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Single-cell mapping of neural and glial gene expression in the developing *Drosophila* CNS midline cells

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Abstract

Understanding the generation of neuronal and glial diversity is one of the major goals of developmental neuroscience. The *Drosophila* CNS midline cells constitute a simple neurogenomic system to study neurogenesis, cell fate acquisition, and neuronal function. Previously, we identified and determined the developmental expression profiles of 224 midline-expressed genes. Here, the expression of 59 transcription factors, signaling proteins, and neural function genes was analyzed using multi-label confocal imaging, and their expression patterns mapped at the single-cell level at multiple stages of CNS development. These maps uniquely identify individual cells and predict potential regulatory events and combinatorial protein interactions that may occur in each midline cell type during their development. Analysis of neural function genes, including those encoding peptide neurotransmitters, neurotransmitter biosynthetic enzymes, transporters, and neurotransmitter receptors, allows functional characterization of each neuronal cell type. This work is essential for a comprehensive genetic analysis of midline cell development that will likely have widespread significance given the high degree of evolutionary conservation of the genes analyzed.

Keywords: CNS; *Drosophila*; Glia; Mesectoderm; Midline cells; Neurogenesis; Neurons

Introduction

The formation of the mature CNS with its diverse assortment of neurons and glia is a complex process that requires a large number of interacting proteins functioning throughout development. Studying this process involves examining how cells acquire their specific fates, divide, migrate, die, extend axons and dendrites, form synapses, and carry-out specific neurophysiological functions. Recent progress on a variety of model systems has utilized genomic techniques to study neural and glial development and function (for example: Blackshaw et al., 2004; Cinar et al., 2005; Freeman et al., 2003; Gray et al., 2004; Reeves and Posakony, 2005; Wenick and Hobert, 2004). However, most systems study terminally differentiated neurons or deal with populations of cells and may miss some of the underlying complexity in neural development. Particularly important is the capability of studying a complex set of neurons and glia in vivo at the single-cell level throughout the entire developmental process. This paper demonstrates that the *Drosophila* CNS midline cells can be studied in such a fashion, and a large number of genes can be assigned to specific cell types at each discrete stage of CNS development.

The mature *Drosophila* CNS midline cells consist of ~22 cells/segment: ~3 midline glia, 2 midline precursor 1 (MP1) neurons, 2 MP3 interneurons (H-cell and H-cell sib), 3 ventral unpaired median interneurons (iVUMs), 3 ventral unpaired median motorneurons (mVUMs), and the median neuroblast (MNB), which generates 7–8 progeny during embryogenesis. The generation of the mature midline cells arises through a series of developmental steps: (1) specification of mesectodermal cells, (2) cell division, (3) acquisition of individual midline cell fates, (4) cell migration, (5) apoptosis, and (6) terminal differentiation resulting in functional neurons and glia. When initially specified during the blastoderm stage, ~8 cells are present in each segment, 4 on either side of the mesoderm, that come together as gastrulation proceeds. These cells are

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characterized by expression of the *single-minded* (*sim*) gene, which acts as a master regulator that is required for all subsequent midline transcription and development (Crews, 2003; Nambu et al., 1991). Five of these 8 cells are the midline precursors (MPs), each will divide only once to generate two neurons. In contrast, the MNB is a stem cell that divides multiple times to produce a characteristic clone of midline neurons (Bossing and Technau, 1994; Schmid et al., 1999), similar to neuroblasts in the lateral CNS. The glial progenitors also undergo multiple rounds of cell division to produce ~10 midline glia, although the exact number of glial progenitors is unknown. During mid-embryogenesis, the midline cells migrate inward to their final positions, and differentiate into mature neurons and glia. The ~10 midline glial cells that arise by mid-embryogenesis are depleted by apoptosis, with 2–3 mature glia remaining to ensheathe the axon commissures (Bergmann et al., 2002; Jacobs, 2000). Thus, the midline cells display a diversity of cell types, modes of cell division, developmental programs, and functions.

Because of their characteristic midline location, identifying genes expressed in CNS midline cells is easy. Using this feature, we employed in situ hybridization to identify and initially characterize the expression of 224 midline-expressed genes at each stage of embryonic development (Kearney et al., 2004); we have subsequently expanded this to include a total of 278 midline-expressed genes (unpublished data). Sixty-four genes are expressed in mesectodermal cells (stages 5–8; staging according to Campos-Ortega and Hartenstein, 1997), 162 in midline primordia (stages 9–12), 65 in mature midline glia, and 131 in mature midline neurons (mature cells span stages 13 to 17). Furthermore, these genes encode 72 transcription factors and 44 signaling proteins, thus representing a large number of genes likely to play roles in midline cell development.

Despite these significant advances in midline gene identification and developmental biology, progress in understanding the genetic basis of how midline neurons and glia are generated has been hindered by the inability to distinguish each midline cell type during their development. This deficiency makes it difficult to interpret genetic experiments. In this paper, this weakness is addressed by combining in situ hybridization, immunostaining, and confocal microscopy to study midline gene expression with single-cell resolution. Here, we mapped 59 genes to individual midline cell types at four developmentally important stages (9, 11, 13, and 17). Included in this set of genes are 27 transcription factors and 9 signaling proteins. We also mapped the expression patterns in neurons of 11 genes that encode peptide neurotransmitters, neurotransmitter biosynthetic enzymes, vesicular and membrane transporters, and neurotransmitter receptors providing insight into their unique neuronal characteristics. These maps allow the construction of detailed developmental pathways for each midline cell type that chart the changes in gene expression that occur during embryogenesis, and illustrate the relationships between midline cells from stages 11 to 17. Many of the genes identified are likely to regulate developmental and transcriptional events that culminate in the mature set of midline neurons and glia. Using the information presented in this paper, genetic analysis of these genes and others have the potential to provide a comprehensive, genome-wide view of neural and glial development and function.

Materials and methods

Drosophila strains

CNS midline cells were visualized using a variety of *lacZ* and *Gal4* lines. These include: *3.7-sim-lacZ* (all early midline cells; Nambu et al., 1991), *C544-Gal4* (MP1s; Landgraf et al., 2003), *MzVUM-Gal4* (mVUMs; Landgraf et al., 2003), *807-Gal4* (mVUMs; A. Brand, unpublished), *per-Gal4* (H-cell sib and iVUMs; Plautz et al., 1997), *TH-Gal4* (H-cell; Friggi-Grelin et al., 2003), and *sim*-Gal4 (all CNS midline cells; Xiao et al., 1996). *UAS* lines included: *UAS-tau-lacZ* (Callahan and Thomas, 1994), *UAS-GFP-lacZ.nls* (Y. Hiromi and S. West, unpublished), and *UAS-tau-GFP*.

Sources of cloned DNA for in situ hybridization

cDNA clones from the Open Biosystems BDGP collection (v1 and v2) (Stapleton et al., 2002) were used to prepare in situ hybridization probes for most of the genes analyzed. Six other genes (*CG33528, CG8394*, *DAT*, *hh*, *SoxN*, and *Tbh*) were PCR-amplified using gene-specific primers from either genomic DNA or cDNA prepared from *Drosophila* embryonic or larval RNA.

In situ hybridization and immunostaining

In situ hybridization and immunostaining were performed as previously described (Kearney et al., 2004). Primary antibodies used were anti-Futsch (MAb 22C10; Developmental Studies Hybridoma Bank, DSHB), mouse anti-β-galactosidase (Promega), rabbit anti-β-galactosidase (Cappel), rabbit anti-Castor (Kambadur et al., 1998), anti-Engrailed MAb 4D9 (Patel et al., 1989a), rabbit anti-Even-skipped (East Asian Distribution Center; EADC; Kosman et al., 1998), rabbit anti-GFP (Abcam), guinea pig anti-Hunchback (EADC), rabbit anti-Paired (EADC), anti-Prospero MAb (DSHB), guinea pig anti-Runt (EADC), rat anti-Single minded (Ward et al., 1998), rabbit anti-Sloppy paired 1 (EADC), mouse anti-Tau (Sigma), and rabbit anti-Sanpodo (O'Connor-Giles and Skeath, 2003).

Results

Experimental approach

From a set of 278 identified CNS midline genes and markers (Kearney et al., 2004), a group of 59 RNA probes, antibodies, and *lacZ* and *Gal4* lines was selected to generate molecular maps at several stages of midline cell development (maps and images available at http://www.unc.edu/~crews). These genes were selected because they encode (1) transcription factors and signaling proteins likely to play important roles in midline cell development, and (2) neural function proteins that mediate the excitable properties of neurons. Four developmental stages (9, 11, 13, and 17) were chosen as they represent useful milestones in the development of these cells. We first identified the gross morphology of the midline cells at each stage and then overlaid gene expression patterns using fluorescent in situ hybridization and immunostaining. Midline cells were identified using a CNS midline-specific driver, *sim-Gal4*, which in combination with *UAS-tau-lacZ* (Callahan and Thomas, 1994) or *UAS-tau-GFP*, marks all midline cells at stages 11–17 (Figs. 2A, 4A, 5A insets). For stage 9, the midline cells were visualized using an antibody against Sim

(Ward et al., 1998) or anti-β-galactosidase staining of *sim-lacZ* embryos (Nambu et al., 1991), both of which mark all midline cell nuclei (Fig. 6A inset).

Relating midline-expressed genes at stage 17 to mature cell types involves correlating three sets of information: (1) DiI-labeled axonal trajectories and cell positions (Bossing and Technau, 1994; Schmid et al., 1999), (2) *Midline enhancer-Gal4 UAS-tau-lacZ* transgenic line axonal trajectories and positions (Kearney et al., 2004), and (3) in situ hybridization or immunostaining (Fig. 1). Various *Gal4* lines that are expressed in subsets of midline cells (*Midline enhancer-Gal4*) were genetically combined with *UAS-tau-lacZ*, and their axonal trajectories compared to those of previously published DiI-labeled cells (Figs. 1A–E) (Kearney et al., 2004). This established in which midline cell type each *Gal4* line was expressed. Colocalization of midline-expressed genes, assayed by in situ hybridization, with *Midline enhancer-Gal4 UAS-tau-lacZ* expression allows assignments of gene expression to specific midline cell types (Figs. 1F–H). Once a gene has been assigned to a specific cell type, it can, itself, be used in colocalization studies with additional genes (Figs. 1I–K).

