Wrapping Glial Morphogenesis and Signaling Control the Timing and Pattern of Neuronal Differentiation in the *Drosophila* Lamina

Journal of Experimental Neuroscience
Volume 12: 1–6

DOI: 10.1177/1179069518759294
SAGE

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ABSTRACT: Various regions of the developing brain coordinate their construction so that the correct types and numbers of cells are generated to build a functional network. We previously discovered that wrapping glia in the *Drosophila* visual system are essential for coordinating retinal and lamina development. We showed that wrapping glia, which ensheath photoreceptor axons, respond to an epidermal growth factor cue from photoreceptors by secreting insulins. Wrapping glial insulins activate the mitogen-activated protein kinase (MAPK) pathway downstream of insulin receptor in lamina precursors to induce neuronal differentiation. The signaling relay via wrapping glia introduces a delay that allows the lamina to assemble the correct stoichiometry and physical alignment of precursors before differentiating and imposes a stereotyped spatiotemporal pattern that is relevant for specifying the individual lamina neuron fates. Here, we further describe how wrapping glia morphogenesis correlates with the timing of lamina neuron differentiation by 2-photon live imaging. We also show that although MAPK activity in lamina precursors drives neuronal differentiation, the upstream receptor driving MAPK activation in lamina precursors and the ligand secreted by wrapping glia to trigger it differentially affect lamina neuron differentiation. These results highlight differences in MAPK signaling properties and confirm that communication between photoreceptors, wrapping glia, and lamina precursors must be precisely controlled to build a complex neural network.

KEYWORDS: *Drosophila* visual system, glia, MAPK, insulins, *Drosophila* explant live imaging

RECEIVED: January 15, 2018. **ACCEPTED:** January 19, 2018.

TYPE: Commentary

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: V.M.F. was supported by Natural Sciences and Engineering Research Council of Canada and Canadian Institutes of Health Research Banting postdoctoral fellowships.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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COMMENT ON: Fernandes VM, Chen Z, Rossi AM, Zipfel J, Desplan C. Glia relay differentiation cues to coordinate neuronal development in Drosophila. Science. 2017 Sep 1;357(6354):886-891. doi: 10.1126/science.aan3174. PubMed PMID: 28860380.

Introduction

Glia-like cells are present in the most evolutionarily ancient bilateria and share common features and functions across divergent species.[1] Although glia constitute over half the volume of the human brain,[2] we have long favored investigating their electrically excitable counterparts, neurons, and neuron-intrinsic processes in brain development and function. This has led to a gross underestimation of the true repertoire of glial functions in the brain. Genetic model systems are at the forefront of uncovering conserved general principles that govern how glial cells function and interact with other cell types in the nervous system. In recent years, several studies, including our own, have added surprising new discoveries to an ever-growing list of glial roles both during nervous system development and in circuit function.[3–10]

For both the fruit fly *Drosophila* and the vertebrate visual systems, light from the 3-dimensional world reaches a 2-dimensional (2D) sheet of photoreceptors in the retina. In flies, photoreceptor axons carry signals from the retina into the optic lobes to a series of neuronal processing layers, which are organized into 4 neuropils: lamina, medulla, lobula, and lobula plate.[11] Each of these neuropils mirrors the 2D spatial organization of the retina. This topographic correspondence across processing layers, termed "retinotopy," is developmentally programmed.[12,13] In our recent work, we uncovered a crucial role for glia in setting up retinotopy.[9] This was unexpected as glia are poorly studied during brain development, and their roles in specifying and organizing neuronal structures have been largely overlooked.

