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Dual role for *Drosophila lethal of scute* in CNS midline precursor formation and dopaminergic neuron and motoneuron cell fate

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SUMMARY

Dopaminergic neurons play important behavioral roles in locomotion, reward and aggression. The *Drosophila* H-cell is a dopaminergic neuron that resides at the midline of the ventral nerve cord. Both the H-cell and the glutamatergic H-cell sib are the asymmetric progeny of the MP3 midline precursor cell. H-cell sib cell fate is dependent on *Notch* signaling, whereas H-cell fate is *Notch* independent. Genetic analysis of genes that could potentially regulate H-cell fate revealed that the *lethal of scute* [*l(1)sc*], *tailup* and *SoxNeuro* transcription factor genes act together to control H-cell gene expression. The *l(1)sc* bHLH gene is required for all H-cell-specific gene transcription, whereas *tailup* acts in parallel to *l(1)sc* and controls genes involved in dopamine metabolism. *SoxNeuro* functions downstream of *l(1)sc* and controls expression of a peptide neurotransmitter receptor gene. The role of *l(1)sc* may be more widespread, as a *l(1)sc* mutant shows reductions in gene expression in non-midline dopaminergic neurons. In addition, *l(1)sc* mutant embryos possess defects in the formation of MP4-6 midline precursor and the median neuroblast stem cell, revealing a proneural role for *l(1)sc* in midline cells. The *Notch*-dependent progeny of MP4-6 are the mVUM motoneurons, and these cells also require *l(1)sc* for mVUM-specific gene expression. Thus, *l(1)sc* plays an important regulatory role in both neurogenesis and specifying dopaminergic neuron and motoneuron identities.

KEY WORDS: bHLH, Dopamine, *Drosophila*, *Lethal of scute*, Neural cell fate, *Tailup*

INTRODUCTION

Complex behaviors require the coordinated action of diverse ensembles of neurons. Each neuron contains a distinct combination of neural function genes, which include genes encoding neurotransmitter biosynthetic enzymes, neuropeptides, vesicular transporters, membrane transporters, neurotransmitter receptors and axon guidance proteins. One of the key goals in developmental neuroscience is to understand how neural gene expression is regulated, and how this defines each neuron and its precursors during development. Not only will this help explain how neuronal diversity is generated, but provide insights into the origins of human nervous system disease and the development of effective therapies.

Efforts to reach these goals have been systematically applied towards studying the development of dopaminergic neurons because of their prominent neurobiological roles in reward, emotion and locomotory pathways, and their importance in neurodegenerative diseases. Parkinson's disease is characterized by the loss of midbrain dopaminergic neurons, and a major goal of neural stem cell research is to generate dopaminergic neurons in vitro for cell replacement therapies to counteract Parkinson's disease. Studies in vertebrates have identified multiple regulatory proteins that are required for controlling gene expression and development of dopaminergic neurons (Smidt and Burbach, 2007). Recent work further showed that an Ets-family transcription factor is evolutionarily conserved between *C. elegans* (AST-1) and mammals (ETV1), and controls dopaminergic neuron gene expression in both organisms (Flames and Hobert, 2009).

In insects, dopaminergic neurons are found in both the nerve cord and brain (Monastirioti, 1999). One of the best-characterized insect dopaminergic neurons is the H-cell (named for its 'H'-like axonal projections), which is present in the CNS midline cells of the nerve cord. The H-cell was first described in grasshopper as one of the two progeny of the Midline Precursor 3 (MP3) cell (Goodman et al., 1981), and shown in the moth *Manduca sexta* to be dopaminergic (Mesce et al., 2001). The H-cell midline interneuron is also present in *Drosophila* (Bossing and Technau, 1994; Budnik and White, 1988; Schmid et al., 1999; Wheeler et al., 2006), and similar to other dopaminergic neurons expresses a set of genes encoding dopamine biosynthetic enzymes, including *pale* (*ple*; which encodes tyrosine hydroxylase) and *dopa decarboxylase* (*Ddc*). The H-cell also expresses a vesicular monoamine transporter (*Vmat*), dopamine membrane transporter (*DAT*) and neurotransmitter receptors that receive input for serotonin (*5-HT1A*), glutamate (*Glu-RI*) and neuropeptide F (*NPFR1*) (Wheeler et al., 2006). This characteristic pattern of gene expression and its 'H' axonal projection, to a large degree, constitute the unique character of the H-cell.

Recent work has provided insight into the origins of midline neurons and glia (Fig. 1). Around the time of gastrulation, the *single-minded* midline master regulatory gene activates the midline developmental program (Crews, 1998), and soon after 3 MP equivalence groups (MP1, MP3, MP4) of five or six cells/each form (Wheeler et al., 2008). *Notch* signaling selects one cell from the MP1 group to become an MP1 and the others become midline glia (MG). The same occurs for the MP3 group, with one cell becoming

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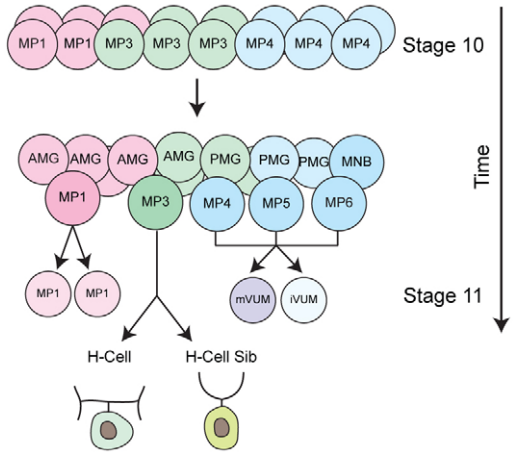

Fig. 1. Schematic summary of *Drosophila* midline neurogenesis. At stage 10, the midline cells comprise three equivalence groups (MP1, MP3 and MP4), each consisting of five or six cells. Six midline neural precursors: MP1, MP3, MP4, MP5, MP6 and MNB form from these equivalence groups. The remaining cells become anterior midline glia (AMG) and posterior midline glia (PMG). At stage 11, each MP divides once to generate two neurons. MP1 generates two identical MP1 neurons, MP3 forms the H-cell and H-cell sib, and MP4-6 each gives rise to an mVUM and iVUM.

an MP3 and the others MG. Development of the MP4 group is more complex, with sequential *Notch*-dependent formation of MP4 followed by MP5, MP6 and the median neuroblast (MNB). Each MP undergoes a single division that leads to two neurons. For MP3-6, this involves binary cell fate decisions: MP3 gives rise to the dopaminergic H-cell and glutamatergic H-cell sib interneurons, and MP4-6 each gives rise to a GABAergic iVUM interneuron and glutamatergic/octopaminergic mVUM motoneuron pair. The differences in MP3-6 neuron cell fate are due to the asymmetric localization of the Numb protein, which is high in H-cell and mVUMs, but low in H-cell sib and iVUMs, and differential Sanpodo localization (Wheeler et al., 2008). Although *Notch* signaling directs H-cell sib and iVUMs to their fates, it is blocked in H-cell and mVUMs due to the presence of Numb (Lundell et al., 2003; Wheeler et al., 2008). Thus, H-cell sib and iVUM cell fate and gene expression are dependent on *Notch* signaling, and a different regulatory program governs H-cell and mVUM fates.

In this paper, we ask the question: what regulatory proteins govern *Notch*-independent H-cell and mVUM fate and gene expression? We also address how the two types of midline precursors, MPs and MNB, form. Proneural genes of the bHLH transcription family have been implicated in controlling neural precursor formation and neuron-specific transcription in both vertebrates and invertebrates. The *Drosophila* bHLH proneural genes, *achaete* (*ac*), *scute* (*sc*), *lethal of scute* [*l(1)sc*] and *atonal* have been implicated in the formation of either sensory cell or CNS neuroblast precursors (Dambly-Chaudiere and Vervoort, 1998; Jimenez and Campos-Ortega, 1990; Martin-Bermudo et al., 1991; Sugimori et al., 2007). Proneural bHLH genes can also direct the formation of specific neuronal cell types, as exemplified by studies in the vertebrate spinal cord (Sugimori et al., 2007) and retina (Hatakeyama and Kageyama, 2004). Neuronal cell type specification is commonly due to the combinatorial action of proneural and homeodomain-containing proteins (Guillemot, 2007). We demonstrate here that three transcription factors: the L(1)sc bHLH protein (Alonso and Cabrera, 1988), Tailup (Tup; Islet) Lim-homeodomain protein (Thor and Thomas, 1997) and the Sox family protein SoxNeuro (SoxN) (Buescher et al., 2002; Cremazy et al., 2000; Overton et al., 2002), work together to control overlapping aspects of H-cell gene expression. In addition, *l(1)sc* regulates mVUM motoneuron gene expression. All three proneural members of the *Drosophila achaete-scute* complex (*AS-C*) [*ac*, *l(1)sc* and *sc*] are expressed in MPs in distinct patterns, and *l(1)sc* is required for the formation of MP4-6 and the MNB. Thus, *l(1)sc* controls both midline precursor formation and, in combination with *SoxN* and *tup*, controls H-cell-specific gene expression and cell fate. Both the *l(1)sc* and *tup* genes may also function together more broadly and control non-midline dopaminergic neuron gene expression.

MATERIALS AND METHODS

Drosophila strains

Drosophila strains used included: sc^{M6} (Gomez-Skarmeta et al., 1995), *Df(1)ase-1* (Gonzalez et al., 1989), *Df(1)sc10-1* (Villares and Cabrera, 1987), *Df(1)sc19* (Campuzano et al., 1985), *Df(1)sc-B57* (Jimenez and Campos-Ortega, 1990), $SoxN^{GA1192}$ (Buescher et al., 2002), tup^{1} (Thor and Thomas, 1997) and $tup^{isl\text{-}1}$ (Thor and Thomas, 1997). *Gal4* and *UAS* lines employed were: *sim-Gal4* (Xiao et al., 1996), *UAS-tau-GFP* (Wheeler et al., 2006), *UAS-l(1)sc* (Parras et al., 1996), *UAS-sc* (Parras et al., 1996) and *UAS-elav* (Koushika et al., 1996).

L(1)sc and Sc antisera

Polyclonal antibodies against full-length L(1)sc and Sc proteins were generated by injecting both guinea pigs and rats (Pocono Rabbit Farm) with N-terminal 6×His-L(1)sc and 6×His-Sc fusion proteins generated in *E. coli*. The specificity of both antibodies was confirmed by the following observations: (1) the lateral CNS staining was identical to published accounts for L(1)sc and Sc (Martin-Bermudo et al., 1991; Skeath et al., 1992); and (2) immunoreactivity in neural precursors was absent in the corresponding mutant strains [*Df(1)sc-B57* for L(1)sc and Sc, and *Df(1)sc10-1* for Sc].

