Homeodomain protein codes specify neuronal diversity in the first ganglion of the *Drosophila* visual system

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Abstract

Recent advances in single-cell transcriptional profiling have enabled us to map diverse neuron types across *Drosophila*, mice, and even human brains, yet we still lack a mechanistic understanding of how these neuron types arise in the brain and expand during evolution. We identified homeodomain transcription factor (HDTF) codes in newborn neurons that specify the five neuron types (L1-L5) in the *Drosophila* lamina, the first ganglion of the visual system. Specifically, we discovered that the pan-lamina HDTF Scro is required for lamina progenitors to exit proliferation and differentiate into lamina neurons. Furthermore, we showed that specific HDTFs are expressed in distinct newborn neurons and specify different lamina neuron types: Zfh1 for L1 and L3, Dve for L2, and Bsh for L4 and L5. Intriguingly, we found that the loss of both Dve and Bsh causes L2, L4, and L5 neurons to revert to L1 and L3 neuron types, potentially representing ancestral lamina neuron types. HDTFs are evolutionarily conserved and broadly expressed in brains across species. Based on these findings, we propose a model in which distinct HDTFs in newborn neurons specify different neuron types in the brain and have been utilized to expand neuron diversity during evolution.

Introduction

Brain function relies on a vast diversity of neuron types, each distinguished by unique gene expression, morphology, and connectivity[1]. While recent advances in single-cell transcriptional profiling have allowed us to map neuron types across species such as *Drosophila melanogaster*, mice, and humans[2–6], we still lack a mechanistic understanding of how these neuron types are specified in the brain and how they expand during evolution. Neuronal fate decisions are made in newborn neurons, and identifying differentially expressed factors in these cells may hold the key to unlocking the codes for neuron type specification. Homeodomain transcription factors (HDTFs) are evolutionarily conserved and broadly expressed in the brains of various species[7–10]. Work in *C. elegans* has shown that each adult neuron type expresses a unique combination of HDTFs [9]. Similarly, in mice, analysis of the transcriptomes from 179 genetically identified neuronal populations revealed that HDTFs exhibit the most robust expression differences across neuron types[10]. These findings position HDTFs as strong candidates for the differentially expressed factors in newborn neurons that specify diverse neuron types.

The *Drosophila* lamina, the first optic ganglion with only five neuron types (L1-L5), serves as an ideal model for studying neuron type specification due to its anatomical simplicity and the availability of genetic tools to manipulate genes in each neuron type (Figure 1 A). Lamina neurogenesis occurs during late larval and early pupal stages, when lamina precursor cells (LPCs) receive Hedgehog signals from photoreceptor axons and undergo terminal divisions[11,12]. After mitosis, LPCs organize into columns along photoreceptor axons and differentiate into lamina neurons. The cell bodies of each lamina neuron type are localized in a layer-specific manner: L2 and L3 cell bodies are intermingled in the most distal layer, while L1, L4, and L5 form distinct layers progressively more proximal[13]. Each lamina neuron type expresses unique TF markers: L1, L2, and L3 neurons express Svp, Bab2, and Erm, respectively, while L4 and L5 neurons express the HDTFs Bsh/Ap and Bsh/Pdm3, respectively [13]. We recently discovered that the HDTF Zfh1 is specifically expressed in, and required for, the generation of L1 and L3 neurons[14]. Furthermore, we showed that the HDTF Bsh specifies L4 and L5 neurons and represses Zfh1 to suppress L3 and L1 fates [14]. Taken together, these findings lead to the hypothesis that distinct HDTFs in newborn neurons may form the codes for generating specific neuron types.

Results

Lamina neurons express a pan-lamina HDTF and neuron type-specific HDTFs.

The first ganglion of the *Drosophila* visual system contains five neuron types (L1-L5). We previously identified the HDTF Zfh1 as necessary for generating L1 and L3 neurons and the HDTF Bsh for specifying L4 and L5 neurons[14]. However, the factor responsible for specifying L2 neurons remained unknown, prompting us to search for additional HDTFs expressed in the *Drosophila* lamina. To investigate this, we performed single-cell RNA sequencing (scRNA-seq) to profile gene transcription across the five adult lamina neuron types (Figure 1 B). From this analysis, we identified

candidate pan-lamina HDTFs expressed in all lamina neurons, as well as neuron type-specific HDTFs expressed in distinct subsets of lamina neurons (Figure 1 C).

Neuronal fate decisions are made in newborn neurons. To confirm whether these HDTFs identified from scRNA-seq data are expressed in newborn lamina neurons, we took two approaches: assessing their protein localization using available antibodies or mRNA expression using available mRNA probes. Using Dac immunostaining to label LPCs and lamina neurons, we found that the pan-lamina HDTF Scarecrow (Scro) is initiated in LPCs and maintained in all lamina neurons, with higher expression levels in the neurons (Figure 1 D-E). Moreover, we discovered that Scro is initiated in LPCs after their terminal mitosis, as Scro+ LPCs lacked phospho-Histone H3 (PH3) staining, which labels cells undergoing mitosis (Figure 1 D-E). In contrast, the HDTFs Zfh2, Exd, Onecut, and Nk7.1 were not expressed in LPCs or lamina neurons during lamina neurogenesis (Supp-Fig. 1), and will not be discussed further.

Next, we analyzed published scRNA-seq data to identify additional HDTFs expressed in lamina neurons. Among them, we found defective proventriculus (Dve) to be expressed in L5 neurons at 40h after pupa formation (APF), based on one RNA-seq dataset[13]. Upon confirming its expression during lamina neurogenesis, we unexpectedly discovered that Dve is expressed exclusively in newborn L2 neurons, but not in other lamina neuron types (Figure 1 F-G). Previously, we identified Zfh1 as a determinant for L1 and L3 neurons, and Bsh for L4 and L5 neurons. Along with Dve, we now identified distinct HDTFs for all newborn lamina neuron types: Zfh1 in L1 and L3, Dve in L2, and Bsh in L4 and L5 (Figure 1 F-G). We also found that newborn lamina neurons are organized in a fixed, layer-specific manner, with Bsh+ newborn L4 and L5 neurons located in the proximal layers, Dve+ newborn L2 in the distal layer, and Zfh1+ L1 and L3 occupying the intermediate layers (Figure 1 F-G). Notably, immature L2 and L3 cell bodies later intermingle with each other (Figure 1 F). Taken together, we uncovered a pan-LN HDTF, Scro, that is initiated in LPCs and maintained in all lamina neurons, as well as neuron type-specific HDTFs that are expressed in specific newborn neurons in the *Drosophila* lamina (Figure 1 H).

