

2014-02-18

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Chaturvedi R, Reddig K, Li H. (2014). Long-distance mechanism of neurotransmitter recycling mediated by glial network facilitates visual function in *Drosophila*. *Neurobiology Publications and Presentations*. <https://doi.org/10.1073/pnas.1323714111>. Retrieved from [https://escholarship.umassmed.edu/neurobiology\\_pp/165](https://escholarship.umassmed.edu/neurobiology_pp/165)

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# Long-distance mechanism of neurotransmitter recycling mediated by glial network facilitates visual function in *Drosophila*

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Edited by Ben A. Barres, Stanford University School of Medicine, Stanford, CA, and approved January 10, 2014 (received for review December 24, 2013)

Neurons rely on glia to recycle neurotransmitters such as glutamate and histamine for sustained signaling. Both mammalian and insect glia form intercellular gap-junction networks, but their functional significance underlying neurotransmitter recycling is unknown. Using the *Drosophila* visual system as a genetic model, here we show that a multicellular glial network transports neurotransmitter metabolites between perisynaptic glia and neuronal cell bodies to mediate long-distance recycling of neurotransmitter. In the first visual neuropil (lamina), which contains a multilayer glial network, photoreceptor axons release histamine to hyperpolarize secondary sensory neurons. Subsequently, the released histamine is taken up by perisynaptic epithelial glia and converted into inactive carcinine through conjugation with  $\beta$ -alanine for transport. In contrast to a previous assumption that epithelial glia deliver carcinine directly back to photoreceptor axons for histamine regeneration within the lamina, we detected both carcinine and  $\beta$ -alanine in the fly retina, where they are found in photoreceptor cell bodies and surrounding pigment glial cells. Downregulating *Inx2* gap junctions within the laminar glial network causes  $\beta$ -alanine accumulation in retinal pigment cells and impairs carcinine synthesis, leading to reduced histamine levels and photoreceptor synaptic vesicles. Consequently, visual transmission is impaired and the fly is less responsive in a visual alert analysis compared with wild type. Our results suggest that a gap junction-dependent laminar and retinal glial network transports histamine metabolites between perisynaptic glia and photoreceptor cell bodies to mediate a novel, long-distance mechanism of neurotransmitter recycling, highlighting the importance of glial networks in the regulation of neuronal functions.

In both vertebrates and insects, glial cells clear neurotransmitters from synaptic clefts (1, 2), thereby increasing the signal-to-noise ratio, enhancing temporal resolution of synaptic transmission, and preventing cross-talk between neighboring neuronal signaling pathways. Once taken into glia, transmitters can be converted into inactive metabolites and sent back to neurons for reuse (3). This glial process of recycling is crucial for neurons to maintain the necessary level of neurotransmitters, such as glutamate and histamine (4, 5), for sustained signal transmission.

A variety of transporters for glutamate, dopamine, GABA, glycine, histamine, and their metabolites have been found in both glial and neuronal membranes (6–8). Metabolic enzymes in the recycling process of transmitters, such as glutamate, GABA, and histamine, are also identified (3, 8, 9). However, our knowledge of the trafficking route of inactive transmitter metabolites to neuronal terminals is still lacking. These metabolites may be released from glial processes in close vicinity to neuronal terminals and recycled in an entirely local manner. Alternatively, they may travel within an intercellular glial network and reach a more proximal part of the neuron. Both mammalian and insect glia form intercellular gap-junction networks that allow free diffusion of small molecules (10, 11). The role of these intercellular networks in neurotransmitter recycling is unknown; we are investigating this using the *Drosophila* visual system as a model.

The *Drosophila* compound eye is composed of hundreds of units named ommatidia. Six peripheral photoreceptors in each ommatidium (12) project axons from the retina to the first layer of visual neuropil, referred to as lamina (5). Within the lamina, photoreceptor axons release histamine upon light stimulation to hyperpolarize projecting large monopolar cells (LMCs) (13). Photoreceptor and LMC axons, and all other neuronal processes in the lamina, are wrapped laterally by epithelial glial cells (14). Whereas the proximal edge of lamina is sealed by marginal glia, the distal edge of laminar neuropil is separated from the retina by four glia layers: two surface (fenestrated and pseudocartridge) glia underneath retina, and distal and proximal satellite glia that wrap cell bodies and initial axon segments of LMCs, respectively (5) (Fig. S1A). Histamine released from photoreceptor axons is removed from the extracellular space by epithelial and probably additional laminar glia (5). In epithelial glia, the *N*- $\beta$ -alanyl-biogenic amine synthetase *Ebony* conjugates histamine to  $\beta$ -alanine to form inactive carcinine for storage and transport (15). Carcinine is then transported back to photoreceptors via an unknown mechanism, where it is hydrolyzed into histamine and  $\beta$ -alanine by a peptidase *Tan* (9). Compared with de novo synthesis by histidine decarboxylase (HDC) in photoreceptors, the recycling of histamine is more energy efficient and is thus considered to be a dominant pathway in maintaining adequate histamine level (16, 17). Indeed, mutations in both *ebony* and *tan* significantly decrease visual histamine level and disrupt fly visual transmission (9).

