *ptc* is expressed in most midline cells, including all MPs, with the exception of two *en*$^{+}$ MG that lie between MP3 and MP4 (Watson et al., 2011). Most striking are the high levels of *ptc* in

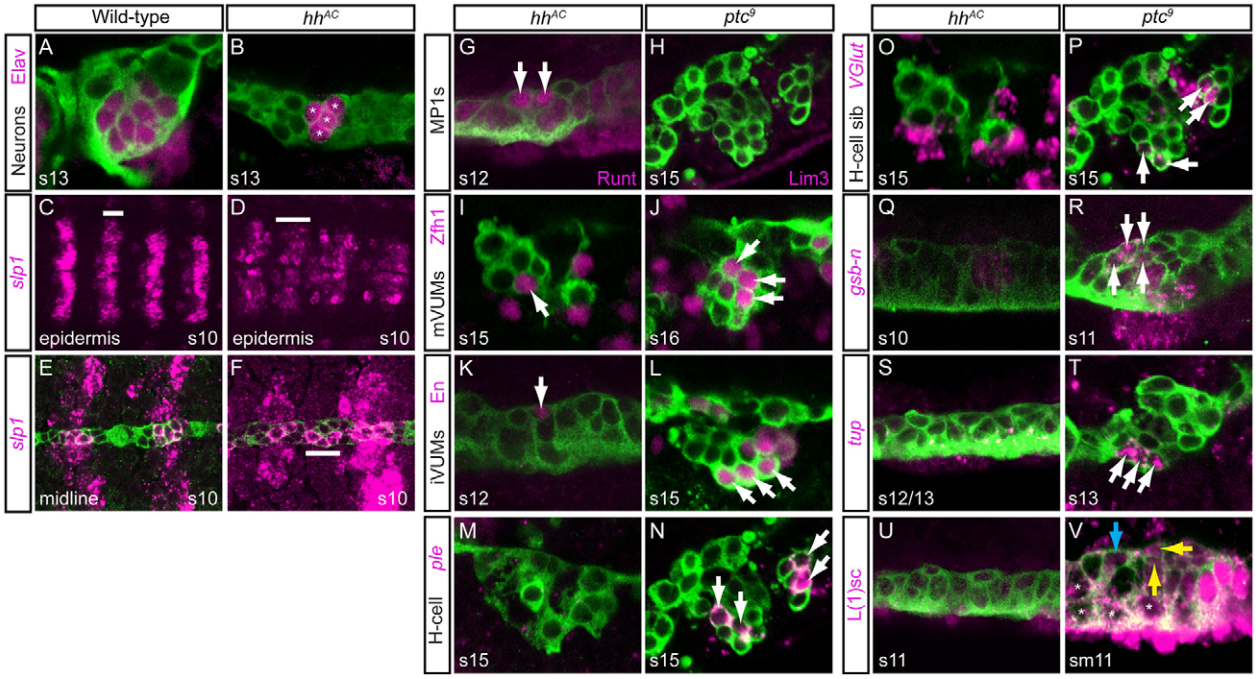


**Fig. 6. *hh* signaling is required for MP3 formation and identity.** Analyses of *sim-Gal4 UAStau-GFP* embryos are shown. (**A**) This wild-type embryo had 11 Elav$^+$ neurons (eight are shown). (**B**) This *hh$^{AC}$* embryo had six Elav$^+$ neurons (asterisks marksfour). (**C**,**D**) *slp1* expression was expanded in the epidermis of *hh$^{AC}$* mutants [compare the width of the white bar in wild-type (C) and mutant (D)]. (**E**,**F**) *slp1* expression was expanded (white bar) in midline cells in *hh$^{AC}$*. (**G**) In *hh$^{AC}$* mutants, two Runt$^+$ MP1 neurons (arrows) were present, as in wild type. (**I**,**K**) *hh$^{AC}$* embryos possessed only (I) one Zfh1+ mVUM and (K) one En+ iVUM (arrows). (**M**,**O**,**Q**,**S**,**U**) In *hh$^{AC}$* mutants, there was an absence of (M) *ple*, (O) high *VGlut*, (Q) *gsb-n*, (S) *tup* and (U) *l(1)sc* expression. (**H**,**J**,**L**) *ptc$^9$* mutants had an absence of Lim3 (H), but a wild-type number of three mVUMs (J) and three iVUMs (L) (arrows). (**N**,**P**) *ptc$^9$* mutant had additional (N) *ple*$^+$ and (P) high *VGlut* cells (arrows); two segments are shown. (**R**) Stage 11 *ptc$^9$* embryo had four *gsb-n*$^+$ cells (arrows). (**T**) *tup* is ectopically expressed in a *ptc$^9$* mutant in three cells (arrows). (**V**) In *ptc$^9$*, L(1)sc is ectopically present in MP1 neurons (blue arrow; only one MP1 neuron is shown), as well as in the H-cell and H-cell sib (yellow arrows) and AMG (white asterisks).

MP3 (Watson et al., 2011). In a *ptc* null mutant (*ptc$^9$*) there were commonly two *ple*$^+$ H-cells (Fig. 6N) and two high *VGlut* H-cell sibs (Fig. 6P). MP1 marker gene expression was absent (Fig. 6H), whereas iVUM and mVUM marker expression resembled that of wild type (Fig. 6J,L). There was also an increase in *gsb-n*$^+$ neurons (H-cell and H-cell sib) in *ptc$^9$* (Fig. 6R), further indicating that *hh* activates *gsb*/*gsb-n* expression in MP3. Consistent with this result, an increase in L(1)sc levels and *tup*$^+$ cells also occurred in *ptc* mutant embryos (Fig. 6T,V). Similar results to the *ptc$^9$* mutant were observed for *sim-Gal4 UAS-hh* embryos, which overexpress *hh* in midline cells (supplementary material Figs S2-S5).