Scro is required for lamina precursor cells to exit proliferation and differentiate into lamina neurons.

Our finding that Scro is initiated in LPCs after their terminal mitosis suggests that Scro may repress LPC proliferation and promote lamina neuron differentiation. To test this hypothesis, we performed conditional Scro-knockdown (Scro-KD) in LPCs and lamina neurons using 27G05-Gal4, Gal80^{ts}, and UAS-Scro-RNAi. We inactivated Gal80^{ts}, which normally suppresses 27G05-Gal4 activity, starting at the third instar stage to knock down Scro during lamina neurogenesis.

First, we evaluated the role of Scro in LPC proliferation. Homothorax (Hth) is found to be expressed in progenitors and is required to promote progenitor proliferation in the *Drosophila* optic lobe[15,16]. Here, in controls, we indeed observed that both Hth and mitotic marker PH3 were present in proximal LPCs outside the columns labeled by Hrp, while Scro was detected in distal LPCs (Figure 2A, 2A', 2C, and 2E). In Scro-KD, Scro expression was largely absent in LPCs and lamina

neurons, as expected (Figure 2A-B'). In contrast to controls, we observed a dramatic increase in Tailless+ (Tll+) LPCs[14] outside the columns. Notably, these expanded LPCs did not express ectopic Hth, although they could be labeled with PH3 (Figure 2A-H). The size of the lamina columns labeled by Hrp decreased due to the expanded LPCs and the reduced number of lamina neurons in Scro-KD. Thus, we conclude that Scro is required to repress proliferation, but not by suppressing Hth expression.

Next, we evaluated the role of Scro in LPC differentiation. We found that in Scro-KD, elav+ lamina neurons were significantly reduced, indicating that LPCs lacking Scro failed to differentiate into lamina neurons (Figure 2A-B', 2I). These LPCs continued to fail to differentiate into lamina neurons throughout development, as lamina neurons remained largely reduced in adult flies (Figure 2J-L). This finding is consistent with observations in hypomorphic Scro mutants, which also show a lack of lamina neurons in adulthood[17]. Furthermore, we found that some of the surviving lamina neurons in Scro-KD express Zfh1, Erm, Bsh, and Pdm3, but show almost no expression of Svp or Ap (Supp-Fig. 2). Notably, Zfh1 and Erm are rarely coexpressed in the same cell, and similarly, Bsh and Pdm3 are rarely coexpressed (Supp-Fig. 2). This suggests that these neurons failed to properly express markers for specific lamina neuron types. Together, our findings reveal a novel role of Scro in LPCs, where it represses proliferation and promotes neuron differentiation (Figure 2M).

Dve specifies the L2 neuron type and represses the L3 fate by inhibiting Zfh1

Neuron type specification is determined in newborn neurons. We identified Zfh1, Dve, and Bsh as distinct HDTFs expressed in specific newborn lamina neurons, suggesting they may act as transcriptional codes for lamina neuron type specification. Supporting this model, our previous work revealed that Zfh1 specifies L1 and L3 neurons, while Bsh specifies L4 and L5 neurons[14]. To further test this model, we investigated the role of Dve in specifying L2 neurons.

In controls, Bsh+ newborn L4 and L5 neurons were positioned in the proximal layers, Dve+ newborn L2 in the distal layer, and Zfh1+ L1 and L3 in the intermediate layers (Figure 3A, 3C). Knocking down Dve resulted in a loss of Dve expression in the lamina, as expected (Figure 3C, 3D). In contrast to controls, Zfh1 was ectopically expressed in newborn L2 neurons within the distal layer, suggesting that these L2 neurons had been re-specified as either L1 or L3 neurons, both of which are normally specified by Zfh1 (Figure 3A-B', 3G, and 3H). To determine whether L2 neurons were switched to L1 or L3 in Dve-KD, we examined available neuron markers and found that the L3 marker Erm, but not the L1 marker Svp, was ectopically expressed in the distal L2 layer. This suggests that L2 neurons were re-specified as L3 in the absence of Dve (Figure 3C-F, 3I). Furthermore, we observed an increase in neurons expressing the L3 markers Zfh1 and Erm, accompanied by a loss of neurons expressing the L2 neuron TF marker Bab2 at 4 days APF, confirming that the switch from L2 to L3 neurons persisted through the completion of development (Figure 3J-O). Notably, Dve is transiently expressed in L2 neurons only until 2 days APF (Supp-Fig. 2). Together, we conclude that Dve specifies the L2 neuron type and represses the L3 fate by inhibiting Zfh1, thereby generating lamina neuronal diversity (Figure 3P).

Loss of both Dve and Bsh transforms all lamina neurons into L1 and L3 neuron types

