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The highly ordered assembly of retinal axons and their synaptic partners is regulated by Hedgehog/Single-minded in the *Drosophila* visual system

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During development of the *Drosophila* visual center, photoreceptor cells extend their axons (R axons) to the lamina ganglion layer, and trigger proliferation and differentiation of synaptic partners (lamina neurons) by delivering the inductive signal Hedgehog (Hh). This inductive mechanism helps to establish an orderly arrangement of connections between the R axons and lamina neurons, termed a retinotopic map because it results in positioning the lamina neurons in close vicinity to the corresponding R axons. We found that the bHLH-PAS transcription factor Single-minded (Sim) is induced by Hh in the lamina neurons and is required for the association of lamina neurons with R axons. In *sim* mutant brains, lamina neurons undergo the first step of differentiation but fail to associate with R axons. As a result, lamina neurons are set aside from R axons. The data reveal a novel mechanism for regulation of the interaction between axons and neuronal cell bodies that establishes precise neuronal networks.

KEY WORDS: Visual system, Lamina, Postsynaptic neuron, *sim*, *hh*, *Drosophila*

INTRODUCTION

Most axons in the brain establish topographic maps in which the arrangement of synaptic connections maintains the relationships between neighboring cell bodies (Udin and Fawcett, 1988). A notable model of topographic map formation is the visual system, where the relay of visual information from the retina to the visual center must be arranged in a spatially ordered manner through the topographic connections of retinal axons with their midbrain target, which is the optic tectum (OT) in lower vertebrates and the superior colliculus (SC) in mammals. This topographic map is termed a retinotopic map. Many studies have shown that Ephrin protein family members, acting through their Eph receptors, play pivotal roles in the establishment of the retinotopic map (reviewed by McLaughlin et al., 2003). In the mouse and the chick, for example, the retinal ganglion cells (RGCs) extend their axons to the OT/SC, and the low-to-high anteroposterior gradient of ephrin A in the target limits the posterior extension of growth cones at various positions, dependent on the EphA level of each RGC.

The *Drosophila* visual system has also provided insight into topographic mapping. In *Drosophila,* the projections of photoreceptor neurons (R cells) themselves induce development of the corresponding postsynaptic neurons (Huang and Kunes, 1996; Huang and Kunes, 1998; Huang et al., 1998; Selleck and Steller, 1991). The *Drosophila* visual system consists of the compound eyes and the three optic ganglia: the lamina, the medulla and the lobula complex. Each of the approximately 750 ommatidial units comprising the compound eye contain six outer photoreceptors (R1-R6) and two inner photoreceptors (R7, R8). R1-R6 cells send their axons to the first optic ganglion, the lamina, whereas R7 and R8 cells send axons through the lamina to the second ganglion, the medulla. R1-R6 cells in each ommatidium make stereotypic connections with particular lamina neurons (Meinertzhagen and Hanson, 1993). Synaptic units in the lamina are referred to as lamina cartridges. During the initial step of the assembly of a lamina cartridge, an arriving photoreceptor axon (R axon) fascicle forms a pre-cartridge ensemble, the 'lamina column', with a set of five lamina neurons. Formation of the ensemble results in a one-to-one correspondence of ommatidia to column units, and is fundamental to the subsequent establishment of intricate synaptic connections (Clandinin and Zipursky, 2002; Meinertzhagen and Hanson, 1993). Development of the lamina is tightly regulated by the projection of R axons. Failure in eye formation results in concurrent loss of the lamina, as in a normal brain, lamina neurogenesis is directly coupled to the arrival of R axons (Fischbach, 1983; Fischbach and Technau, 1984; Meyerowitz and Kankel, 1978; Power, 1943; Selleck and Steller, 1991; Steller et al., 1987). Both R cell differentiation and ommatidial assembly progress in a posterior-to-anterior direction across the eye disc. Differentiated R cells begin to send their axons to the brain in the same sequential order, reflecting their position in the retina along the anteroposterior and the dorsoventral axes. Wnt signaling plays a role in regulating projections along the dorsoventral axis (Sato et al., 2006).

As the axons from each new row of ommatidial R cell clusters arrive in the lamina, a corresponding group of lamina precursor cells (LPCs) undergo a final division and initiate differentiation into lamina neurons. In the first step of their neurogenesis, direct contact with R axons triggers the transition of G1-phase LPCs into S phase (Selleck et al., 1992). Both the G1-S transition and the initial specification into a lamina neuron are induced by Hedgehog (Hh), which is delivered by arriving R axons (Huang and Kunes, 1996), and the next step in lamina differentiation is induced by the Spitz signaling molecule, which is also delivered by R axons (Huang et al., 1998). Hh expressed in R cells (Lee et al., 1992) functions as a signal for photoreceptor development as well: secreted Hh induces anterior precursor cells to enter the pathway of R cell specification (for a review, see Heberlein and Moses, 1995).

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Thus, the retinotopic map along the anteroposterior axis of the lamina seems to be established autonomously and in a posterior-to-anterior order, as newly specified R cells send their axons to the lamina layer and make lamina columns. Each ommatidial unit sends a set of R axons as a single bundle to the lamina along the pre-existing fascicle that has been just projected. Then, the axon bundles are enveloped by the processes of newly induced lamina neurons (Meinertzhagen and Hanson, 1993). This step is key to forming the one-to-one associations between R axon bundles and their corresponding lamina neurons. We show here that the activity of Single-minded (Sim) is required for developing lamina neurons to establish an association with the corresponding R axons and, hence, to form the lamina column. *sim* encodes a basic-helix-loop-helix-PAS (bHLH-PAS) transcription factor and is induced by Hh provided by the R axons. In *sim* mutant brains, the developing lamina neurons fail to associate with R axon bundles, resulting in a failure to establish connections between R axons and lamina neurons. We infer that *sim* programs developing lamina neurons to express a molecule(s) that is required for the association with R axons.

Retinotopic mapping in *Drosophila* provides unique insights into neuronal network formation not only because of its tight coupling to the control of development, the molecular mechanisms of which were revealed by Kunes and colleagues (Huang and Kunes, 1996; Huang and Kunes, 1998; Huang et al., 1998), but also because of the interactions between axons and neuronal cell bodies, as revealed in this study. The interactions we observed stand in sharp contrast to what has been found for other models of axon guidance, where the growth cones of axons respond to a variety of attractive or repulsive guidance cues to navigate to their synaptic target cells. The cues include the netrins, Slits, semaphorins and ephrins (reviewed by Dickson, 2002; Guan and Rao, 2003; Tessier-Lavigne and Goodman, 1996; Yu and Bargmann, 2001), and the restricted expression pattern of these cues and the reactivity of growth cones play pivotal roles in the establishment of the proper synaptic connections. In this context, postsynaptic cells are seen as mere providers of guidance/adhesion molecules, passively awaiting the arrival of a growth cone. In other words, it is conceivable that presynaptic growth cones seek their targets dynamically, whereas postsynaptic cells remain static. Unlike the roles of presynaptic axons, the cellular behaviors of postsynaptic cells in the establishment of synaptic targeting are poorly understood. Here, we propose another possible model for the establishment of topographic neuronal connections in which postsynaptic cells dynamically interact with presynaptic axons.