One issue regarding the *hh* and *ptc* mutant and *hh* overexpression experiments is whether the effects of *hh* are due to alterations in ectodermal patterning and are not autonomous to midline cells. This was addressed by examining *sim-Gal4 UAS-ci.VP16*, in which the *hh* signaling pathway is only active in midline cells (*ci* encodes the transcriptional effector of the *hh* signaling pathway). These experiments also showed an increase in H-cell and H-cell sib at the expense of MP1 (supplementary material Figs S2-S4).

Since the *hh* and *wg* signaling pathways are known to regulate each other (Hatini and DiNardo, 2001) it is possible that the *wg* MP3/H-cell phenotype only reflects a reduction in *hh* signaling and is not due to a direct effect of *wg*. To address this issue, *ptc wg* double-mutant embryos were examined, as they lack *wg* function but have constitutively active *hh* signaling. The results showed that expression of *gsb-n*, *odd*, *ple* and *slp1* was absent (supplementary material Fig. S7A,B,D,G), whereas *zfh1* mVUM expression and *en* iVUM expression were expanded (supplementary material Fig. S7C,E,F). Thus, *wg* is required for *gsb-n* MP3 expression. Similarly, the ability of activated *hh* in anterior midline cells to generate additional *gsb-n*$^+$ MP3s via *sim-Gal4 UAS-Ci.VP16* indicates that *hh* can activate *gsb-n* expression. In summary, the *hh* mutant and misexpression experiments demonstrate that *hh* signaling emanating from outside the midline is directly responsible for MP3 cell fate. When overexpressed, *hh* signaling has the ability to convert MP1 into MP3, indicating that MP1 and MP3 derive from a developmentally similar ground state.

## Genes controlling MP3 development are also required for the development of other *Drosophila* dopaminergic neurons

We have demonstrated that *gsb*/*gsb-n*, *hh*/*ptc*, *wg* and *slp1/2* are required for MP3/H-cell development. Do these genes also control the development of DA neurons in other lineages? In the embryonic VNC, two additional DA neurons exist per hemisegment in addition to the H-cell (Lundell and Hirsh, 1994). These are the paramedial DA neurons and dorsal lateral DA neurons (Fig. 7A). Interestingly, most of these neurons are collinear with the H-cell suggesting that the same segmentation genes that control H-cell development also play a role in paramedial and dorsal lateral neuron development. Consistent with this view, recent work (Tio et al., 2011) has indicated that both DA neurons are *wg*$^+$, which overlaps in lateral CNS expression with *gsb* and *slp1/2*

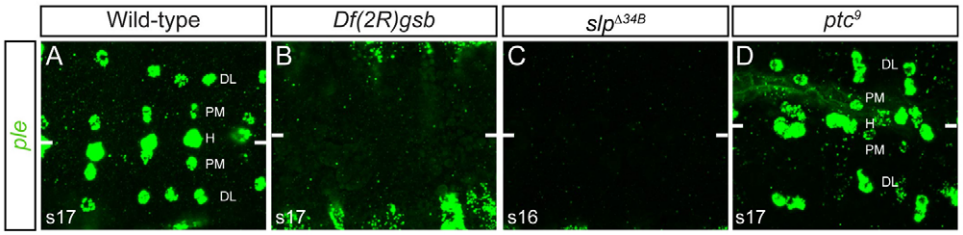


**Fig. 7. Genes controlling H-cell development also affect non-midline DA neuronal development.** Horizontal views of the CNS of stage 14-16 *sim-Gal4 UAS-tau-GFP* embryos stained for *ple* expression. (**A**) Wild-type embryo showing the ventral nerve cord (VNC) of dopaminergic (DA) neurons: H-cell (H), paramedial DA neurons (M) and dorsal lateral DA neurons (DL). (**B-D**) DA neuron *ple* expression was absent from *Df(2R)gsb* (B) and $slp^{\Delta 34B}$ (C) but expanded in $ptc^{9}$ (D). The $ple^{+}$ staining in B is in epidermal stripes. White bars indicate the midline.

(supplementary material Fig. S1A-C,E,F). We looked at *ple* expression in the paramedial DA and dorsal lateral DA neurons in *Df(2R)gsb*, $ptc^{9}$ and $slp^{\Delta 34B}$ embryos at stage 16. In *Df(2R)gsb* and $slp^{\Delta 34B}$ embryos (Fig. 7B,C) *ple* expression was absent in all lateral DA neurons. In $ptc^{9}$ embryos (Fig. 7D) there were additional $ple^{+}$ dorsal lateral neurons in 57% of hemisegments scored ($n$=42) and additional paramedial DA neurons in 52% of hemisegments ($n$=50). These results are similar to the effects seen on the H-cell and provide an initial indication that different *Drosophila* DA neurons might share a common set of genes for their development.

## DISCUSSION

The results presented here and in the literature (Stagg et al., 2011; Wheeler et al., 2008) have identified key regulators of *Drosophila* MP3/H-cell development, as summarized in Fig. 8.