We previously showed that the loss of Bsh caused L4 and L5 neurons to be re-specified as L3 and L1, respectively, reducing the neuronal diversity in the *Drosophila* lamina to only three types: L1, L2, and L3. This prompted us to investigate the effects of simultaneously knocking down both Bsh and Dve. To explore this, we performed Bsh/Dve double knockdown (Bsh/Dve-DKD) specifically in the *Drosophila* lamina during lamina neurogenesis. In controls, we observed that the HDTFs Zfh1, Dve, and Bsh are expressed in distinct newborn lamina neurons, with Dve in neurons of the distal layer, Bsh in those of the proximal two layers, and Zfh1 in neurons of the intermediate layers (Figure 4A, 4A'). In contrast, we found that Zfh1 was expressed in all newborn neurons in Bsh/Dve-DKD (Figure 4A-B', 4G, 4H). Furthermore, Zfh1 expression in all newborn neurons indeed led to the specification of only L1 and L3 neuron types in the *Drosophila* lamina, as L1 marker Svp and L3 marker Erm were initiated in all lamina neuron layers (Figure 4C-F). This is consistent with our findings that L2 neurons were re-specified as L3 in Dve-KD and that L4 and L5 neurons were re-specified as L3 and L1, respectively, in Bsh-KD. Furthermore, we found that the lamina of Bsh/Dve-DKD contained only L1 (~40%) and L3 (~60%) neuron types at 4 days APF, confirming that the switch from L2, L4, and L5 neurons to L1 and L3 neurons persisted through the completion of development (Figure 4I-N). Taken together, we conclude that Zfh1, Dve, and Bsh act as HDTF codes in newborn neurons that specify distinct lamina neuron types (Figure 4O). Our findings also raised the hypothesis that the ancient lamina may have contained only L1 and L3 neuron types, which respond to light increments and decrements, respectively. The addition of Dve and Bsh may have led to the generation of three new neuron types: L2, L4, and L5 (see Discussion).

Discussion

Deciphering the transcriptional codes for neuron type specification is crucial not only for understanding how diverse neuron types are generated in the brain, but also for providing insights into regenerating specific neuron types to restore brain function after neuron loss. However, we still lack a model to identify these transcriptional codes. Here, we show distinct HDTFs in newborn neurons act as the codes that specify the diverse neuron types in the *Drosophila* lamina, the first ganglion of the visual system. Specifically, we identified a pan-lamina HDTF, Scro, which is necessary for LPCs to differentiate into lamina neurons. Additionally, we discovered distinct HDTFs that are expressed in specific newborn neurons and are responsible for specifying distinct lamina neuron types: Zfh1 for L1 and L3, Bsh for L4 and L5, and Dve for L2. Moreover, we found crosstalk between these HDTF codes, with Dve and Bsh both inhibiting Zfh1 to suppress L1 and L3 fates (Figure 5A). HDTFs are evolutionarily conserved and widely expressed in brains across species. Notably, we found that mouse retinal bipolar cells (BCs) also express pan-BC HDTFs and neuron type-specific HDTFs (unpublished data). Based on these findings, we propose a model in which distinct HDTFs in newborn neurons act as transcriptional codes that specify distinct neuron types in

the brain (Figure 5B). As brain transcriptome data across species become increasingly available, our model will help identify the transcriptional codes within these data, revealing how diverse neuron types arise in the brain.

In *C. elegans, cut* homeobox genes are expressed in all neurons and regulate pan-neuronal gene expression[18]. In contrast, in *Drosophila* and mice, we did not identify pan-neuronal HDTFs. Instead, we found pan-lamina HDTF Scro in *Drosophila* and pan-BC HDTFs Vsx2 and Otx2 in mice. These HDTFs are expressed in progenitors, where they are required for the generation of lamina neurons and BCs[19], and are maintained in neurons through development into adulthood. It would be interesting to explore whether they control pan-lamina and pan-BC gene expression during development and maintenance. In *Drosophila,* Scro is also expressed in other brain regions, such as the central complex and medulla, where it is identified as a late temporal TF[20]. Notably, the loss of Scro expression increases progenitor number in these central brain regions[20], consistent with our finding that Scro is necessary for LPCs to exit proliferation and differentiate into neurons. Future work should investigate the downstream target genes of Scro and how this pathway integrates with extracellular cues, such as insulin-like peptides from wrapping glia, which are involved in lamina neuron differentiation[21].

We found that Dve specifies the L2 neuron type. In Dve-KD, Zfh1 is ectopically expressed and re-specifies L2 as the L3 neuron type, but not L1. Similarly, in Bsh-KD, Zfh1 is ectopically expressed and re-specifies L4 as the L3 neuron type and L5 as the L1 neuron type[14] (Figure 5A). What drives this one-to-one fate switch (L2>L3; L4>L3; L5>L1)? We hypothesize that L2, L3, and L4 are Notch-ON neurons. This hypothesis is based on our finding that Bsh specifies the L4 neuron type in the presence of Notch signaling in newborn neurons, while in its absence, Bsh specifies the L5 neuron type[22]. In Bsh-KD, ectopic Zfh1, together with Notch signaling, specifies L3, while in the absence of Notch signaling, it specifies L1[22] (Figure 5A). These results suggest that HDTF codes can be further modified by Notch signaling, enabling a single HDTF code to specify two distinct neuron types.

Recent advances in comparative scRNA-seq analysis across species offer tremendous potential for understanding brain evolution, including identifying both conserved and species-specific neuron types. However, how new neuron types emerge during evolution remains unclear. We hypothesize that HDTF codes for neuron type specification have driven the generation of new neuron types during evolution. This hypothesis is based on our finding that, in the absence of Bsh and Dve, which specify L4, L5, and L2, these neurons revert to L1 or L3 identities - responding to light increments and decrements, respectively. This suggests an evolutionary trajectory where L1 and L3, as two ancestral neuron types, expanded into five distinct neuron types through the addition of Bsh and Dve (Figure 5A). Consistently, the "core" L1-L3 neurons, but not L4 or L5, receive direct synaptic input from photoreceptors. Furthermore, imaging and electrophysiology studies reveal that L5 neurons are absent in honeybees and bumblebees[23–25]. Future work will explore the identity of lamina neuron types in more distantly related insects, such as silverfish and cricket.

Acknowledgments

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Declaration of interests

The authors declare no competing financial or non-financial interests.

Data Availability

All data will be deposited in databases for open access, and/or by request.