Because *Ebony* is expressed specifically in epithelial glia in the lamina (18), this glial type was thought to be the only laminar glia mediating histamine recycling until now (Fig. S1A). Given that

## Significance

Neurons communicate at synapses through neurotransmitters. For sustained neuronal signaling, neurotransmitters are recycled after release from neuronal terminals. During this process, perisynaptic glial cells take in and convert neurotransmitters such as glutamate, GABA, and histamine into inactive metabolites for transport. It has been assumed that inactive metabolites are delivered directly into neighboring neuronal terminals for neurotransmitter regeneration. Our work in the fruit fly, however, demonstrates that a gap junction-dependent multicellular glial network transports metabolites of histamine between perisynaptic glia and cell bodies of photoreceptor neurons, and this is required for visual signal transmission and alert behavior. Thus, by mediating this novel, long-distance recycling of neurotransmitters, intercellular glial networks play an important role in the maintenance of neuronal functions.

Author contributions: R.C. and H.-S.L. designed research; R.C. and K.R. performed research; R.C. and H.-S.L. analyzed data; and R.C. and H.-S.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1323714111/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1323714111/-DCSupplemental).

all laminar glia may form an intercellular network through gap junctions, as evident in a larger fly species *Musca* (19), it is important to investigate the role of other laminar glia cells in the recycling of histamine. By examining the levels and distributions of histamine, carcinine, and  $\beta$ -alanine in both wild-type and genetically modified flies, we provide evidence that recycling of histamine involves the entire laminar glial network, as well as retinal glia pigment cells.

## Results

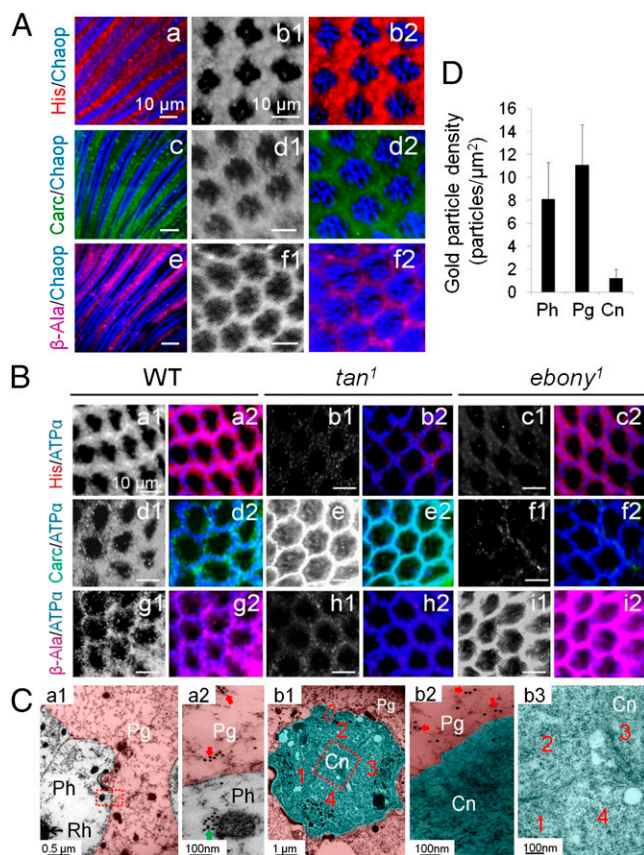
**Retinal Pigment Cells Contain High Levels of Histamine, Carcinine, and  $\beta$ -Alanine.** Previous work has shown that carcinine and  $\beta$ -alanine are present in both the retina and lamina (20). By immunostaining, we confirmed the presence of all three molecules (i.e., histamine, carcinine, and  $\beta$ -alanine) in the retina of wild-type flies (Fig. S1 B and C). The histamine signal was absent in a null HDC mutant (*hdc<sup>jk910</sup>*) (16) and diminished in a *tan<sup>1</sup>* mutant fly. Carcinine expression was dramatically reduced in both *hdc<sup>jk910</sup>* and *ebony<sup>1</sup>* mutants, whereas  $\beta$ -alanine levels were significantly lower in *tan<sup>1</sup>* and a *Su(r<sup>1</sup>);r<sup>c</sup>;b<sup>1</sup>* triple mutant (Fig. S1 B and C), which is deficient in two pathways of endogenous  $\beta$ -alanine synthesis (21, 22). Moreover, carcinine expression was increased in the retinal and distal laminar regions of *tan<sup>1</sup>* mutants, whereas  $\beta$ -alanine exhibited stronger staining in the whole visual system of *ebony<sup>1</sup>*.

We examined cellular localizations of these three molecules in the retina by colabeling with chaoptin, a cell adhesion protein that strongly labels the apical photosensitive microvilli of photoreceptors (the rhabdomere). When costained with chaoptin, the majority of histamine, carcinine, and  $\beta$ -alanine signals in the retina were visualized between chaoptin-labeled rhabdomere bundles (Fig. 1A). In cross-retina sections, all three molecules showed a honeycomb-like distribution that did not overlap with chaoptin. These staining patterns suggest that all three molecules reside in the basal part of photoreceptor cell bodies and/or in secondary pigment cells (Fig. 1A), which are a specialized glia type forming the lateral boundary of each ommatidium.