### *sim* and the functional role of master regulatory genes

The *Drosophila sim* gene is a master regulator of CNS midline cell development. *sim* mutants fail to develop midline neuronal and glial precursors, and midline transcription of almost all genes normally expressed in the midline is absent (Nambu et al., 1990). Similarly, ectopic expression of *sim* in the neuroectoderm transforms the entire CNS into midline cells (Nambu et al., 1991). However, here we suggest a further refinement of *sim* function and propose that *sim* commits cells to an MP4 neural precursor fate that is followed by a series of signaling events that act on these cells to generate a diverse group of midline neuronal precursors and glia. This concept extends the notion of master regulator to posit a specific function for *sim* in initiating MP4 fate while subsequently working combinatorially with other transcription factors to control midline cell type-specific gene expression (Ma et al., 2000).

### *wg*, *slp1/2* and *hh* establish a midline anterior neural precursor domain

The midline cells initially appear morphologically and molecularly uniform, as characterized by the expression of *sim* in all mesectodermal cells. In our model, *sim* initially commits mesectodermal cells to an MP4 neural precursor fate (Fig. 8A). This is followed by *wg* signaling that establishes an anterior domain in which cells are committed to an MP1 fate (Fig. 8B). This is mediated by activation of *slp1/2* expression in anterior cells. Signaling by *hh* also maintains a distinct *slp1/2* anterior-posterior boundary. One important aspect of *slp1/2* function is the repression of *en* in the anterior region, as experimentally inducing *en* in wild-type anterior cells disrupts MP3 and MP1 neuronal development. In this sense, *slp1/2* play a role in midline neural precursor development that is conceptually similar to that of *runt* in MG development (Watson et al., 2011). The major function of *runt* in MG is to repress *en* expression in ensheathing glia (AMG) and ensure that AMG do not become $en^{+}$ non-ensheathing glia (PMG).

### *hh* signaling specifies MP3 identity

Both *hh* mutant and misexpression/overexpression experiments indicate that *hh* signaling is required for MP3 identity (Fig. 8C). The influence of *hh* on MP3 identity occurs largely, if not completely, by *hh* activation of *gsb*/*gsb-n* expression. *hh* is also required for expression of the bHLH factor L(1)sc in MPs, and both *hh* and *l(1)sc* mutants have similar proneural phenotypes with regard to the formation of MP4-6 and the MNB (Stagg et al., 2011). However, *l(1)sc* does not play a proneural role in MP3, even though it is expressed in MP3 (Stagg et al., 2011). There are two interpretations of the *hh* mutant results. In one scenario, MP3 fails to form and divide in an *hh* mutant, and thus *hh* plays an MP3 proneural role. Since MP3 formation is unaffected in *gsb*/*gsb-n* and *l(1)sc* mutants, the proneural function of *hh* may act through direct activation of proneural target genes by the *hh* pathway transcriptional effector Ci, or it could be through indirect Ci activation of additional transcription factors. Another interpretation is that MP3 is transformed in an *hh* mutant into an MP4-6-like cell, and MP4-6 fail to form. In this case, *hh* would not be acting as an MP3 proneural gene.

It is important to note that *hh* signaling is postulated to convert a group of about five cells to an MP3 fate (Fig. 8C). The selection of the single MP3 found in each segment is through *Notch* signaling (Fig. 8D) (Wheeler et al., 2008). In this manner, *Delta-Notch* lateral inhibition results in the appearance of a single MP3, while the remaining cells become AMG and PMG. However, the division of MP3 is dependent on *hh*, and not *Notch*, signaling, as both MP3 and MP1 divide and differentiate in *Delta* mutant embryos (Wheeler et al., 2008). When *hh* signaling is activated in all midline cells in either *ptc* mutants or by *hh* pathway gene overexpression, cells destined to become MP1 instead become MP3. This suggests that, in wild-type embryos, the Hh morphogen is insufficiently active to direct the anteriormost cells to become MP3 even though these cells have the intrinsic ability to become MP3 if *hh* signaling is activated. Future studies will address the pathway by which *hh* controls MP3 formation, how MP1 is specified, and how *hh* signaling is inhibited in the cells that give rise to MP1.

### *gsb*/*gsb-n* direct MP3 cell fate

*gsb* and *gsb-n* are targets of *hh* signaling and act to specify MP3 cell fate. Although not required for MP3 delamination or division, *gsb*/*gsb-n* mutant embryos did show a delay in the timing of MP3 division, which can be considered an aspect of cell fate. Both genes