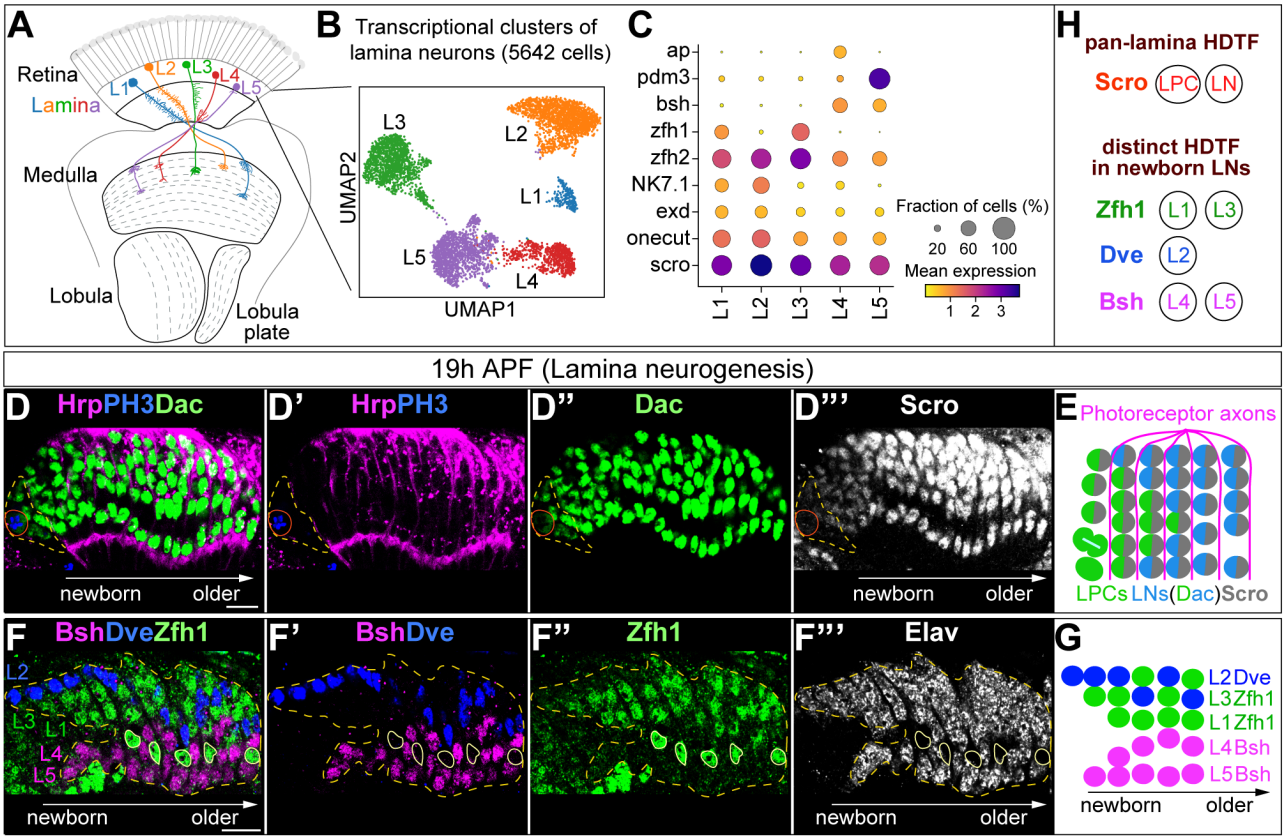


Figure 1. Lamina neurons express a pan-lamina HDTF and neuron type-specific HDTFs.

(A) Schematic of *Drosophila* visual system.

(B) UMAP dimensionality reduction of single-cell RNA sequencing data for lamina neurons. Each point corresponds to a single cell, and colors represent the five lamina neuron cell types, as determined by Leiden clustering.

(C) HTDF expression in the five lamina neuron types from single-cell RNA sequencing data in (B). The size of each circle depicts the percentage of cells in the cluster in which the marker was detected, and its color depicts the average normalized gene expression in expressing cells.

(D-E) Scro is initiated in LPCs and maintained in all lamina neurons. Dac labels LPCs and lamina neurons. PH3 is a mitotic marker. Hrp labels photoreceptor axons, which form lamina columns. The dashed circle outlines the LPCs outside the column. The red circle outlines the mitotic cell. Here and below: scale bar, 10 µm, n≥5 brains.

(F-G) Zfh1, Dve, and Bsh are expressed in specific newborn lamina neurons. Dve is in L2; Zfh1 is in L1 and L3; Bsh is in L4 and L5. The dashed circle outlines lamina neurons. The yellow circles outline Zfh1+ glial cells.

(H) Summary. LPC: lamina precursor cell; LN: lamina neuron.