In situ hybridization, immunostaining, microscopy, and statistical analysis

Embryo collection, in situ hybridization, immunostaining and confocal imaging were performed as previously described (Kearney et al., 2004; Wheeler et al., 2006; Wheeler et al., 2008). Digoxygenin-labeled or biotin-labeled antisense RNA probes for *5-HT1A*, *BarH1*, *CG13565*, *CG15236*, *CG16778*, *DAT*, *dgk*, *Glu-RI*, *NPFR1*, *nubbin*, *ple*, *pdm2*, *Tbh*, *wor*, *wrapper* and *VGlut* for in situ hybridization were generated from cDNA clones from the *Drosophila* Gene Collection (Open Biosystems). The clone used to make the *tup* probe was derived from PCR of *Drosophila* genomic DNA, and the *Vmat* probe was derived by PCR of *Drosophila* adult head cDNA. Primary antibodies used were: rabbit anti-Cas (Kambadur et al., 1998), guinea pig anti-Eve (Asian Distribution Center for Segmentation Antibodies), mouse and rat anti-Elav (Developmental Studies Hybridoma Bank), rabbit anti-GFP (Abcam), guinea pig anti-Lim3 (Broihier and Skeath, 2002), rabbit anti-SoxN (John Nambu), mouse anti-Tau (Sigma), rat anti-Tup (Broihier and Skeath, 2002), and guinea pig anti-Zfh1 (Fortini et al., 1991). Alexa Fluor-conjugated secondary antibodies were used (Molecular Probes), and the Tyramide Signal Amplification System (Perkin Elmer) was employed for some experiments. Abdominal segments 1-8 were analyzed for each experiment. Statistical analysis was performed using Fisher's Exact Test. Percentages and *P* values for each experiment are listed in Tables 1, 2 and Tables S1, S2 in the supplementary material.

RESULTS

Expression of H-cell neural function and transcription factor genes

MP3 divides at early stage 11 (~5 hours post-fertilization) to give rise to H-cell and H-cell sib. Expression of H-cell neural function genes that are involved in dopamine biosynthesis,

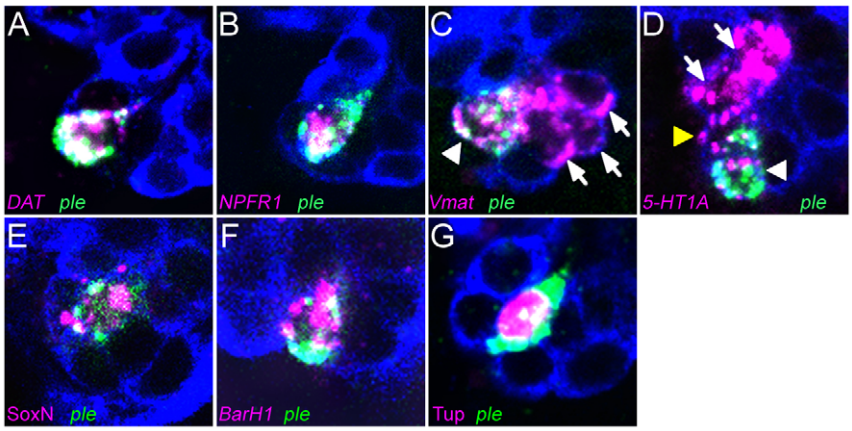

Fig. 2. H-cell gene expression. Sagittal views of single segments of *sim-Gal4 UAS-tau-GFP* embryos at stage 14. Embryos were stained for GFP or Tau (blue; midline cell cytoplasm is GFP$^+$ and cell nuclei are GFP$^-$) and additional antibody and in situ hybridization probes. Italicized gene names represent RNA and unitalicized capitalized names represent protein. (A-G) In all panels, *ple* expression (green) indicates the H-cell. (**A-D**) The neural function genes (A) *DAT*, (B) *NPFR1*, (C) *Vmat* and (D) *5-HT1A* (all magenta) colocalize with *ple* in the H-cell. (C) The *Vmat* gene is expressed in the H-cell (arrowhead) and in three mVUMs (arrows). (D) The *5-HT1A* gene is expressed in the H-cell (white arrowhead), H-cell sib (yellow arrowhead) and two MP1 neurons (arrows). (**E-G**) The transcription factor genes (E) SoxN, (F) *BarH1* and (G) Tup (all magenta) colocalize with *ple* in the H-cell.

dopamine transport and neurotransmitter receptors begins at late stage 13 (~10 hours post-fertilization). Fig. 2A-D shows the expression of the *ple*, *DAT*, *NPFR1*, *Vmat* and *5-HT1A* neural function genes in the H-cell. These genes were previously mapped to the H-cell by in situ hybridization (Wheeler et al., 2006). Previous expression analysis identified 36 transcription factor genes known to be expressed in midline cells (Wheeler et al., 2006; Wheeler et al., 2008), and identified *SoxN* as being expressed in the H-cell (Wheeler et al., 2006) (Fig. 2E). In addition, both the *BarH1* (Reig et al., 2007) and *tup* (Thor and Thomas, 1997) transcription factor genes had previously been shown to be expressed in the H-cell (Fig. 2F,G; Fig. 3A-D). In this paper, we further examine the expression of each gene during H-cell development, and also demonstrate that two members of the *AS-C* gene family, *l(1)sc* and *sc*, are expressed in the H-cell (Fig. 3E-J; see Fig. S1A-E in the supplementary material). The genes and proteins analyzed were assigned to specific midline cell types by the use of co-staining with midline cell type-specific markers in *sim-Gal4 UAS-tau-GFP* embryos [data not shown; see Wheeler et al. (Wheeler et al., 2008) for details]. The *BarH1*, *SoxN*, and *tup* genes are each expressed at stage 11 and remain on through the end of embryonic development. *BarH1* is not expressed in MP3, but is present in the H-cell beginning at late stage 11 (Fig. 2F, Fig. 3I), and is absent in the H-cell sib. *SoxN* is expressed in both the H-cell and H-cell sib until the end of stage 13, and then becomes localized to only the H-cell (Fig. 2E). *tup* is absent in MP3, initially present in both the H-cell and H-cell sib, but by late stage 11 is preferentially localized to only the H-cell (Fig. 2G and Fig. 3A-D). Expression is summarized in Fig. 4. Each of these genes is expressed before the appearance of H-cell neural function gene expression, and could potentially regulate their expression either directly or indirectly.

AS-C genes are expressed in midline precursors and their neuronal progeny

The *AS-C* consists of three proneural basic-helix-loop-helix (bHLH) genes, *ac*, *l(1)sc* and *sc* (Campuzano and Modolell, 1992), and the *asense* (*ase*) bHLH neural precursor gene (Brand et al., 1993; Dominguez and Campuzano, 1993; Gonzalez et al., 1989). The proneural bHLH genes play important roles in CNS and sensory cell development (Bertrand et al., 2002). Previous work has shown that *l(1)sc* was expressed in midline cells (Bossing and Brand, 2006; Kearney et al., 2004) and we earlier described the expression of *ase* in MPs and MNB (Wheeler et al., 2006). However, detailed expression analysis of the *ac*, *l(1)sc* and *sc* genes had not been carried out. Consequently, we examined their expression by immunostaining with antibodies we generated against the L(1)sc and Sc proteins, as well as an existing Ac monoclonal antibody.

L(1)sc

L(1)sc is present in all midline neuronal precursors, including MP1, MP3, MP4, MP5 and MP6 at stages 10-11 and MNB at stages 10-12 (Fig. 3E-H,J). L(1)sc remains present in the newly divided neurons of MP3 (the H-cell, H-cell sib), MP5 (mVUM5, iVUM5) (Fig. 3F,G) and MP6 (mVUM6, iVUM6) (not shown). Although both the H-cell and H-cell sib initially possess high levels of L(1)sc protein (Fig. 3F), the amount is greatly reduced in the H-cell sib as stage 11 progresses, whereas levels remain high in the H-cell (Fig. 3G-I). Similar L(1)sc dynamics are observed for the VUMs: L(1)sc is initially present in both mVUM5 and iVUM5 (Fig. 3F), but levels later become higher in mVUM5 with respect to iVUM5 (Fig. 3G) (the same dynamics apply to mVUM6 and iVUM6; not shown). By contrast, L(1)sc is present in MP1 and MP4 (Fig. 3E,F), but is undetectable in their progeny (Fig. 3F-H). By the end of stage 11, L(1)sc is no longer detectable in midline neurons (Fig. 3J), including H-cell and mVUMs. L(1)sc is present in PMG from stages 10-12 (Fig. 3E-H,J) and occasionally appears weakly in 2 AMG (data not shown).

Sc

Sc is present in the MP1, MP3, MP4, MP5, MP6 and MNB precursors (see Fig. S1A-D in the supplementary material). Unlike L(1)sc, the Sc protein is absent in the newly divided MP and MNB neurons. Sc appears in the H-cell beginning at stage 14 (see Fig. S1E in the supplementary material) and remains on throughout embryonic development. Sc is not present in any other midline neurons. Sc is present in PMG from stages 10-11, but absent in AMG (see Fig. S1A-C in the supplementary material).