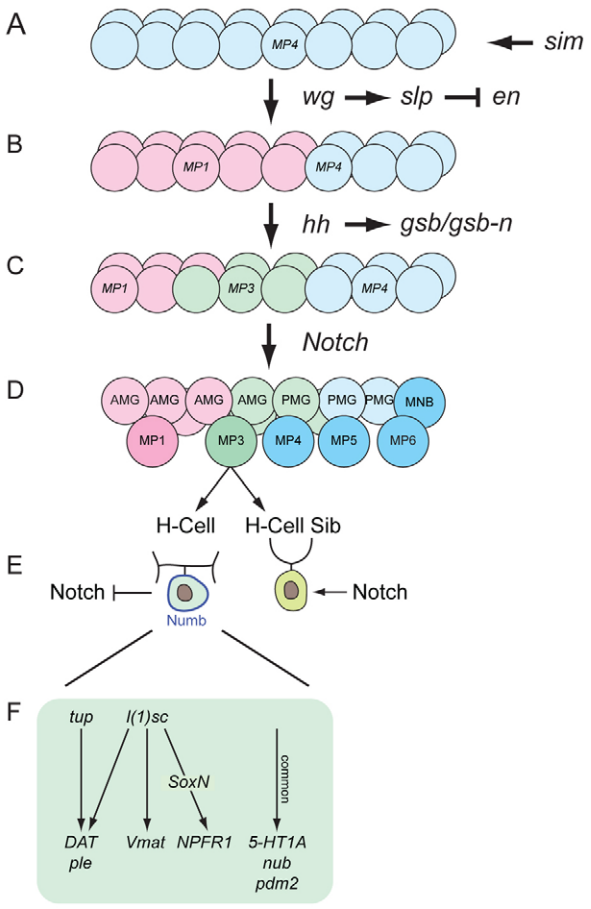


**Fig. 8. Major steps in MP3 and H-cell development.** A single segment is shown. (**A**) *sim* commits mesectodermal cells to an MP4 fate. (**B**) *wg* signaling activates *slp1/2* expression in the anterior region, which represses *en* and commits cells to an MP1 fate (pink). (**C**) *hh* signaling directs a group of anterior cells to an MP3 fate (green) by activating expression of *gsb/gsb-n*. (**D**) *Notch* signaling selects one cell in the MP3 cluster to become MP3. (**E**) H-cell and H-cell sib fates are determined by *Notch* signaling and asymmetric localization of Numb. *gsb/gsb-n* activate expression of *l(1)sc* and *tup* in the H-cell. (**F**) *l(1)sc* and *tup* control H-cell-specific gene expression and differentiation. Genes expressed in common between the H-cell and H-cell sib are regulated by a different pathway.

are expressed in MP3 and each plays a role in MP3 cell fate specification. Embryos homozygous mutant for *gsb* show defects in MP3 development, but these are less severe than in *gsb gsb-n* double-mutant embryos, indicating functional roles for both genes. One function of *gsb* is to activate expression of *gsb-n* in MP3, indicating that these genes might function in a hierarchical manner. The role of *gsb-n* was reinforced from misexpression experiments, in which MP1 was transformed into MP3.

Upon division of MP3, the two progeny, i.e. the H-cell and H-cells sib, acquire their distinct identities due to Numb asymmetric localization and *Notch* signaling (Fig. 8E) (Wheeler et al., 2008). Whereas H-cell sib differentiation is dependent on *Notch* signaling, H-cell differentiation is largely dependent on the L(1)sc and Tup transcription factors (Fig. 8F). Genetically, *gsb*/*gsb-n* function is required for expression of *l(1)sc* and *tup*, linking cell fate to differentiation. Interestingly, *l(1)sc* also controls mVUM-specific gene expression in addition to H-cell-specific gene expression. This raises the question of how the same transcription factor, L(1)sc, controls two distinct developmental programs. Since the H-cell and mVUMs differ in their MP precursors, one possibility is that Gsb/Gsb-n combinatorially interact with L(1)sc to control H-cell transcription and differentiation, whereas L(1)sc interacts with an unknown MP4-6 cell fate factor to control mVUM transcription and differentiation.

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**Competing interests statement**
The authors declare no competing financial interests.