Neuronal birth and specification must be coordinated across different regions of the developing brain to generate the correct number and type of neurons that constitute neural circuits. One of the best characterized examples of such coordination is that between the retina and the lamina, the first neuropil to receive input from photoreceptors.[13] For each of the 800 unit eyes in the retina, there is a corresponding lamina unit (called a column or cartridge) in the optic lobe, made up of 5 lamina neuronal types (L1-L5, named for the medulla layers in which they arborize in the adult and marked by the pan-neuronal marker, embryonic lethal abnormal vision [Elav]), and multiple glial subtypes.[14] Seminal work by the Kunes lab established that photoreceptors induce the differentiation of lamina neurons, their neuronal target field, via 2 secreted cues: The first cue, Hedgehog (Hh), specifies lamina precursors and their assembly into columns.[15–17] The second cue, epidermal growth factor (EGF), was believed to specify the 5 lamina neuronal types directly in a spatiotemporal sequence.[18]

A long-standing discrepancy with this model was the spatiotemporal order of lamina neuronal differentiation, which could not be easily explained by induction through photoreceptor EGF.[18] In each assembled column of naïve lamina precursor cells, the most proximal and most distal cells differentiate first into L5 and L2, respectively; differentiation then proceeds in a

distal-to-proximal order, L3 forming next followed by L1 then L4.[19] Here, we present a brief summary of our recent work, which uncovered an unexpected role for glia in inducing neuronal differentiation in the visual system of *Drosophila melanogaster*.[9] In addition, we use live imaging to show how wrapping glia extend their processes progressively into the developing optic lobe and describe how this supports our model of sequential differentiation of lamina precursors into L1 to L4 neurons. We also describe data suggesting that the kinetics of the same signaling pathway effector, mitogen-activated protein kinase (MAPK), differentially affect the fate outcome of lamina neurons. Finally, we demonstrate signaling ligand specificity for their ability to generate the appropriate timing and pattern of neuronal differentiation when secreted by wrapping glia.

Materials and Methods

Immunohistochemistry and Drosophila strains

Eye-brain complexes were dissected from early pupae (10-15 hours after puparium formation) in 1× phosphate-buffered saline and then fixed and stained according to standard protocols.[20] We used the following antibodies for this study: mouse anti-DAC 2-3 (mAbdac2-3 was deposited to the Developmental Studies Hybridoma Bank [DSHB] by Rubin, G.M. [DSHB Hybridoma Product mAbdac2-3]); Rat anti-Elav (Rat-Elav-7E8A10 anti-Elav was deposited to the DSHB by Rubin, G.M. [DSHB Hybridoma Product Rat-Elav-7E8A10 anti-Elav]); Rabbit anti-GFP (green fluorescent protein) (Cell Signaling); and goat anti-HRP (horseradish peroxidase). Secondary antibodies were used at 1:400 and were obtained from Jackson ImmunoResearch or Invitrogen. Confocal images were acquired using a Leica SP5 microscope. Images were processed and assembled using ImageJ, Adobe Photoshop, and Illustrator.

The following genotypes were used in this study: {;;*R94A08-Gal4/UAS-CD8::GFP*} (R94A08-Gal4 = wrapping glia Gal4, BL40673), {;*UAS-CD8::GFP/13XLexAop6XmCherry* (BL52 272); *Repo-Gal4*[21]*/R27G05-LexA*[22]}, {;;*rho3*$^{PLLb/PLLb}$} (a gift from B. Shilo), {;*UAS-rl*^{Sem}*/+; rho3*^{PLLb}, *UAS-CD8::GFP/rho3*^{PLLb}, *UAS-CD8::GFP, R27G05-Gal4*} (R27G05-Gal4 = lamina-Gal4; BL48073), {;*UAS-InR*^{ACT}*/+; rho3*^{PLLb}, *UAS-CD8::GFP/rho3*^{PLLb}, *UAS-CD8::GFP, R27G05-Gal4*}, {;*UAS-EGFR*^{ACT}*/+; rho3*^{PLLb}, *UAS-CD8::GFP/rho3*^{PLLb}, *UAS-CD8::GFP, R27G05-Gal4*}, {;*UAS-ilpX/+; rho3*^{PLLb}, *UAS-CD8::GFP/rho3*^{PLLb}, *UAS-CD8::GFP, R94A08-Gal4*} (IlpX = Ilp2, Ilp3, Ilp6, and Ilp7; gifts from E. Hafen). Crosses were raised at 18°C for 7 days and shifted to 29°C for 28 hours prior to dissection.