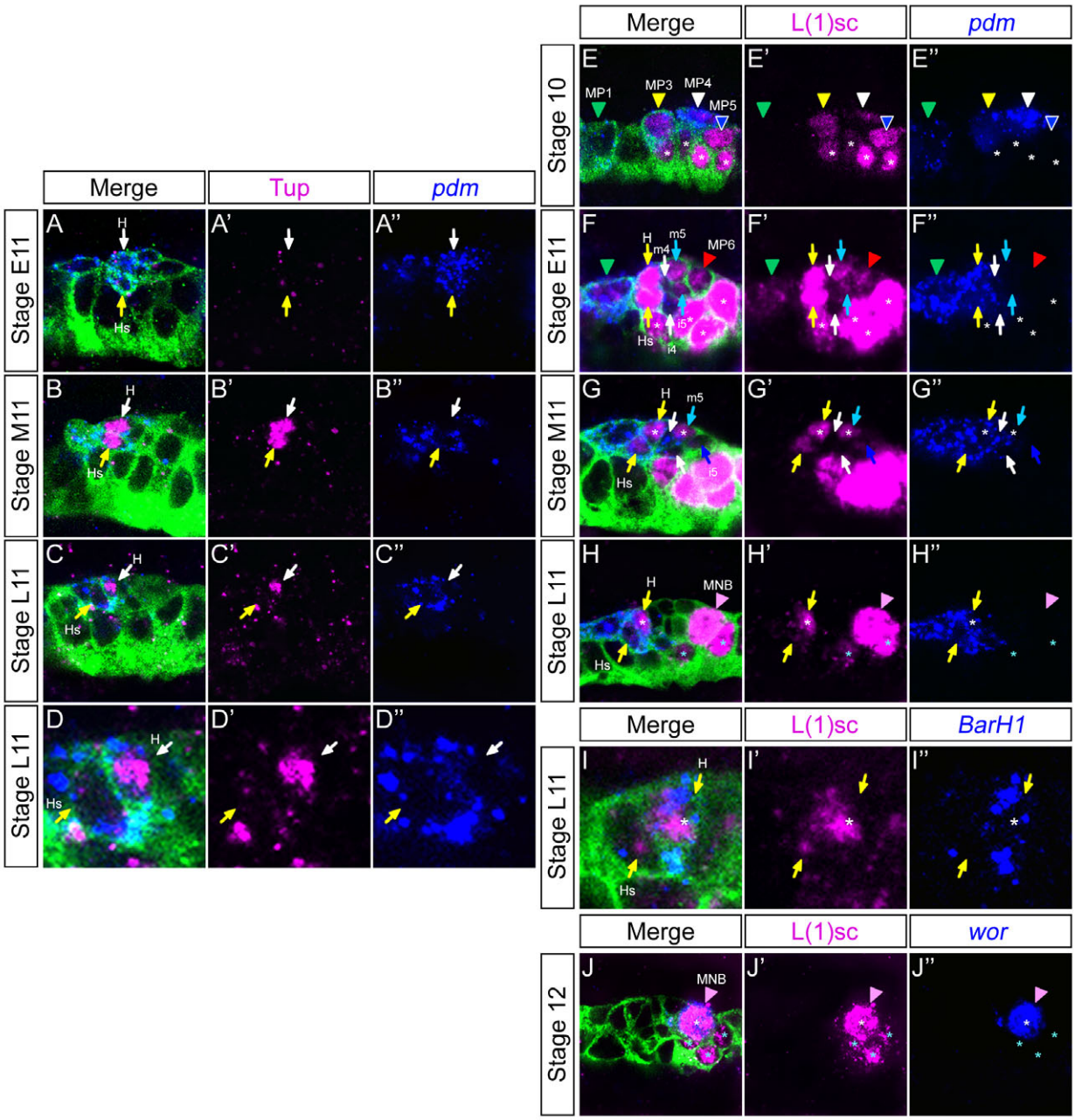

Fig. 3. The *tup* and *l(1)sc* genes are expressed in midline precursors and their neuronal progeny. Sagittal views of single segments of *sim-Gal4 UAS-tau-GFP* embryos stained with (A-D) anti-Tup (magenta), (E-J) anti-L(1)sc (magenta), (A-J) anti-GFP (green), (A-H) *pdm2/nubbin* (blue), (I) *BarH1* (blue) and (J) *wor* (blue). Embryonic stages are indicated on the left: 10, early 11 (E11), mid-11 (M11), late 11 (L11) and 12. Embryos are progressively older moving from top to bottom. Each segment shows the magenta and blue channels separately, along with a merge image. Arrowheads indicate MPs and arrows indicate neurons. In all panels the following designations apply: MP1 (green arrowhead), MP3 (yellow arrowhead), MP4 (white arrowhead), MP5 (blue arrowhead), MP6 (red arrowhead), MNB (purple arrowhead), H-cell (H), H-cell sib (Hs), mVUM4 (m4), iVUM4 (i4), mVUM5 (m5) and iVUM5 (i5). (A-I) Combined *pdm2* and *nubbin* expression (*pdm*; blue) marks MP1, MP3, MP4 and their neuronal progeny, but is absent in MP5 and MP6 and their progeny, in MNB and in MG. (**A-A″**) At early stage 11 immediately after MP3 division, Tup is absent from the H-cell (white arrows) and H-cell sib (yellow arrows). (**B-B″**) Later during stage 11, Tup is present in both the H-cell and H-cell sib. (**C-C″**) By the end of stage 11, Tup is present in the H-cell, but is absent from the H-cell sib. (**D-D″**) Higher magnification view of C. (**E-E″**) At stage 10, L(1)sc is present in *pdm*$^{+}$ MP3 (yellow arrowheads), *pdm*$^{+}$ MP4 (white arrowheads), *pdm*$^{-}$ MP5 (blue arrowheads), and MP6, MNB and PMG (*; these cell types cannot be distinguished at this time). L(1)sc is absent in MP1 (green arrowheads). (**F-F″**) At early stage 11, L(1)sc is present at low levels in MP1 (green arrowheads) and at high levels in the newly divided MP3 neurons (H-cell and H-cell sib; yellow arrows). It is absent in the MP4 neurons (white arrows) and present in both MP5 neurons (mVUM5 and iVUM5; blue arrows) and MP6 (red arrowheads). Levels are high in the PMG and MNB (*). (**G-G″**) L(1)sc levels are higher in the H-cell (*, yellow arrows) than in the H-cell sib (yellow arrows), absent in VUM4s (white arrows) and present in mVUM5 (*, blue arrows), but not iVUM5 (blue arrows). (**H-H″**) L(1)sc is present in the H-cell (white asterisk), but levels are greatly reduced in the H-cell sib, absent in VUMs, and present in MNB (purple arrowhead) and PMG (blue; *). (**I-I″**) High-magnification view of a late stage 11 embryo stained for L(1)sc and BarH1. *BarH1* expression is restricted to the H-cell, and this image demonstrates the higher levels of L(1)sc in the *BarH1*$^{+}$ H-cell (*) compared with the H-cell sib. (**J-J″**) Expression of L(1)sc overlaps with *wor*, which is expressed in the MNB (white asterisks, purple arrowheads) and is also present in PMG (blue asterisks).

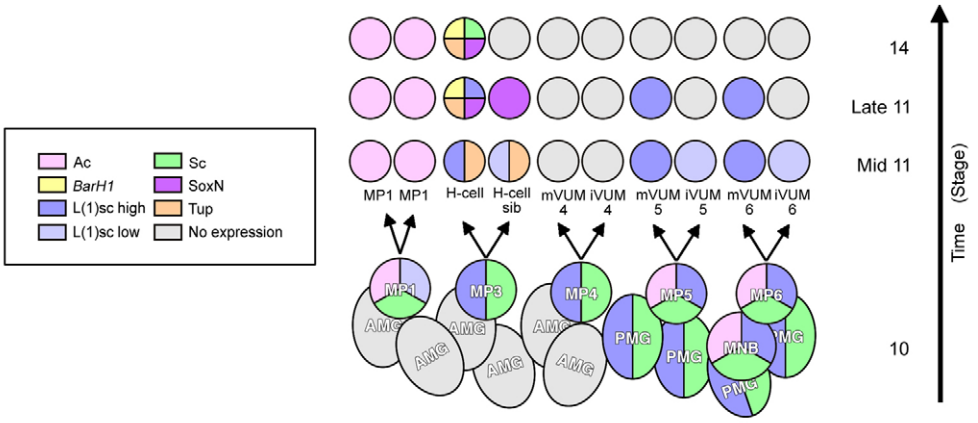

Fig. 4. Summary of H-cell transcription factor gene expression. Schematic summary of transcription factor gene expression in midline cells from stages 10-14.

Ac

Ac is present in MP1 and transiently in MP5, MP6 and MNB at stages 10-11 (see Fig. S1F-H in the supplementary material). Ac prominently remains on in the MP1 neurons after division throughout embryogenesis (see Fig. S1H-J in the supplementary material). By contrast, Ac is absent in all other midline neurons and MG.

In summary, the three *AS-C* proneural genes are all expressed in the midline cells in a dynamic manner (see Fig. 4 for a schematic summary). Although they partially overlap in expression, each gene has a unique pattern of midline expression. In the case of *ac* and *sc*, this contrasts with their expression in embryonic lateral neuroblasts and sensory organ precursors, where their expression closely overlaps, owing to use of a shared cis-regulatory region (Skeath and Carroll, 1991; Skeath et al., 1992). Regarding H-cell development, *l(1)sc* and *sc*, but not *ac*, are expressed in MP3 and could be involved in MP3 formation. Most significantly, *l(1)sc* is expressed early in H-cell and H-cell sib development, and could influence cell fate in either cell type, although it remains on longer in H-cell. The *sc* gene is also expressed specifically in H-cell, but relatively late in development. Similarly, expression of *l(1)sc*, *ac* and *sc* in MP1, MP4, MP5, MP6 and MNB could influence MP formation and division, and *l(1)sc* could control mVUM cell type-specific expression, as it is preferentially expressed in mVUMs compared with iVUMs.

l(1)sc is required for H-cell gene expression

Based on the expression of *l(1)sc* and *sc* (but not *ac*) in the H-cell and their potential roles in H-cell development, we analyzed mutants of the *AS-C* to assess their role in H-cell development and gene expression. The mutants analyzed included: (1) *Df(1)sc-B57* [mutant for *ac*, *l(1)sc*, *sc* and *ase*], (2) *Df(1)sc19* [mutant for *ac*, *l(1)sc* and *sc*], (3) sc^{M6} (mutant for *sc*), (4) *Df(1)sc10-1* (mutant for *ac* and *sc*) and (5) *Df(1)ase-1* (mutant for *ase*). In *Df(1)sc-B57* mutant embryos, there was an absence of expression (ranging from 43-76% of segments analyzed) for the H-cell neural function genes *DAT*, *NPFR1*, *ple* and *Vmat* (Fig. 5A-D,H-K; Table 1). In wild-type embryos, *Vmat* was expressed in the pdm^+ H-cell and the three pdm^- mVUMs (Fig. 5D). In *Df(1)sc-B57* embryos (Fig. 5K), *Vmat* was absent in the H-cell in 43% of segments, absent from all mVUMs in 39% of segments, present in only one mVUM in 43% of segments and present in two mVUMs in 17% of segments (Tables 1, 2). This result indicated that *AS-C* genes are required for H-cell and mVUM gene expression. *BarHI*, *SoxN* and *tup* transcription factor gene expression was assayed in *Df(1)sc-B57* stage 14-16 mutant embryos, when expression of these genes is restricted to only the H-cell in wild type (Fig. 5E-G,L-O; Table 1). *BarH1* expression was absent in 63% of segments, and *SoxN* was absent in 47% of segments. By contrast, Tup protein was present in *Df(1)sc-B57* embryos in one cell in 71% of segments, two cells in 27% of segments and was absent in 2% of segments. Both Tup^+ cells are also pdm^+, so the identity of the two Tup^+ cells are likely to be the H-cell and H-cell sib, but this is unproven as all H-cell markers were reduced or absent in *Df(1)sc-B57*, and we did not detect colocalization between Tup and the *CG13565* H-cell sib marker when analyzed together (0/7 segments). In wild-type embryos, *CG13565* is present in H-cell sib in 54% of segments (Table 1). In *Df(1)sc-B57* mutant embryos, *CG13565* was present in 46% of segments, only slightly lower than wild type (Table 1). Thus, the H-cell sib was not transformed into an H-cell in most embryos. However, it remains possible that H-cell sib neurons lose *CG13565* expression when coupled with ectopic *tup* in *Df(1)sc-B57* mutant embryos.