Stage 17

Stage 17 midline cells consist of two major cell groups, the mature midline glia and midline neurons, which reside at characteristic positions. The midline glia ensheathe the axon commissures along the dorsal side of the CNS, while the midline neurons reside ventral to the glia (Fig. 2A).

Midline glia

Most commonly, the 3 midline glia are arranged in a triangle, in which two are dorsal and one is ventral to the commissures (Fig. 2A, inset), and each glial cell extends its cytoplasm around one or both of the commissures. DiI labeling experiments have provided data arguing that mature midline glia arise from either one or two precursor cells (Bossing and Brand, 2006; Bossing and Technau, 1994). Using confocal analysis, we analyzed 9 genes and markers expressed in all mature midline glia (Supplementary Table 1; only 4 are included in Fig. 2A). Among these are the transcription factor genes *runt*, *sim*, *snail* (*sna*), *SoxNeuro* (*SoxN*), and *ventral veinless* (*vvl*), and signaling protein genes *argos* (Fig. 2B), *slit* (*sli*), and *wrapper* (Fig. 2C). No genes expressed in subsets of midline glia at stage 17 were observed, suggesting that all mature midline glia are functionally equivalent.

MP1 neurons

The 2 lineally related MP1 neurons have identical axonal trajectories and patterns of gene expression suggesting that they are functionally similar, and possibly, identical. The axonal morphology and position of the cells labeled by *C544-Gal4* are identical to those of the MP1 neurons indicating that *C544-Gal4* is expressed in MP1s (Fig. 1A). Expression of both the *odd-skipped* (*odd*) transcription factor gene and *C544-Gal4* colocalize, confirming that *odd* is also expressed in MP1

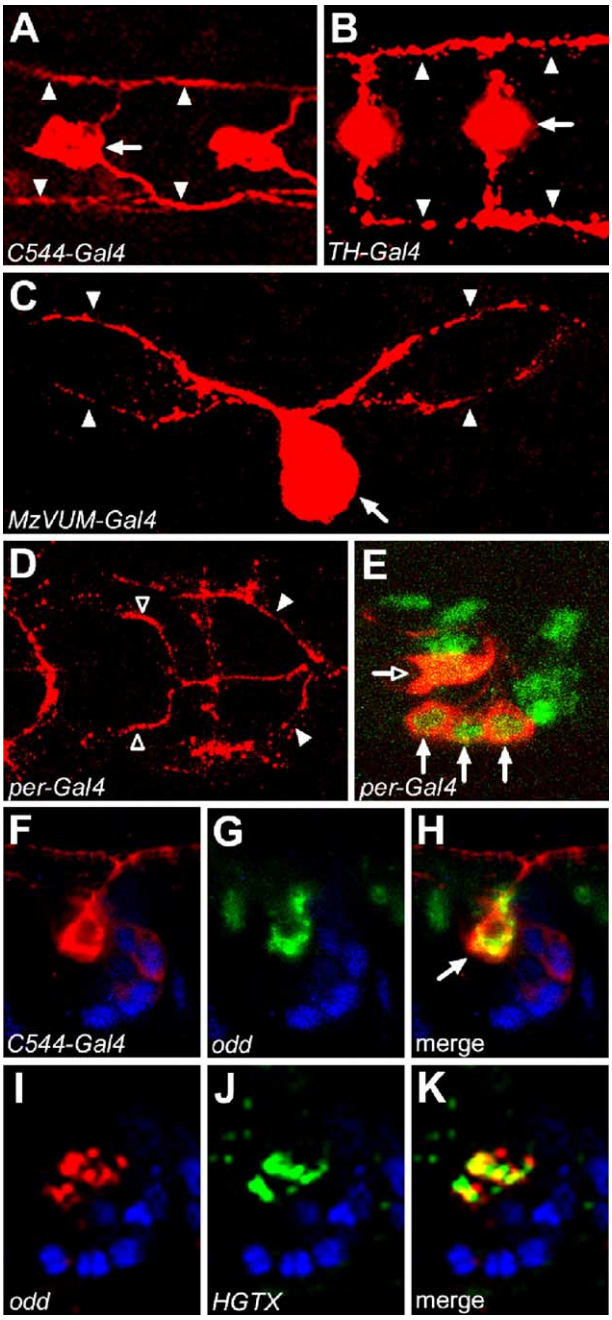
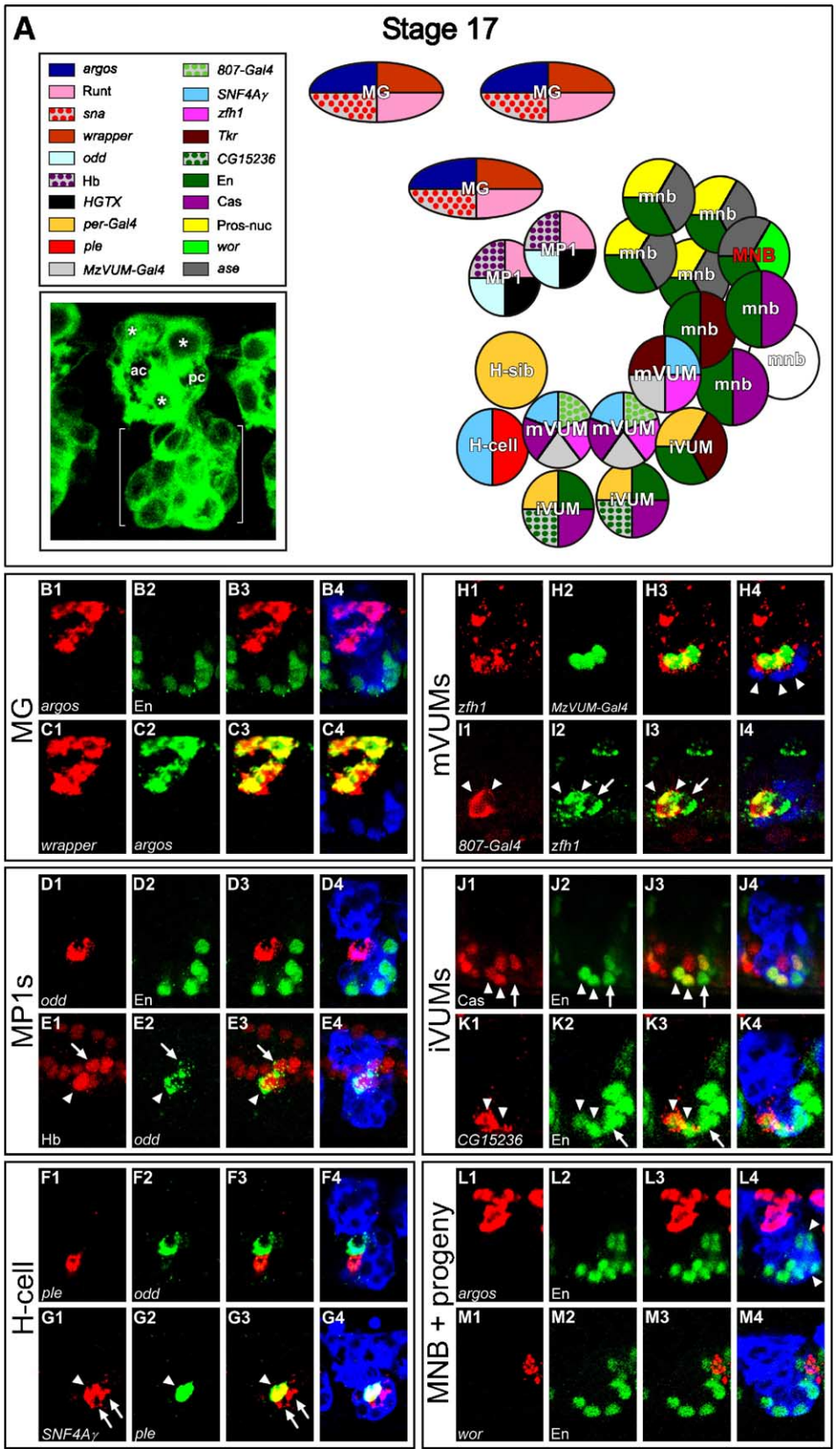


Fig. 1. *Gal4* transgenic reporters and midline cell identification. (A–D) Ventral and (E) sagittal views of stage 15–17 *Midline enhancer-Gal4*; *UAS-tau-lacZ* embryos stained with anti-β-galactosidase. Midline cell bodies (arrows) and corresponding axons (arrowheads) are shown. *Gal4* drivers are indicated at the bottom of each panel. The midline cells defined by each Gal4 line are: (A) MP1s, (B), H-cell, (C) mVUMs, and (D, E) H-cell sib (open arrow and arrowheads) and iVUMs (filled arrows and arrowheads). In panel D, only the axons are shown, the cell bodies are out of the plane of focus, whereas in panel E, the cell bodies of H-cell sib (open arrow) and the 3 iVUMs (filled arrow) are shown. (E) Embryo is also stained with anti-Sim (green) showing that H-cell sib and iVUMs are Sim^{+}. (F–K) Sagittal views of individual multi-labeled segments; En staining (blue) indicates the position of the iVUMs and MNB progeny (see Fig. 2). (F–H) *odd* RNA (green) colocalizes with *C544-Gal4* (red) in the soma (arrow) of the MP1 neurons. (I–K) Colocalization of *odd* (red) and *HGTX* (green) RNA illustrates that *HGTX* is also expressed in MP1 neurons.

neurons (Figs. 1F–H; Kearney et al., 2004), as suggested by others (Spana et al., 1995). Using *odd* colocalization, we identified 9 additional genes expressed in MP1 neurons at stage 17 (Supplementary Table 1). These include the transcription factor genes *forkhead* (*fkh*), *hunchback* (*hb*) (Fig. 2E), *HGTX* (Fig. 1K) and *runt*.