**Supplementary material**
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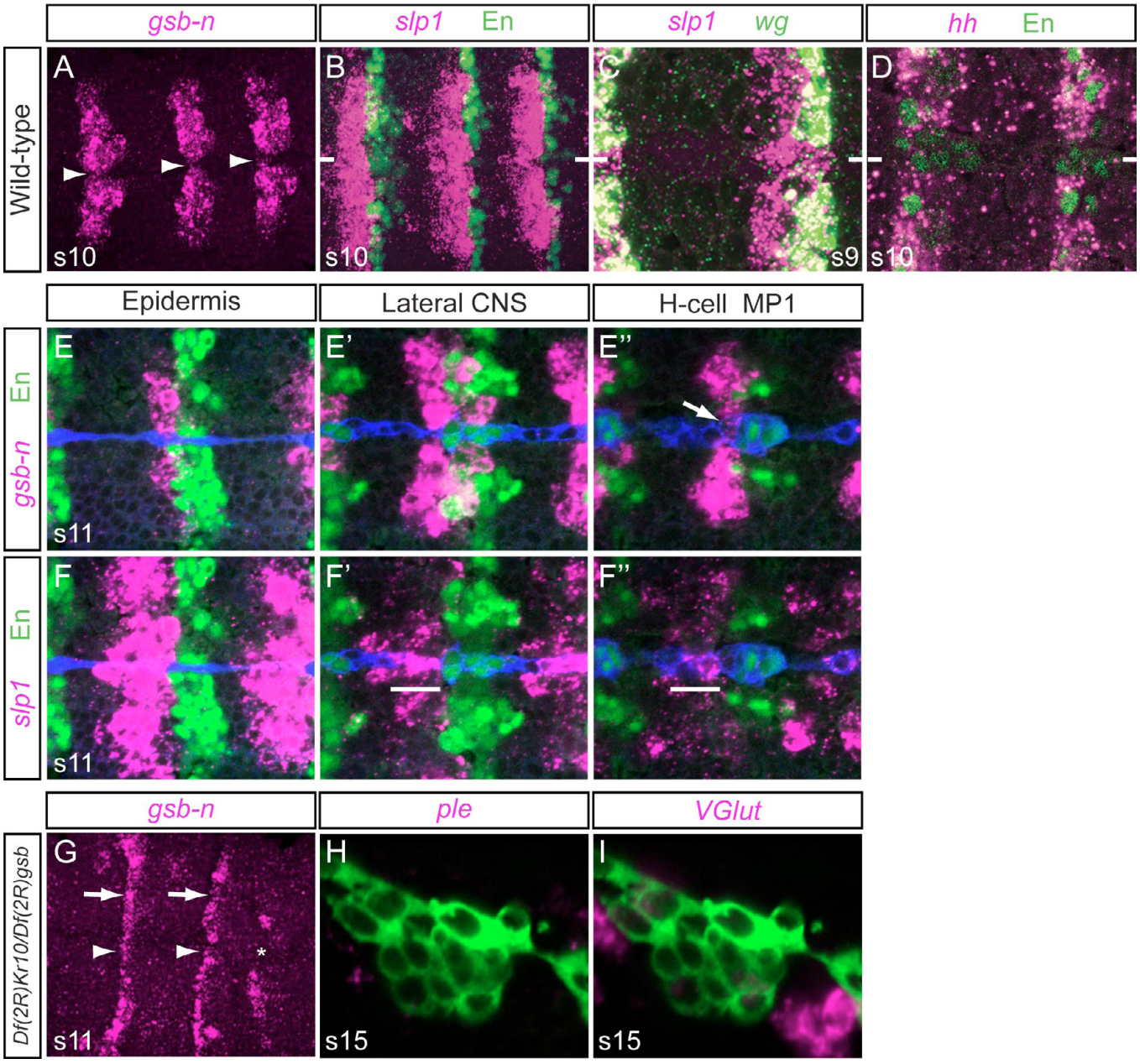


**Fig. S1. CNS midline and ectodermal expression of *Drosophila* segmentation genes.** Horizontal views are shown with anterior to the left. Short white lines mark the location of the midline in some panels. (**A**) At stage 10, *gsb-n* is expressed in MP3 (arrowheads) and in broader collinear neuroectodermal stripes. Three segments are shown. Expression of *gsb* is similar but broader (data not shown). (**B**) *slp1* is expressed in anterior midline cells at stage 10. (**C**) Expression of *wg* at stage 9 is largely absent from midline cells, but is present in a stripe of cells perpendicular to the midline cells. *wg* overlaps in expression with the posteriormost *slp1*$^{+}$ cells. (**D**) The *hh* gene is expressed in a stripe perpendicular to the midline cells at stage 10, but is absent from the midline cells. The *hh* stripe overlaps the En stripe. (**E-F″**) Horizontal views of stage 11 *sim-Gal4 UAS-tau-GFP* embryos stained for (E) *gsb-n* RNA or (F) *slp1* RNA in addition to anti-En and anti-Tau (blue). Three different focal planes reveal the epidermis, lateral CNS, and midline H-cell and MP1 neurons. (E-E″) *gsb-n* expression in the lateral CNS is broader than midline expression, which is restricted to H-cell and H-cell sib (arrow). (F-F″) *slp1* is expressed in anterior midline cells (above the white line) and is adjacent to, but does not overlap, En. (**G**) *Df(2R)Kr10/Df(2R)gsb* embryos are homozygous mutant for *gsb* and lack one copy of *gsb-n*. Lateral CNS expression of *gsb-n* (arrows) is reduced compared with wild type (A). Midline expression of *gsb-n* is present in some segments (arrowheads) and absent in others (asterisks). (**H**,**I**) Both *ple* and *VGlut* expression are frequently absent in *Df(2R)Kr10/Df(2R)gsb* embryos.

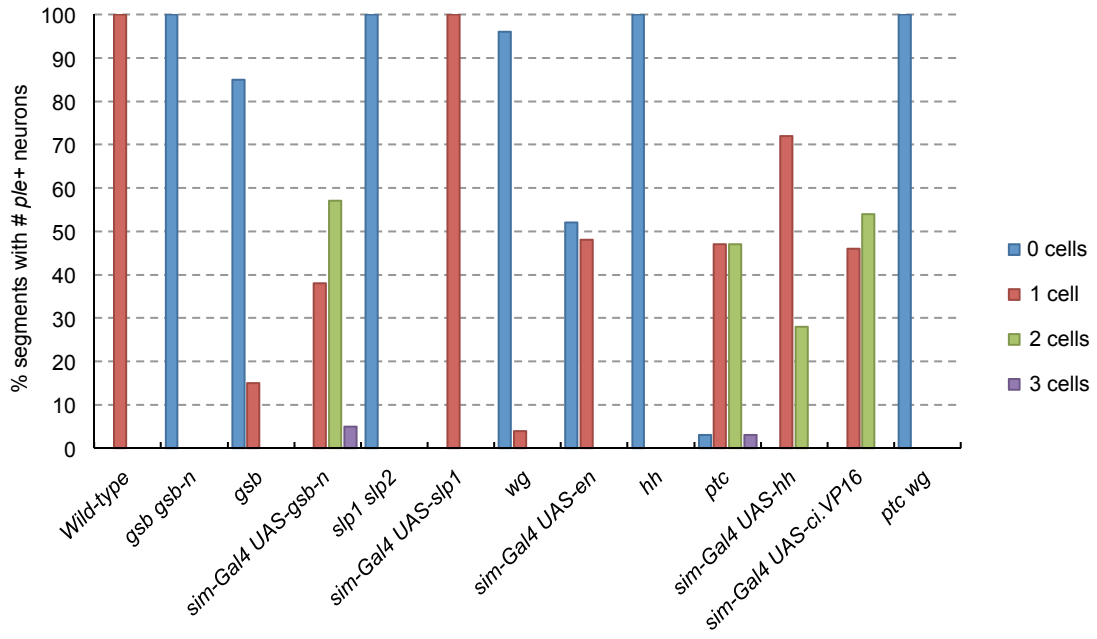


**Fig. S2. Summary of genetic experiments analyzing H-cell development.** The number of *ple*$^{+}$ midline cells observed in each segment was counted and expressed as a percentage. Segments scored for each mutant or misexpression genotype