MATERIALS AND METHODS

Fly stocks

The following mutant and transgenic strains were used in this study. *y w* flies were used as wild-type controls. *tkv-lacZ* is a reporter construct that has an insertion of a P element carrying *lacZ* in the promoter region of the *tkv* gene (Tanimoto et al., 2000). *NP6099-GAL4* is a lamina specific *GAL4* driver (Hayashi et al., 2002; Yoshida et al., 2005). *UAS-ARNT*^{DN} was described by Ohshiro and Saigo (Ohshiro and Saigo, 1997), UAS-*sim* was described by Chang et al. (Chang et al., 2003). *UAS-nlsGFP* encodes a nuclear-targeted form of GFP. *smo*^{1} is an extreme hypomorphic allele (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). *dac*^{3} is an amorphic allele of the *dac* mutant, *egfr*^{CO} is an amorphic allele of a *egfr* mutant (Price et al., 1989). And finally, *sim*^{2} and *sim*^{ry75} are an amorphic allele and a deletion removing the late promoter of *sim* (Pielage et al., 2002).

Clonal analysis

Wild-type, *smo*, *dac* and *sim* clones were preferentially induced in the optic lobe using P(y^{+}) *FRT40A, smo*^{1} *FRT40A*, *dac*^{3} *FRT40A*, *FRT82B egfr*^{CO}, *FRT82B sim*^{2}, *ubiGFP FRT40A*, *FRT82B ubiGFP* and *6099-GAL4 UAS-flp*.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Huang and Kunes, 1996; Takei et al., 2004). The following monoclonal antibodies were provided by the Developmental Studies Hybridoma Bank (DSHB): mAb24B10 (1:10), mouse anti-Sim (Single-minded mAb, 1:100), mouse anti-Dac (mAbdac2-3, 1:1000), rat anti-Elav (7E8A10, 1:25), mouse anti-Flamingo (1:10), and mouse anti-LacZ (40-1a, 1:200). Goat Cy3 anti-HRP (Accurate Chemical and Scientific) was used at a dilution of 1:200. Rat anti-Dac was raised against synthetic peptides (Hokudo) and diluted 1:40 or 1:200. Secondary antibodies (Jackson) were used at the following dilutions: anti-mouse Cy3, 1:200; anti-mouse Cy5, 1:200; anti-mouse FITC, 1:200; anti-rat Cy3, 1:200; and anti-rat Cy5, 1:200. Specimens were mounted with vectashield mounting media (Vector) and viewed on a Zeiss LSM510 confocal microscope.

RESULTS

Lamina neurons lacking Hh signaling are excluded from the developing lamina cartridges

Hh signaling is required for the development of both photoreceptor and lamina neurons (Heberlein and Moses, 1995; Huang and Kunes, 1996). Hh delivered by newly arriving R axons initiates the entry of G1-phase lamina precursor cells (LPCs) into S phase and induces the expression of several markers of differentiation, including the nuclear protein Dachshund (Dac), at the lamina furrow (Fig. 1A-C). Soon after LPCs start to differentiate, they form close associations with newly arriving R axons and make stereotyped ensembles, known as lamina columns, which consist of sets of fasciculated axons from each ommatidium and corresponding lamina neurons (Fig. 1C) (Meinertzhagen and Hanson, 1993).

In an effort to elucidate the mechanisms underlying lamina column formation, we examined the requirement for Hh signaling in LPCs. We used a mutant form of *smoothened* (*smo*) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), which encodes an essential component of the Hh receptor, to block the ability of LPCs to receive and transduce the Hh signal. Furthermore, we used the Flp-mediated mitotic recombination technique (Golic and Lindquist, 1989; Xu and Rubin, 1993) to generate 'twin spots' composed of sibling *smo*$^{-}$/*smo*$^{-}$ (*smo* mutant) and *smo*$^{+}$/*smo*$^{+}$ clones in a *smo*$^{-}$/*smo*$^{+}$ animal. Cells of each genotype could be distinguished by the expression of a marker protein, GFP. *smo* mutant clones were marked by the absence of a GFP marker residing on the *smo*$^{+}$ chromosome and *smo*$^{+}$/*smo*$^{+}$ clones were marked with two copies of GFP. We also used *NP6099-GAL4*, which is specifically expressed in the lamina neurons (Yoshida et al., 2005), and *UAS-flp*, to induce mitotic recombination specifically in the lamina neurons. In this system, the autonomous effect of a given mutation in lamina neurons can be examined without the effects of R axon inputs.

In *smo* mutant clones, expression of Dac was abolished in a cell-autonomous manner, consistent with previous reports (Fig. 1H) (Huang and Kunes, 1998). Here, we divide the lamina into two domains, one anterior to the newly arriving R axons and the other posterior. We will refer to the regions as the pre-assembling and assembling domains, respectively (Fig. 1C). It was observed that *smo* clones were recovered in the pre-assembling domains contiguous with the region called the outer proliferation center (OPC), where neuroblasts of lamina and outer medulla arise. However, *smo* clones were not seen in the assembling domain (100%, *n*=17; Fig. 1G, arrow for OPC), whereas control clones were recovered there (100%, *n*=13; Fig. 1D-F). The data are consistent with a previous observation (Huang et al., 1998), and suggest that Hh activity is required for developing lamina neurons to be assembled into the lamina columns (Huang et al., 1998). Even at an early stage, *smo* clones induced were never recovered in the