We also identified some of the machinery that confers MP1 neuronal function. The MP1 neurons in segments A7-8 express *Pigment-dispersing factor* (*Pdf*) (Fig. 3B; expression of neural function genes is summarized in Fig. 3A), which encodes a neuropeptide that controls adult circadian locomotion (Renn et al., 1999). *Pdf* is initially expressed at stage 15 in most MP1 neurons, but by stage 17 is restricted to A7-8. The MP1 neurons also express the *5-HT1A* serotonin receptor gene, indicating serotonergic input. The MP1 neurons in CNS ganglia S3-A4 undergo programmed cell death at late stage 17 (Miguel-Aliaga and Thor, 2004). Thus, the anterior MP1 neurons may only play a role in pioneering axon guidance (Jacobs and Goodman, 1989) and not in neural function. In contrast, the expression of *Pdf* and *5-HT1A* in stage 17 A7-8 MP1 neurons suggests that these MP1 neurons function in neurotransmission.

H-cell

Previous work in *Drosophila* revealed a pair of clonally derived neurons whose axonal morphology is similar to the grasshopper H-cell and H-cell sib, and are likely to be homologous cell types (Bossing and Technau, 1994; Schmid et al., 1999). In grasshopper, they are the progeny of the MP3 cell (Goodman, 1982), and the *Drosophila* neurons (also called unpaired median interneurons (UMIs) (Bossing and Technau, 1994)) are also likely to be progeny of an MP3 cell. Both *Drosophila* and *Manduca sexta* have a single dopaminergic neuron along the midline that contains tyrosine hydroxylase (TH) (Budnik and White, 1988; Kearney et al., 2004; Mesce et al., 2001), a key enzyme in dopamine synthesis. In *Drosophila,* TH is encoded by the *pale* (*ple*) gene (Neckameyer and White, 1993), and the *TH-Gal4* line drives reporter gene expression in a single midline neuron. This cell shows axonal morphology characteristic of the H-cell (Fig. 1B), indicating that the H-cell is dopaminergic.

Using *ple* colocalization, 8 additional genes were shown to be expressed in the H-cell (Supplementary Table 1). These genes encode SoxN and SNF4/AMP-activated protein kinase gamma subunit (SNF4Aγ) (Fig. 2G). Multiple neurotransmission genes are expressed in the H-cell (Fig. 3A). Consistent with dopamine production, the H-cell expresses the *dopamine transporter* (*DAT*) gene (Fig. 3C) and *CG33528*, a vesicular monoamine transporter (VMAT) (Fig. 3D) (Greer et al., 2005). The H-cell also expresses the *neuropeptide F receptor* (*NPFR1*) gene (Fig. 3E), a serotonin receptor (*5-HT1A*) (Fig. 3F), and the glutamate receptor (*Glu-RI*). This indicates that the dopamine-producing H-cell has serotonergic, peptidergic, and glutamatergic synaptic inputs. *NPFR1* expression is restricted to thoracic segments, indicating segmental differences in H-cell function.

H-cell sib

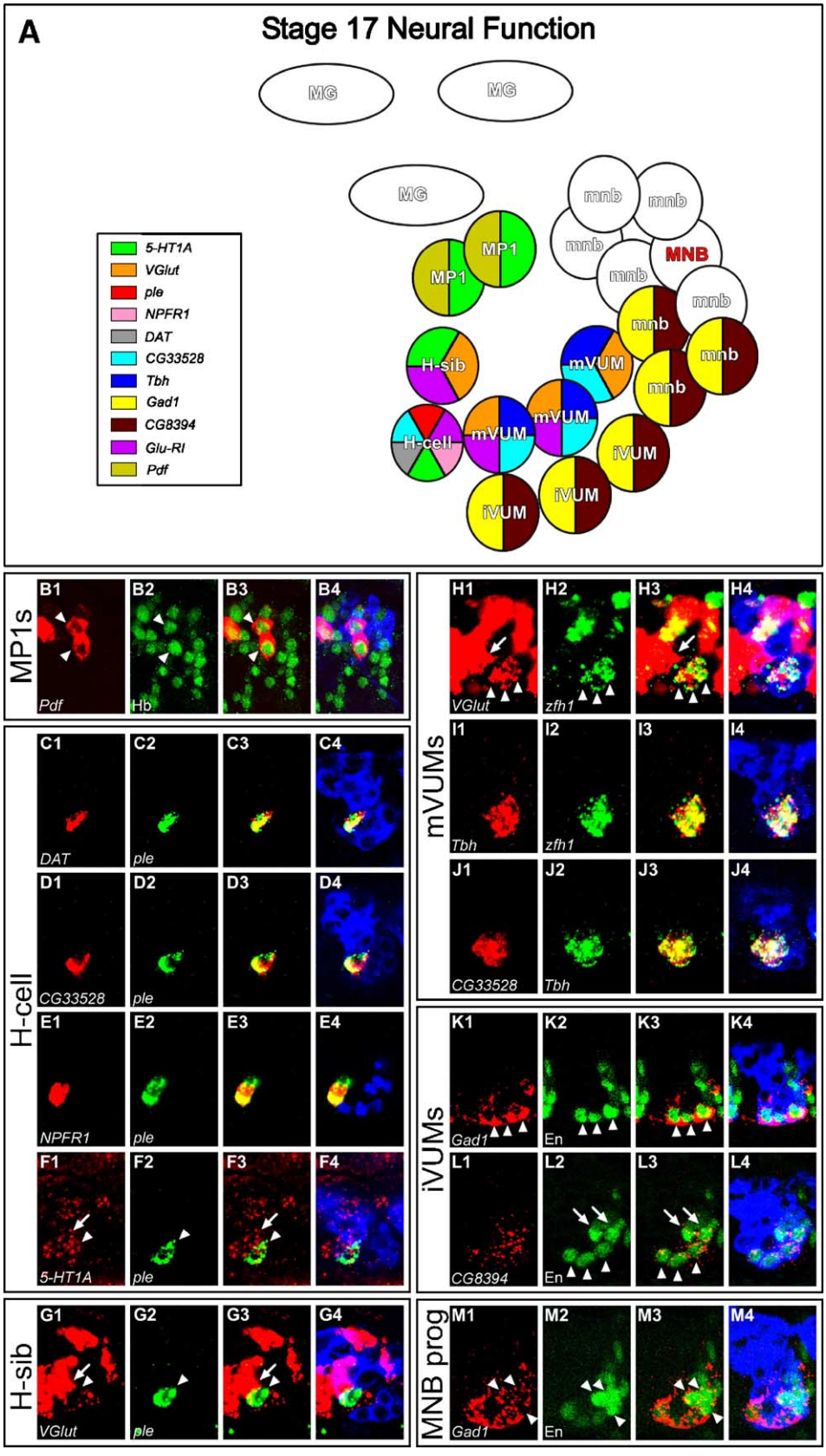
H-cell sib lies adjacent to the H-cell, but little is known about this cell other than its axonal trajectory. Expression of *period* (*per*)-*Gal4* shows that the axonal morphology of one of the 4 *per-Gal4* expressing midline cells is identical to previous reports describing the axonal trajectories of H-cell sib (Figs. 1D, E) (Bossing and Technau, 1994; Schmid et al., 1999). Two transcription factor genes whose expression colocalizes with *per-Gal4* are *forkhead* (*fkh*) and *sim*. These are particularly noteworthy since neither is expressed in the H-cell. H-cell sib expresses the *CG9887 vesicular glutamate transporter* (*VGlut*) gene (Daniels et al., 2004), indicating that H-cell sib is glutamatergic (Fig. 3G). Since H-cell sib appears to be an interneuron, it presumably releases glutamate onto excitatory central synapses. In addition, H-cell sib expresses *5-HT1A* (Fig. 3F) and *Glu-RI*, indicating that, like the H-cell, this neuron receives input from serotonergic and glutamatergic neurons.

VUM motorneurons

The mVUMs are distinguished by expression of the *MzVUM-Gal4* enhancer trap line, which shows the characteristic axonal pattern of mVUMs when driving expression of *UAS-tau-lacZ* (Fig. 1C) (Kearney et al., 2004; Landgraf et al., 2003). Several genes are expressed in all 3 mVUMs: these include the *Zn finger homeodomain 1* (*zfh1*) transcription factor gene (Fig. 2H), *SNF4Aγ*, and *futsch*, which encodes a cytoskeletal protein found in axons and dendrites. Previous dye-labeling studies showed that each mVUM arises from a separate midline precursor and is lineally related to an iVUM. It remains unclear if each mVUM is located at the same anterior–posterior position as its sibling iVUM. These studies further provided evidence for mVUM diversity: axons from two of the mVUMs exit the intersegmental nerve and innervate medial and dorsal muscles, while the other mVUM axon exits the segmental nerve and innervates ventral muscles (Bossing and

Fig. 2. A molecular map of the midline cells at stage 17. (A) Schematic of stage 17 CNS midline neurons (circles) and glia (ovals) shown in sagittal view. Each cell type expresses a characteristic set of genes (see key at left). Inset—confocal projection of a single anti-β-galactosidase-stained stage 17 abdominal segment from a *sim-Gal4*; *UAS-tau-lacZ* embryo. Midline glia (asterisks denote nuclei) surround the anterior commissure (ac) and posterior commissure (pc), and are positioned dorsal to all midline neurons (brackets). (B–M) Single segments stained for the expression of the genes or markers listed in each panel; sagittal views are shown with anterior left and dorsal up. Columns 1 and 2 show gene or *Gal4* expression, column 3 merges these channels, and column 4 shows gene expression compared to all midline cells that are defined by *sim-Gal4*; *UAS-tau-lacZ* (B4, D4–G4, J4–M4) or En (C4, H4, I4) staining. (B) *argos* expression (red) is restricted to midline glia and is distinct from midline neurons stained with En (green). (C) *wrapper* (red) and *argos* (green) are coexpressed in midline glia. (D) *odd* expression (red) is restricted to the MP1s residing just below the midline glia, and do not overlap with En^+ neurons (green). (E) Hb (red) and *odd* (green) overlap in the MP1s (arrowhead). MP2 neurons in the lateral CNS also express Hb and *odd* (arrow). (F) *ple* (red) is expressed in the H-cell, which lies below the odd^+ (green) MP1s. (G) *SNF4Aγ* (red) is expressed in the mVUMs (arrows), and the *ple* (green)-expressing H-cell (arrowhead). (H) *zfh1* (red) is expressed in all mVUMs, as shown by overlap with *MzVUM-Gal4* (green), and not in the En^+ (blue) iVUMs (arrowheads). (I) *807-Gal4* is expressed in the 2 anterior-most mVUMs (arrowheads), and not in the posterior-most $zfh1^+$ mVUM (arrow). (J) Cas (red) is localized to the 2 anterior-most iVUMs (arrowheads); the most posterior iVUM (arrow) is En^+ but not Cas^+. (K) *CG15236* (red) is expressed in the 2 anterior-most iVUMs (arrowheads) and not in the posterior-most En^+ (green) iVUM (arrow). (L) En (green) and *argos* (red) show the relative locations of the MNB progeny (between the arrowheads) and midline glia. (M) *wor* (red) is expressed in the En^+ (green) MNB.