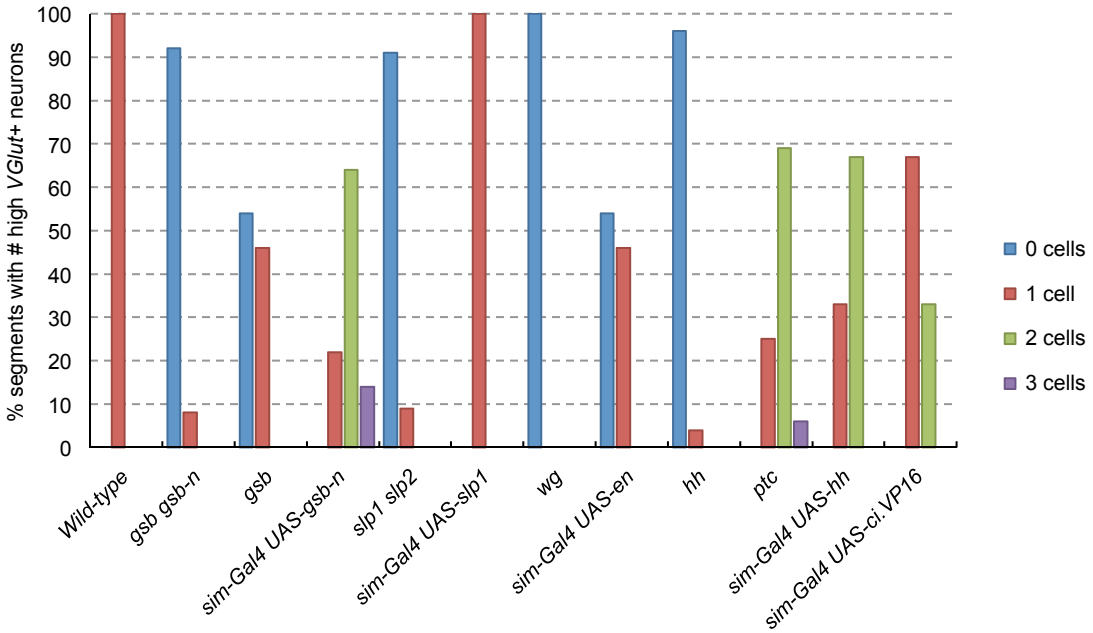


**Fig. S3. Summary of genetic experiments analyzing H-cell sib development.** The number of high *VGlut* cells observed in each segment was counted and expressed as a percentage. Segments scored for each mutant or misexpression genotype ranged between nine and 22.

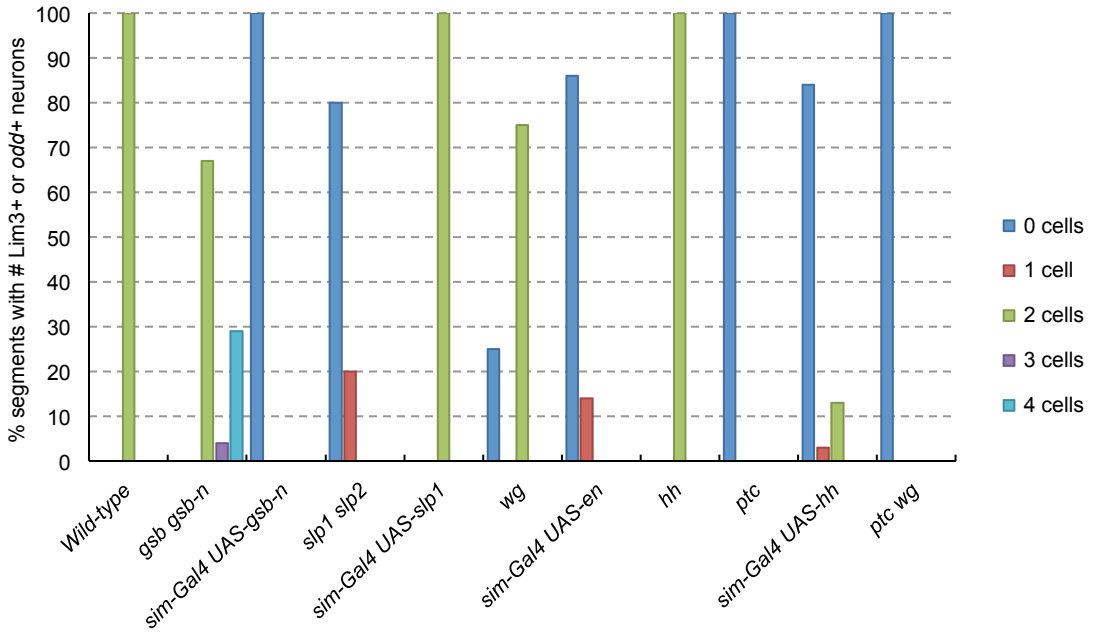


**Fig. S4. Summary of genetic experiments analyzing MP1 neuron development.** The number of Lim3$^{+}$ or *odd*$^{+}$ cells observed in each segment was counted and expressed as a percentage. Segments scored for each mutant or misexpression genotype ranged between eight and 30.