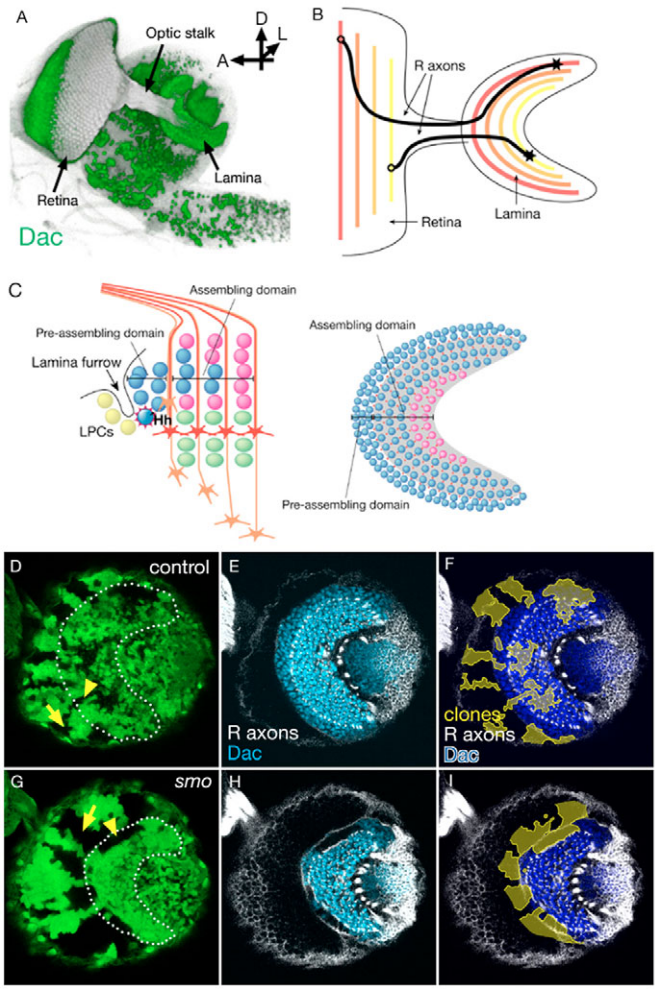

Fig. 1. *smo* mutant clones are not recovered between R axon bundles. Unless otherwise noted, all images are late third instar, oriented with anterior left and dorsal up. (**A**) Developing visual system of the third instar larva. R axons (white) and Dac-positive neural cell precursors (green) are labeled; the developing retina, optic stalk and lamina are shown. (**B**) A schematic drawing of the R axon projection. Photoreceptor cells differentiate as a row in a synchronized stepwise fashion and in a posterior to anterior order, and extend their axons to the lamina via the optic stalk. R axon projection induces lamina neuron differentiation; hence lamina neurons differentiate coordinately in the same manner. Colored bars represent rows of developing photoreceptors and lamina neurons; the same colors roughly represent axonal connections. (**C**) Schematic illustration of lamina column assembly from the horizontal perspective (left) and lateral perspective (right). As R axon innervations trigger the differentiation of lamina precursor cells in a posterior-to-anterior direction in a stepwise manner, the lamina furrow progresses anteriorly. The G1-phase lamina precursor cells (yellow) contact the most recently arriving R axons, receive Hh provided by the axons, and differentiate into the lamina neurons (light blue) at the trough of the lamina furrow. In older columns (at the posterior of the lamina), a subset of lamina neurons express neuronal markers (pink) as they become mature lamina neurons L1-L5. The R1-R6 axons (orange lines in horizontal and dots in lateral) stop between rows of glial cells (light green), whereas the R7 and R8 axons proceed to deeper target regions in the medulla. We have divided the lamina into two domains, one anterior to the newly arriving R axons and one posterior, and refer to the domains as pre-assembling and assembling, respectively. (**D-I**) Clones were induced by *NP6099-GAL4 UAS-flp* and marked by the absence of GFP. The optic lobe with wild-type (D-F) and *smo* (G-I) clones is shown. The developing lamina was demarcated by white dots (D,G). Developing lamina neurons express Dac (cyan in E,H; blue in F,I). R axons are visualized by the anti-HRP antibody (white in E,F,H,I). (G) *smo* clones are recovered in OPC (arrow) and in the pre-assembling domains (arrowhead). GFP-negative clones are outlined in F and I (yellow). (G,H) Dac expression is abolished in *smo*^{1} clones cell autonomously. (I) In contrast to control (F), *smo*^{1} clones were not recovered in the assembling domain.

assembling domain (data not shown), suggesting that the phenotype was not caused merely by a defect in the proliferation of lamina neuron precursors but rather by a defect in an activity required for interaction between R axons and lamina neurons. However, there is an alternative interpretation; namely, that *smo* is required for the survival of lamina neurons in the assembling domain. To address this possibility, we used the MARCM technique (Lee and Luo, 1999) to investigate whether the forced expression of the caspase inhibitor, p35, could recover the *smo* clones in the assembling domain. We found that this was not the case (data not shown).

Lamina column formation during third larval instar

To study lamina column formation, we took a close look at interactions between R axon bundles and lamina neurons of third larval instar. We staged lamina development based on the progress of R cell differentiation. Lamina neurons begin to differentiate when approximately five rows of R axon bundles are innervated into the brain (4-5 row stage; Fig. 2A,E). Even after lamina neurons have differentiated, they do not begin to assemble into lamina columns until the 11-12 row stage (Fig. 2F,G). Right after this stage, lamina neurons are intermingled with R axon bundles and assemble into lamina columns (Fig. 2D,H). We thus infer that lamina column formation does not progress autonomously; instead, it appears to require an activity that takes time to become functional during lamina development.

Next, we sought to address the behavior of R axons that have just arrived in and innervated the optic lobe. Flamingo (Fmi; Stan – FlyBase) is transiently expressed in R8 axons as they enter the optic lobe (Lee et al., 2003). We took advantage of anti-Fmi antibody staining to distinguish the most-anterior (most recently arriving) row of R axon bundles from the rest (Fig. 2I). The anterior-most row of R axon bundles makes contact with Fmi-negative R axon bundles (Fig. 2J,J′,K,K′). By contrast, there were only a few cases in which neighboring Fmi-negative R axon bundles make contact with each other in the posterior lamina (Fig. 2J,J′). This is consistent with previously reported electron microscopy (EM) work on early pupal brains (Meinertzhagen and Hanson, 1993). The EM observation also revealed that the two youngest developing R axon bundles associate with each other at the interface between the pre-assembling and the assembling domain in early pupal brains. Furthermore, we observed that R axons from the two youngest developing R-cell clusters project closely to each other to the lamina (Fig. 2K). These observations support the idea that newly projecting R axons innervate the lamina along the existing R axon bundles, and then the neighboring two R axon bundles are separated by lamina neurons.

dac and *egfr* are not required for lamina column formation

The *dac* gene, which encodes a transcription factor (Mardon et al., 1994), is induced by Hh (Huang and Kunes, 1996) and may be required for column formation. To examine this possibility, *dac* null