Technau, 1994; Landgraf et al., 1997; Sink and Whitington, 1991). This diversity is also reflected in gene expression. The *807-Gal4* enhancer trap line (Fig. 2I) and Castor (Cas) protein are localized to the 2 anterior-most mVUMs, while the *Tyrosine kinase-related protein* (*Tkr*) gene, which encodes a putative transcription factor, is expressed in the most posterior mVUM.



Since the gene expression profiles of the 2 anterior-most mVUMs are similar, and distinct from the posterior-most mVUM, it is reasonable to propose that axons from the 2 anterior-most mVUMs exit the intersegmental nerve, and the posterior-most mVUM axons exit the segmental nerve, but this has not been directly demonstrated.

Glutamate is the primary neurotransmitter used by insect motorneurons, and, not surprisingly, the mVUMs express *VGlut* (Fig. 3H). In addition to being glutamatergic, the mVUMs express *Tyramine β-hydroxylase* (*Tbh*), which encodes the key biosynthetic enzyme for the production of octopamine, a modulatory monoamine neurotransmitter (Fig. 3I). In addition, the mVUMs express *CG33528*, a vesicular monoamine transporter gene (Fig. 3J). These neurons synapse widely throughout the somatic musculature, consistent with a neuromodulatory role in controlling movement (Landgraf et al., 1997; Sink and Whitington, 1991). The two anterior mVUMs express *Glu-RI*, suggesting that these cells receive glutamatergic input, and further distinguishes these cells from the posterior-most mVUM, which does not express *Glu-RI*.

VUM interneurons

The 3 iVUMs are the ventral-most midline neurons, and *engrailed* (*en*) serves as a reliable marker for these cells (Siegler and Jia, 1999). Using *en* colocalization and cellular position, we identified 10 additional markers localized to iVUMs (Supplementary Table 1). The *fkh* and *sim* transcription factor genes are expressed in all iVUMs, along with *per-Gal4* (Figs. 1D, E). Despite having identical initial axonal trajectories (Bossing and Technau, 1994), iVUMs exhibit differences in gene expression. Both Cas protein (Fig. 2J) and *CG15236* expression (Fig. 2K) are localized to the 2 anterior-most iVUMs, while *Tkr* is expressed in the most posterior iVUM. Expression of neural function genes in iVUMs provides insight into their physiological roles. All 3 iVUMs are inhibitory GABAergic neurons as they express *Gad1*, the gene encoding a key enzyme in GABA biosynthesis (Fig. 3K), and *CG8394*, a putative GABA vesicular transporter gene (VIAAT) (Fig. 3L). While present in all iVUMs, the levels of *Gad1* generally appear higher in the anterior-most and posterior-most cells, and lowest in the middle cell.

MNB and progeny

The MNB is the one midline neuronal cell that undergoes multiple rounds of cell division. By stage 17, 7–8 clonally related MNB progeny reside within a tight cluster of cells in the posterior of the ganglion. The MNB is likely present at stage 17 because one cell in the MNB cluster expresses *worniu* (*wor*) (Fig. 2M) and *asense* (*ase*), two transcription factor genes that mark the MNB earlier in development (see stage 13). The MNB and 6–7 of its progeny express *en* (Fig. 2L), while ~1 progeny cell in each segment does not. The progeny of the MNB also display other differences in gene expression. The Prospero (Pros) transcription factor is localized to nuclei in 2–4 MNB progeny that are adjacent to the MNB, while Cas protein is present in two progeny that do not contain Pros. In the lateral CNS, Pros is observed in ganglion mother cells (GMCs) and transiently in their neuronal progeny (Spana and Doe, 1995). Thus, the $Pros^+$ MNB progeny are likely recently born neurons, while the Cas^+ $Pros^-$ cells are older MNB progeny. Consistent with this interpretation, Cas protein localizes to a subset of $Pros^+$ MNB progeny at stage 13. In addition, *Tkr* is expressed in a single progeny cell that does not contain either Cas or Pros, suggesting that there are at least 3 distinct subsets. The expression of *ase*, *en*, *fkh*, and *per-Gal4* in some, but not all, MNB progeny suggests that there may be further distinctions among MNB progeny.

Three to five MNB progeny express *Gad1* (Fig. 3M) and the *CG8394* GABA vesicular transporter gene (Fig. 3L), indicating that these cells are GABAergic neurons. These neurons are relatively ventral in the MNB progeny cluster, but their exact relationship to other MNB progeny markers has not been determined. We did not detect expression of genes encoding dopaminergic, glutamatergic, or octopaminergic biosynthetic enzymes or transporters in the remaining MNB progeny. Either these cells use a different transmitter or they have not yet fully differentiated into functional neurons.

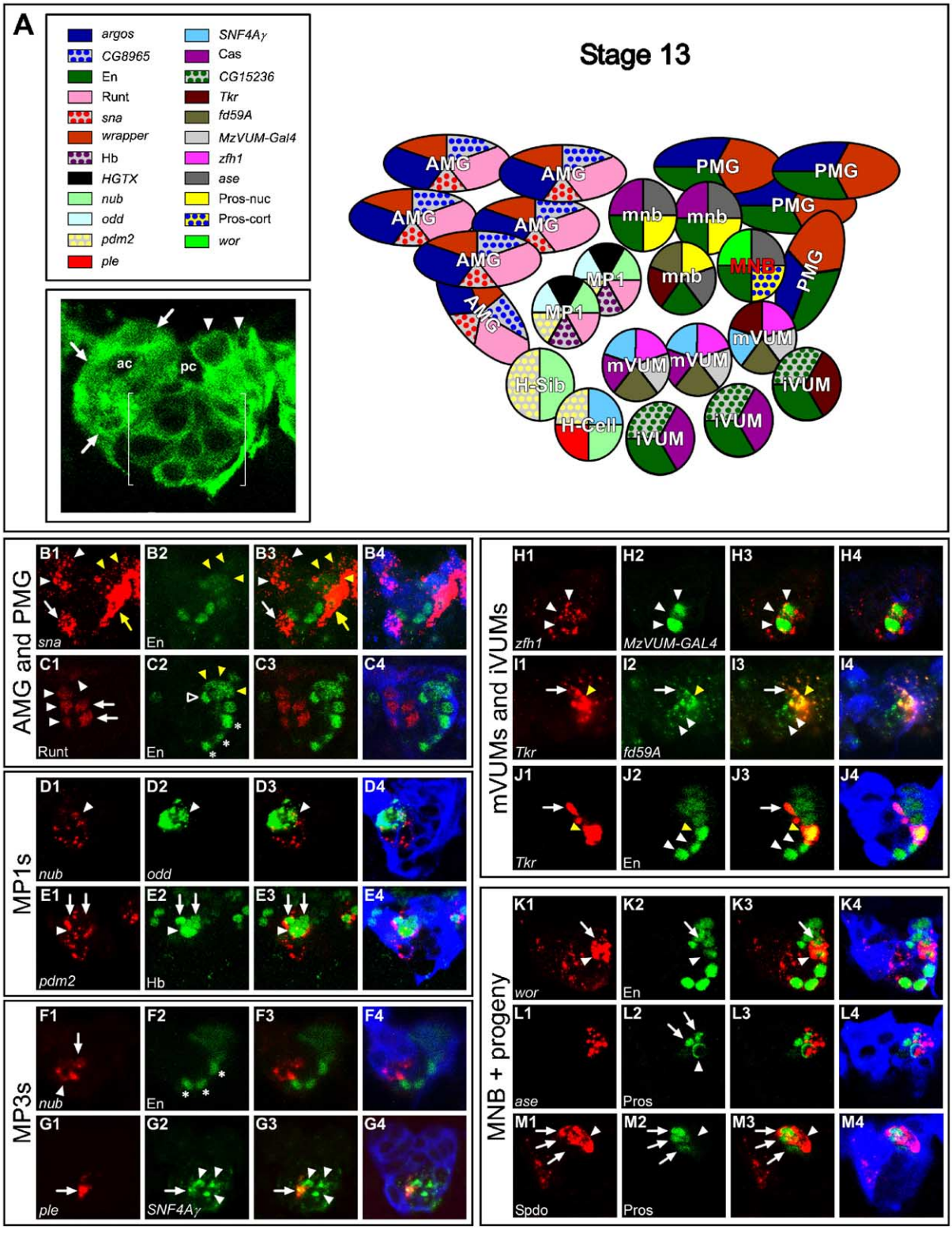
Stage 13

The stage 13 CNS represents an intermediate stage of development in which most midline neurons and glia have formed and axonal outgrowth has begun. All of the neurons express *embryonic lethal, abnormal vision* (*elav*), a marker for postmitotic neurons (Robinow and White, 1991). However, the cells are immature and not fully differentiated. The midline cells at stage 13 have undergone all of their MP and glial divisions, but the MNB has only undergone 1–2 divisions, with 2–3 more remaining during embryogenesis. In general, the midline neurons at stage 13 have the same position as they do at stage 17 (Fig. 4A, inset). This conserved arrangement aided in the identification of distinct neurons and glia. Moreover, many genes expressed at stage 13 are also expressed at stage 17 (31/39), further facilitating the identification of midline cell types.