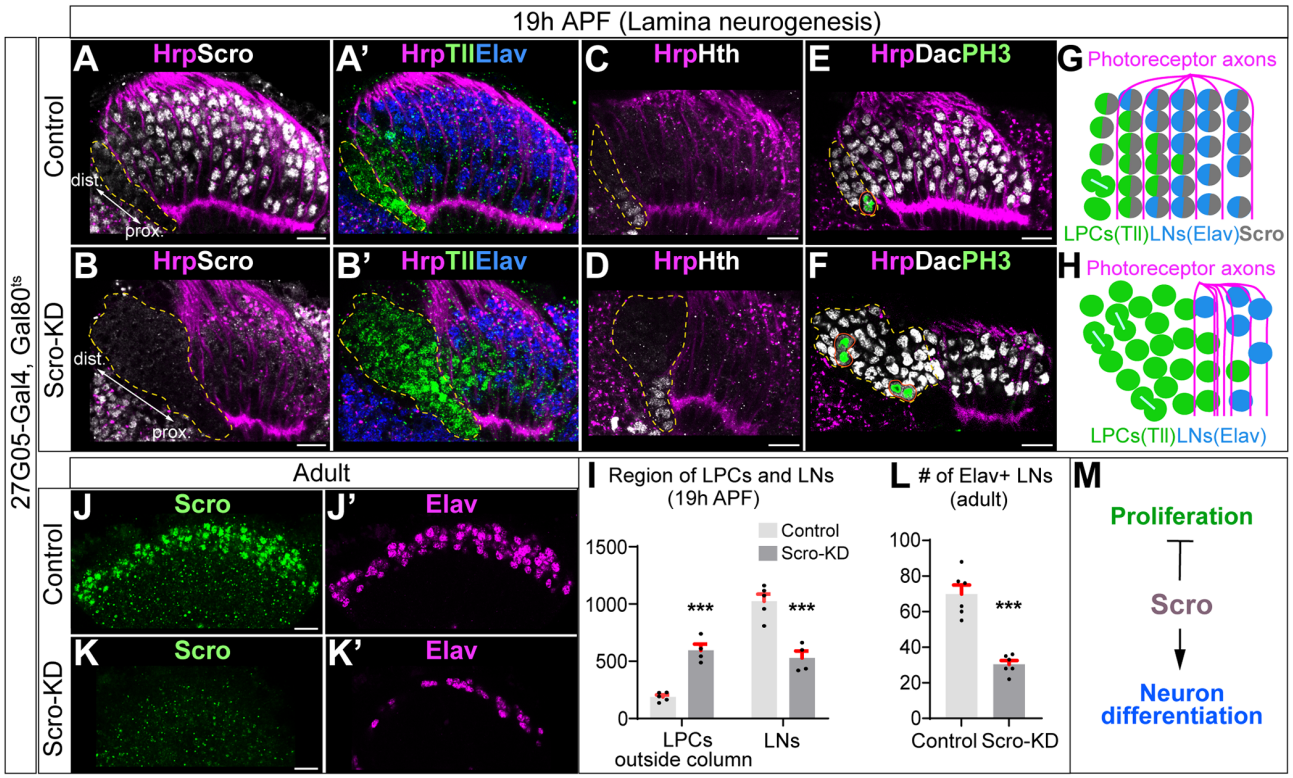


Figure 2. L1 neurons express Delta and activate Notch signaling in adjacent L4 neurons.
(A-B', I) Scro-KD shows expanded LPCs outside the lamina columns and reduced lamina neurons within the columns. Hrp labels photoreceptor axons, which form lamina columns. Tll labels LPCs. Elav labels neurons. Lamina neurons are Elav+ cells within lamina columns. The dashed circle outlines the LPCs outside the column. Here and below: scale bar, 10 µm, n≥5 brains. (I) Quantification.
(C-H) PH3 but not Hth is detected in expanded LPCs outside the columns in Scro-KD. PH3 is a mitotic marker. The dashed circle outlines the LPCs outside the column. The red circles outline PH3+ LPCs.
(J-L) Scro-KD shows reduced lamina neurons in adults. (L) Quantification.
(M) Summary: Scro inhibits proliferation and promotes neuron differentiation.