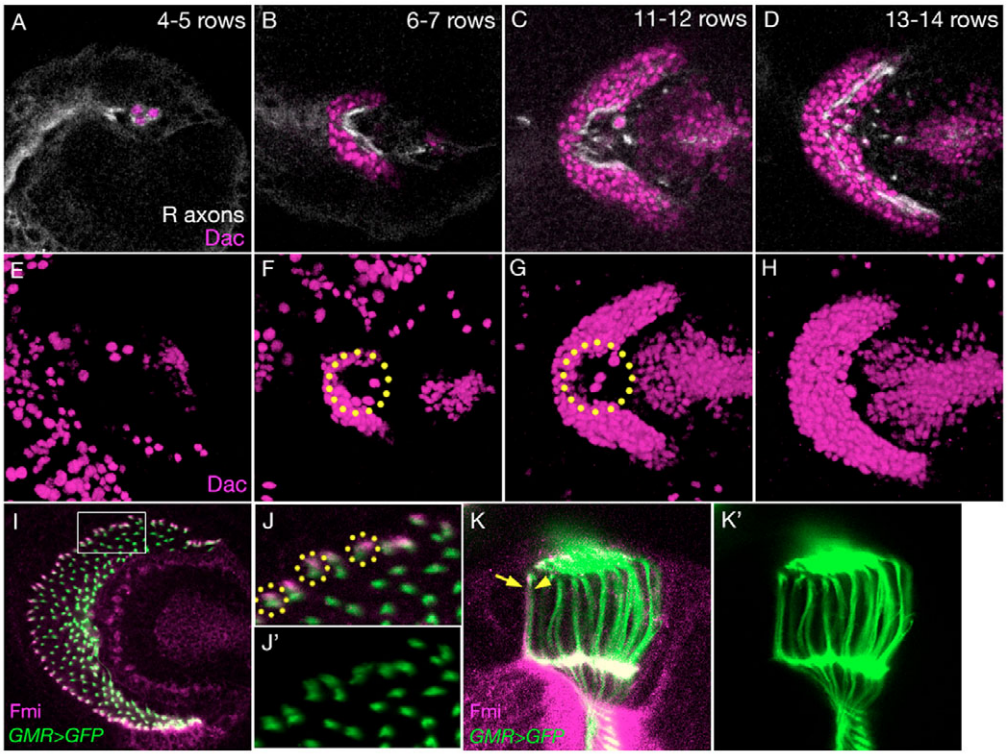

Fig. 2. Development of lamina neurons and R axon projection. (**A-H**) Positional relationship between R axons and lamina neurons in early to mid third instar larval brains. Single optical sections (A-D) and 3D reconstructions (E-H) of distinct developmental timing through the third instar larval stage. Lamina neurons visualized by Dac (magenta) and R axons visualized by anti-HRP (white) are shown. In early stages of four to five (A,E), six to seven (B, F) and 11 to 12 (C,G) rows of ommatidial differentiation, lamina neurons are not present in the assembling domain (encircled by yellow dots in F,G). Lamina neurons are detected in the assembling domain when 13 to 14 rows of ommatidia have differentiated (D,H), and at all later stages. (**I-K′**) Newly arriving axons make contact with existing axons. R axons are visualized by *GMR-GAL4 UAS-GFP* (green), whereas only anterior-most (most recently arriving) R axon bundles are detected by anti-Flamingo antibody (magenta). Images are from a lateral perspective (I,J,J′) and a horizontal perspective (K,K′). A magnified image of the dorsal area (white-lined box) in I is shown in J with Fmi signal, and in J′ without Fmi signal. Fmi-positive R axon bundles (arrow in K) make contact with Fmi-negative bundles (arrowhead in K) all along the entire lamina layer.

mutant (dac^3) clones were induced in the lamina. In contrast with the results of *smo* mutant clones, *dac* mutant clones were recovered in the assembling domain (100%, *n*=7; Fig. 3A-C), indicating that *dac* is not required for column formation. Another candidate could be *egfr*, which is also induced by Hh and is required for neuronal differentiation of lamina neurons (Huang et al., 1998). *egfr* null mutant ($egfr^{CO}$) clones were also recovered in the assembling domain (100%, *n*=7; Fig. 3D-F, see also Fig. S1 in the supplementary material). Recently, it was reported that *dac* regulates the expression of Egfr and a neuronal differentiation marker, Elav (Chotard et al., 2005). Taken together, the results with *dac* and *egfr* strongly suggest that Hh regulation of column formation acts through target(s) other than the pathway involving *dac* and *egfr*.

Single-minded is expressed in lamina under the control of Hh

In an effort to identify factors that do regulate column formation under the control of Hh, we carried out enhancer trap screening. We found that the *lacZ* reporter of the enhancer trap line for *single-minded* (*sim*) gene is expressed specifically in lamina neurons (data not shown). Moreover, anti-Sim antibody staining shows expression of Sim in developing lamina neurons and only modest expression in the eye disc (Fig. 4A,B,D) (Pielage et al., 2002). The *sim* gene encodes a bHLH-PAS-type transcription factor (Crews et al., 1988). It has been shown that *sim* acts as a master regulator of CNS midline cell development in *Drosophila* embryos (Nambu et al., 1991). Sim forms a heterodimer with another bHLH-PAS protein, dARNT (also known as Tgo), that translocates into the nucleus and functions as a transcription regulator (Sonnenfeld et al., 1997). It is worth pointing out that nuclear localization is more obvious in the posterior lamina (Fig. 4C,C′). The correlation between nuclear localization of Sim and maturation of the lamina neurons implies that Sim gradually becomes functional as lamina neurons mature. To investigate whether *sim* expression in lamina neurons is regulated by the Hh signal, we examined Sim expression in *smo* mutant clones. Sim

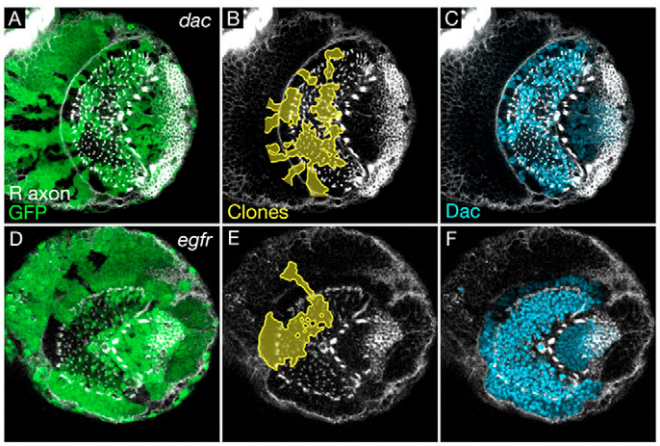

Fig. 3. Incorporation of lamina neurons into the assembling domain is independent of *dac* or *egfr*. (**A-F**) *dac* (A-C) and *egfr* (D-F) mutant clones are normally recovered in the assembling domain. Clones are visualized by the absence of GFP (A,D; green), R axons by anti-HRP (A-F; white) and lamina neurons by anti-Dac (C,F; cyan). Mutant clones in lamina are shown (B,E; yellow).

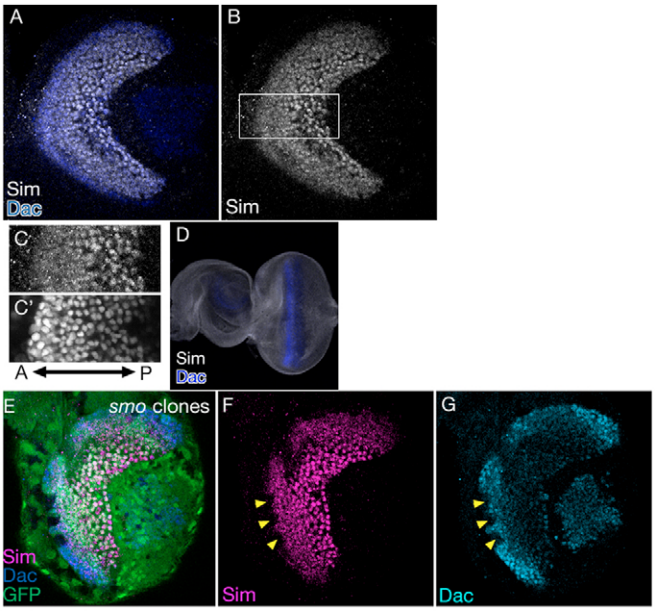

Fig. 4. Single-minded is specifically expressed in lamina under the control of Hh signaling. (**A**,**B**) Single-minded (Sim) is expressed specifically in lamina neurons. Sim (white) and Dac (blue) protein are visualized by immunofluorescence microscopy. (**C**) A magnified image of the boxed area of B. Nuclei of the lamina neurons were visualized by *NP6099-GAL4 UAS-nlsGFP* in C′. Note that nuclear localization of Sim is obscure in the anterior (left side) and obvious in the posterior (right side) region. (**D**) A marginal level of Sim expression (white) is detected in the eye disc. Dac (blue) is expressed at the morphogenetic furrow. (**E-G**) Sim expression is cell-autonomously abolished in *smo* mutant clones. *smo* mutant clones (arrowheads) are visualized by the absence of GFP (green); Sim (magenta) and Dac (blue in E; cyan in G) were visualized by immunofluorescence microscopy.