The *5-HT1A, pdm2* and *nub* genes are expressed in both H-cell and H-cell sib (Wheeler et al., 2006), and their expression was unaffected in *Df(1)sc-B57* mutants (Fig. 5H,M,P,R). *Lim3* is expressed in MP1 neurons, and its expression was also unaffected in *Df(1)sc-B57* mutant embryos (Fig. 5Q,S). These data suggest that genes with dual expression in both H-cell and H-cell sib and genes expressed in MP1 neurons are not regulated by the *AS-C*.

The *Df(1)sc-B57* mutant strain provided strong evidence for defects in H-cell gene expression, but is mutant for four regulatory genes [*ac*, *ase*, *l(1)sc*, *sc*]. Similar defects in *DAT* and *ple* expression were observed in another mutant strain, *Df(1)sc19* [mutant for *ac*, *l(1)sc* and *sc*] (data not shown). The analysis of additional mutations in the *AS-C* locus suggested that the *l(1)sc* gene is the principal *AS-C* gene required for H-cell gene expression, as H-cell gene expression was largely unaltered in *Df(1)sc10-1* (ac^- sc^-), sc^{M6} (sc^-) and *Df(1)ase-1* (ase^-) (see Fig. S2A,B,E,F and Table S1 in the supplementary material). In sc^{M6} (sc^-), *BarH1*, *DAT*, *NPFR1*, *ple*, *SoxN* and *tup* expression was unaffected (see Fig. S2I-K,M-P and Table S1 in the supplementary material), but *ple* expression was absent in 2% of segments and *Vmat* expression absent in 8% of segments (see Fig. S2L and Table S1 in the supplementary material). This indicates that *sc* may play a minor role in regulating H-cell gene

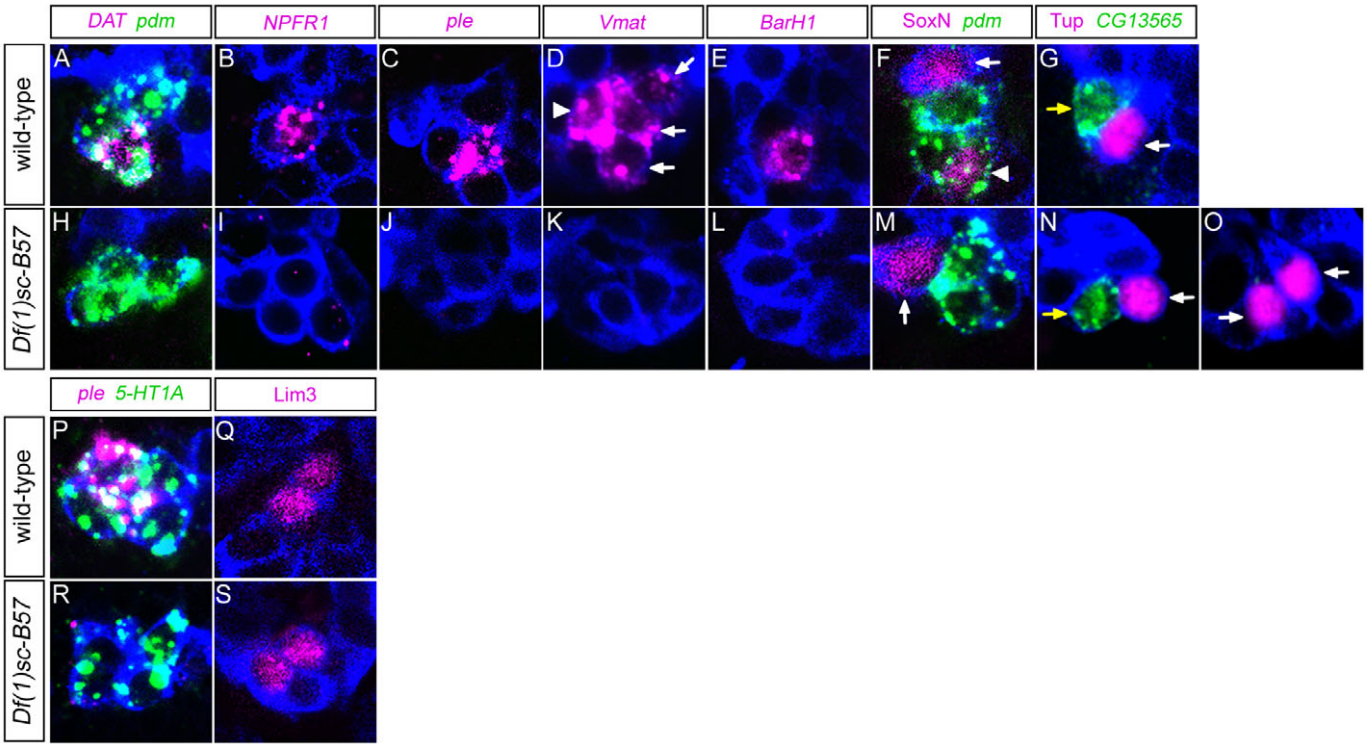

Fig. 5. H-cell gene expression is affected in *l(1)sc* mutants. Confocal images of stage 14-16 (A-G,P,Q) wild-type and (H-O,R,S) *Df(1)sc-B57* embryos. All embryos contained *sim-Gal4 UAS-tau-GFP* and were stained for GFP (blue), and all segments (except G,N-S) were stained for *pdm2/nub* (*pdm*; green) to identify the H-cell, H-cell sib and MP1 neurons. For simplicity, *pdm2/nub* staining is omitted from most panels, except for A,F,H,M. (**A-F**) In wild-type control embryos, (A) *DAT*, (B) *NPFR1*, (C) *ple*, (D) *Vmat*, (E) *BarH1* and (F) *SoxN* expression (all magenta) was present in the H-cell. (**H-M**) In *Df(1)sc-B57* embryos, (H) *DAT*, (I) *NPFR1*, (J) *ple*, (K) *Vmat*, (L) *BarH1* and (M) *SoxN* expression was absent from the H-cell in the segments shown. In A, *pdm* staining was present to illustrate that MP1 neurons, the H-cell and H-cell sib can be identified. Only one pdm^+ MP1 is shown in this focal plane. *DAT* expression overlaps with *pdm* in the H-cell. In wild type (D), *Vmat* expression is present in the H-cell (arrowhead; identified by *pdm* staining, which is not shown) and mVUMs (arrows), whereas in *Df(1)sc-B57* (K), *Vmat* expression was absent in the H-cell and mVUMs. (F) SoxN was present in the wild-type pdm^+ H-cell (arrowhead) and pdm^- MG (arrow); in *Df(1)sc-B57* (M), SoxN was absent from pdm^+ cells, including the H-cell, but present in pdm^- MG (arrow). (**G**,**N**) In wild type (G), Tup was present in the H-cell (white arrow) and *CG13565* was present in the H-cell sib (yellow arrow), whereas in *Df(1)sc-B57* (N), Tup was present in the H-cell (white arrow) and the H-cell was not transformed to the H-cell sib (yellow arrow) as the H-cell sib-specific gene expression (*CG13565*; green) was absent in the H-cell. (**O**) In *Df(1)sc-B57*, Tup was present in an additional cell in 27% of segments scored. (**P**,**R**) Expression of the *5-HT1A* gene, which is present in the H-cell, H-cell sib and MP1 neurons in wild-type embryos, was unaffected in *Df(1)sc-B57*. Note the absence of *ple* expression in *Df(1)sc-B57*. (**Q**,**S**) Lim3 was present in the two MP1 neurons in both wild-type and *Df(1)sc-B57* mutant embryos.

expression, but, more generally, demonstrates that *ac*, *sc* and *ase* do not significantly contribute to the *Df(1)sc-B57* defects by themselves. Without a *l(1)sc* single-gene mutant available, we attempted transgenic *l(1)sc* RNAi experiments, but these failed to significantly reduce *l(1)sc* levels or H-cell gene expression. However, rescue experiments employing *sim-Gal4 UAS-l(1)sc* successfully rescued the *Df(1)sc-B57* phenotype. In these experiments, ple^+ H-cells were present in 92% of rescue segments, compared with only 24% ple^+ segments that were present in *Df(1)sc-B57* (see Table S2 in the supplementary material). These data indicate that *l(1)sc* is a key regulator of H-cell gene expression. This is consistent with the prominent H-cell-specific localization of the L(1)sc protein.

Misexpression of *l(1)sc* activates H-cell gene expression

To gain further insight into *l(1)sc* and *sc* function, we individually misexpressed both genes in all midline cells in *sim-Gal4 UAS-l(1)sc* and *sim-Gal4 UAS-sc* embryos, and assayed stage 14-16 embryos for alterations in H-cell gene expression. In *sim-Gal4 UAS-l(1)sc* embryos, L(1)sc protein was present in all midline cells through mid-stage 11, but was absent in midline neurons by late stage 11, while persisting in MG past stage 16. When *l(1)sc* was misexpressed, *ple* expression was observed in an extra cell in addition to the H-cell in 18% of segments analyzed (Fig. 6A,B; Table 1). The additional ple^+ cell was an H-cell sib based on co-expression of *ple* with *CG13565*, an H-cell sib-specific marker (Fig. 6B). As *CG13565* was still expressed in the ple^+ cell, this indicated that H-cell sib was not completely transformed into an H-cell in all segments, but rather that *l(1)sc* was able to activate H-cell-specific gene expression in H-cell sib. Ectopic *ple* expression was not observed in any other midline cell type besides H-cell sib, suggesting that only H-cell sib has the requisite transcriptional co-activators, chromatin structure or other factors required for L(1)sc to activate H-cell-specific transcription. *l(1)sc* misexpression also induced the expression of additional H-cell specific genes, including *BarH1*, *DAT*, *NPFR1*, *SoxN*, *tup* and *Vmat* in an extra cell in 14-24% segments analyzed (Fig. 6C-H; Table 1). In all cases, the two cells were an H-cell and H-cell sib. The *Vmat* gene, which is expressed in both the H-cell and mVUMs in wild-type embryos, showed an increase in *l(1)sc* misexpression embryos