Live imaging

Eye-brain complexes from late L3 larvae of genotype {;*UAS-CD8::GFP/13XLexAop6XmCherry; Repo-Gal4/R27G05-LexA*} were dissected in imaging medium (Schneider's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 0.25% insulin) on ice. Eye-brain complexes were then embedded in 0.4% low temperature gelling agarose and incubated in imaging medium for the duration of image acquisition with an Olympus FV1000MPE multi-photon laser scanning microscope (at 920 nm). Movie was processed using ImageJ software packages.

Results

Wrapping glia induce lamina neuron differentiation

To investigate the origin of the spatiotemporal pattern of lamina neuronal differentiation we perturbed gene expression in specific cell types with modern genetic tools.[23,24] We found that, surprisingly, communication between photoreceptors and lamina precursors is indirect (Figure 1)[9]; instead, photoreceptors signal to wrapping glia, which then directly induce lamina neuronal differentiation.[9] Our ability to uncover these interactions was dictated almost entirely by the genetic tractability of *Drosophila*: the MAPK pathway is characterized extensively in flies and there are numerous tools available including mutants, overexpression constructs, and cell type–specific (photoreceptor, glial, and lamina) driver lines.[23,24] We took considerable advantage of the *rhomboid 3* (*rho3*) mutant, in which photoreceptors cannot secrete the EGF ligand Spitz (Spi) from their axons but are able to do so from their cell bodies in the retina so that photoreceptor recruitment, specification, and targeting are unaffected.[26,27] Rhomboids are a family of proteases that cleave Spi into an active form for secretion. Rho3 is localized specifically to photoreceptor axons, thus *rho3* mutants specifically lack photoreceptor axon-derived EGF.[26] In these mutants, L1 to L4 neurons are absent, implicating photoreceptor axon-derived EGF in lamina neuron differentiation.

We combined various cell type–specific driver lines (eg, photoreceptor-specific, wrapping glia-specific, and lamina-specific Gal4 drivers)[23,24] with the *rho3* loss-of-function mutant to perform a series of rescue experiments that uncovered which cells induce lamina neuronal differentiation and by what signals.[9] We showed that wrapping glia but not lamina precursors respond to EGF from photoreceptors.[9] In response to EGF receptor (EGFR) activation, wrapping glia send a signal to the lamina via insulin-like peptides (Ilps), which activate the insulin receptor (InR) in lamina precursors.[9] MAPK activation downstream of InR leads to the differentiation of L1 to L4 neurons, but not L5 neurons, which follow a distinct, and as yet unresolved, developmental program (Figure 1).[9]

Such a signaling relay appears to fulfill 2 functions: first, it specifies the spatial pattern of differentiation, which appears critical for generating L1 to L4 lamina neuronal cell types, and second, it introduces a temporal lag to allow lamina precursors to assemble into the correct stoichiometry and physical alignment before differentiating.[9]

Wrapping glia morphogenesis determines lamina neuron differentiation

Because wrapping glia are central to inducing neuronal differentiation in the lamina, we focused on understanding their