was abolished in *smo* mutant clones in a cell-autonomous manner (Fig. 4E-G), indicating that Sim expression in lamina neurons is regulated by the Hh signal.

sim mutant neurons fail to make lamina columns

If Sim is a target of Hh required for column formation, *sim* mutant clones should not be recovered in the assembling domain, similar to what we found for *smo* clones. Indeed, *sim* clones were rarely recovered there (the average number of recovered clones in assembling domain per brain=1.6, *n*=18; Fig. 5A-C, compare with Fig. 1D,F). We noted that in the pre-assembling domain, *sim* clones were larger than *smo* clones and expressed Dac (compare Fig. 5A with Fig. 1G, arrowheads), suggesting that neither proliferation nor the first step of differentiation of lamina neurons was impaired by the loss of *sim* activity.

We next made use of a heteroallelic combination of *sim* mutations to further investigate Sim function. In sim^{2}/sim^{ry75} mutant larval brains, more developing lamina neurons stayed in the pre-assembling domain than normal (100%, *n*=25; Fig. 5G,LM compare with Fig. 5D,J,K, respectively), and R axons that projected to the smaller assembling domain contained much fewer than the normal number of lamina neurons (Fig. 5D-I). *sim* mutant brains were comparable to control brains with regard to the number of differentiated ommatidia and the total number of Dac-positive lamina neurons (Fig. 5G compare with 5D). We also examined the pattern of cell divisions in the pre-assembling domain by using an anti-E2F antibody as a marker (Bosco et al., 2001). In control brains, strong E2F signals were observed at the lamina furrow. The E2F expression pattern at the lamina furrow in *sim* mutant brains was indistinguishable from that of the control brain, although the expression in the IPC domain (posterior to the assembling domain) is greatly reduced in *sim* mutant brain (see Fig. S2 in the supplementary material). This supports the idea that the expanded cell population in the pre-assembling domain does not result from the overproliferation of lamina precursor cells but rather from the accumulation of postmitotic cells. These findings suggest a requirement for *sim* in the interaction of lamina neurons with R axons. Furthermore, only cells in the assembling domain expressed Elav, which is known to be induced by Spitz provided by R axons (see Fig. S1 in the supplementary material) (Huang et al., 1998).

Ectopic expression of a dominant-negative form of dARNT, the partner of Sim, phenocopies *sim* loss of function

In order to rigorously examine Sim function in lamina neurons, we attempted to perturb Sim function specifically in lamina neurons, without affecting the development of R cells. Sim has been shown to function as a heterodimer with dARNT (Sonnenfeld et al., 1997; Ward et al., 1998), and, thus, a dominant-negative form of dARNT ($dARNT^{DN}$) (Ohshiro and Saigo, 1997) was expressed in lamina neurons with a developing lamina neuron-specific *GAL4* driver, *NP6099,* in order to disrupt Sim function (Fig. 6A-C). The resulting phenotype was more extreme than that of *sim* mutants: all of the R axon bundles were packed tightly in the posterior domain of the lamina region and almost all of the developing lamina neurons were located outside of the R axon bundles (100%, *n*=20; Fig. 6D-F; see also Fig. 8A). To determine whether the effects of expression of $dARNT^{DN}$ are caused by an inhibition of Sim function, wild-type *sim* was co-expressed with $dARNT^{DN}$. In brains expressing both $dARNT^{DN}$ and *sim* in lamina neurons, both the arrangement of lamina neurons and the spacing of R axon bundles were almost indistinguishable from those of control brains (71%, *n*=10; Fig. 6G-I). Suppression of the effects of $dARNT^{DN}$ by *sim* overexpression indicates that $dARNT^{DN}$ adversely affects lamina neurons by perturbing endogenous Sim function. Therefore, lamina neuron specific perturbation of endogenous Sim function showed a defect similar to that seen in *sim* mutant brains. The result reveals an autonomous requirement for Sim function in lamina neurons for their assembly with R axons. Additionally, the data show that the spacing of R axon bundles is dependent upon a contribution from the lamina neurons.

Sim overexpression causes precocious lamina column formation

We next examined the effect of *sim* overexpression. When *sim* was overexpressed in lamina neurons using the *NP6099-GAL4* driver, the number of Dac-expressing lamina neurons in the pre-assembling domain was reduced (Fig. 7A-C). This can be interpreted as resulting from a premature incorporation of lamina neurons between the R axon bundles (assembling domain). We confirmed this idea by using an enhancer trap line carrying the *lacZ* gene inserted into the *thickveins* (*tkv*) locus. The *tkv* gene encodes a type I receptor for *decapentaplegic*, a member of the Tgfβ family (Brummel et al., 1994; Nellen et al., 1994; Penton et al., 1994), and is useful for visualization of the lamina precursor cells at the lamina furrow. In the control brain, *tkv-lacZ*-positive lamina neurons posterior to the lamina furrow migrate apically along the furrow. *tkv-lacZ* expression is gradually lost as cells move posteriorly (Fig. 7G, yellow broken