To determine whether histamine and its metabolites exist in pigment cells, we costained with the  $\alpha$  subunit of  $\text{Na}^+/\text{K}^+$  ATPase (ATP $\alpha$ ). ATP $\alpha$  resides in the pigment cell membrane and the basal membrane of photoreceptors, and thus can be used to mark the boundary of ommatidia. Any histamine/carcinine/ $\beta$ -alanine signal detected within the staining region of ATP $\alpha$  would be in pigment cells, whereas signals on the border or outside of the ATP $\alpha$  staining region are considered to be in photoreceptors. The results indicated that  $\beta$ -alanine was more highly present in pigment cells than in photoreceptors, which is consistent with a previous report (20). Importantly, both pigment cells and photoreceptors contained carcinine and histamine (Fig. 1B and Fig. S2). Next, we performed immunogold labeling of carcinine using a white-eye wild-type strain (*cn<sup>1</sup>, bw<sup>1</sup>*). EM revealed the presence of gold particles in pigment cells as well as in photoreceptor cell bodies (Fig. 1C and D), confirming the localization of carcinine in both cell types.

**Pigment Cells Accumulate Carcinine and  $\beta$ -Alanine in *tan<sup>1</sup>* and *ebony<sup>1</sup>* Mutants, Respectively.** To test whether the presence of histamine, carcinine, and  $\beta$ -alanine in pigment cells is relevant to the recycling of histamine, we costained these molecules with ATP $\alpha$  in the retina of *ebony<sup>1</sup>* and *tan<sup>1</sup>* mutants (Fig. 1B). In the retina of *tan<sup>1</sup>*, which is defective in the hydrolysis of carcinine, both histamine and  $\beta$ -alanine levels were reduced as expected, and the level of carcinine increased significantly. The carcinine level was approximately threefold higher than wild type, with the highest increase observed in the region of pigment cells (Fig. 1B and Fig. S2). In contrast, carcinine was only slightly increased in the lamina of this mutant (Fig. S1B), suggesting that pigment cells in the retina serve as a major storage site for excess carcinine in the fly visual system.

In the *ebony<sup>1</sup>* mutant, which is defective in carcinine synthesis, carcinine levels drastically decreased in both pigment cells and



**Fig. 1.** Carcinine and  $\beta$ -alanine are present in pigment cells and photoreceptor cell bodies of the retina. (A) Colabeling of histamine (a–b2), carcinine (c–d2), and  $\beta$ -alanine (e–f2) with chaoptin (blue) in the retina of WT flies. All three molecules are localized outside of chaoptin-labeled rhabdomeres, and their single-channel staining signals are shown in grayscale images b1, d1, and f1. Both lateral (a, c, and e) and cross-section (b, d, and f) views are presented. (B) Colabeling of histamine, carcinine, and  $\beta$ -alanine with ATP $\alpha$  (blue) in the retina of WT, *tan<sup>1</sup>*, and *ebony<sup>1</sup>* mutants. All three molecules exist in both photoreceptor and pigment cells. Grayscale images show single-channel signals of respective molecules. Note that *tan<sup>1</sup>* mutants accumulate carcinine but have reduced  $\beta$ -alanine level in pigment cells, whereas *ebony<sup>1</sup>* mutants show the opposite. Both mutants have reduced histamine level in the retina. (C) Immunogold labeling of carcinine in cross eye sections of *cn<sup>1</sup>, bw<sup>1</sup>* flies. Boxed areas in a1 and b1 are enlarged in a2, b2, and b3, which show the presence of immunogold particles (arrowheads) in both pigment (Pg, pink) and photoreceptor (Ph, gray) cells, but not in cone cells (Cn, green). (D) Quantification of immunogold particles in photoreceptor, pigment, and cone cells.

photoreceptors (Fig. 1B). Both cell types also exhibited a reduction in histamine level (Fig. 1B). In contrast, the level of  $\beta$ -alanine increased significantly in pigment cells of *ebony<sup>1</sup>* (Fig. 1B and Fig. S2). Thus, pigment cells serve as a repository for both  $\beta$ -alanine and carcinine in the visual system.

**The Gap-Junction Protein *Inx2* Functions in Laminar Glia.** Given that histamine is released from photoreceptors in the neuropil region of the lamina and is converted into carcinine in the epithelial glia, it is intriguing to observe a high level of carcinine in retinal pigment cells, which do not express *Ebony* and are separated from epithelial glia by fenestrated, pseudocapillary, and satellite glia. One explanation is that carcinine and its metabolites are transported between retina and lamina through membrane transporters (7) and within the laminar glia network through gap junctions (10).

Glia throughout lamina are connected into an intercellular network by gap junctions, which allow free diffusion of molecules

smaller than 2 kDa (23, 24). In *Drosophila*, gap junctions are assembled with innexin (Inx) proteins. Several innexins, such as *inx1* and *inx2*, are essential to the fly survival (25, 26). Because all available gap junction inhibitors are nonspecific and could kill the fly, we decided to identify and study innexin proteins in laminar glia using RNAi. We knocked down all eight innexins in glia using a pan-glia driver *repo-Gal4* (27), and found the knockdown (KD) fly of *Inx2* is embryonic lethal. This KD effect of *Inx2* was observed with two independent RNAi lines of distinct targeting sequences: JF02446 and KK111067. In contrast, when *Inx2* was knocked down in neurons using a neuronal driver *elav-Gal4*, we did not see any defect in either fly viability (Fig. 2A and Fig. S3) or visual signal transmission, as observed in electroretinogram (ERG) recordings (Fig. S4A and B). Moreover, the lethality observed in *Inx2* mutants was partially rescued by overexpressing a *UAS-Inx2-eGFP* transgene in glia using the *repo-Gal4* driver (Fig. 2A and Fig. S3). These rescued flies showed normal visual transmission (Fig. S4C and D), excluding a role of neuronal *Inx2* in visual transmission. Thus, *Inx2* functions in glia.