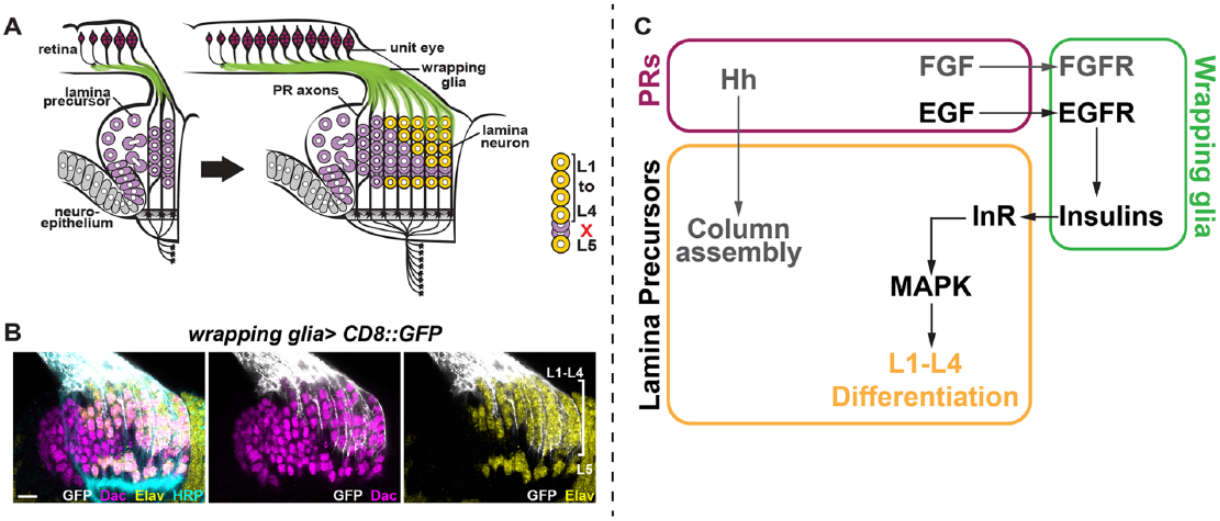


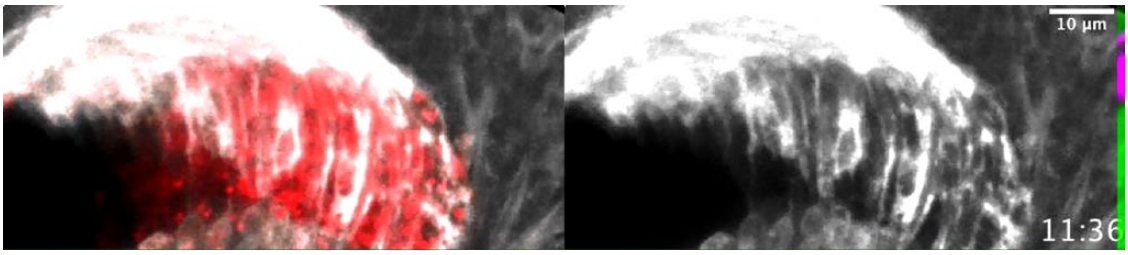
Figure 1. Progressive wrapping glial process extension induces sequential lamina neuron differentiation. (A) Schematic of lamina development in the optic lobes. Hh secreted from incoming PR axons causes lamina precursors (magenta) to organize into columns, each of which consists of 6 to 7 cells.[15–17] Following column assembly, lamina precursors differentiate in an invariant spatiotemporal sequence (yellow).[14] Photoreceptor axons also secrete FGF to induce glial morphogenesis and ensheathment of PR axons.[25] Wrapping glial processes (green) extend along lamina columns progressively. The depth of wrapping glial process extension depends on the age of each column. Older columns (rightmost in the figure) have glial processes that extend further than younger columns.[9] (B) Horizontal view of a late larval eye disc and optic lobe showing the progressive innervation of wrapping glial processes (GFP, white) down PR axons that are marked by HRP (cyan). The number of embryonic lethal abnormal vision (Elav, yellow) differentiated lamina neurons in each column correlates with the depth of glial process extension. Note that L5 neurons located at the bottom of each column differentiate independent of wrapping glial process extension.[9] All lamina cells, including differentiated and undifferentiated lamina precursors, are marked by Dachshund (Dac, magenta). (C) Summary of known signaling pathways active between PRs, glia, and lamina precursors during lamina development. Photoreceptors induce lamina precursors to assemble into columns and wrapping glia morphogenesis through Hh and FGF signaling. EGF secreted from PR axons activates EGFR in glia, causing them to secrete insulins that activate the InR on lamina precursors. Activation of MAPK in lamina precursors leads to lamina neuronal differentiation. EGF indicates epidermal growth factor; EGFR, EGF receptor; Elav, embryonic lethal abnormal vision; FGF, fibroblast growth factor; GFP, green fluorescent protein; Hh, Hedgehog; HRP, horseradish peroxidase; Ilps, insulin-like peptides; InR, insulin receptor; MAPK, mitogen-activated protein kinase; PR, photoreceptor.