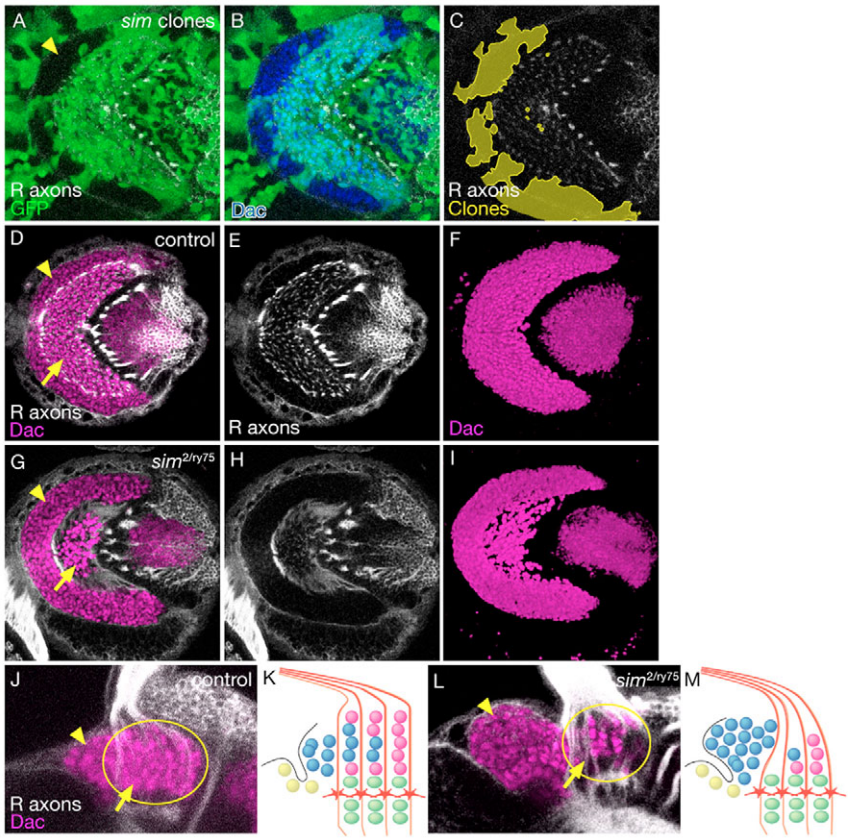

Fig. 5. *sim* mutant lamina neurons are not incorporated into the assembling domain. (**A-C**) *sim*^{2} clones are not recovered in the assembling domain. Clones are visualized by the absence of GFP (green in A,B), R axons by anti-HRP (white) and lamina neurons by anti-Dac (blue in the merged image B). Mutant clones in lamina are shown in C (yellow). (**D-M**) Fewer lamina neurons are incorporated into the assembling domain in the mutant. Wild-type (D-F) and *sim*^{2}/*sim*^{ry75} mutant (G-I) lamina neurons are marked by Dac (magenta in D,F,G,I) and R axons are visualized by anti-HRP (white in D,E,G,H). 3D reconstructions of Dac expression in D and G are shown in F and I, respectively. In contrast to wild type (D-F), in *sim*^{2}/*sim*^{ry75} mutant brains, a smaller number of lamina neurons are present in the assembling domain (compare G with D, arrows), leaving more lamina neurons behind in the pre-assembling domain (compare G with D, arrowheads). In *sim*^{2}/*sim*^{ry75} mutant brains, the assembling domain is smaller (H) than in wild type (E). (J-M) Wild-type (J) and *sim*^{2}/*sim*^{ry75} mutant (L) lamina viewed from a horizontal perspective. Dac expression (magenta) and R axons (white) are visualized with the respective antibodies. Assembling domains are encircled by a yellow line. The number of lamina neurons in the assembling domain is reduced (arrows in J,L) and the number of lamina neurons in the pre-assembling domain is increased (arrowheads in J,L) in *sim*^{2}/*sim*^{ry75}. (K,M) Schematic illustration of J and L, respectively. Colors of cells are as noted in Fig. 1C.

line) (Huang and Kunes, 1996; Selleck et al., 1992). Note that no lamina neurons express high levels of *tkv-lacZ* in the assembling domain in the control and the *sim*^{2}/*sim*^{ry75} mutant brains (Fig. 7D,F,G,I). However, in the brain where *sim* was overexpressed in lamina neurons, apical migration was not observed; instead, *tkv-lacZ*-positive cells were found in the assembling domain (Fig. 7E,H). Thus, we postulate that anterior lamina neurons overexpressing Sim precociously acquire the ability to interact with the R axon bundles. Together with the results with *dARNT*^{DN}, the data indicate that Sim regulates a set of genes required for the first step of the lamina column assembly, presumably for interaction with R axons.

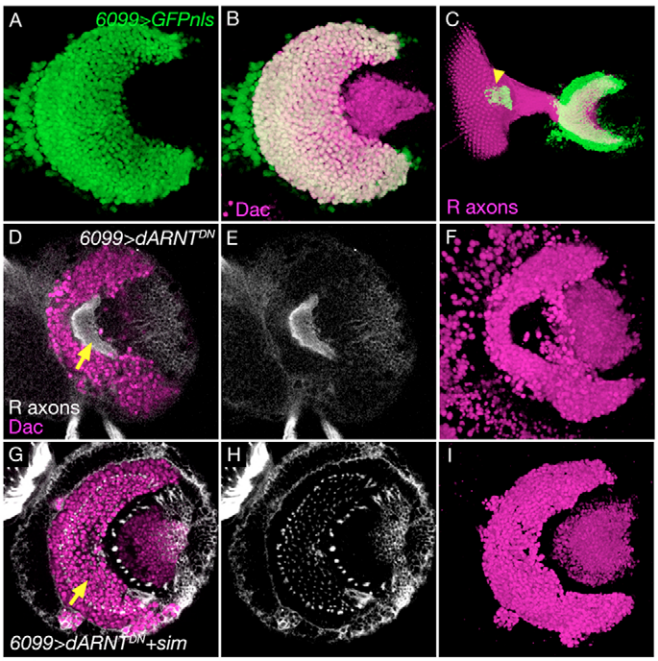

Fig. 6. Targeted expression of dominant-negative dARNT prevents lamina neurons from assembling lamina columns. (**A-C**) *NP6099-GAL4* expression in the optic lobe visualized using *UAS-nlsGFP* (green) and anti-Dac (magenta in merged image B). *NP6099-Gal4* is also expressed in a small part of the most posterior eye disc, including in about 10 ommatidia (arrowhead in C). R axons are visualized by 24B10 (magenta in C). (**D-F**) A dominant-negative form of dARNT inhibits the incorporation of lamina neurons into the assembling domain; Dac, magenta and R axons, white. 3D reconstruction of Dac expression in D is shown in F. Lamina neurons are almost never seen in the assembling domain (F, arrow in D). (**G-I**) Effect of a dominant-negative form of dARNT expression is suppressed by co-expression of *sim*; Dac, magenta and R axons, white. 3D reconstruction of Dac expression in G is shown in I. Lamina neurons are incorporated in the assembling domain (arrow in G) and lamina columns as in the wild type.

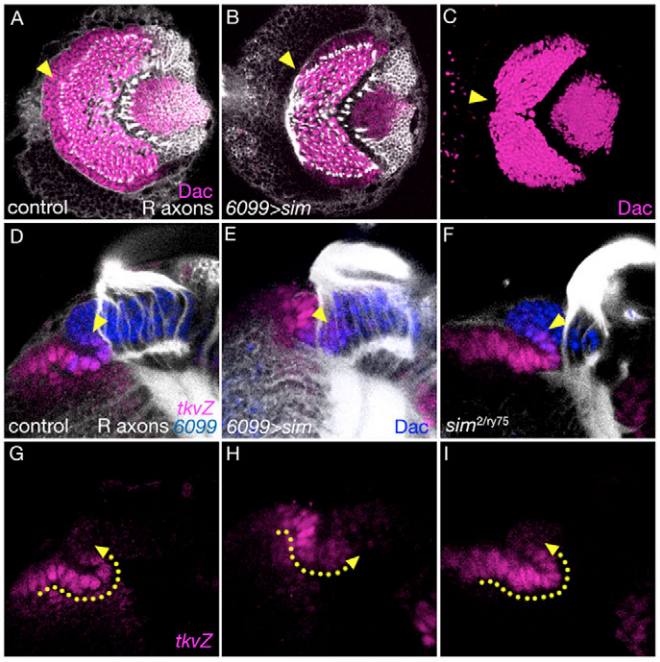

Fig. 7. Overexpression of *sim* causes the premature incorporation of lamina neurons into the assembling domain.
(**A-C**) Overexpression of *sim* in lamina neurons using *NP6099-GAL4* (B,C). Lamina neurons are marked by Dac (magenta) and R axons are visualized by anti-HRP (white in A,B). *NP6099-GAL4* driving *GFP* alone was used as a control (A). 3D reconstruction of Dac expression in B is shown in C. The number of lamina neurons in the pre-assembling domain is reduced (arrowheads in A-C). (**D-I**) Horizontal views of wild-type and mutant lamina at third larval instar. In contrast to wild type (D,G) and *sim* mutants (F,I), lamina neurons overexpressing *sim* (E,H) do not migrate apically; instead, they enter the assembling domain directly (arrowheads in D-F; yellow broken lines in G-I). The lamina precursor cells at the lamina furrow are visualized with *tkv-lacZ* (magenta). Lamina neurons are marked by *NP6099-GAL4 UAS-nlsGFP* (blue in D) or Dac (blue in E,F), and R axons by anti-HRP (white). G-I are single channel of *tkv-lacZ* for D-F, respectively. The migratory route is shown by arrows.