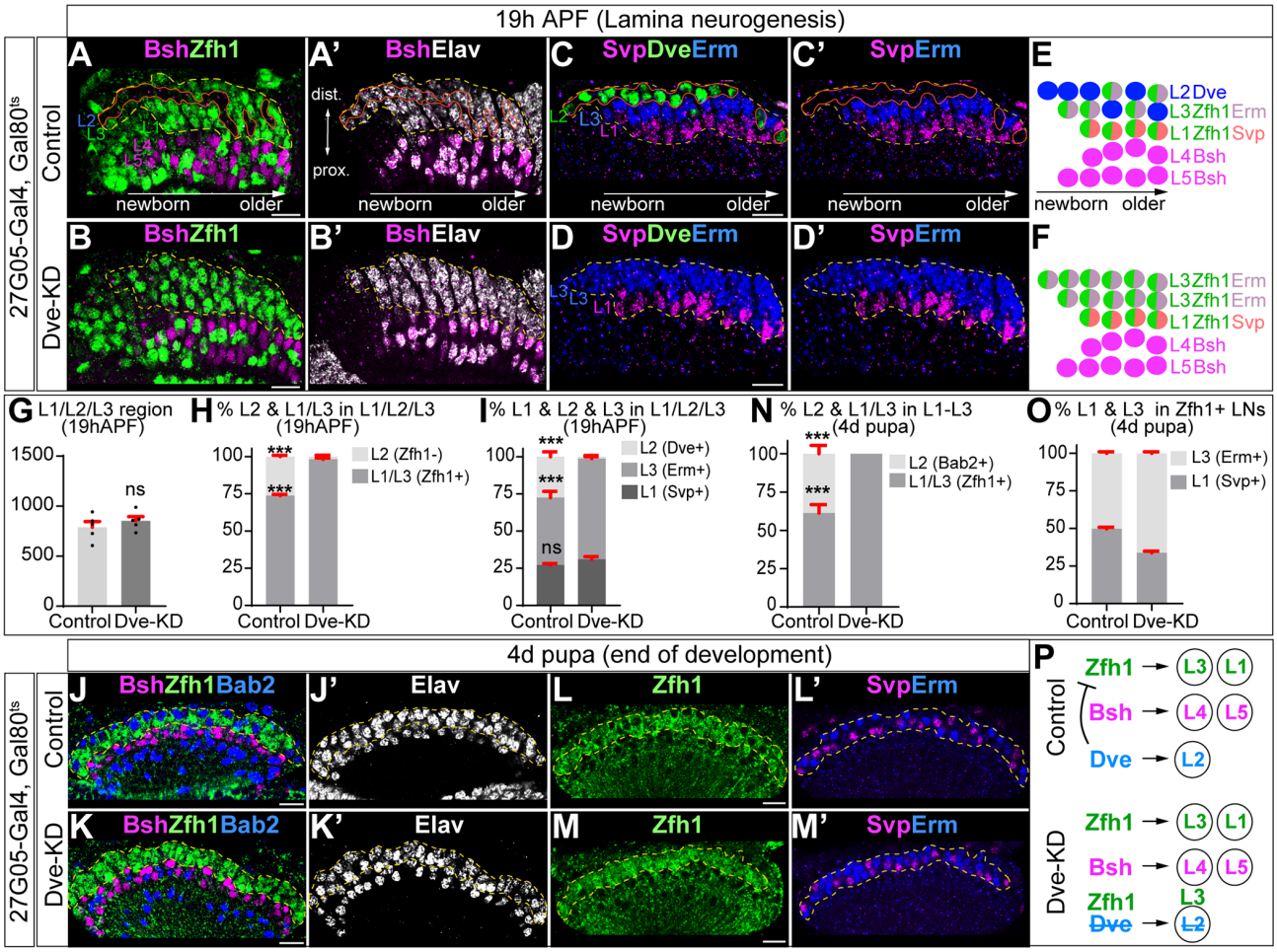


Figure 3. Dve specifies the L2 neuron type and represses the L3 fate by inhibiting Zfh1.
(A-B', G, H) Dve-KD causes Zfh1 to become expressed in all L1, L2, and L3 neurons during lamina neurogenesis. The dashed circle outlines L1, L2, and L3 neurons. The red circle outlines L2 neurons. Zfh1 labels L1 and L3 neurons. Bsh labels L4 and L5 neurons. Here and below: scale bar, 10 µm, n≥5 brains. (G, H) Quantification.
(C-F, I) Dve-KD shows that L2 neurons are re-specified as L3 neuron type during lamina neurogenesis. Svp and Erm are L1 and L3 markers, respectively. (I) Quantification.
(J-O) Dve-KD shows that L2 neurons are lost and L3 neurons are increased in adults. Bab2 is L2 marker. (O) Quantification.
(P) Summary: Dve specifies the L2 neuron type and represses the L3 fate by inhibiting Zfh1.

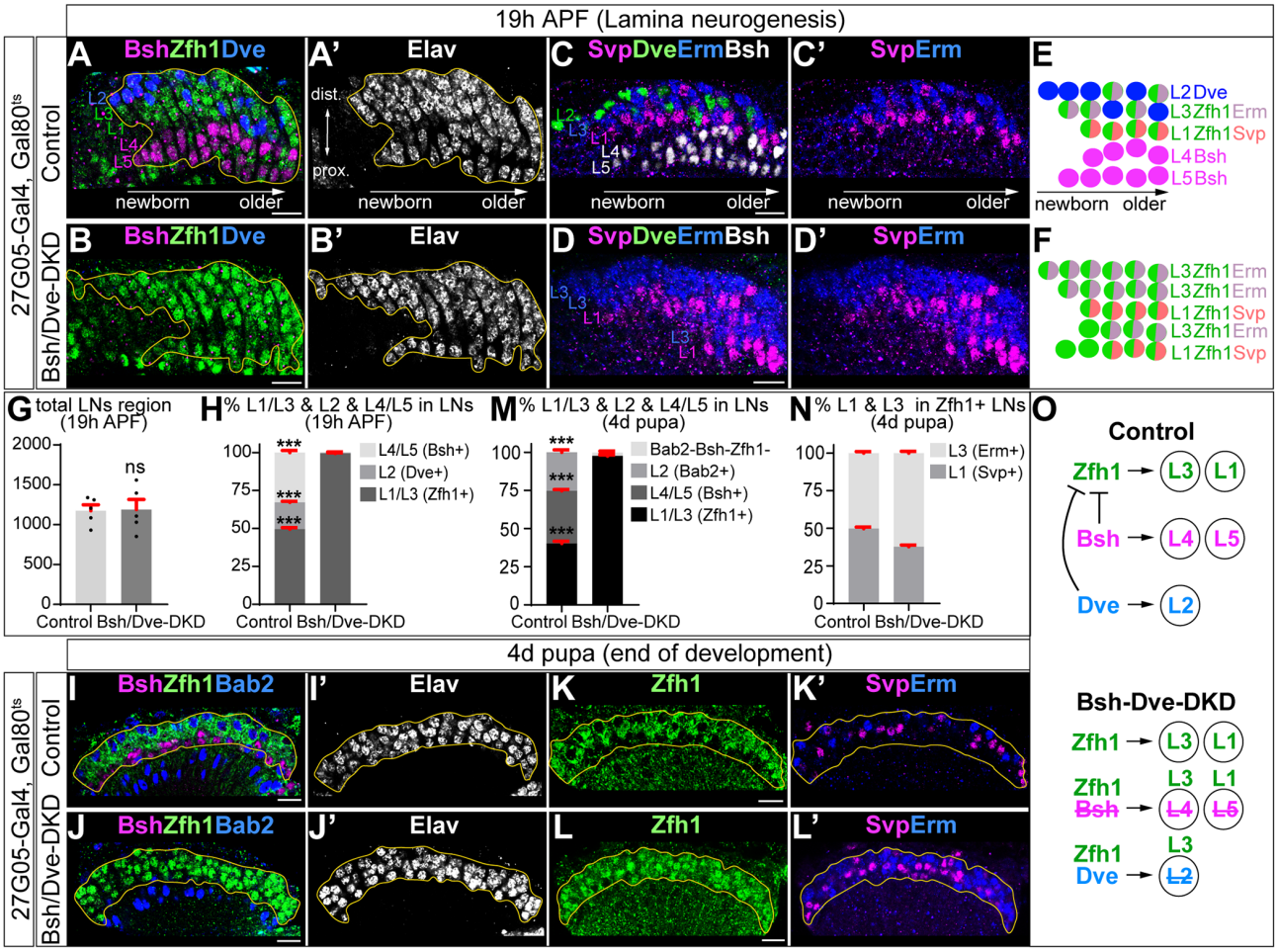


Figure 4. Loss of both Dve and Bsh transforms all lamina neurons into L1 and L3 neuron types.

(A-B', G, H) Bsh/Dve-DKD shows that Zfh1 becomes expressed in all lamina neurons during lamina neurogenesis. The yellow circle outlines lamina neurons. Zfh1 labels L1 and L3. Dve labels L2. Bsh labels L4 and L5 neurons. Here and below: scale bar, 10 µm, n≥5 brains. (G, H) Quantification.

(C-F) Bsh/Dve-DKD shows that all lamina neurons are specified as L1 and L3 neuron types. Svp and Erm are L1 and L3 markers, respectively.

(I-N) Bsh/Dve-DKD results in only L1 and L3 neuron types in adults, in contrast to the five lamina neuron types in controls. (M, N) Quantification.

(O) Summary: Bsh and Dve generate lamina neuronal diversity.