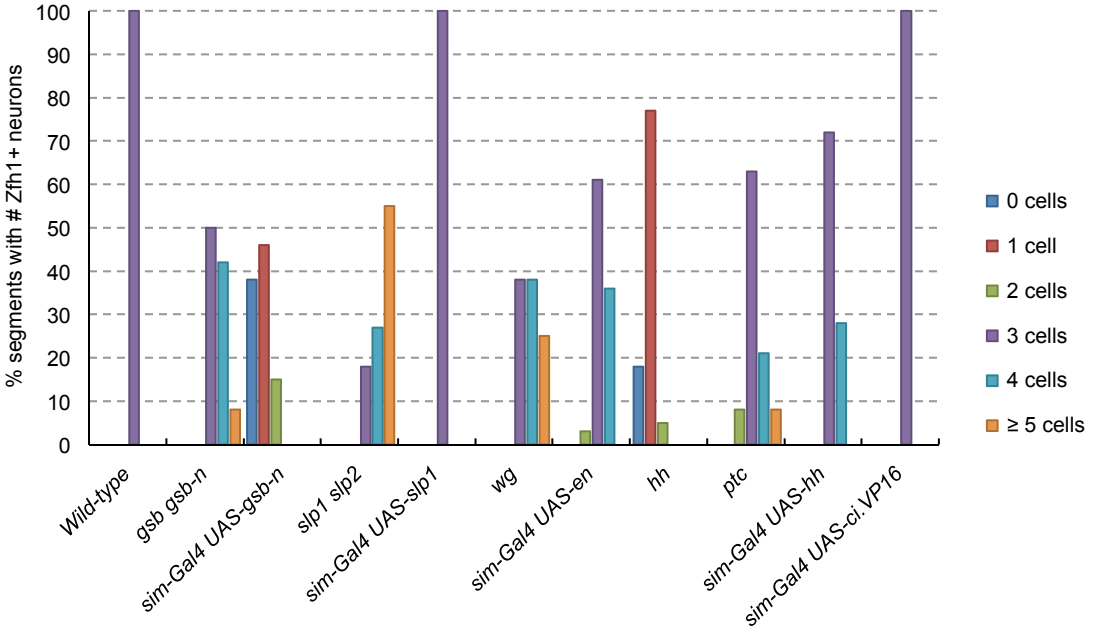


**Fig. S5. Summary of genetic experiments analyzing mVUM neuron development.** The number of Zfh1$^{+}$ cells observed in each segment was counted and expressed as a percentage. Segments scored for each mutant or misexpression genotype ranged between eight and 44.