To test whether *Inx2* is expressed in the lamina, we stained wild-type fly brains using an *Inx2* antibody and observed strong *Inx2* expression in the layers of laminar pseudocartridge and satellite glia (Fig. 2B). The localization of *Inx2* in these glia was confirmed by colabeling of a membrane-associated GFP in flies that express a *UAS-CD8::GFP* transgene through *repo-Gal4* (Fig. 2C). In addition, epithelial glia that wrap photoreceptor axon bundles also contained a relatively low level of *Inx2* (Fig. 2D).

**Satellite Glial *Inx2* Is Required to Maintain the Carcinine Level in both the Retina and Lamina.** To test whether *Inx2* gap junctions mediate transport of carcinine from the lamina to retinal pigment cells, we knocked down *Inx2* specifically in satellite glia, which separate the laminar neuropil region from surface glia and retina. In the lamina, a *Gal4* line *mz0709-Gal4* (28) drives expression in satellite glia (Fig. S5). Expression of an *Inx2* RNAi line JF02446 using *mz0709-Gal4* did not cause obvious morphological change to satellite glia (Fig. S5A), excluding their dependence on *Inx2* for development or survival. Immunostaining of these *Inx2* KD flies, henceforth referred to as *Inx2<sup>RNAi</sup>* flies, revealed an overall reduction of carcinine signal in both the retina and lamina. In

wild type, carcinine was present at higher levels in marginal and distal laminar glia and retinal pigment cells (Fig. 3A and B). In *Inx2<sup>RNAi</sup>* flies, however, only a few (30% of cases) tiny patches of staining were observed in distal laminar glia. The intensity of staining dramatically decreased in all glia layers, including retinal pigment cells (Fig. 3A and B and Fig. S6A). Thus, *Inx2* expression in satellite glia is essential for maintaining the overall level of carcinine in the visual system.

In addition, we compared the relative staining intensities of carcinine in different laminar glia layers among wild type, *Inx2<sup>RNAi</sup>*, and flies defective in histamine metabolic enzymes, i.e., *hdc<sup>jk910</sup>*, *ebony<sup>1</sup>*, and *tan<sup>1</sup>* mutants (Fig. 3B). The result indicated that, in all layers of glia, the reduction of carcinine in *Inx2<sup>RNAi</sup>* flies was at least as severe as in the *ebony<sup>1</sup>* mutant.

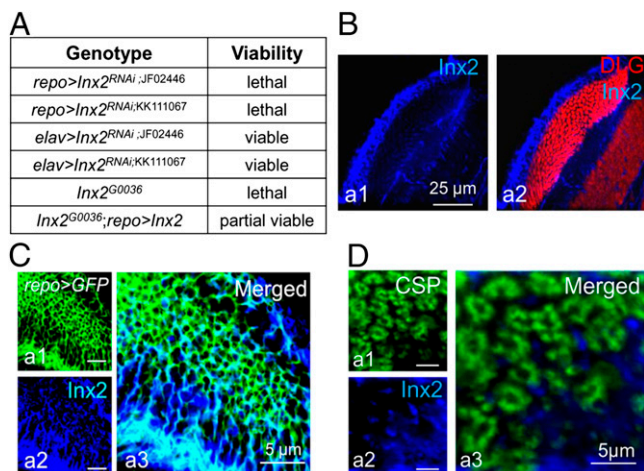
***Inx2* KD in Satellite Glia Caused  $\beta$ -Alanine Accumulation in the Retina and Changed the Histamine Level and Distribution.** The reduction of carcinine in laminar areas proximal to satellite glia, i.e., epithelial and marginal glia, suggests that *Inx2* KD may not only block the transport of carcinine but also affect its synthesis. One explanation is that *Inx2* KD in satellite glia prevents the transport of  $\beta$ -alanine from the retinal pool to laminar epithelial glia, the site of carcinine synthesis. We examined the level and distribution of  $\beta$ -alanine in *Inx2<sup>RNAi</sup>* flies and found that the  $\beta$ -alanine level was significantly increased in the retina (Fig. 3C and Fig. S6). Comparison of staining intensities also revealed a significant reduction of  $\beta$ -alanine level in laminar areas of epithelial and marginal glia (Fig. 3E). Taken together, these data suggest a role of *Inx2* in the transport of  $\beta$ -alanine from the retina to the lamina.

Because the carcinine synthesis in lamina is important for the visual system to maintain its histamine level, we next stained histamine in *Inx2<sup>RNAi</sup>* flies. As expected, the overall level of histamine in these flies was significantly lower than wild type, with drastic reductions seen in the retina and distal laminar glia (Fig. 3D and E and Fig. S6B). However, considerable accumulation of histamine was observed in regions of epithelial glia in  $\sim 55\%$  of *Inx2<sup>RNAi</sup>* flies (Fig. 3D), leading to a small but significant increase of histamine level in the laminar neuropil area containing epithelial and marginal glia (Fig. 3E).