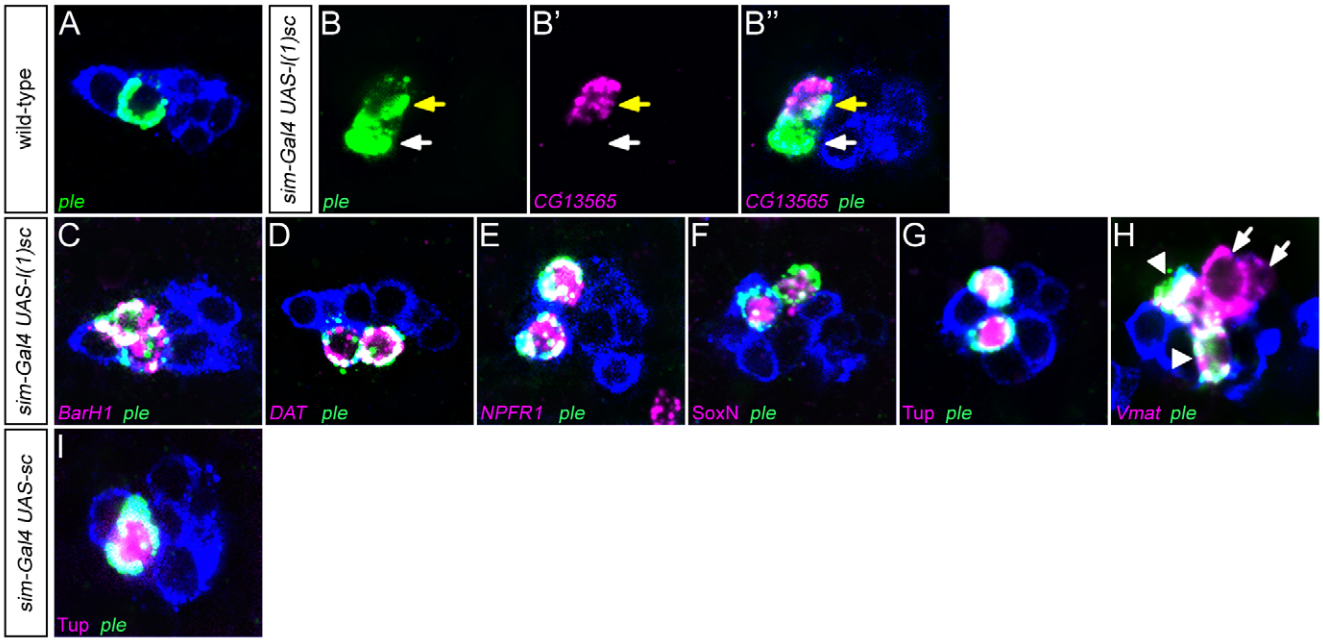

Fig. 6. *l(1)sc* activates H-cell gene expression. Horizontal views of single segments of stage 14-16 embryos. (A) Wild-type, (B-H) *sim-Gal4 UAS-l(1)sc* and (I) *sim-Gal4 UAS-sc* embryos stained for *ple* expression (green) and various H-cell-expressed genes (magenta). All embryos were *sim-Gal4 UAS-tau-GFP* and were stained for GFP (blue). (**A**) The single wild-type *ple*$^{+}$ H-cell. (**B-B″**) Misexpression of *l(1)sc* resulted in two *ple*$^{+}$ cells in 18% of segments scored. One cell was the H-cell (white arrow), and the additional cell was *CG13565*$^{+}$ (magenta; yellow arrow), which indicated that it was the H-cell sib. (**C-G**) Misexpression of *l(1)sc* resulted in the appearance of (C) *BarH1*, (D) *DAT*, (E) *NPFR1*, (F) SoxN and (G) Tup in an additional *ple*$^{+}$ cell in 14-24% of segments scored. (**H**) Misexpression of *l(1)sc* resulted in the expansion of *Vmat* expression from four cells in wild type to five to eight cells. In this segment, there were five *Vmat*$^{+}$ cells, two of which were *ple*$^{+}$ (arrowheads; H-cell and H-cell sib) and the other three were mVUMs (arrows; only two *Vmat*$^{+}$ mVUMs appear in this focal plane). (**I**) Misexpression of *sc* had no affect on *ple* or *tup* expression; only H-cell expression was observed.

from four cells in wild type to five to eight cells (Fig. 6H). One of the additional cells was an H-cell sib, based on co-expression with *ple*; the others were presumably iVUMs that ectopically expressed *Vmat*. This demonstrated that *l(1)sc* can ectopically activate both H-cell and mVUM gene expression. By contrast, misexpression of *sc* did not result in expanded *ple* or *tup* expression (Fig. 6I), even though Sc protein was present in all midline cells until mid-stage 11, similar to L(1)sc protein dynamics in *l(1)sc* misexpression experiments. These results indicated that *l(1)sc* has the ability to activate most, if not all, genes specifically expressed in H-cell, whereas *sc*, despite its close sequence homology to *l(1)sc*, was not able to induce ectopic H-cell specific gene expression. Use of *elav-Gal4* to express *l(1)sc* in post-mitotic neurons failed to elicit ectopic H-cell gene expression, suggesting that the presence of high levels of *l(1)sc* in MP3 or its newly-born progeny is required to ectopically activate H-cell-specific gene expression.

SoxN and tup regulate aspects of H-cell gene expression

Two other transcription factors that regulate H-cell gene expression are SoxN and Tup. The *tup* gene, like *l(1)sc*, is expressed in H-cell and H-cell sib, and then localizes to only the H-cell (Fig. 3A-D). Previous work has demonstrated that *tup* was required for *ple* and *ddc* expression (Thor and Thomas, 1997). Genetic analyses of *tup*^{1} and *tup*$^{isl-1}$ mutant embryos with additional neural function genes indicated that *tup* function is required for *ple* and *DAT* expression

Table 1. Summary of H-cell, H-cell sib and dorsal lateral dopaminergic neuron genetic data

		Wild type			*Df(1)sc-B57*			*sim-Gal4 UAS-l(1)sc*		
Gene	Cell type	No cells	One cell	Two cells	No cells	One cell	Two cells	No cells	One cell	Two cells
BarH1	H-cell	0	50 (100)	0	17 (63)	10 (37)	0	0	14 (82)	3 (18)
DAT	H-cell	0	50 (100)	0	14 (70)	6 (30)	0	0	15 (83)	3 (17)
NPFR1	H-cell	0	46 (100)	0	10 (45)	12 (55)	0	0	19 (76)	6 (24)
ple	H-cell	0	92 (100)	0	13 (76)	4 (24)	0	0	117 (82)	26 (18)
SoxN	H-cell	0	50 (100)	0	7 (47)	8 (53)	0	0	25 (86)	4 (14)
tup	H-cell	0	44 (100)	0	1 (2)	42 (71)	16 (27)	0	25 (78)	7 (22)
Vmat	H-cell	0	34 (100)	0	10 (43)	12 (52)	1 (4)	0	19 (86)	3 (14)
CG13565	H-cell sib	6 (46)	7 (54)	0	34 (54)	29 (46)	0	6 (46)	7 (54)	0
DAT	DL DA neuron	0	49 (100)	0	33 (83)	7 (17)	0			
ple	DL DA neuron	0	44 (100)	0	24 (67)	12 (33)	0			

Cell type refers to the wild-type cell in which each gene is expressed. DL DA neuron refers to dorsal lateral dopaminergic neuron. Values represent the number of segments in which no, one or two cells expressed the gene. Percentages are indicated in parentheses. In *Df(1)sc-B57*, there was a single appearance of two *Vmat*$^{+}$ *pdm*$^{+}$ cells, indicating that the two cells were probably the H-cell and H-cell sib, and not VUM neurons. $P<0.0001$ for all *Df(1)sc-B57* and rescue experiments, except *tup* ($P=0.4943$) and *CG13565* ($P=0.7622$). $P<0.0163$ for the *sim-Gal4 UAS-l(1)sc* experiments, except for *Vmat* ($P=0.0556$).

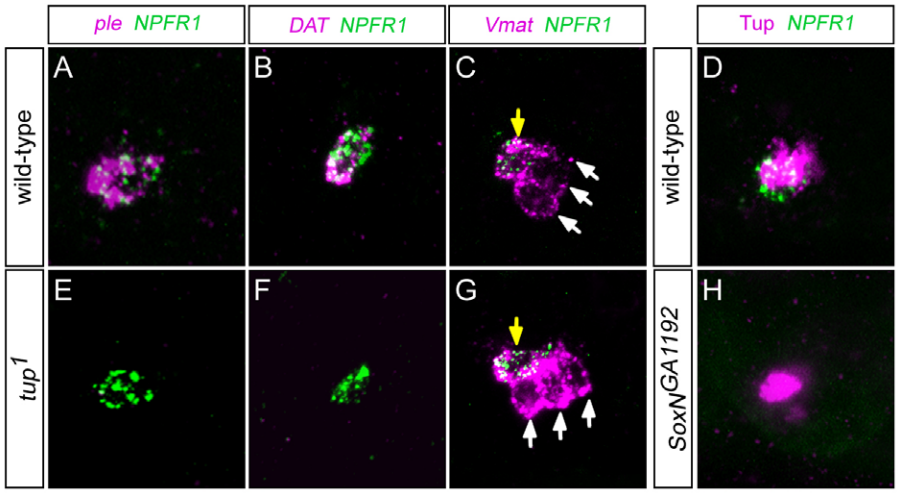

Fig. 7. *SoxN* and *tup* control different aspects of H-cell gene expression. Confocal images of stages 14-16 (A-D) wild-type, (E-G) *tup*^{1} embryos and (H) *SoxN*^{GA1192} embryos. (**A-D**) Wild-type embryos show H-cell expression of *ple*, *NPFR1*, *DAT*, *Vmat* and *tup*. (**E-G**) *tup*^{1} mutant embryos showed an absence of (E) *ple* and (F) *DAT* expression, but (E-G) *NPFR1* and (G) *Vmat* expression was unaffected. In *tup*^{1} mutants, *NPFR1* expression indicates the presence of the H-cell. (C,G) In *tup*^{1}, *Vmat* expression was present in both the H-cell (*NPFR1*$^{+}$; yellow arrows) and mVUMs (*NPFR1*$^{-}$; white arrows). (**H**) *SoxN* mutant embryos lacked *NPFR1* expression, but *tup* expression was unaffected.

(Fig. 7A,B,E,F), but *Vmat* and *NPFR1* levels are not reduced in thoracic segments (Fig. 7A-C,E-G; although in some abdominal segments there is a derepression of *NPFR1*; A.R.G., unpublished). In particular, *l(1)sc* expression was unaffected in *tup* mutant embryos (data not shown). As *tup* is expressed transiently in H-cell sib, *tup* mutant embryos were analyzed for expression of *CG13565*, *sim* and *VGlut*, which are expressed in H-cell sib in wild-type embryos. Expression of these three genes was not significantly affected (data not shown). In addition, expression of *nub*, *pdm2*, *5-HT1A* and *Glu-RI*, which are expressed in both the H-cell and H-cell sib, were unchanged between wild-type and *tup* embryos (data not shown). These results indicate that *tup* regulates expression of three genes (*DAT*, *ddc*, *ple*) involved in dopamine biosynthesis and transport, but not in other aspects of H-cell or H-cell sib development. Consequently, *tup* has a more limited role in H-cell development than *l(1)sc*.