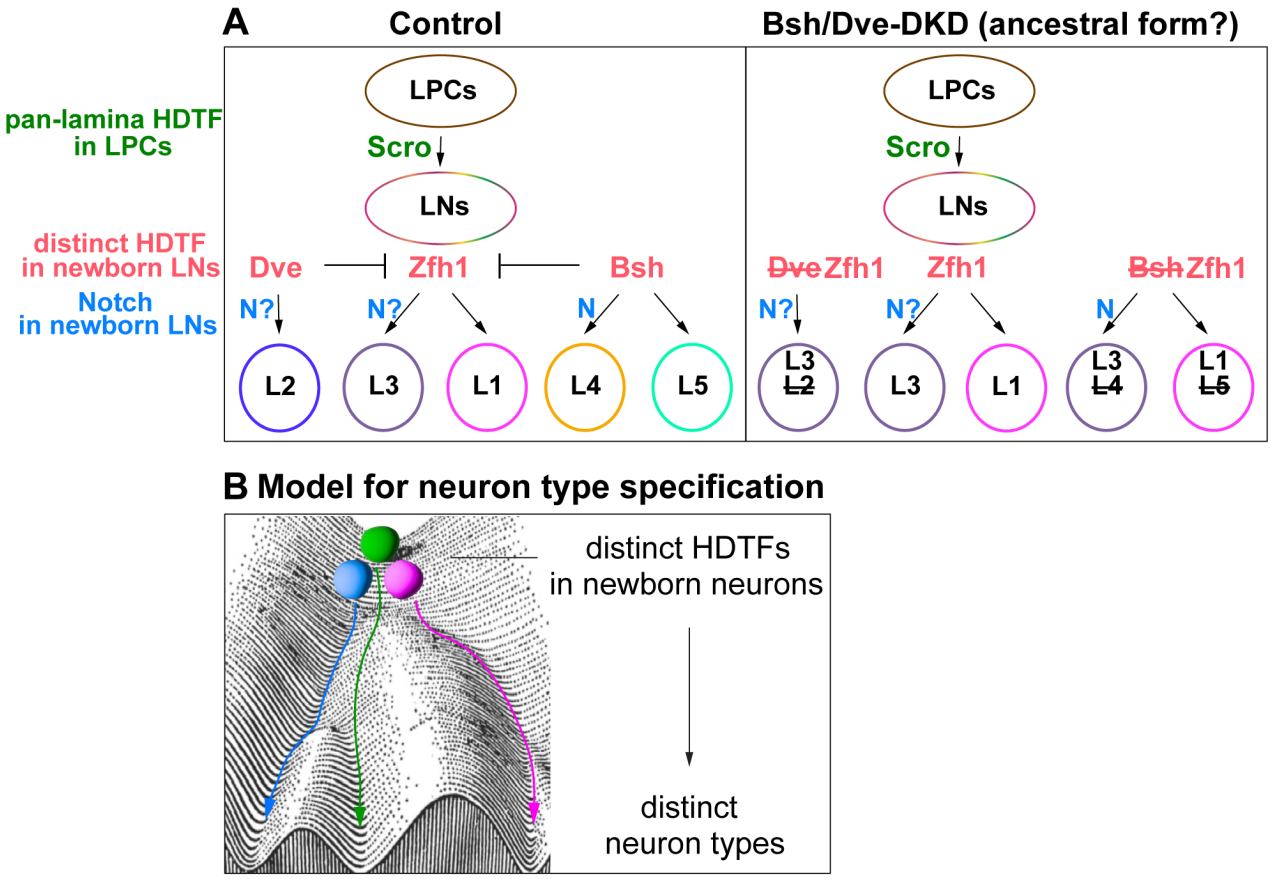
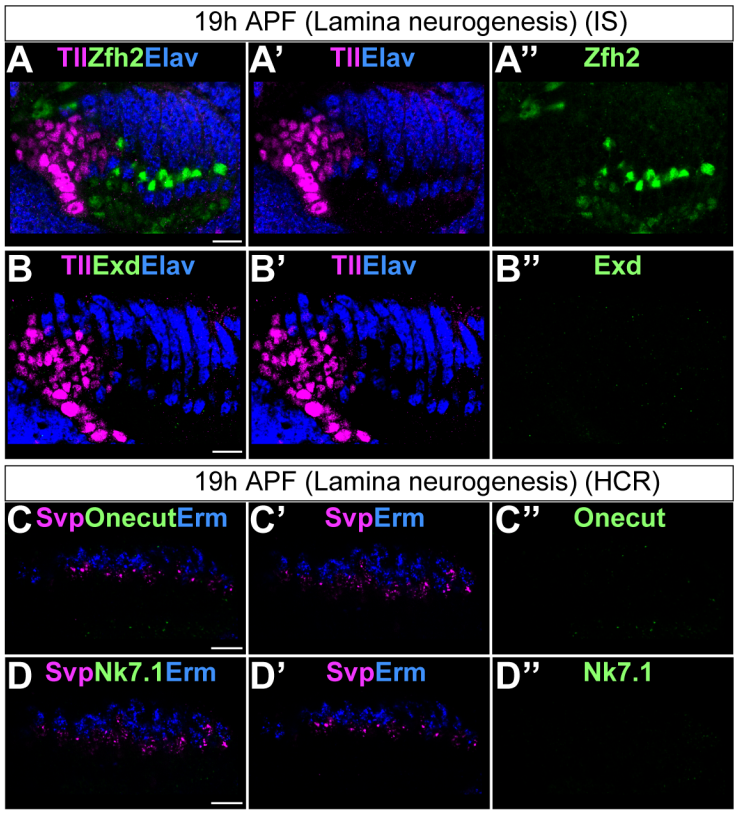


Figure 5. Summary

(A) Scro is required for LPCs to differentiate into lamina neurons. Distinct HDTFs (Dve, Zfh1, and Bsh) are expressed in specific newborn lamina neurons and specify the five lamina neuron types. Moreover, these HDTF codes can be modified by Notch signaling, allowing a single HDTF to specify two distinct neuron types (see Discussion). In Bsh/Dve-DKD, L2, L4, and L5 neurons revert to L1 and L3 neuron types. This suggests an evolutionary trajectory where L1 and L3, as two ancestral neuron types, expanded into five distinct neuron types through the addition of Bsh and Dve. LNs: lamina neurons.