DISCUSSION

Drosophila lamina formation provides a notable model for the study of how a precise topographic map is formed in the brain. A series of studies by Kunes and his colleagues has revealed an elaborate inductive mechanism in which the signaling molecules Hh and Spi from R cells are transmitted along the R axons and regulate the proliferation and development of lamina neurons (Huang and Kunes, 1996; Huang et al., 1998; Kunes, 2000; Salecker et al., 1998). This builds up lamina columns, each with a set of R axons from a single ommatidium and five lamina neurons, which serve as the basic units of precise synaptogenesis in later stages, forming a retinotopic map (Clandinin and Zipursky, 2000; Clandinin and Zipursky, 2002; Meinertzhagen and Hanson, 1993; Prakash et al., 2005). A more precise understanding of the mechanisms of lamina column formation is still elusive; for example, how the number of lamina neurons matches the number of ommatidia (the set of axon bundles) and how a set of R axons associates with a corresponding set of lamina neurons are not known.

sim is required for lamina column assembly

Here we show that Sim, a target of Hh, is required for at least the first step of lamina column formation; namely, the incorporation of developing lamina neurons into the area where R axons project and lamina columns mature, an area we refer to as the assembling domain. We base our model for Sim on four observations. First, sim^2/sim^{ry75} brains had a reduced number of lamina neurons in the assembling domain, leaving an abnormally large number of premature lamina neurons behind in the pre-assembling domain. Second, in clonal analysis, sim^2 clones fail to be recovered in the assembling domain (similar to smo^1 clones). Third, lamina neuron-specific inhibition of Sim function caused R axon bundles to be tightly packed and lamina neurons to be excluded from R axon bundles. And fourth, overexpression of *sim* in lamina neurons caused precocious incorporation of lamina neurons into the assembling domain.

In this last case, neither expansion of the assembling domain nor increase in the number of lamina neurons relative to the number of R axon bundles was observed, even though lamina neurons prematurely incorporated into the assembling domain. This is probably because a reduced number of lamina neurons were generated. In fact, we observed loss of E2F expression at the lamina furrow in *NP6099-GAL4 UAS-sim* brains (see Fig. S2 in the supplementary material). The onset of incorporating lamina neurons into the assembling domain might be linked to an inhibition of cell proliferation. However, we think this is unlikely for two reasons: (1) lamina neurons did not show any extra E2F signal in the *sim* mutant brain in spite of an increase in unincorporated lamina neurons (see Fig. S2 in the supplementary material); and (2) lamina neurons ectopically expressing a cell cycle-braking factor, the *Drosophila* p21/p27 homolog *dacapo* (*dap*) (Lane et al., 1996), did not cause the precocious incorporation of lamina neurons (data not shown). Thus, a direct link between cell cycle regulation and the incorporation of lamina neurons is less plausible.

We propose an alternative model, the ‘time lag’ model. There appears to be a lag between the onset of *sim* expression and the onset of incorporation of lamina neurons. Differentiating lamina neurons are held temporarily in the pre-assembling domain and then the proper amount of lamina neurons are coordinately integrated into columns as more R axons are projected (Fig. 8B). Thus, we speculate that a certain degree of accumulation of the Sim/dARNT heterodimer in nuclei is needed to exert cellular function. Consistent with this idea, graded accumulation of Sim is observed, with lower Sim levels in anterior (younger) lamina neuron nuclei and higher levels in posterior (older) lamina neuron nuclei (Fig. 4C). Overexpression of Sim in lamina neurons would thus cause higher levels of accumulation of the protein in young lamina neurons and facilitate their incorporation into the assembling domain. Interestingly, overexpression of the wild-type dARNT did not have any detectable effects, suggesting that Sim accumulation is a limiting factor for cell incorporation (data not shown).

The mechanism of neuronal maturation and that of assembly of lamina neurons are independent, although both are under the control of Hh signaling (Fig. 8C). Disruption of *sim* did not affect the differentiation and proliferation of lamina neurons (see Results). Correspondingly, neither the incorporation of lamina neurons into the lamina column nor the expression of *sim* were affected by *dac* mutation (Fig. 3A-C, see also Fig. S3 in the supplementary material). We still do not understand the cellular function required for assembling the column or the function of Sim at the cellular level. Electron microscopic observations by Meinertzhagen and Hanson revealed an intriguing behavior of lamina neurons at the early pupal stage; large processes extending from lamina neurons engulf R1 and R6 axons of newly incoming R axon bundles (Meinertzhagen and Hanson, 1993). This may be the key step in lamina column formation and interaction between the R axons and

lamina neurons. Sim may regulate genes required for process formation, interaction with R axons and/or events that follow shortly after, as lamina neurons seem to fail to make interactions with R axons from the beginning in the *sim* mutant background. Sim is expressed in the midline cells of the CNS throughout neurogenesis in the *Drosophila* embryo and is required for the proper differentiation of the midline cells into mature neurons and glial cells (Nambu et al., 1991). Midline precursor cells undergo synchronized cell division and then transform into the bottle-shaped cells, in which the nuclei migrate internally and leave a cytoplasmic projection joined to the surface of the embryo. The *sim* mutant midline cells fail to delaminate from the epidermal cell layer. Cells do not make the normal bottle-like shape and, instead, they appear rounded (Nambu et al., 1991). In addition, overexpression of *sim* can induce other cell types to exhibit midline morphology (Nambu et al., 1991). *sim* may thus regulate the transcription of a set of genes required for morphological changes, which in turn are required for interaction between cells, both in the lamina and during embryonic CNS development.

Although we have shown that *sim* expression is regulated by Hh signaling, this does not answer the question of whether *sim* function is sufficient to confer on cells the ability to be incorporated into the assembling domain. We examined whether *smo* mutant clones can be recovered in the assembling domain by forcing *sim* expression in *smo* clones using the MARCM technique. However, *smo* mutant clones expressing *sim* were not recovered in the assembling domain (data not shown). This suggests that additional factors are involved in lamina neuron assembly. Hh may also contribute to specification of the difference in affinity between lamina neurons and R axons and/or between anterior and posterior lamina neurons. In *Drosophila* wing discs, the Hh signal differentiates the affinity of anterior compartment cells from that of the posterior compartment cells, thereby maintaining the compartment border (Blair and Ralston, 1997; Rodriguez and Basler, 1997; Wang and Holmgren, 2000).