SoxN, one of the two *Drosophila Sox* transcription factor genes related to the mammalian Group B Sox genes (Cremazy et al., 2000), was assayed for effects on H-cell transcription by analyzing *SoxN*^{GA1192}-null mutant embryos (Fig. 7D,H). Expression of the *NPFR1* neuropeptide receptor gene was absent in *SoxN* mutant embryos, whereas *BarH1*, *DAT*, *ple*, *tup* and *Vmat* expression were unaffected (data not shown). Thus, *l(1)sc* regulates all H-cell-specific gene expression, whereas *SoxN* acts downstream and *tup* in parallel of *l(1)sc* to regulate subsets of H-cell gene expression: dopamine biosynthetic enzyme genes and a membrane transporter in the case of *tup*, and a neurotransmitter receptor in the case of *SoxN*.

l(1)sc is required for formation of VUM midline precursors and mVUM gene expression

The *l(1)sc* gene is important for H-cell gene expression. However, it does not affect MP1 and MP3 formation or their division into neurons, as the two MP1 neurons, the H-cell and H-cell sib are present in *Df(1)sc-B57* mutant embryos based on positive staining for *Lim3* (MP1 neurons), *tup* (H-cell), *CG13565* (H-cell sib), *5-HT1A* and *nub*/*pdm2* (MP1 neurons, H-cell, H-cell sib) (Fig. 5H,M-O,R,S). The *l(1)sc*, *ac* and *sc* genes are expressed in MP4-6, MNB and PMG, and could potentially play a role in their development. Consequently, we assayed those cell types in *AS-C* mutant embryos for alterations in cell number and gene expression. In stage 12 wild-type embryos, there are 10 Elav^{+} neurons present (*n*=10 segments) (Fig. 8A). They are the progeny of the five MPs, as the MNB is just beginning to generate neurons at this time (Wheeler et al., 2006; Wheeler et al., 2008). The number of Elav^{+} neurons increases to an average of 14.1 neurons at stage 14 (*n*=9 segments) (Fig. 8B) and 15.6 neurons at stage 16 (*n*=8 segments) (Fig. 8C) with the additional neurons being MNB progeny. In stage 12 *Df(1)sc-B57* mutants, there are an average of 5.4 Elav^{+} cells (*n*=8 segments) (Fig. 8F), and this number is maintained at stages 14 (5.9 cells, *n*=8 segments) and 16 (5.7 cells, *n*=6 segments) (Fig. 8G,H). Although *AS-C* genes, including *l(1)sc* are expressed in PMG, analysis of *Df(1)sc-B57* mutant embryos revealed no alterations in MG number, type (AMG and PMG) or gene expression (*wrapper*) (Fig. 8D,I). The reduction of midline neurons observed in *Df(1)sc-B57* was not seen in *Df(1)sc10-1*, *Df(1)ase-1* or *sc*^{M6} mutant embryos (see Fig. S2A-P and Table S1 in the supplementary material) and *sim-Gal4 UAS-l(1)sc Df(1)sc-B57* rescue embryos had a wild-type number of neurons (see below). Accordingly, the loss of midline neurons in *Df(1)sc-B57* is due to loss of *l(1)sc*, and not to loss of *ac*, *sc* or *ase*.

Examination of *Df(1)sc-B57* embryos at stages 10-11 revealed only three Elav^{+} cells (instead of six in wild type) with the appearance of MPs (see Fig. S3 in the supplementary material), and two of those cells were MP1 and MP3 (with the other cell MP4, MP5 or MP6). This indicates that the missing neurons in *Df(1)sc-B57* are due to a failure of MP4-6 and the MNB to delaminate and divide, which is a proneural phenotype. Analysis with neuron-specific markers further confirmed that the missing neurons of *Df(1)sc-B57* were VUMs. We examined the expression of three VUM-expressed genes: (1) *Tyramine* β *hydroxylase* (*Tbh*), which is expressed in mVUM4-6 and encodes an octopamine biosynthetic enzyme; (2) *castor* (*cas*), which is present in iVUM4,5 and mVUM4,5; and (3) *CG16778* (also *Tyrosine kinase-related*, *Tkr* and *Jim Lovell*), which is expressed in iVUM6 and mVUM6 (Fig. 8K-M,O-Q,S-U; Table 2) (Wheeler et al., 2006; Wheeler et al., 2008). In *Df(1)sc-B57* mutant embryos, *Tbh* expression was absent

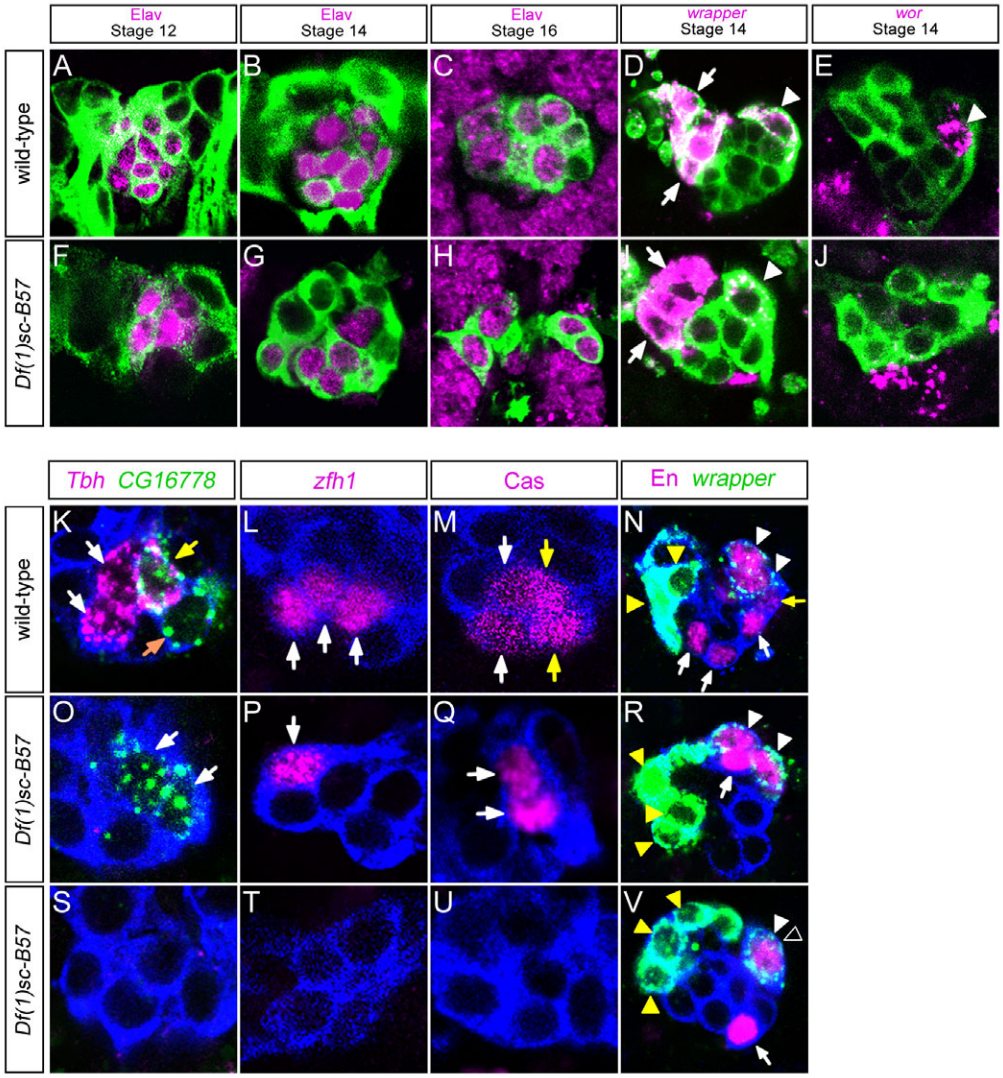

Fig. 8. *l(1)sc* controls VUM neuron and MNB formation. Confocal images of (A-E,K-N) wild-type and (F-J,O-V) *Df(1)sc-B57 sim-Gal4 UAS-tau-GFP* embryos are shown with the embryonic stage indicated at the top. All views are sagittal (except C and H, which are horizontal views). Embryos were stained with anti-GFP (A-J, green; K-V, blue). (**A-C**) In wild-type embryos, there were an average of 10.0 Elav$^+$ neurons at stage 12, 14.1 Elav$^+$ neurons at stage 14 and 15.6 Elav$^+$ neurons at stage 16 (not all Elav$^+$ neurons can be observed in the focal planes shown). (**F-H**) In *Df(1)sc-B57* embryos, there were an average of 5.4, 5.9 and 5.7 Elav$^+$ neurons at stages 12, 14 and 16. (**D**,**I**) Expression of *wrapper* was high in AMG (arrows) and low in PMG (arrowheads) in both wild-type and *Df(1)sc-B57* embryos, and the same number of MG were present in wild-type and mutant embryos. (**E**,**J**) Expression of *wor* was present in the MNB (arrowhead) in wild-type embryos, but was absent in *Df(1)sc-B57* embryos. (**K**) In wild type, *CG16778* is present in mVUM6 (yellow arrow) and iVUM6 (orange arrow). *Tbh* expression marks the three mVUMs (white and yellow arrows). (**O**,**S**) *CG16778* was present in two cells (and once in three cells) in 65% of *Df(1)sc-B57* segments (O), indicating the presence of two VUM6 neurons (white arrows), but was absent or in one cell in 35% of segments (S). (**L**,**P**,**T**) In wild type (L), *zfh1* was expressed in the three mVUMs (arrows), but in *Df(1)sc-B57* it was commonly expressed in only one cell (P; white arrow) or was absent (T). (**M**) In wild type, Cas was present at intermediate levels in mVUM4 and iVUM4 (white arrows) and at higher levels in mVUM5 and iVUM5 (yellow arrows), but was absent in mVUM6 and iVUM6. (**Q**,**U**) In *Df(1)sc-B57*, Cas was present in (Q) two cells (white arrows) in 50% of segments (either VUM4 or VUM5) and was (U) absent in 50% of segments. (**N**) In wild type, En was present in $wrapper_{low}$ PMG (white arrowheads) and in $wrapper^-$ MNB (yellow arrow) and iVUMs (white arrows). The $wrapper_{high}$ AMG are indicated by yellow arrowheads. (**R**,**V**) In *Df(1)sc-B57* embryos, En was present in $wrapper_{low}$ PMG (white arrowheads; empty arrowhead indicates that PMG is out of the focal plane) and in a single $wrapper^-$ cell, which was probably an iVUM (white arrow). AMG are indicated by yellow arrowheads.

in 82% of segments, compared with its presence in three neurons in wild type (Fig. 8K,O,S). *CG16778* was absent in 24% of segments and present in only one cell in 12% of segments (Fig. 8K,O,S), instead of two cells observed in wild type. Expression of *cas* was absent in 50% of segments, and reduced to only two cells in another 50% of segments (instead of four cells in wild type) (Fig. 8M,Q,U). These observations demonstrated that *l(1)sc* is required for the generation of MP6 and VUM6 neurons, and either MP4/VUM4s or MP5/VUM5s or both [we cannot distinguish these cell types in *Df(1)sc-B57*].