Fig. 3. A map of neural function genes expressed at stage 17. (A) Schematic of stage 17 CNS midline cells summarizing neural function gene expression. (B) Ventral and (C–M) sagittal views are shown with anterior left and dorsal up. (B) *Pdf* (red) and Hb (green) colocalize in the MP1s (arrowheads) of segment A7. (C–F) Colocalization with *ple* (green) shows that the following genes (red) are expressed in the H-cell: (C) *DAT*, (D) *CG33528*, (E) *NPFR1*, and (F) *5-HT1A* (arrowhead). *5-HT1A* is also expressed in the ple^- H-cell sib (arrow). (G) *VGlut* (red) is expressed in H-cell sib (arrow), but not in the adjacent ple^+ (green) H-cell (arrowhead). (H) *VGlut* (red) is expressed in the $zfh1^+$ (green) mVUMs (arrowheads) and the H-cell sib (arrow). (I) *Tbh* (red) is expressed in the $zfh1^+$ (green) mVUMs. (J) *CG33528* (red) is expressed in the Tbh^+ (green) mVUMs. (K) *Gad1* (red) is expressed in the En^+ (green) iVUMs (arrowheads). (L) *CG8394* (red) is expressed in En^+ (green) iVUMs (arrowheads), as well as a subset of En^+ MNB progeny (arrows). (M) *Gad1* (red) is expressed in ~3 En^+ (green) MNB progeny (arrowheads).

Midline glia

Unlike stage 17, at stage 13 there exist two populations of midline glia, anterior midline glia (AMG) and posterior midline glia (PMG), which can be identified based on their position, morphology, and marker gene expression. A subset of the AMG will persist to stage 17 to become mature midline glia, while the remainder of the AMG and all of the PMG will undergo apoptosis (Bergmann et al., 2002; Dong and Jacobs, 1997). There is currently no data describing the lineal relationships among PMG or between AMG and PMG. Using colocalization with *argos* and *wrapper*, which mark all midline glia, we have identified a total of 14 genes expressed in midline glia



(Supplementary Table 2; Fig. 4A). Ten are expressed in all midline glia including: (1) *Dichaete*, *sim*, *sna* (Fig. 4B), *SoxN*, and *vvl*, which encode transcription factors, (2) *argos* and *slit*, which encode signaling proteins, (3) *wrapper*, which encodes an adhesion protein, and (4) *CG31145* and *CG32244*, two genes of unknown function. Four genes are expressed in subsets of stage 13 midline glia. *CG8965* and *runt* (Fig. 4C) are expressed in AMG, while *Cad74A* and *en* (Figs. 4B, C) are expressed in PMG, indicating that the two populations of midline glia are molecularly distinct. However, the genes we examined did not distinguish subpopulations of either AMG or PMG.

MP1 and MP3 neurons

The 2 MP1 neurons are positioned just posterior and ventral to the AMG, and the 2 MP3 progeny reside ventral to the MP1 neurons and just anterior to the VUM neurons (Fig. 4A). By stage 13, the MP1 and MP3 progeny express *elav* and are extending axons, indicating that they are in the process of neural differentiation. The stage 13 MP1 neurons have a similar expression profile to those at stage 17, and they express 9 of the 11 genes found at stage 17 (Supplementary Tables 1 and 2), including *odd* and *hb* (Figs. 4D, E). Furthermore, the two stage 13 MP1 neurons show an identical profile of gene expression to one another, as also observed at stage 17. The MP3 progeny, H-cell and H-cell sib, can be distinguished at stage 13 by differences in gene expression, indicating they have acquired their respective cell fates by this time of development: 4 genes are expressed only in the H-cell, 2 genes are expressed only in H-cell sib, and 4 genes are expressed in both cells (Supplementary Table 2). The immature nature of the MP1 and MP3 neurons is reflected in the observation that only 2 neural function genes expressed in these cells at stage 17 are expressed at stage 13: *ple* (Fig. 4G) and *CG33528*. One of the major differences between stages 13 and 17 involves the transcription factor-encoding genes *nubbin* (*nub*) (Figs. 4D, F) and *POU domain protein 2* (*pdm2*) (Fig. 4E) that are expressed in both MP1 and MP3 neurons at stage 13 but are absent at stage 17.

VUM neurons

Like the MP1 and MP3 neurons, the expression profiles of the stage 13 VUMs indicate that they are immature neurons. The VUMs are $elav^+$ and can be distinguished as mVUM or iVUM based on position and gene expression, but they do not yet express most of the neural function genes observed at stage 17. The stage 13 mVUMs share the expression of most genes (8/9) with their stage 17 counterparts, with *forkhead domain 59A* (*fd59A*) as an exception, whereas all of the stage 13 iVUM genes examined are also expressed at stage 17. At stage 13, differences among the mVUMs can be recognized: only the posterior-most mVUM expresses *Tkr* (Fig. 4I), and only the two anterior-most mVUMs contain Cas protein. The iVUMs differ in exactly the same way (Fig. 4J), indicating that at stage 13, just like stage 17, the anterior-most 4 VUMs differ from the posterior-most 2 VUMs.

MNB and progeny

By stage 13, the MNB has generated about half of its progeny. The MNB can be recognized due to its asymmetric cortical localization of Pros (Fig. 4L), a characteristic of actively dividing neuroblasts (Spana and Doe, 1995). At stage 13, there are 2–4 MNB progeny in each segment, indicating that at least two additional MNB stem cell divisions must follow to generate the 7–8 MNB progeny observed at stage 17. The MNB expresses 5 transcription factors genes, *ase* (Fig. 4L), *en* (Fig. 4K), *pros* (Figs. 4L, M), *sim*, and *wor* (Fig. 4K), in addition to *sanpodo* (*spdo*) (Fig. 4M), which functions in *Notch* signaling (O'Connor-Giles and Skeath, 2003). Unlike the MNB, the MNB progeny possess nuclear Pros (Fig. 4L) and a subset is $elav^+$. Similar to stage 17, two MNB progeny are Cas^+ and one expresses *Tkr* (Fig. 4J). While both en^+ and en^- MNB progeny are found at stage 17, all of the MNB progeny observed at stage 13 express *en* indicating that either *en* is extinguished in some of the stage 13 progeny, as observed in grasshopper (Jia and Siegler, 2002), or that some progeny generated after stage 13 do not express *en*.

Stage 11

Stage 11 (Fig. 5A) represents a critical stage of development, of which little is known. Most midline glial division has occurred by mid-stage 11, but the MNB has yet to divide. Patterns of gene expression are complex indicating that cells are acquiring specific fates, and considerable cell movement is occurring. Consequently, we have mapped the expression of genes encoding transcription factors and signaling proteins that likely mediate the development of the midline cells at this stage. Midline cells at stage 11 can be grouped into three broad categories: (1) glial cells, which express *argos* and *wrapper*, (2)

Fig. 4. A molecular map of the midline cells at stage 13. (A) Schematic of midline cells at stage 13. Inset—confocal projection of a single anti-β-galactosidase-stained stage 13 abdominal segment from a *sim-Gal4*; *UAS-tau-lacZ* embryo. Midline neurons (brackets) reside between the 2 populations of midline glia, AMG (arrows) and PMG (arrowheads). ac—anterior commissure, pc—posterior commissure. (B) *sna* (red) is expressed in both AMG (white arrowheads and arrow) and the En^+ PMG (yellow arrowheads and arrow). In this segment, some AMG (white arrow) and PMG (yellow arrow) have not yet migrated to their final dorsal position. (C) Runt (red) is localized to the AMG (white arrowheads) and MP1 neurons (arrows), and does not overlap with the En^+ (green) PMG (yellow arrowheads). (D) *nub* (red) and *odd* (green) are coexpressed in the MP1 neurons (arrowhead). (E) The MP1 neurons (arrowhead) express both *pdm2* (red) and Hb (green). Both *pdm2* and Hb are also expressed in the lateral CNS (arrows). (F) *nub* (red) is expressed in the H-cell (arrowhead) and H-cell sib (arrow) that lie just dorsal to the En^+ iVUMs (*). (G) The H-cell (arrow) expresses both *ple* (red) and *SNF4Aγ* (green). *SNF4Aγ* is also expressed in mVUMs (arrowheads). (H) *zfh1* (red) and *MzVUM-Gal4* (green) expression colocalize in mVUMs (arrowheads). (I) All of the mVUMs (arrowheads) express *fd59A* (green), while only the posterior-most mVUM (yellow arrowhead) expresses *Tkr* (red). *fd59A* and *Tkr* are coexpressed in one progeny of the MNB (arrow). (J) All 3 iVUMs (arrowheads) express En (green), while the most posterior iVUM (yellow arrowhead) and 1 MNB progeny coexpress *Tkr* (red) and En. (K) *wor* (red) is expressed in the En^+ (green) MNB (arrowhead), and is often observed in MNB progeny (arrow). (L) The MNB (arrowhead) expresses *ase* (red) and shows cortical localization of Pros protein (green). MNB progeny (arrows) show nuclear Pros localization and are occasionally observed with *ase* expression. (M) Spdo is expressed in the MNB (arrowhead) and the nuclear $Pros^+$ (green) MNB progeny (arrows).

neurons, which possess nuclear Pros, and (3) the MNB, which is a large cell containing cortical Pros. The midline neurons and MNB lie between 2 populations of glial cells along the A–P axis (Fig. 5B). Note that stage 11 is a dynamic stage with respect to gene expression, cell division, and cell movement; the description below and the stage 11 molecular map (Fig. 5A) refer to mid-stage 11, while Supplementary Table 3 also describes transient expression at early and late stage 11.