morphogenesis. Indeed, wrapping glial morphogenesis appears tightly linked to the pattern of differentiation in the lamina. Our experiments showed that differentiation of lamina neurons in *rho3* mutants could be rescued by expressing Ilps in photoreceptors.[9] However, in this instance, we observed that differentiation did not follow the normal pattern, where older columns differentiate first and progressively but instead lamina neurons differentiated out of sequence.[9] The pattern of neurogenesis was only restored when Ilps were expressed from the wrapping glia themselves. These results imply that wrapping glia instruct the lamina pattern of differentiation, and we speculate that this is due to their progressive morphogenesis[9] (Figure 1A and B; Movie 1). Wrapping glia interact intimately with photoreceptors. They are a glial subtype that originates from dedicated glial precursors in the optic stalk.[25] These precursors proliferate and migrate into the developing retina, where they begin to ensheath nascent photoreceptor axons in response to Thisbe, an FGF8-like ligand, which photoreceptors secrete.[25] Wrapping glia ensheath photoreceptor axons progressively, with a slight delay relative to axonal growth.[28,29] Photoreceptor axons arrive in the optic lobe, induce lamina precursor terminal divisions, and organize columns as "naked" axons[30] (Figure 1B).

Through live imaging, we showed that wrapping glia arrived in the lamina progressively, after column assembly (Movie 1). Wrapping glial processes penetrated the lamina such that the oldest photoreceptor axons (associated with the oldest lamina columns) were wrapped more extensively, and glial processes extended deeper along older columns relative to younger ones. This resulted in glial processes extending into the lamina in a manner that correlates with the spatiotemporal order of lamina neuron differentiation for L1 to L4.[9] These observations are consistent with our hypothesis that the rate of lamina differentiation is limited by the morphogenesis of the wrapping glia. This is further supported by our previous observation that preventing wrapping glial morphogenesis by blocking fibroblast growth factor receptor (FGFR) activity led to a disrupted differentiation pattern, such that the only lamina cells to differentiate were at the distal ends of columns, adjacent to the wrapping glia that abutted but did not enter the lamina.[9]

Upstream receptors have different effects on signaling through MAPK

A striking feature of the photoreceptor-to-wrapping glia-to-lamina signaling relay is the reiterative use of MAPK signaling. Although a variety of activating ligands (FGF, EGF, or Ilps) are used in combination with different receptors (FGFR, EGFR, or InR), all pathways seemingly converge on MAPK signaling.[31] However, the use of different ligands and receptors



Movie 1. Time-lapse 2-photon imaging of a late L3 eye-optic lobe brain complex in which glia were labeled by expression of a membrane-targeted green fluorescent protein (GFP, white) driven by the pan-glial Gal4 driver, *Repo-Gal4*.[21] The developing lamina was labeled by expression of mCherry (red) driven by *lamina-LexA* (*R27G05-LexA*).[22] Wrapping glial processes extended progressively along lamina columns. Note that glial processes extended deepest in the oldest columns (rightmost in the movie), whereas newer columns showed progressive growth of glial processes down the column. Lamina and glia development were observed over the course of ~12 hours (scale bar = 10 µm).