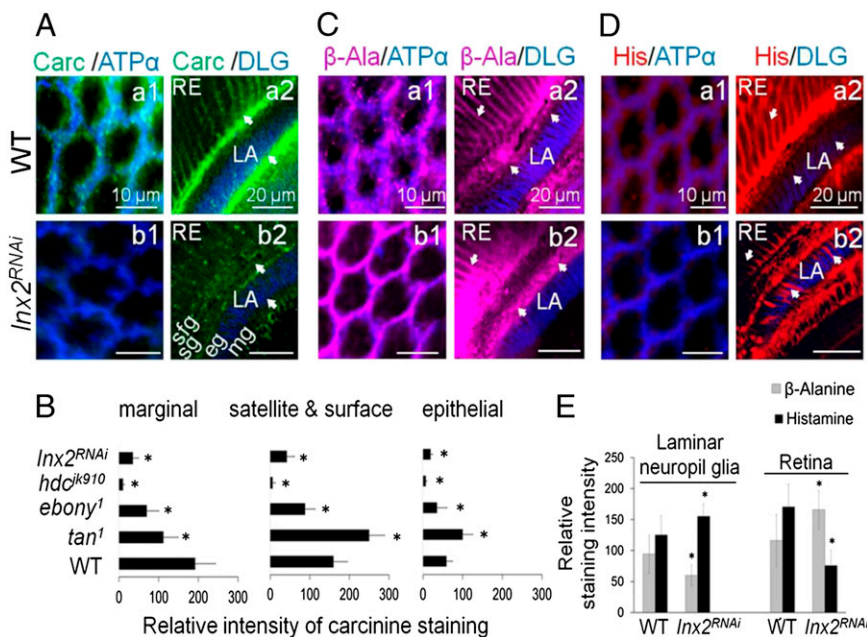
**Satellite Glial *Inx2* Is Essential for Visual Synaptic Transmission.** Because histaminergic vesicles of photoreceptor termini represent the majority of synaptic vesicles in the lamina, a decrease in the histamine levels may reduce the overall density of laminar synaptic vesicles. To test this probability, we stained fly laminae using an antibody against the synaptic vesicle marker cysteine string protein (CSP). In *Inx2<sup>RNAi</sup>* flies, CSP staining was significantly lower than in wild type (Fig. 4A and B and Fig. S7A). A similar reduction in CSP signal was observed in *tan<sup>1</sup>* and *hdc<sup>jk910</sup>* mutants (Fig. 4B and Fig. S7A), whereas *ebony<sup>1</sup>* mutants exhibited only a small, but still significant, reduction in CSP staining (Fig. 4B and Fig. S7A).

We also conducted EM to directly examine the density of vesicles in photoreceptor terminals. Under EM, *Inx2<sup>RNAi</sup>* flies had normal arrays of ommatidia (Fig. S8) and cartridges (Fig. S9) in the retina and lamina, respectively. In the lamina, fine structures such as capitate projections and T-bar synapses appeared normal in photoreceptor terminals (Fig. S9). However, photoreceptor terminals exhibited a reduced number of synaptic vesicles (Fig. 4C and Fig. S7B). We counted and calculated the density of vesicles in photoreceptor terminals (29), and compared densities of *Inx2<sup>RNAi</sup>*, wild type, and histamine metabolism enzyme mutants. The result indicated that the density of synaptic vesicles in *Inx2<sup>RNAi</sup>* flies was  $\sim 50\%$  of wild type (Fig. 4D), a larger reduction than that in *ebony<sup>1</sup>* mutants, which have previously been found deficient in vesicle number (30). The maximum loss of vesicles is observed in *hdc<sup>jk910</sup>* mutants, followed by *tan<sup>1</sup>*, *Inx2<sup>RNAi</sup>*, and *ebony<sup>1</sup>* flies (Fig. 4D and Fig. S7B).

To test whether disruption of the *Inx2*-mediated laminar glial network impairs synaptic transmission, we examined ON and OFF transients in ERG. ON and OFF transients represent the



**Fig. 2.** Gap-junction protein *Inx2* is present in laminar glia. (A) Viabilities of different *Inx2* KD, mutant, and rescue flies. (B) Immunolocalization of *Inx2* (blue; a1) and its colabeling (a2) with a photoreceptor terminal marker disc-large (DLG) (red) in WT lamina. (C) Costaining of glial GFP (green; a1) and *Inx2* (blue; a2) in the lamina of *repo-Gal4>UAS-mCD8::GFP* flies showing the localization of *Inx2* in laminar glia. (D) Immunolocalization of CSP (green, axon marker; a1) and *Inx2* (blue; a2) in the lamina of WT flies. The merged image (a3) shows localization of *Inx2* in epithelial glia.