Midline glia

The stage 11 glia can be identified based on their oval morphology and expression of genes that are characteristic of

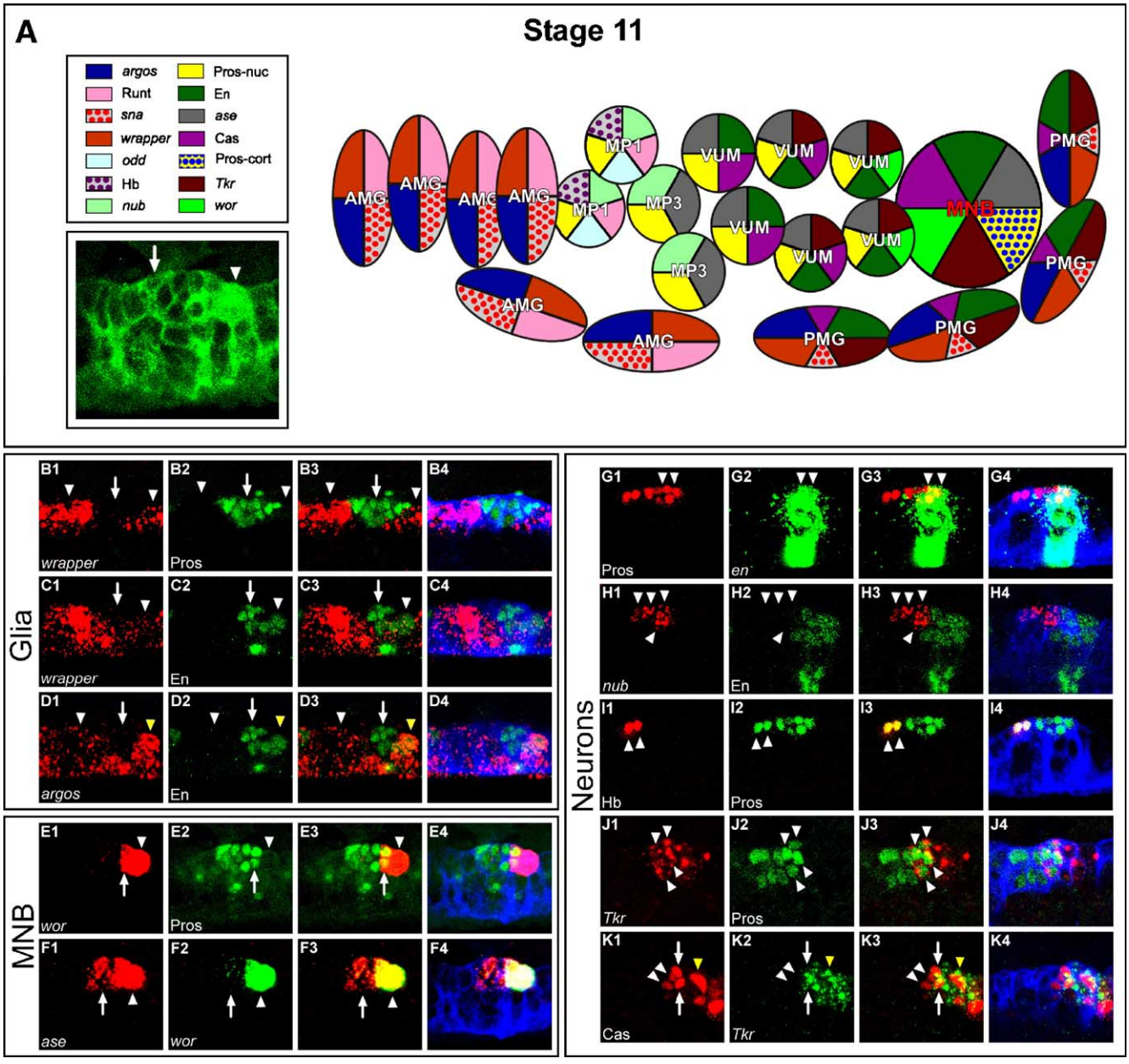


Fig. 5. A molecular map of the midline cells at stage 11. (A) Schematic of stage 11 midline cells. Inset—confocal projection of an anti-β-galactosidase-stained stage 11 abdominal segment from a *sim-Gal4*; *UAS-tau-lacZ* embryo. The wedge-shaped MP1s (arrow) can be identified by their morphology, and the MNB (arrowhead) can be identified by its large size and uniform Tau-lacZ staining. (B) The *wrapper*$^{+}$ (red) AMG and PMG (arrowheads) flank the MNB and Pros^{+} (green; arrow) neurons. (C) The *wrapper*$^{+}$ (red) PMG (arrowhead) are also En^{+} (green). En^{+} Pros^{+} neurons that do not express *wrapper* (arrow) are likely to be the VUMs and MNB. (D) *argos* (red) is expressed in the En^{-} AMG (arrowhead) and En^{+} PMG (yellow arrowhead) but not the cluster of MNB and En^{+} *wrapper*$^{-}$ neurons (green; arrow). (E) *wor* (red) is expressed in the MNB (arrowhead), a large cell with cortical Pros (green). Two *wor*$^{+}$ neurons (arrow), likely VUMs, with nuclear Pros reside just anterior to the MNB. (F) *ase* (red) and *wor* (green) are coexpressed in the MNB (arrowhead) while *ase* marks additional midline neurons (arrow). (G) The 6 posterior nuclear Pros^{+} (red) VUM neurons (arrowheads) express *en* (green). (H) *nub* (red) is expressed in 4 anterior En^{-} (green) VUM neurons (arrowheads). (I) Hb protein (red) is expressed in the 2 anterior-most nuclear Pros^{+} (green) neurons (arrowheads), which are MP1s. (J) *Tkr* (red) is expressed in the 4 posterior-most Pros^{+} (green) VUM neurons (arrowheads). (K) Cas protein (red) is localized to the 4 anterior-most VUM neurons, which are nuclear Pros^{+} and En^{+}. The two most anterior Cas^{+} neurons do not express *Tkr* (white arrowheads), while the 2 posterior Cas^{+} neurons (arrows) do express *Tkr* (green). Cas and *Tkr* are also coexpressed in the MNB (yellow arrowhead) and PMG.

stages 13 and 17 midline glia, including *wrapper* (Figs. 5B, C), *argos* (Fig. 5D), *CG32244*, *sna*, and *vvl*. There are ~4 glia located in the posterior of the segment that contain Cas and express *Tkr* and *en* (Fig. 5D): we conclude that they are PMG based on location and expression of *en*, which is a marker characteristic of PMG at stage 13. The AMG consist of ~6 cells that reside in the anterior part of the segment, and express *runt*, but not *en*. Thus, even at stage 11, the two populations of midline glia are distinct.

Midline neurons

The 10 midline neurons found at mid-stage 11 are likely to be the immature MP1, MP3, and VUM neurons. These cells are characterized by *elav* expression, indicating that they are neurons (Robinow and White, 1991), and by nuclear Pros, indicating that they have not matured (Spana and Doe, 1995). However, they can be divided into subgroups based on differential gene expression. One observation is that patterns of gene expression generally come in pairs: this suggests that at mid-stage 11, the 2 neurons derived from each MP are not yet distinct. Thus, expression of these different fates occurs between mid-stage 11 and stage 13. The 6 posterior nuclear Pros$^+$ neurons express *en* (Fig. 5G), while the 4 anterior-most Pros$^+$ neurons express *nub* (Fig. 5H). The *nub*$^+$ cells can be further subdivided into 2 anterior, wedge-shaped cells (Fig. 5A, inset) that express *hb* (Fig. 5I), *odd*, and *runt*, whereas the adjacent, more posterior 2 cells do not express any of these genes. The anterior cells are likely MP1 neurons, since the only neurons at stages 13 and 17 that express *hb*, *odd*, and *runt* are the MP1 neurons. Similarly, the 2 posterior *nub*$^+$ cells are likely to be the MP3 progeny, since they also express *pdm2*, and at stage 13, only H-cell and H-cell sib express *nub* and *pdm2*, besides the MP1 neurons.

The 6 En$^+$ nuclear Pros$^+$ neurons that lie between the MNB and the MP3 neurons are likely to be VUM cells. Consistent with this interpretation, these cells express *Tkr* (Figs. 5J, K) and contain Cas Protein (Fig. 5K), markers which also label subsets of mature VUM neurons at stage 13. Specifically, Cas protein is localized in the 4 anterior-most VUM neurons (Fig. 5K) at both stages 11 and 13. *Tkr* is expressed in the 4 posterior-most En$^+$ VUM neurons (Fig. 5J) at stage 11, and the 2 posterior-most VUM neurons at stage 13. By the end of stage 11, *en* is expressed in only 3 of the 6 VUMS; these become the iVUMs. Similar results regarding *en* expression have been shown for the grasshopper MP4–6 progeny, which are likely VUM equivalents (Jia and Siegler, 2002).