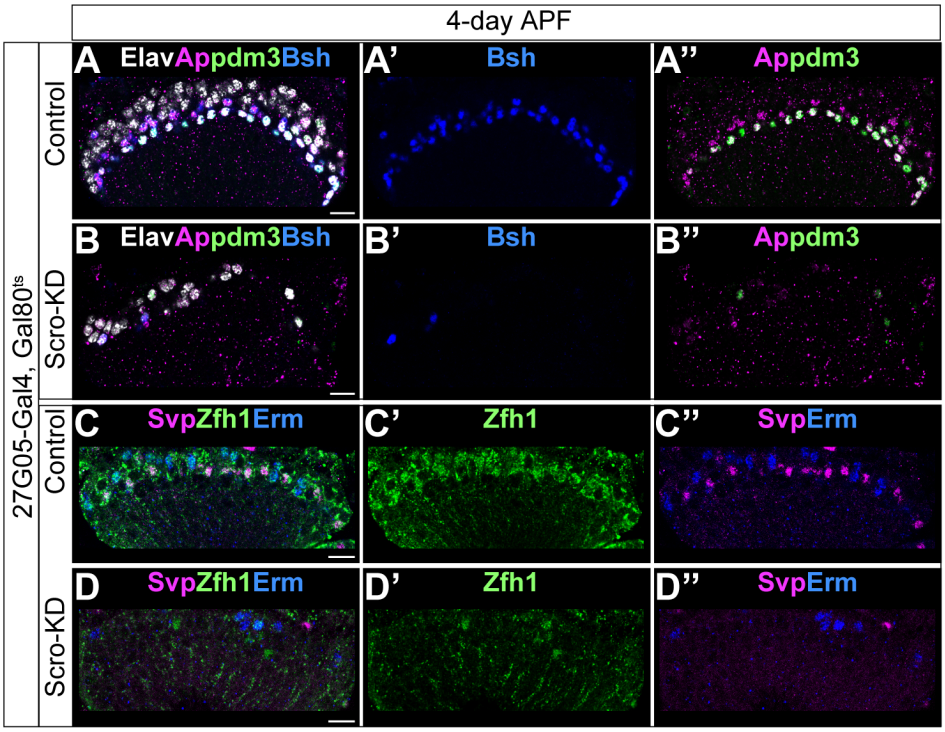
(B) We propose a model in which distinct HDTFs in newborn neurons act as transcriptional codes that specify distinct neuron types in the brain.



Supp-Figure 1. Zfh2, Exd, Onecut, and NK7.1 are not expressed in the lamina during lamina neurogenesis.

(A-B") Zfh2 and Exd proteins are not detected in lamina neurons during lamina neurogenesis. Tll labels LPCs. Elav labels neurons. Here and below: scale bar, 10 µm, n≥5 brains.

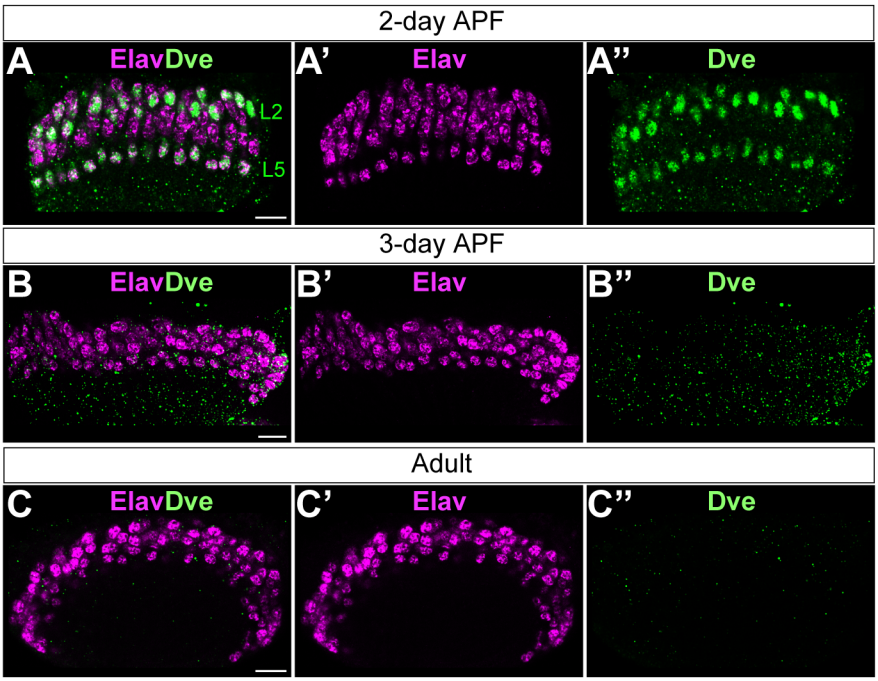
(C-D") Onecut and NK7.1 mRNA are not detected in the lamina during lamina neurogenesis. Svp and Erm are L1 and L3 markers, respectively.



Supp-Figure 2. The survived lamina neurons in Scro-KD failed to properly express markers for specific lamina neuron types.

(A-B") Pdm3 and Bsh are expressed in very few lamina neurons in Scro-KD, and they are rarely co-expressed. Ap is not expressed in lamina neurons in Scro-KD. Here and below: scale bar, 10 µm, n≥5 brains.

(C-D") Erm and Zfh1 are expressed in very few lamina neurons in Scro-KD, and they are rarely co-expressed. Svp is not expressed in lamina neurons in Scro-KD.



Supp-Figure 3. Dve expression is initiated and maintained in L2 neurons until 2-day APF.
(A-A") Dve is expressed in L2 and L5 neurons at 2-day APF. Here and below: scale bar, 10 µm, n≥5 brains.
(B-C") Dve is not expressed in lamina neurons at 3-day APF or in adults.

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