Roles of postsynaptic cells for topographic mapping

We propose an active role for postsynaptic cells in making a topographic map of the *Drosophila* visual system. Targeted expression of the dominant-negative form of the Sim partner in the lamina neurons clearly showed a role for postsynaptic cells in assembling lamina columns. This presumably affects an early step of assembly, as mentioned above. We do not know if Sim function is also required for later steps in more mature lamina neurons. The forced expression of the dominant-negative Sim partner in the posterior lamina neurons had no effect, although it may simply be that the level of expression of the dominant-negative form of dARNT was not sufficient to have an observable effect on Sim function (data not shown). In the lamina column, the R axon bundle associates with a precisely arranged row of five lamina neurons. No mechanisms for the development and formation of this stereotypic structure have been revealed. Another signal might be provided from the R axons with lamina neurons, and/or intrinsic structures of the R axons might play a role in this architecture. Chiba and colleagues observed an intriguing property of postsynaptic muscle cells for axonal targeting: the muscle cells bear numerous postsynaptic filopodia (‘myopodia’) during motoneuron targeting (Ritzenthaler et al., 2000). They showed that postsynaptic cells actively contribute to synaptic matchmaking by direct, long-distance communication. Together with what has been learned about myopodia in neuromuscular synapse formation, our findings reveal an active role for postsynaptic cells for the establishment of precise neural networking.

A possible conserved role for the Sim transcription factor in insects and mammals

Sim belongs to the family of bHLH-PAS transcription factors, whose members function in many developmental and physiological processes, including neurogenesis (Michaud et al., 1998; Nambu et

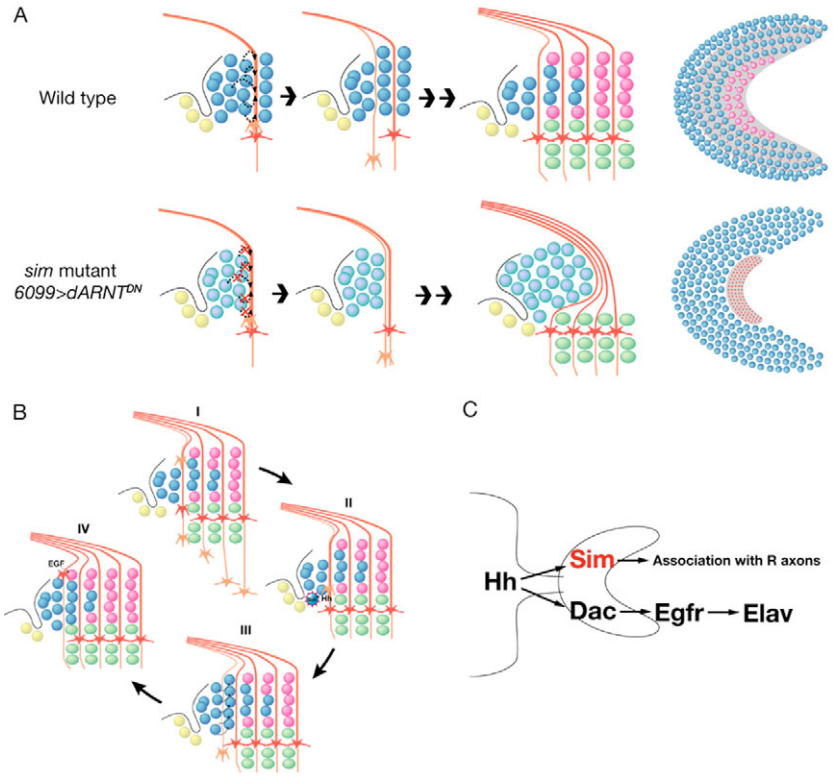

Fig. 8. A model for the onset of lamina column formation, which depends on Single-minded induced by Hh delivered from R axons. (**A**) Schematic illustrations of the *sim* mutant phenotype. Wild-type (upper) and *sim* mutant (lower) lamina. *sim* mutant neurons (pale blue circles) fail to be incorporated into the assembling domain and accumulate in the pre-assembling domain. Lateral perspective of the wild-type and *sim* mutant lamina are also illustrated (right). (**B**) A model for the incorporation of the lamina neurons into the process of lamina column formation. Newly arriving axons project to the lamina layer along existing R axons (I). G1-phase lamina precursor cells receive Hh from R axons, which triggers Dac and Sim expression and differentiation to lamina neurons (II). Sim enables lamina neurons to associate with one of the paired R axon bundles (III). The R axon bundle is enveloped by lamina neurons and is incorporated into the lamina column. Lamina neurons become mature by receiving Spitz, an Egf ligand, which is also provided by R axons at a later stage. (**C**) Hh delivered from R axons induces *dac*, *egfr* and *sim* independently.

al., 1991), tissue development (Wilk et al., 1996), toxin metabolism (Schmidt and Bradfield, 1996), circadian rhythms (Panda et al., 2002), response to hypoxia (Lavista-Llanos et al., 2002; Maltepe et al., 1997), and hormone receptor function (Ashok et al., 1998). bHLH-PAS proteins usually function as dimeric DNA-binding protein complexes. The most common functional unit is a heterodimer. These heterodimers consist of one partner that is broadly expressed, and another whose expression is regulated spatially, temporally or by the presence of inducers. Sim and the bHLH-PAS protein dARNT heterodimerize to bind to their responsive element, the CME (CNS midline enhancer element), to activate target gene transcription (Sonnenfeld et al., 1997). In this complex, dARNT is the general dimerization partner and Sim is the tissue-specific partner.

The *Drosophila* Sim and mammalian Sim1 and Sim2 proteins are highly conserved in their amino-terminal halves, which contain a bHLH and a PAS domain (Ema et al., 1996a; Fan et al., 1996). Murine Sim1 and Sim2 are also expressed in both proliferative and postmitotic zones of the central nervous system at different stages of neural development. These zones of expression include the longitudinal basal plate of the diencephalon (Sim1 and Sim2), the mesencephalon (Sim1), the zona limitans intrathalamica (Sim1 and Sim2) and the portion of the spinal cord that flanks the floor plate (Sim1) (Ema et al., 1996a; Ema et al., 1996b; Fan et al., 1996). Sim2 maps to the region responsible for Down Syndrome (DS) on Chromosome 21 (Chrast et al., 1997; Dahmane et al., 1995; Muenke et al., 1995). Interestingly, Sim2 is also expressed in non-neuronal tissues, including branchial arches and the developing limb, which are primordia of tissues and organs where DS abnormalities are frequently observed (Rachidi et al., 2005).

Given the important roles of *sim* in *Drosophila* development and the expression of Sim2 in cell types that are affected in DS individuals, it was proposed that Sim2 may play a causative role in DS. However, because of a lack of direct evidence and the existence of other candidate genes (Antonarakis et al., 2004), this remains speculative. Cells expressing *sim* during *Drosophila* development and Sim2-positive cells affected in DS seem to be able to migrate (Rachidi et al., 2005). The conserved role of Sim may enable cells to migrate and/or interact with surrounding cells in the various tissues, including the central nervous system. It will thus be intriguing to search for extra cellular targets of Sim regulation with the hope of elucidating mechanisms that underlie the behavior of Sim-expressing cells.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/5/791/DC1

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