Median neuroblast

The MNB is readily distinguished because it is larger than other midline cells (Figs. 5A, inset, E, F). In *sim-Gal4 UAS-tau-lacZ*-stained embryos, the Tau-LacZ protein is often not cortical, but uniformly present throughout the cytoplasm, likely reflecting the redistribution of microtubules just prior to cell division (Fig. 5A, inset). Consistent with this interpretation, mid-stage 11 MNBs that have uniform Tau-LacZ distribution also exhibit asymmetric, cortical localization of Pros protein

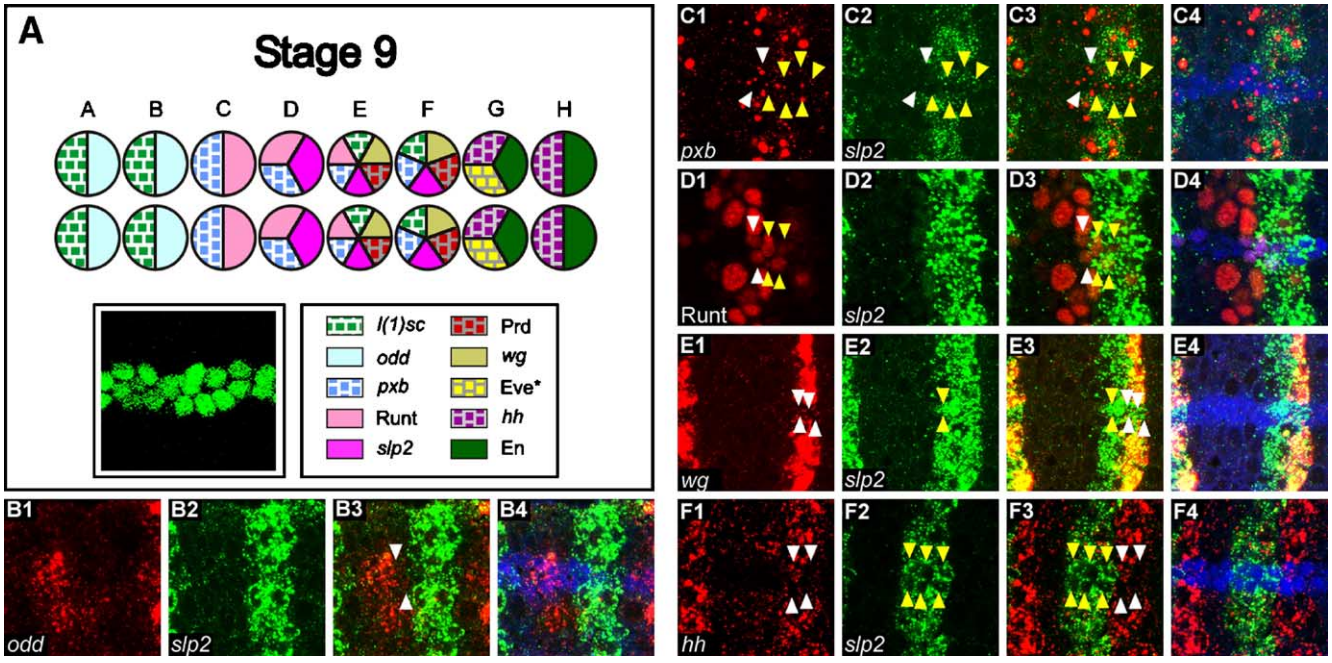


Fig. 6. A molecular map of the midline cells at stage 9. (A) Schematic of a ventral view of stage 9 midline cells illustrating 8 rows (A–H) of 2 cells each. Inset—confocal projection of an anti-β-galactosidase-stained stage 9 abdominal segment from a *sim-lacZ* embryo. (B–F) Ventral views, anterior left, blue staining (anti-Sim) shows all midline cells. (B) Comparison of *odd* expression (red; rows A and B) and *slp2* expression (green; rows D–F) reveals an unlabeled single pair of cells in row C (arrowheads). (C) *pxb* (red) is expressed in 8 cells. The most anterior pair of *pxb*$^+$ cells is in row C (white arrowheads), and does not express *slp2* (green), while the remaining 6 cells (rows D–F, yellow arrowheads) are *slp2*$^+$. (D) Runt protein (red) is localized to 6 cells. Two Runt$^+$ cells (row C; white arrowheads) do not express *slp2* (green), and 4 Runt$^+$ cells (rows D and E; yellow arrowheads) are coexpressed with *slp2*. (E) *wg* (red) is expressed in 4 cells (rows E and F; white arrowheads) that are also *slp2*$^+$. The anterior-most *slp2*$^+$ cells (row D; yellow arrowhead) do not express *wg*. (F) *hh* (red) is expressed in the 2 posterior-most rows (rows G and H, white arrowheads), since the 6 *slp2*$^+$ cells (green) in rows D–F (yellow arrowheads) do not overlap with *hh*.

(Fig. 5E), which is a hallmark of asymmetrically dividing lateral CNS neuroblasts (Spana and Doe, 1995). Cortical localization of Pros is transient, as it is not observed in all stage 11 MNBs examined. The expression of 8 additional transcription factor genes was localized in the MNB, including *ase* (Fig. 5F), *cas*, *en*, *lethal of scute* (*l(1)sc*), *pros*, *Tkr*, *wor* (Fig. 5E), and *zfh1*. Several of these genes are not specific for the MNB, but their combination distinguishes the MNB from other midline cells.

Stage 9

The stage 9 midline cells represent an early stage of development in which the midline cells do not show obvious signs of neural or glial differentiation, yet it is possible that important cell fate decisions are occurring at this time. At stage 9, the midline cells have recently divided and are loosely arranged in two columns of ~8 cells each (Fig. 6A). There is variability in the number of cells (~16 cells, varying from 14–20, using En staining as a segmental marker) and relative position of specific midline cells within each segment. To simplify the schematic of the molecular map at this stage (Fig. 6A), we illustrate the stage 9 midline cells as two perfectly spaced columns of 8 cells each and depict gene expression patterns in paired cells. However, variability causes gene expression in individual segments to commonly differ from this idealized view.

We examined the expression of 17 genes (Supplementary Table 4), most of which are expressed in patterns that are orthogonal to the A–P axis. These genes were selected based on their well-established roles in intrasegmental patterning of the epidermis (Nusslein-Volhard and Wieschaus, 1980), as well as other roles in segmentation and neurogenesis. The *l(1)sc*, *odd*, *runt*, *sloppy paired 1* (*slp1*), *sloppy paired 2* (*slp2*), *paired* (*prd*), *even skipped* (*eve*), and *en* genes encode transcription factors, and *pxb*, *wingless* (*wg*), and *hedgehog* (*hh*) encode signaling proteins. The two anterior-most pairs of cells (rows A and B) express *l(1)sc* and *odd* (Fig. 6B), while the third pair (row C) expresses both *pxb* (Fig. 6C) and *runt* (Fig. 6D). The next three pairs of cells (rows D, E, and F) all express *slp2* (Figs. 6B–F) and *pxb* (Fig. 6C), and can be subdivided into three distinct types. The most anterior of these rows (row D) also expresses *runt* (Fig. 6D), whereas row E expresses *l(1)sc*, *prd*, *runt*, and *wg* (Fig. 6E), and row F expresses *l(1)sc*, *prd*, and *wg*, but not *runt*. The posterior-most 2 pairs of cells (rows G and H) express both *en* and *hh* (Fig. 6F), and row G also expresses *eve* in alternating segments. Together, the expression patterns of these genes define 7 molecularly distinct types of midline cells arrayed along the A–P axis. Because of the dynamic nature of gene expression at stage 9 and the lack of genes that are expressed consistently from stage 9 onward, the relationships between these cells and their stage 11, 13, and 17 counterparts are unclear.

Discussion

The major goal of this paper is to provide a series of gene expression maps, derived from a large gene set, that describe the development of the *Drosophila* CNS midline cells in molecular terms. While previous research identified the various midline cell types, relatively little is known regarding the molecular mechanisms that govern midline cell development. Our study followed gene expression throughout development, and inspection of the data suggests relationships between midline cells and potential molecular pathways of development. Many of the genes analyzed are well-known transcription factors and signaling proteins that are likely to play important developmental roles. Furthermore, the mapping of genes encoding peptide neurotransmitters, neurotransmitter biosynthetic enzymes, neurotransmitter receptors, and transporters will be useful for understanding CNS midline neural function and its regulation. This work will be invaluable for continuing genome-wide assignment of gene expression to individual midline cell types, for genetic analysis, and for study of transcriptional control during development. Because many of these genes are conserved across species, these data will provide important insight for understanding neural development in metazoans.

CNS midline cell relationships and transcriptional cascades

Analysis of gene expression in midline cells reveals how the stage 11 primordial cells develop into mature neurons and glia. Thus, AMG, PMG, MNB, and immature MP1s, MP3s, and VUMs can all be identified at stage 11 based on their similarities in gene expression to stages 13 and 17. While not proof of cell relationships, examination of gene expression for multiple genes expressed in each cell type provides a consistent view. In contrast, the relationship between specific midline cells at stage 9 and earlier to mature midline cell types is unclear, although recent work has provided some insight (Bossing and Brand, 2006). An early model proposed a detailed scheme of midline cell development, and indicated that midline cell types acquire their individual identities prior to the division from 8 to 16 cells (embryonic stage 8) (Klämbt et al., 1991). However, critical experimental support was lacking. In contrast, Bossing and Brand (2006) proposed a model in which the identities of some midline cell types are specified by a combination of transcription factors and signaling pathways after the 16 cell stage. In this model, *wg* and *hh* signaling define a group of cells in the anterior of the segment that initiate *en* expression during stage 10 and are integrated into the next anterior segment. It is suggested that this group of cells gives rise to the en^+ MNB and VUM neurons. However, Bossing and Brand (2006) do not account for the PMG, the en^+ group of 3–4 midline glia that are present at the posterior of each segment beginning at stage 11 (Figs. 4, 5; Dong and Jacobs, 1997; Kearney et al., 2004). Thus, the exact identities of the cells that arise from this cluster are uncertain. Our gene expression analysis indicates at least 7 different pairs of midline cells at stage 9. However, expression of most of these genes does not persist past stage 10, and those that do (*en*, *l(1)sc*, *odd*, and *runt*) are dynamically expressed and, therefore, are unreliable lineage markers. Thus, our data do not provide insight into lineage relationships between stages 9 and 11, but provides molecular markers with which models can be tested.

The maps detailed in this work allow the identification of individual midline neuronal and glial lineages across embryonic development. This ability reveals potential genetic and physical interactions, as well as regulatory relationships. Coexpressed genes can be assayed for common DNA binding sites and transcription factors can be assessed for combinatorial contributions to gene regulation. This is illustrated in timelines of expression for the MP1 and MP3 neurons (Fig. 7).