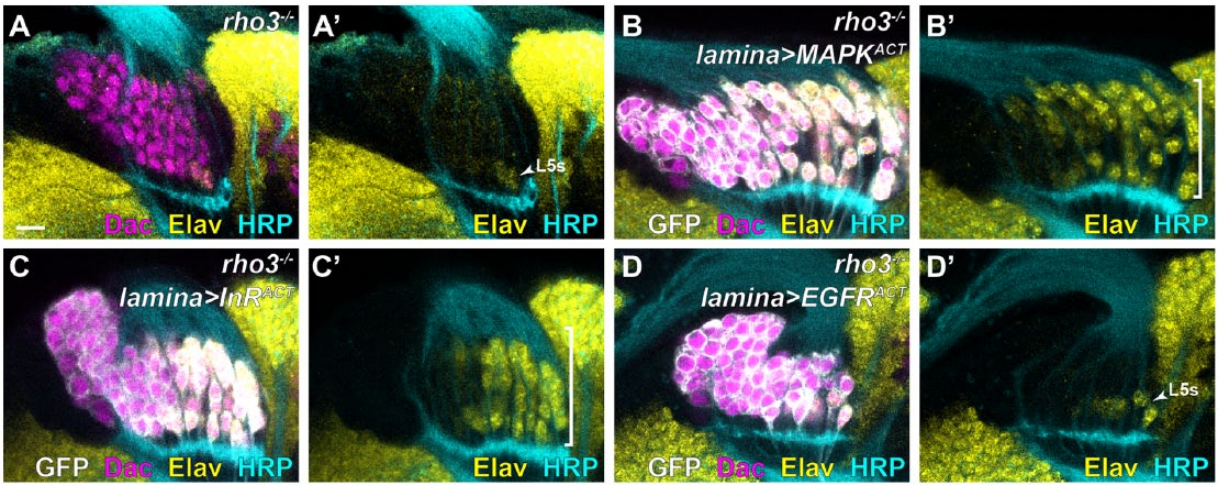


Figure 2. Receptors upstream of MAPK differentially rescue lamina neuronal differentiation. (A, A′) In a *rho3* mutant, lamina precursors marked by Dac expression (magenta) organize into columns defined by PR axons marked by HRP (cyan) but do not differentiate (lack of Elav, yellow). Note that precursors at the bottom of each column differentiate into presumptive L5 neurons but with a delay as this process occurs independent of EGF from photoreceptors and wrapping glial Ilps.[9] (B, B′) Activation of MAPK in the lamina (white, marked by GFP coexpression) in a *rho3* mutant rescued lamina neuron differentiation (Elav, yellow).[9] (C, C′) Similarly, expression of a dominant-active InR in the lamina (GFP, white) rescued differentiation (Elav, yellow).[9] (D, D′) Expression of dominant-active EGFR in the lamina (GFP, white) did not lead to differentiation of lamina precursors except for presumptive L5 neurons located at the bottom of oldest columns. EGF indicates epidermal growth factor; EGFR, EGF receptor; Elav, embryonic lethal abnormal vision; GFP, green fluorescent protein; HRP, horseradish peroxidase; Ilps, insulin-like peptides; InR, insulin receptor; MAPK, mitogen-activated protein kinase; PR, photoreceptor.

suggests some specificity in the signaling properties of each combination.[31]

We showed previously that lamina-specific expression of activated MAPK or activated InR could rescue neuronal differentiation in the *rho3* mutant.[9] Moreover, we showed that InR activity in the lamina acted through MAPK to induce lamina neuron differentiation (Figure 2B and C).[9] We therefore tested whether expressing an activated form of EGFR in lamina cells could drive lamina neuronal differentiation. We predicted that if activating MAPK was sufficient for differentiation, then expressing a dominant-active EGFR should also drive neurogenesis. Strikingly, we found that dominant-active EGFR could not autonomously rescue lamina neuron differentiation in a *rho3* mutant (Figure 2D). These data point to receptor-specific effects on MAPK activation. Such specificity may arise due to differences in the kinetics of MAPK activation; for example, the InR may lead to a more sustained or a higher level of MAPK activation compared with MAPK activation by EGFR. Indeed, differences in MAPK kinetics have been reported in other systems when different receptor tyrosine kinase ligands and receptors are used: activation of EGFR via EGF versus activation of nerve growth factor (NGF) receptor by NGF lead to transient compared with sustained MAPK activation in PC-12 cells, respectively.[31,32] These differential kinetics of MAPK activity cause distinct cell fate outcomes, similar to the different outcomes on lamina neuronal differentiation we observed when rescuing the *rho3* mutant with EGFR compared with InR.