**Fig. 3.** *Inx2* KD in satellite glia leads to reduction of visual carcinine, and accumulation of  $\beta$ -alanine and reduction of histamine in the retina. (A) Colabeling of carcinine with ATP $\alpha$  (blue; a1 and b1, in cross-retina sections) or DLG $\alpha$  (blue; a2 and b2, in both retina (RE) and lamina (LA)). Arrows point to areas of reduced carcinine level in *Inx2<sup>RNAi</sup>* flies. sfg, sg, eg, and mg indicate layers of surface, satellite, epithelial, and marginal glia. (B) Quantification of the staining intensity for carcinine in different laminar glia layers of WT, histamine metabolism mutants, and *Inx2<sup>RNAi</sup>* flies. (C and D) Colabeling of  $\beta$ -alanine and histamine with ATP $\alpha$  (blue; a1 and b1) or DLG $\alpha$  (blue; a2 and b2). Arrows point to areas where the respective molecule has changed level in *Inx2<sup>RNAi</sup>* flies. (E) Quantification of staining intensities for  $\beta$ -alanine and histamine in laminar neuropil glia and the retina of WT and *Inx2<sup>RNAi</sup>* flies (\* $P < 0.05$ , compared with WT, two-tailed *t* test,  $n = 10$ , for each quantification).

electrical activity of laminar LMC neurons at the onset and the end of light stimulation, respectively, and depend on the primary, histaminergic visual synaptic transmission (31, 32). A failure in either histamine release from photoreceptors or the reception of histamine by laminar neurons will cause loss of both transients. Though wild-type flies displayed normal ON and OFF transients in ERG recordings, no transients were visible in mutants of histamine metabolism enzymes, i.e., *tan<sup>1</sup>*, *ebony<sup>1</sup>*, and *hdc<sup>jk910</sup>* (Fig. S7C). Similarly, when *Inx2* was knocked down in satellite glia, both ON and OFF transients disappeared (Fig. 4E). Quantifications of ON and OFF transients showed that the phenotype in *Inx2<sup>RNAi</sup>* flies was as severe as in the enzyme mutants and as in *ort<sup>1</sup>*, a null mutant of the LMC histamine receptor (Fig. 4F). In contrast, normal ON and OFF transients were detected in *black<sup>1</sup>* and *Su(r<sup>1</sup>),r<sup>c</sup>;b<sup>1</sup>* mutants (Fig. 4F), which retain virtually normal levels of histamine. These results suggest that disruption of *Inx2* gap junctions in satellite glia impairs synaptic transmission from photoreceptor to LMCs, most likely due to the reduction in histamine level.

***Inx2* KD in Satellite Glia Altered Visual Alert Behavior.** To examine whether disruption of the *Inx2*-mediated satellite glial network causes any abnormalities in fly visual behavior, we designed an assay for the visual alert response (VAR). In this assay (Fig. 5A), 80% of wild-type flies responded to a moving block by immediately freezing (Movie S1), and 20% showed brief pauses (Fig. 5B). In contrast, a *norPA<sup>7</sup>* mutant that has no visual transduction in photoreceptors (33), and thus served as a blind control, showed 90% no response. These flies continued to move up and pass the block. When *Inx2* was knocked down in satellite glia, 55% of flies had no response (Movie S2), 30% showed a delayed response of 0.5~1 s, and 15% responded with only a very brief pause (Fig. 5B). Thus, disruption of *Inx2* function in satellite glia alters visual behavior in *Drosophila*.

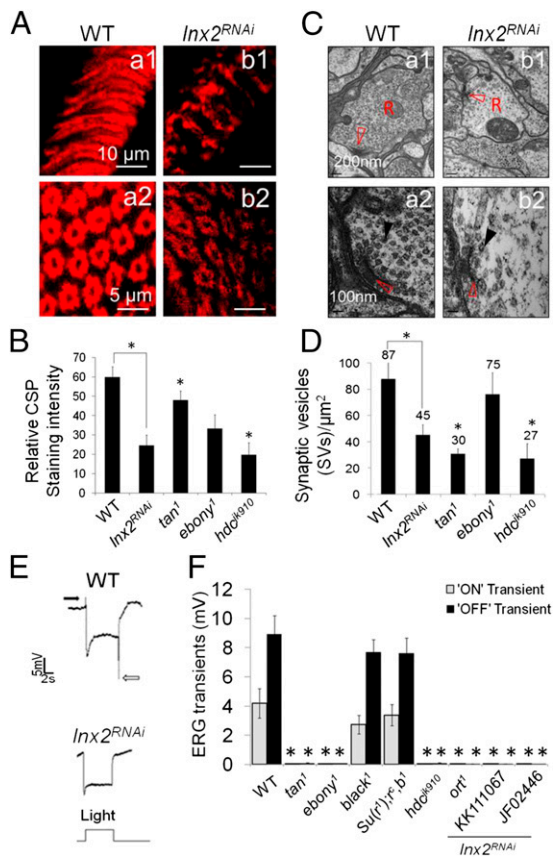
For comparison, we examined the VAR in flies with mutations in histamine metabolic enzymes. Sixty percent of *hdc<sup>jk910</sup>* mutants showed no response; 25% exhibited a delayed response, and a brief-pause response was observed in 15% of flies. Some 65% of *tan<sup>1</sup>* mutants displayed no response, and a delayed response 25% of the time, whereas 40% of *ebony<sup>1</sup>* mutants exhibited no response, 20% showed a delayed response, and 24% showed a brief-pause response (Fig. 5B). With a relatively high level of histamine (Fig. S1B), *Su(r<sup>1</sup>),r<sup>c</sup>;b<sup>1</sup>* mutants showed wild type-like freezing responses in 60% of flies and was delayed responsive

in only 20% of flies. Together, these data suggest that the reduction in histamine levels correlates with impaired VARs in flies.