Analysis of mVUM and iVUM gene expression in *Df(1)sc-B57* mutant embryos indicated that the two surviving VUMs (on average) were likely to be an iVUM/mVUM pair in which mVUM-specific gene expression was reduced. Not only are the

Table 2. Summary of mVUM, iVUM and MNB genetic data

		Wild type					*Df(1)sc-B57*				
Gene	Cell type	No cells	One cell	Two cells	Three cells	Four cells	No cells	One cell	Two cells	Three cells	Four cells
Tbh	mVUM4-6	0	0	0	44 (100)	0	14 (82)	3 (18)	0	0	0
Vmat	mVUM4-6	0	0	0	34 (100)	0	9 (39)	10 (43)	4 (17)	0	0
zfh1	mVUM4-6	0	0	0	34 (100)	0	11 (65)	4 (23)	2 (12)	0	0
en	iVUM4-6	0	0	0	42 (100)	0	3 (30)	7 (70)	0	0	0
CG16778	mVUM6 and iVUM6	0	0	44 (100)	0	0	4 (24)	2 (12)	10 (59)	1 (5)	0
cas	mVUM4,5 ,5 and iVUM4	0	0	0	0	34 (100)	6 (50)	0	6 (50)	0	0
wor	MNB	0	42 (100)	0	0	0	13 (93)	1 (7)	0	0	0

Values represent the number of segments in which no, one, two, three or four cells expressed the gene. Percentages are indicated in parentheses. $P<0.0002$.

levels of the mVUM marker *Tbh* greatly reduced in *Df(1)sc-B57* (Fig. 8O,S), but the expression of *Vmat*, *zfh1* and *dgk*, which is present in mVUMs but not in iVUMs (Wheeler et al., 2006; Wheeler et al., 2008), was reduced in *Df(1)sc-B57* (Fig. 5D,K; Fig. 8P,T; see Fig. S4A,C in the supplementary material). By contrast, iVUM-expressed genes, including *en* and *CG15236* (Wheeler et al., 2006), were not affected (Fig. 8N,R,V; see Fig. S4B,D in the supplementary material). Expression of *Tbh* was unaffected in *Df(1)sc10-1* or *Df(1)ase-1* mutant embryos, suggesting that the defects in mVUM gene expression seen in *Df(1)sc-B57* mutant embryos were dependent on *l(1)sc* function (see Fig. S2C,G; Table S1 in the supplementary material).

The *l(1)sc* gene plays an important proneural role in the formation of neuroblasts in the *Drosophila* embryonic CNS (Jimenez and Campos-Ortega, 1990; Younossi-Hartenstein et al., 1997). As it is also prominently expressed in the MNB, we assayed *Df(1)sc-B57* embryos for defects in MNB formation by assaying *wor* expression, a gene required for NB formation (Ashraf et al., 1999; Ashraf and Ip, 2001; Cai et al., 2001). Analysis of *Df(1)sc-B57* mutant embryos indicated that *wor* expression was absent from the MNB in 93% of segments (Fig. 8E,J; Table 2), consistent with the observation by Jiménez and Campos-Ortega (Jiménez and Campos-Ortega, 1990) that the MNB was absent in *Df(1)sc-B57* embryos. Expression of *wor* was unaffected in *Df(1)sc10-1* or *Df(1)ase-1* mutant embryos and rescued in *sim-Gal4 UAS-l(1)sc Df(1)sc-B57* embryos, indicating that MNB formation is dependent on *l(1)sc* (see Fig. S2D,H and Table S2 in the supplementary material). These results are consistent with the reduced number of Elav$^+$ cells observed in *Df(1)sc-B57*.

Previous work indicated that an *AS-C* deficiency resulted in loss of midline *en* expression, although the midline cell types affected were not analyzed in detail (Bossing and Brand, 2006). Consistent with their results, our data indicated that in *Df(1)sc-B57* mutants, most of the *en*$^+$ iVUMs, MNB and MNB progeny were absent (Fig. 8N,R,V). By contrast, we analyzed the *en*$^+$ PMG in *Df(1)sc-B57*: these cells were present and *en*$^+$ (Fig. 8N,R,V). Thus, *l(1)sc* does not regulate *en* expression in PMG and the reduction of neuronal *en* reflects the loss of *en*$^+$ cells.

AS-C is required for dorsal lateral dopaminergic neuron gene expression

As *l(1)sc* is required for neuronal gene expression in two types of midline neurons, the dopaminergic H-cell and mVUM motoneurons, this raises the question: does *l(1)sc* commonly regulate gene expression in non-midline CNS neurons? The answer to this question is largely 'no'. Co-staining the embryonic CNS for both Elav and L(1)sc revealed that newly born Elav$^+$ neurons (stages 11-12) were rarely L(1)sc$^+$, except in the midline cells (see Fig. S5A,B in the supplementary material). Examination of specific lineages confirms this observation. NB4-2 gives rise to GMC4-2a that divides into RP2 and RP2 sib. *Notch* signaling is required for RP2 sib fate, whereas RP2 is *Notch* independent (Buescher et al., 1998). When the developing CNS was stained for L(1)sc and Even-skipped (Eve), which is present in GMC4-2a, RP2 and RP2 sib, it was observed that neither GMC4-2a nor its neuronal progeny were L(1)sc$^+$ (see Fig. S5C,D in the supplementary material).

Although *l(1)sc* may not be commonly required for embryonic CNS neuronal-specific gene expression, does it control gene expression in other dopaminergic neurons? There is a dorsal lateral dopaminergic neuron in each hemisegment in the embryonic CNS (Fig. 9A-B). Previously, Thor and Thomas (Thor and Thomas, 1997) showed that this cell is *tup*$^+$, and that *ple* expression is dependent on *tup* function, similar to the H-cell. We examined dorsal lateral dopaminergic neuron *DAT* and *ple* expression in *Df(1)sc-B57* embryos. Expression of *DAT* was absent in 83% of hemisegments (Fig. 9C, Table 1) and *ple* expression was absent in 67% of segments (Fig. 9D, Table 1). These results suggest that *AS-C* function, potentially *l(1)sc*, is required for gene expression in the dorsal lateral dopaminergic neurons, although whether it mechanistically functions similar to its role in the H-cell will depend on more detailed studies of the dorsal lateral dopaminergic neuronal cell lineage, L(1)sc localization and genetic analyses.

DISCUSSION

l(1)sc selectively controls midline precursor formation

The formation of midline neural precursors (five MPs and the MNB) is a dynamic, yet stereotyped process. The MPs undergo cellular changes in which their nuclei delaminate from an apical position within the ectoderm and move to the basal (internal) surface. There they divide after orienting their spindles. The precursors arise in a distinct order: MP4→MP3→MP5→MP1→MP6→MNB (Wheeler et al., 2008). We demonstrated that the *l(1)sc* gene is required for the formation of the MP4-6 and MNB precursors and their neuronal progeny (Fig. 10A). As we cannot definitively distinguish MP4 from MP5 in *Df(1)sc-B57*, there is some uncertainty whether both cell types are regulated by *l(1)sc*. However, as most segments only possess two VUMs, and those are VUM6s in over 60% of segments, it is likely that both MP4 and MP5 are commonly affected in *Df(1)sc-B57*, in addition to MP6. The *ac* and *sc* genes are both expressed in MPs and MNB, yet do not appear to play a significant role in MP and MNB formation. Although *l(1)sc* is the major proneural gene that controls formation of embryonic neuroblasts (Jimenez and Campos-Ortega, 1990; Younossi-Hartenstein et al., 1997), relatively little is known about how it functions and the identity

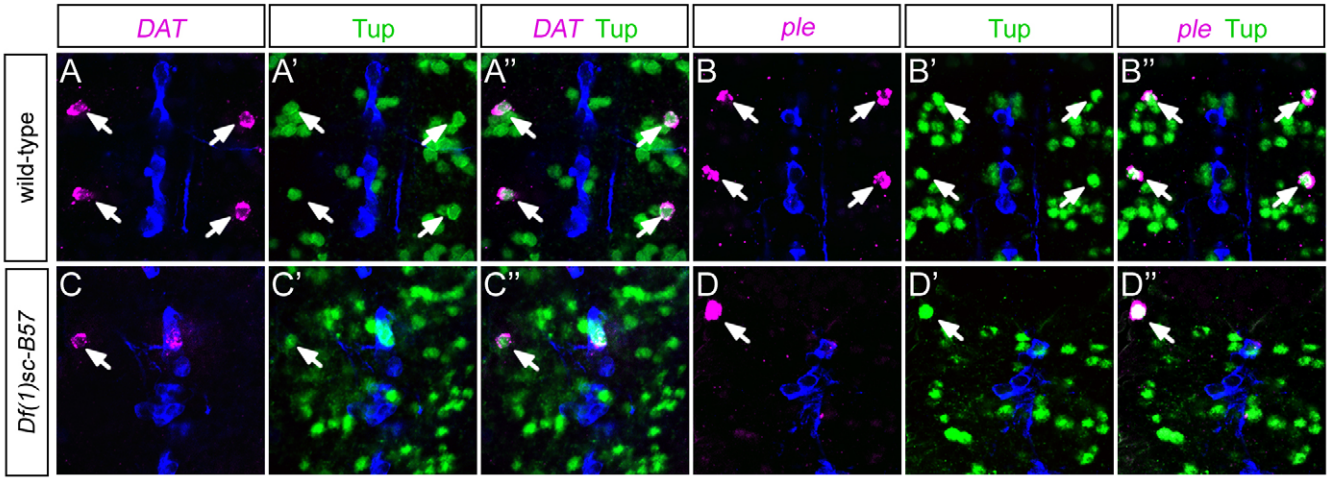

Fig. 9. Reduction of *DAT* and *ple* expression in *Df(1)sc-B57* dorsal lateral dopaminergic neurons. Horizontal views of two segments of stage 16 (A-B″) wild-type and (C-D″) *Df(1)sc-B57* embryos. All embryos contained *sim-Gal4 UAS-tau-GFP* and were stained for GFP (blue). (**A-B″**) In wild-type embryos, (A-A″) *DAT* and (B-B″) *ple* (both magenta) expression was present in the dorsal lateral dopaminergic neuron (arrows) in each of the four hemisegments shown and colocalized with Tup (green). (**C-D″**) In *Df(1)sc-B57* embryos, (C-C″) *DAT* and (D-D″) *ple* expression was present in the dorsal lateral dopaminergic neuron (arrows) in only one hemisegment, and was absent in the other three hemisegments.

of relevant target genes. In one study, it was shown that morphological changes that accompany neuroblast formation were dependent on *l(1)sc* function (Stollewerk, 2000). This is likely to be the case for *l(1)sc* and MP4-6 and MNB development, as MP4-6 and MNB delamination or division was commonly absent in *Df(1)sc-B57*. One key question is what activates or maintains *l(1)sc* expression in MP3-6 and MNB? Signaling by *hedgehog* (*hh*) is likely to be important, as no midline *l(1)sc* expression is present in *hh* mutant embryos (Bossing and Brand, 2006).