MP1 neurons

At stage 11, the MP1 neurons express *elav* and contain nuclear Pros, which together characterize immature post-mitotic neurons in the lateral CNS. At this time, the MP1 neurons are characterized by expression of 8 additional transcription factor genes: *Dichaete*, *hb*, *HGTX*, *nub*, *odd*, *pdm2*, *runt*, and *sim*. By stage 13, an additional transcription factor, *fkh*, is expressed. By stage 17, genes, such as *Pdf* and *5-HT1A*, whose expression is characteristic of terminally differentiated neurons, are expressed in the MP1 neurons. Expression of many of the transcription factor genes observed at stages 11 and 13 is still present at stage 17, although *Dichaete*, *nub*, *pdm2*, and *sim* expression are extinguished. These data suggest that the maintenance of some transcription factors and the downregulation of others may be key events in terminal differentiation and expression of neural function genes.

MP3 neurons

The MP3 lineage differs from the MP1 lineage in that immature MP3s give rise to two different neurons, H-cell and H-cell sib. Similar to the MP1s, the MP3 neurons at stage 11 are *elav*$^+$ and have nuclear Pros, indicative of immature post-mitotic neurons. The 2 MP3s have nearly identical patterns of gene expression at mid-stage 11, indicating that they acquire their distinct fates afterwards. The first indication that individual cell fates have been acquired is revealed by expression of *SoxN* in only one of the two MP3 neurons (most likely the H-cell), at late stage 11. The differences are clearly apparent by stage 13, in which the MP3 cells also begin to express neural function genes, such as the H-cell-expressed *ple* gene, which encodes tyrosine hydroxylase, a dopamine biosynthetic enzyme. Both the *nub* and *pdm2* transcription factor genes are expressed in the immature MP3 neurons, and their expression levels decline as these neurons begin terminal differentiation. It will be interesting to see whether reduction in their expression in both MP1s and MP3s is required for terminal differentiation to occur. Similarly, it will be useful to investigate whether the *SoxN*, *fkh*, and *sim* transcription factor genes regulate differences between H-cell and H-cell sib, since each is expressed in only one of the two cells.

Comparison of gene function between the CNS midline cells and the lateral CNS

Many genes that function during development of the lateral CNS are also expressed in the CNS midline cells. One of the key early differences between the midline cells and lateral CNS is that expression of *sim* imparts a midline identity to the mesectodermal cells (Crews, 2003), whereas a similar function is carried out in the lateral CNS by genes, such as *ventral nerve cord defective* (*vnd*), *intermediate nerve cord defective* (*ind*), and *muscle segment homeodomain* (*msh*) (Skeath and Thor, 2003). At the same time, the expression patterns of the transcription factors and signaling proteins encoded by segmentation genes subdivide both the midline and lateral

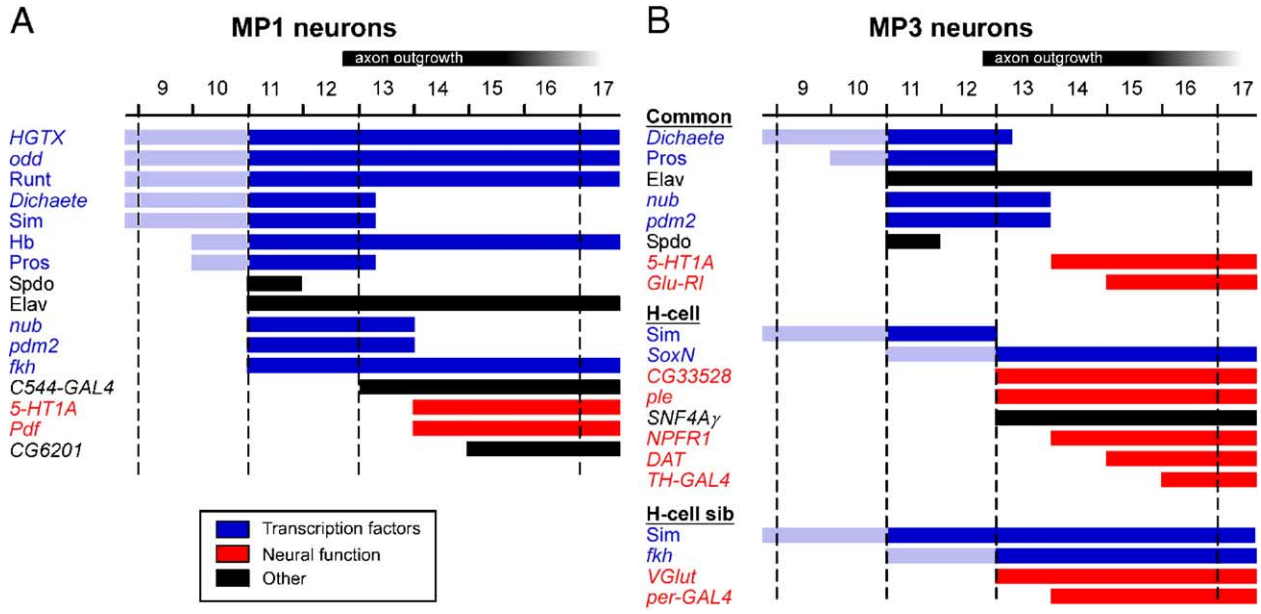


Fig. 7. Timelines of developmental gene expression. Schematic progression of gene expression in the: (A) MP1 neurons and (B) MP3 neurons from stages 9 to 17. Gene expression is depicted as horizontal bars representing transcription factor genes (blue), neural function genes (red), or other genes (black). For simplicity, genes that initiate expression at any point during a particular stage are shown as expressed throughout the stage. Light blue bars indicate expression prior to the ability to identify MP1 or MP3 neurons (A), or to distinguish between the H-cell and H-cell sib neurons (B). The MP3 expressed genes include those expressed in both cells (common), only H-cell, and only H-cell sib. The "axon outgrowth" bar indicates the period of CNS axonogenesis.

CNS cells along the anterior–posterior axis. In the lateral CNS, genetic studies have demonstrated that these segmentation genes impart distinct identities on different rows of neural precursors (Bhat, 1999; Chu-LaGraff and Doe, 1993; Skeath et al., 1995). In the midline cells, genetic and transplantation studies have suggested roles for *wg* and *hh* signaling in the control of midline cell development (Bossing and Brand, 2006; Hummel et al., 1999; Patel et al., 1989b). In the stage 9 midline molecular map (Fig. 6A), the combinatorial expression pattern of the 17 genes examined subdivides the 8 rows of midline cells into 7 different types of cells. These transcription factors and signaling proteins, while not all segmentation genes, may act in addition to or in combination with *hh* and *wg* to impart different fates and functions on different midline cells.

The *snail* family of zinc finger proteins, which includes *sna*, *esg*, and *wor*, is expressed in lateral CNS neuroblasts (Cai et al., 2001). *sna* and *wor*, and to a lesser extent *esg*, work together to control neuroblast asymmetry (including Pros localization) and neuroblast cell division (Ashraf and Ip, 2001; Cai et al., 2001). *sna* and *wor* are expressed in a large number of neuroblasts, but not ganglion mother cells (GMCs); *esg* neuroblast expression is considerably more limited. In the midline, *wor* and *esg* are expressed in the MNB and other neurons, resembling their expression in the lateral CNS. However, *sna* is not expressed in midline neurons or the MNB, but only in glial cells from stages 11 to17, indicating a significant difference between the midline and lateral CNS.

In the lateral CNS, neuroblasts divide as stem cells giving rise to a succession of GMCs that each divide once to generate two neuronal progeny (Skeath and Thor, 2003). The identity of GMCs and their progeny within a single neuroblast lineage is largely imparted by differences in a temporal cascade of transcription factors following the order: *hb* → *kr* → *nub* → *cas* (Kambadur et al., 1998; Pearson and Doe, 2004). We have examined expression of these genes during midline development, and the MNB is clearly distinct from this pathway. Neither *hb*, *kr*, nor *nub* is expressed in the MNB, although they are expressed in other midline cell types. However, *cas* is prominently expressed in the MNB, although it is also expressed in additional midline neurons and glia. Since there exist 4 (of 30) neuroblasts in the lateral CNS that also differ from the canonical *hb* cascade (Isshiki et al., 2001), and begin cell division with a cas^{+} GMC, the MNB and these small number of neuroblasts may represent a similar mode of development.

The Pros protein is asymmetrically localized to the basal side of neuroblasts in the lateral CNS, where it translocates to the nuclei of GMCs and their daughter cells, and regulates gene expression (Spana and Doe, 1995). In the midline, the MNB expresses *pros*, and Pros protein is found as a cortical crescent. Moreover, the MNB progeny also show nuclear Pros localization at stages 13 and 17. Thus, with respect to Pros localization, the MNB lineage is similar to lateral neuroblast lineages. Like lateral CNS GMCs and the non-midline MP2 neural precursor, the MP1, MP3, and VUM precursors divide only once to generate 2 neurons. Additionally, there is evidence that midline precursors and lateral CNS GMCs use similar mechanisms to generate asymmetric neuronal cell fates (Lundell et al., 2003, SRW unpublished data). Based on these similarities, one might expect Pros to be localized to midline precursors and transiently in their neuronal progeny. However, *pros* is not expressed in midline precursors but is detected only in their progeny. Thus, while Pros may play a similar role in lateral CNS and midline neuronal development, it is exclusively functioning post-mitotically in MP1, MP3, and VUM lineages.

In summary, many of the genes involved in development of the neuroblasts, GMCs, and neurons of the lateral CNS are also expressed in the midline cells. However, their expression suggests similarities in function in some cases, and differences in others. Not surprisingly, genes expressed in the MNB and progeny, such as *pros*, *sna*, *wor*, and *cas* have the highest likelihood of conserved function, since the development of these midline cells closely resembles that of some lateral CNS lineages. In contrast, the midline glia are a cell type whose development (to the extent known) is unique.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.03.016.

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