One InR but distinct responses to different Ilps

Another surprising aspect of this system is the use of insulin/insulin-like factor signaling, which is best known for activating phosphoinositide 3-kinase (PI3K) to systemically regulate whole-animal metabolism. Although the *Drosophila* genome encodes 7 Ilps (*ilp1-7*), these act redundantly through the sole InR.[33,34] A small set of neurons in the central brain, called the insulin producing cells, secrete systemic Ilps, which regulate systemic growth and carbohydrate metabolism.[35] Of the 7 Ilps,

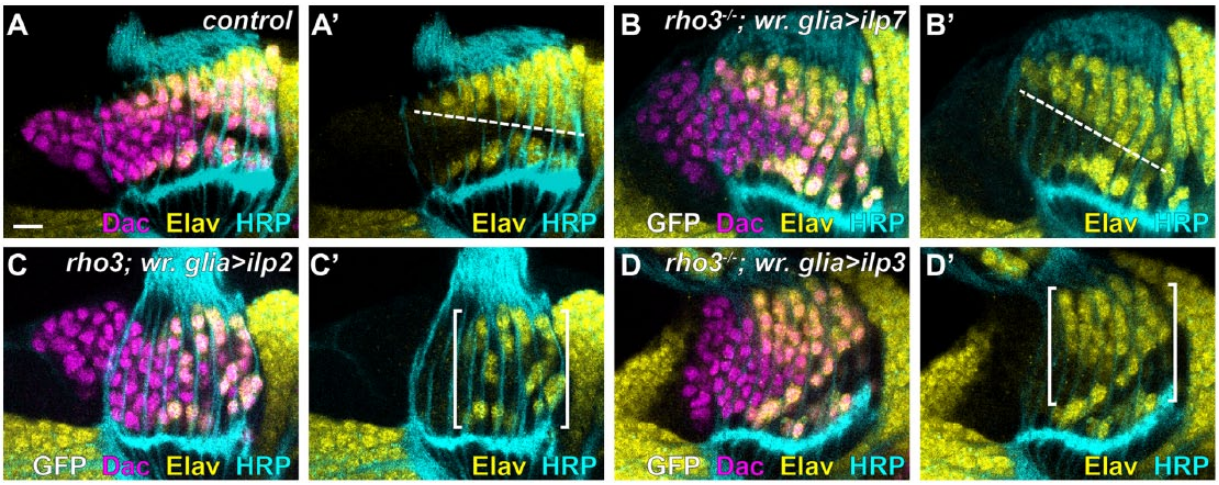


Figure 3. Choice of insulin expression by wrapping glia affects the timing of lamina neuronal differentiation. (A, A′) In a control L3 brain, lamina precursors marked by Dac expression (magenta) are organized into columns by PR axons (HRP, cyan) and differentiate in a triangular pattern when viewed horizontally (white dashed line demarcates the bottom of the triangle and Elav (yellow) marks differentiated lamina neurons. Note that older columns (rightmost columns) have more differentiated neurons than newer formed columns. (B, B′) In a *rho3* mutant, wrapping glial expression of *ilp7* (GFP, white) rescued the triangular front of lamina neuron differentiation. (C, C′ and D, D′) However, wrapping glial expression of systemic *ilps*, such as *ilp2* (C, C′) or *ilp3* (D, D′), lead to differentiation that was less robust (C, C′) and not in a triangular pattern (C, C′ and D, D′). Elav indicates embryonic lethal abnormal vision; GFP, green fluorescent protein; HRP, horseradish peroxidase; Ilps, insulin-like peptides; PR, photoreceptor.