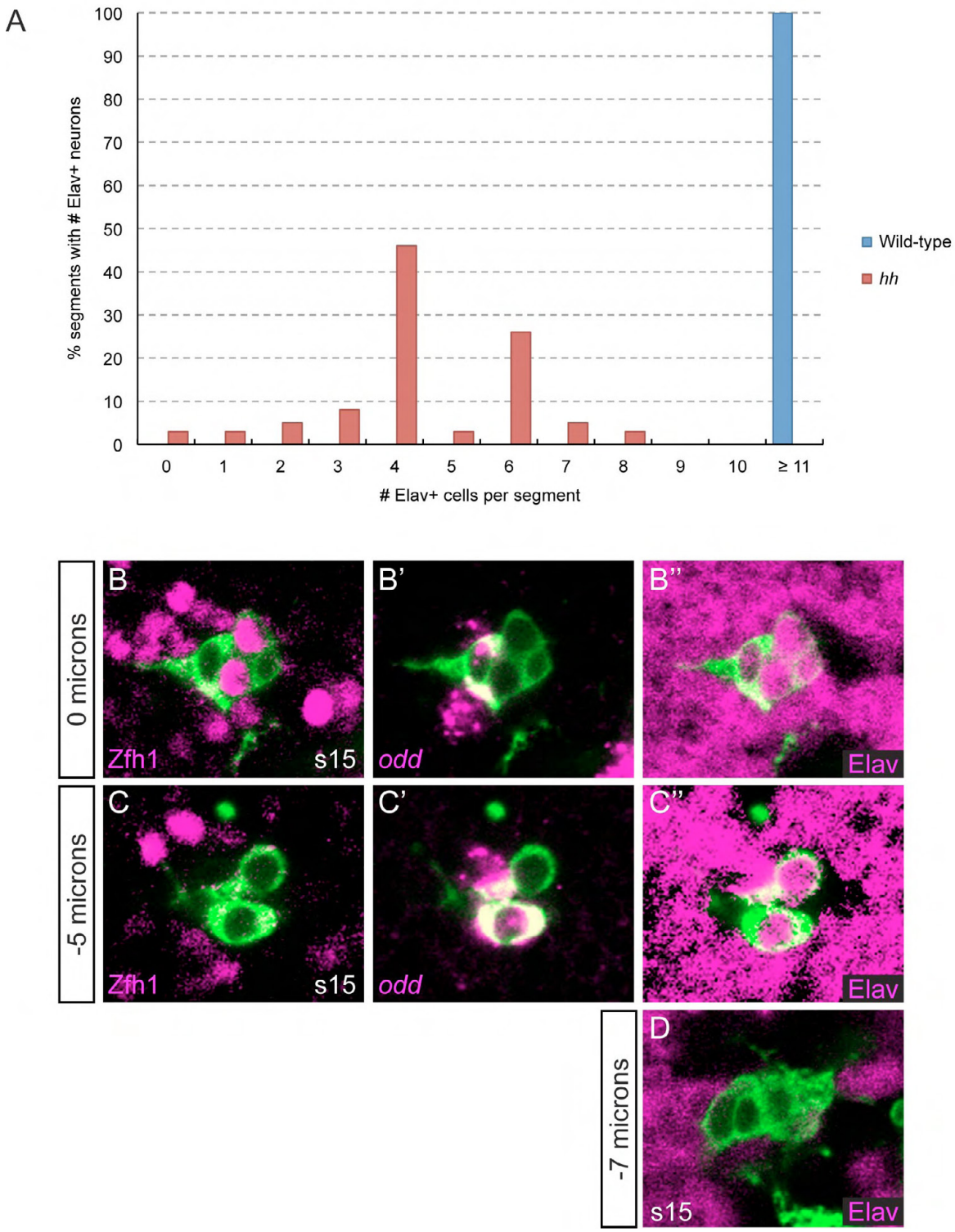


**Fig. S6. Number and identity of midline neurons in *hh* mutant embryos.** (**A**) The number of Elav$^{+}$ neurons observed in each segment of wild-type (*n*=22 segments) and $hh^{AC}$ mutant (*n*=39 segments) embryos was counted and expressed as a percentage. Most mutant segments had either four or six neurons, whereas wild-type segments had 11 or more. (**B-D**) Horizontal views of a single segment of a stage 15 $hh^{AC}$ *sim-Gal4 UAS-tau-GFP* mutant embryo shown at three focal planes (difference in distance is indicated on the left). The embryo was stained for anti-Tau (green), Zfh1, *odd* and Elav (all magenta). Imaging throughout the segment revealed that only six Tau-GFP$^{+}$ cells were Elav$^{+}$. Two of these were Zfh1$^{+}$ (mVUMs) and two were *odd*$^{+}$ (MP1s). The other two are likely to be iVUMs.

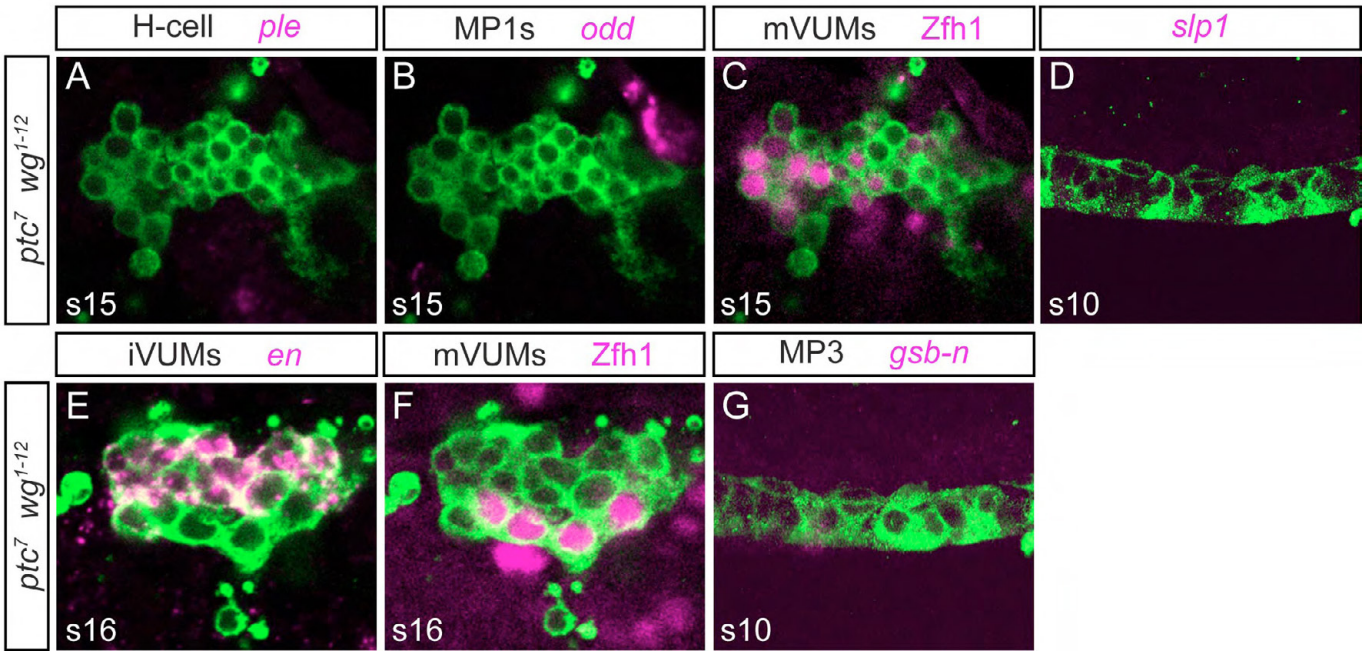


**Fig. S7. Both *wg* and *hh* are required for activation of *gsb-n* in MP3.** (**A-C**) In the same *ptc*$^{7}$ *wg*$^{1-12}$ double-mutant embryo, expression of (A) *ple* (H-cell) and (B) *odd* (MP1 neurons) was absent, and (C) Zfh1 (mVUMs) was expanded. (**D**) *slp1* was absent in *ptc*$^{7}$ *wg*$^{1-12}$ embryos. (**E**,**F**) In another embryo, both (E) *en* and (F) Zfh1 were expanded. In the midline cells shown (which might constitute more than one fused segment), there were 20 En$^{+}$ cells and ten Zfh1$^{+}$ cells (not all can be seen in this focal plane). Together, they constitute all of the midline cells present. (**G**) *gsb-n* expression was absent in *ptc*$^{7}$ *wg*$^{1-12}$.