## Discussion

During light stimulation, fly photoreceptors constantly release histamine to hyperpolarize LMC neurons in the lamina neuropil. It is estimated that without recycling, vesicular histamine in photoreceptor termini would be depleted within approximately 10 s (30). To sustain the tonic visual transmission, the fly depends on Ebony in epithelial glia and Tan in photoreceptors to inactivate and regenerate histamine, respectively (5). This recycling process was previously assumed to occur locally between epithelial glia and photoreceptor axons (9, 18). Capitate projection, a special membrane invagination of epithelial glia formed within photoreceptor axons (14, 34), has been hypothesized to mediate uptake of carcinine/histamine into photoreceptors in addition to its potential function in synaptic vesicle recovery (35).

Our observations here, however, suggest that the *Inx2*-dependent laminar glial network and retinal pigment cells together mediate an additional, long-distance mechanism of histamine recycling that is essential to fly vision. In this novel pathway (Fig. 5C), carcinine synthesized in epithelial glia is transported across laminar glial network to retinal pigment cells, and then delivered into cell bodies of photoreceptor in the retina, where it is hydrolyzed into histamine and  $\beta$ -alanine by Tan. Though the regenerated histamine diffuses to the photoreceptor axon for vesicle reloading,  $\beta$ -alanine is sent through pigment cells and distal laminar glia back to epithelial glia for new carcinine synthesis. This model is collectively supported by observations in this work and from previous findings. First, both distal laminar glia and retinal pigment cells contain high levels of carcinine. Second, pigment cells, but not epithelial glia, accumulate carcinine upon mutation of the photoreceptor enzyme Tan, suggesting an obstructed transport of carcinine from pigment cells to the photoreceptor cell body. Third, compared with the axon, the cell body of photoreceptor contains a larger amount of Tan protein (36) and thus could be the major site of histamine regeneration. Fourth,  $\beta$ -alanine is concentrated in pigment cells (20). This pool of  $\beta$ -alanine is further increased when Ebony activity is impaired and thus may represent a major source of  $\beta$ -alanine for carcinine synthesis. Fifth, when the gap-junction protein *Inx2* is knocked down in satellite glia,  $\beta$ -alanine is accumulated in the retina and the level of carcinine is reduced drastically, which suggests that  $\beta$ -alanine trafficking across distal laminar glia is essential for



**Fig. 4.** Satellite glial *Inx2* is essential for fly visual synaptic transmission. (A) CSP staining signal is reduced in the lamina of *Inx2<sup>RNAi</sup>* flies. (a1–b1 and a2–b2) Lateral and cross-views of lamina, respectively. (B) Quantification of laminar CSP immunostaining intensities. (C) EM images of photoreceptor terminals in the lamina. The red arrowhead-marked T-bar synapses in a1 and b1 are magnified in images a2 and b2, respectively. Black arrowheads point to synaptic vesicles. (D) Quantification of photoreceptor synaptic vesicles at the EM level (\* $P < 0.05$ , compared with WT, two-tailed  $t$  test,  $n = 10$ , for each quantification). (E) *Inx2<sup>RNAi</sup>* flies lost both ON (black arrow) and OFF transients (white arrow) of ERG that represent histaminergic synaptic transmission activities. (F) Comparison of ON and OFF transient amplitudes among WT, *Inx2<sup>RNAi</sup>* flies, and histamine metabolism mutants. Two independent *Inx2* RNAi lines were tested (\* $P < 0.001$ , compared with WT, two-tailed  $t$  test,  $n = 20$ , for each quantification).

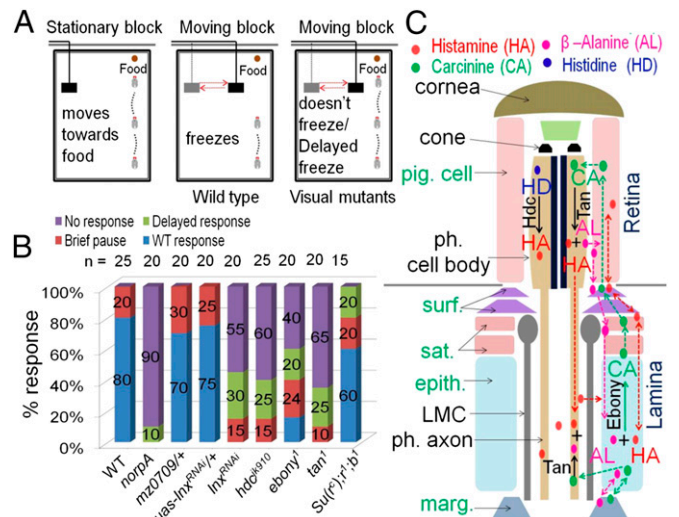
carcinine synthesis in epithelial glia. Finally, although histamine appears to accumulate in epithelial glia of *Inx2<sup>RNAi</sup>* flies, the density of histaminergic vesicles in photoreceptor terminals is much lower than wild type, suggesting that local and direct recycling mechanisms are insufficient to maintain the level of vesicular histamine.