Ilp6 shows closer homology to insulin-like growth factor than to insulin and is more locally and developmentally expressed.[34,36] Ilp7 is less studied but shows regional expression outside of the central brain.[37–39] We previously showed that wrapping glial expression of Ilp6 could rescue L1 to L4 neuronal differentiation in a *rho3* mutant in the appropriate spatiotemporal pattern.[9] Therefore, we sought to test whether other Ilps could also induce the appropriate pattern of lamina neuronal differentiation or whether Ilp6 had specific signaling effects.

We overexpressed Ilp2, Ilp3, and Ilp7 in wrapping glia in a *rho3* mutant and observed that each of these factors was sufficient to restore lamina neuronal differentiation (Figure 3). However, unlike Ilp6 or Ilp7, expressing the systemic Ilps resulted in out of sequence rather than sequential differentiation[9] (Figure 3B to D). Moreover, the pattern of differentiation was less robust and showed greater variability. Therefore, the type of ligand produced by wrapping glia affects the pattern of neurogenesis, even though all signal through the same receptor, InR. These data raise the possibility that the different Ilps may vary in diffusivity or that they may interact with their receptor differently, for instance, at lower concentration thresholds.

Discussion

In recent years, there has been an increasing appreciation that glia are active players in nervous system development and function.[40,41] They can provide neurotrophic support, guide axon outgrowth, regulate synaptogenesis and plasticity, and eliminate cellular debris and bias stem cell divisions toward neuronal outcomes.[40,42–44] Glia are also increasingly regarded as essential components of neural stem cell niches, providing growth factors for neural stem cell proliferation at neurogenic sites in the adult brain or in response to injury.[40] Here, rather than providing self-renewal signals for lamina precursors, which are postmitotic, wrapping glia establish a "differentiation niche" that provides differentiation signals to postmitotic cells. Our findings parallel those of adult hippocampal astrocytes, which instruct the generation of neuronal progeny in coculture with neural stem cells.[8] Mammalian sites of adult neurogenesis are extremely restricted, and only astrocytes derived from these restricted regions can promote neural stem cell proliferation and neurogenesis.[8,40,45] It remains to be determined whether relevant astrocyte-derived signals depend on cues from other cell types, as we describe here. Indeed, we show that the neuronal inducing properties of wrapping glia in the lamina arise from their response to photoreceptor cues. Thus, it is possible that astrocytes in adult neurogenic regions of mammalian brains, such as wrapping glia in the *Drosophila* optic lobes, instruct neurogenesis by conveying relevant spatiotemporal information to neural stem cells, perhaps via signal relays from other cells types such as other neurons, glia, or endothelial cells in the vasculature.[40,45]

Different regions of the brain follow distinct neurogenic programs. For example, while lamina neuronal differentiation is dictated by naïve precursors responding to extrinsic signals, *Drosophila* medulla neurons are the products of temporal patterning of neural progenitors.[20,46] Distinct modes of neurogenesis have different developmental requirements, implying that neurons and glia interact in different ways. Future studies will reveal the general principles underlying these interactions.

Acknowledgements

The authors thank Claude Desplan, Marc Amoyel, and Desplan lab members for comments and suggestions and Ben Shilo, Ernst Hafen, Larry Zipursky, the Bloomington Drosophila Stock Center, and Developmental Studies Hybridoma Bank for reagents.

Author Contributions

VMF conceived the project, performed all experiments described in Figures 1-3, analyzed the data, and wrote the manuscript. AMR prepared and performed the live imaging experiment, analyzed the data, and wrote the manuscript.

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