Although all MPs and MNB express *l(1)sc*, only MP4-6 and MNB were affected in mutants – formation of MP1 and MP3 were unaffected. These differences are unlikely to be solely due to different levels of L(1)sc protein or to a combination of Ac, L(1)sc

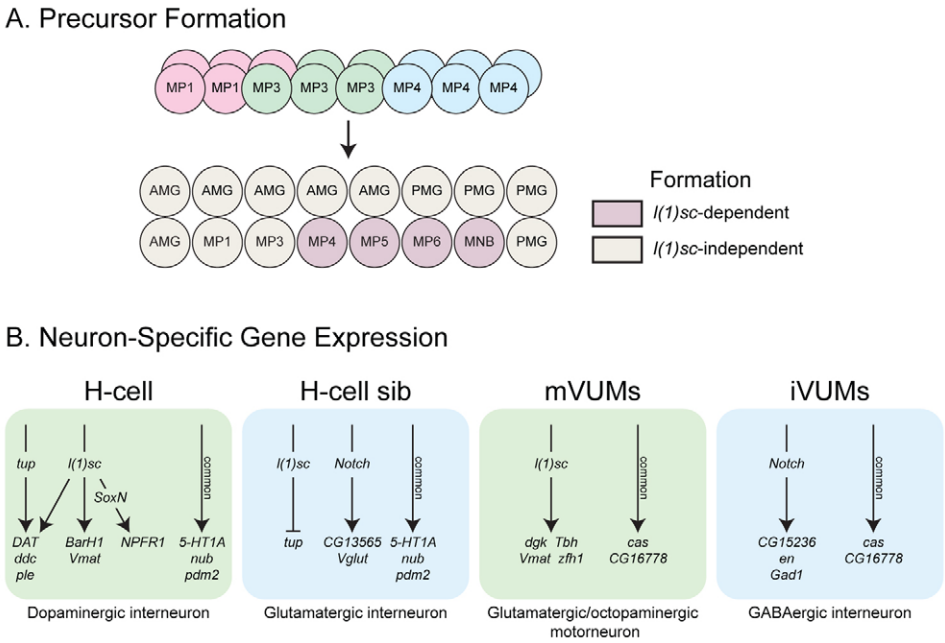

Fig. 10. Summary of midline precursor formation and neuron-specific gene expression. The genetic results presented in this paper. (**A**) From a group of three neural precursor equivalence groups (MP1, MP3 and MP4), *Notch* signaling partitions midline precursors at stage 10 into MPs, MNB, AMG and PMG. As shown here, *l(1)sc* acts as a proneural gene to promote the formation of MP4-6 and MNB, bt not MP1, MP3, AMG or PMG. (**B**) The H-cell, H-cell sib, mVUMs and iVUMs are represented along with their regulatory pathways and target genes. The *tup* and *l(1)sc* genes play important roles in H-cell-specific gene expression, and *l(1)sc* plays an important role in mVUM-specific gene expression. H-cell sib and iVUM-specific gene expression is largely controlled by *Notch* signaling. Gene expression in common to H-cell and H-cell sib or to mVUMs and iVUMs is controlled by separate pathways.

and Sc. L(1)sc protein levels are relatively constant among all five MPs and MNB, both L(1)sc and Sc are present in all MPs and MNB, and Ac, L(1)sc and Sc are present in MP1 as well as MP5,6 and MNB, yet no defects in MP1 and MP3 delamination or cell division were observed. Instead, the ability of *l(1)sc* to direct development of some MPs and not others may reflect the different cell states (and distinct co-factors) of the precursor populations from which each MP arises (Powell and Jarman, 2008). Similarly, *l(1)sc* controls expression of different genes in the H-cell compared with mVUMs, probably based on their different origins (MP3 versus MP4-6). Variability in the genetic control of midline MP formation extends to the non-midline MP2 cells. The MP2s require both *ac* and *sc* for MP formation and differentiation, whereas *l(1)sc* does not play a role (Skeath and Doe, 1996). Thus, MP2 and midline MPs (MP4-6) each require *AS-C* gene activity for proneural and differentiation functions, but use different *AS-C* family members.

Multiple regulatory pathways control H-cell gene expression

At least two distinct genetic programs control H-cell gene expression: (1) H-cell-specific gene expression is controlled by *l(sc)*, *tup* and *SoxN*, and (2) unknown factors control gene expression that is present in both the H-cell and H-cell sib (Fig. 10B). All H-cell-specific gene expression requires *l(1)sc* function. *tup* acts in parallel to control important aspects of H-cell gene expression, including the *DAT*, *ddc* and *ple* genes. *SoxN* acts downstream of *l(1)sc* to control *NPFR1* expression. H-cell neural function gene expression begins at stage 13, well after *l(1)sc* expression is absent, indicating that *l(1)sc* is unlikely to directly regulate these genes. However, Tup is present after stage 13 and could directly regulate *DAT*, *ddc* and *ple*; SoxN is also present and could directly regulate *NPFR1*. The *l(1)sc* gene regulates mVUM gene expression in a manner similar to its control of H-cell expression (Fig. 10B), but does so independently of *tup*, which is not expressed in mVUMs. We note that L(1)sc protein is present at higher levels in H-cell than mVUMs (Fig. 3F-H), although the significance of this is unclear. Expression of genes common to both H-cell and H-cell sib cells, including *5-HT1A*, *Glu-RI* and *tup*, were not affected in *l(1)sc* or *Notch* pathway mutants (Wheeler et al., 2008), indicating a second distinct regulatory pathway. This was also observed for genes expressed in common between mVUMs and iVUMs.

l(1)sc and *tup* regulation of H-cell and H-cell sib gene expression

The relationship between *l(1)sc* and *tup* in controlling H-cell-specific gene expression is complex. Both genes are initially expressed in the H-cell and H-cell sib after MP3 division, but expression of both is soon restricted to the H-cell. Misexpression of *l(1)sc* resulted in the ectopic expression of *tup* in the H-cell sib, similar to other H-cell-specific genes. However, in *l(1)sc* mutants, *tup* expression was not absent in the H-cell, but instead *tup* expression remained present in the H-cell and sometimes in two cells: one was the H-cell and the other was (probably) the H-cell sib. In addition, *l(1)sc* expression was not affected in *tup* mutants. These results indicated that: (1) *l(1)sc* and *tup* act in parallel in the H-cell to regulate dopaminergic pathway gene transcription; and (2) *l(1)sc* downregulates *tup* in the H-cell sib, indicating a role for *l(1)sc* in H-cell sib development. The best marker for the H-cell sib is *CG13565*, although it is expressed in wild type in only 54% of segments. In *Df(1)sc-B57* mutant embryos, *CG13565* was expressed in 46% of segments, similar to wild type. However, given its variability of gene expression in *Df(1)sc-B57* mutants (Table 1) and the normal variability of *CG13565* expression, it remains possible that *l(1)sc* (and *tup*) may play roles in H-cell sib development. Additional experiments are necessary to determine how *l(1)sc* and *tup* function together to control H-cell-specific gene expression.

How common is *l(1)sc* control of neuron-specific gene expression?

Within midline cells, *l(1)sc* plays important roles in controlling H-cell and mVUM gene expression, while playing relatively insignificant roles in MP1, H-cell sib and iVUM neuronal gene expression. We have begun to address whether non-midline neuronal gene regulation is regulated by *l(1)sc*. Significantly, *Df(1)sc-B57* mutant embryos show a strong reduction in *DAT* and *ple* expression in the non-midline dorsal lateral dopaminergic neurons. Thor and Thomas (Thor and Thomas, 1997) showed that *ple* expression in these cells was also reduced in *tup* mutant embryos. Although more detailed cellular and genetic studies are required to bolster these observations, these data raise the possibility that both *l(1)sc* and *tup* may regulate gene expression in both midline and non-midline dopaminergic neurons. More generally, *l(1)sc* control of neuron-specific gene expression is likely to be uncommon. This is based on the observation that in the developing CNS, there is little L(1)sc protein colocalizing with newly divided Elav$^+$ neurons or GMCs.

Evolutionary aspects of *Drosophila* dopaminergic neuron regulation

Because of the key neurobiological and medical importance of dopaminergic neurons, there has been intensive analysis of the regulatory factors that control their development in vertebrates and *C. elegans*. Are the regulatory programs involved in dopaminergic neuron differentiation conserved between insects, worms, and mammals? The two key regulatory proteins that control *Drosophila* H-cell dopamine differentiation are *l(1)sc* and *tup*. In vertebrates the bHLH genes mouse achaete-scute homolog [*Mash1*; homolog of *l(1)sc*] and neurogenin 2 (*Ngn2*) play roles in midbrain dopaminergic neuron development, although the role of *Mash1* is secondary to *Ngn2*, which has a key function in dopaminergic differentiation (Kele et al., 2006). However, *Mash1* (as well as *Ngn2*) can initiate neurogenic programs of other neuronal cell types. This was emphatically demonstrated in recent work in which forced expression of *Mash1* and two other transcription factor genes converted murine fibroblast cells to neurons (Vierbuchen et al., 2010). The mammalian orthologs of *Drosophila tup*, *Isl1* and *Isl2*, play important roles in motoneuron differentiation (Tsuchida et al., 1994), but have not been reported to influence dopaminergic neuron development and gene expression. Recently, *C. elegans* and vertebrate ETS family transcription factor genes were shown to directly regulate dopamine pathway gene expression (Flames and Hobert, 2009). It will be important to identify the transcription factors in *Drosophila* that directly regulate dopaminergic neural function genes and connect them to the regulatory genes we identified in this paper.

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