*Inx2*, a functional analog of mammalian gap-junction protein connexins (24), may mediate transport of  $\beta$ -alanine, carcinine, and histamine within the laminar glial network. KD of *Inx2* in satellite glia causes  $\beta$ -alanine to accumulate in retinal and distal laminar glia, suggesting that *Inx2* is required to transport  $\beta$ -alanine across satellite glia. Because the low level of carcinine in *Inx2<sup>RNAi</sup>* flies can be explained by limited carcinine synthesis in the absence of laminar  $\beta$ -alanine, direct evidence for the *Inx2* mediation of carcinine trafficking is lacking. However, based on the molecular size of carcinine (molecular weight 182.2), it is plausible that this small dipeptide may diffuse through *Inx2* gap junctions in wild-type flies. The accumulation of histamine in epithelial glia of *Inx2<sup>RNAi</sup>* flies, but not in *ebony<sup>1</sup>* mutants, suggests that expression of *Inx2* in satellite glia is required to transport histamine from epithelial glia to distal laminar glia and retina, two potential storage sites of histamine.

A variety of membrane transporters may participate in the process of histamine recycling. The *Drosophila* vesicular monoamine transporter B is required to store histamine or carcinine in subretinal fenestrated glia and to maintain the overall level of visual histamine (37). ATP binding cassette transporters encoded by genes *white*, *brown*, and *scarlet* in pigment cells appeared to modulate the level of visual histamine, with the mechanisms unknown (5, 38). In addition, a transporter encoded by the gene *inebriated* is proposed to clear carcinine from the extracellular space of laminar neuropil (39); however, its carcinine transporting activity has yet to be demonstrated directly (40). Identification and localization of a carcinine transporter at either the cell body or the axon part of photoreceptors will reveal the site of carcinine delivery and help to confirm the long-distance trafficking and/or neuropil-confined shuttling of carcinine, respectively.

Glial network-mediated long-distance recycling of neurotransmitters may have multiple advantages over local recycling. First, delivery of neurotransmitter metabolites to a proximal part of the neuron may avoid interference with the fusion and retrieval of synaptic vesicles at the axon terminal. Second, the large capacity of glial networks may help to store neurotransmitters and metabolites, thereby regulating the transmitter level in neurons and preparing them for varied signaling intensities. Third, the intercellular glial network may redistribute neurotransmitters among parallel projection neurons to balance strengths of synaptic transmission.

Mammalian glial cells, such as astrocytes, also connect into gap-junction networks, which are known to mediate nutrient transport and ion buffering (10). It is plausible that glial networks in mammals may recycle neurotransmitters in a long-distance manner, similar to the fly visual system. This idea is supported by the following observations: first, disconnected



**Fig. 5.** *Inx2* KD in satellite glia alters fly visual alert response due to blockage in histamine recycling through a visual glia network. (A) Illustration of VAR behavior assay. In stationary condition of the block, fly exhibits antigeotaxis and climbs up for food in a vertical chamber. In moving condition of the block, wild-type fly freezes at a certain distance after seeing the block moving toward it. A visual defective fly, however, is unable to see the moving block from the same distance as WT and shows no response, delayed freezing, or brief pause response. (B) VARs of WT, *norPA* (blind), parent controls (*mz0709-Gal4/+* and *UAS-Inx2<sup>RNAi</sup>/+*), *Inx2<sup>RNAi</sup>* flies, and histamine recycling-defective mutants. The number within colored bars represents percentage of flies showing the respective type of response. (C) Model showing that retinal pigment cells and the laminar glial network of *Drosophila* is actively involved in storage and recycling of histamine. After histamine is converted into carcinine by epithelial glia, carcinine moves through the laminar glial network to reach pigment (pig.) cells and enters the cell bodies of photoreceptor (ph.).  $\beta$ -alanine, however, moves in the opposite direction to reach epithelial glia.

hippocampal astrocytes in connexin knockout mice are unable to clear glutamate from extracellular spaces (11); second, gap junctions in astrocytes are permeable to neurotransmitters such as glutamate and ATP (41, 42); third, sodium-dependent amino acid transporter (SNAT)7, a transporter for the glutamate metabolite glutamine, is concentrated in neuronal cell bodies in mouse cortex and spinal cord (43), suggesting the involvement of neuronal cell bodies in the recycling of glutamate; fourth, a rat glycine transporter GlyT1 is found in the cell body and dendrites of hippocampal pyramidal neurons and in the cell body of retinal amacrine neurons (44). Confirming the involvement of mammalian glial networks in neurotransmitter recycling will not only advance our knowledge of glial physiology but also help us to understand the pathology of glia-involved neurological diseases.

## Methods

**Fly Stocks.** Fly lines Canton-S (as WT), *repo-Gal4*, *elav-Gal4*, *tan<sup>1</sup>*, *ebony<sup>1</sup>*, *hdc<sup>JK910</sup>*, *Su(r<sup>1</sup>),r<sup>c</sup>;b<sup>1</sup>*, *ort<sup>1</sup>*, *black<sup>1</sup>*, *pyd<sup>1</sup>*, and *norpA<sup>2</sup>* were obtained from the

Bloomington Drosophila Stock Centre. The *mz0709-Gal4* flies were obtained from the Freeman laboratory at the University of Massachusetts Medical School, and *UAS-Inx2-eGFP* flies and *Inx2* antibody were received from Reinhard Bauer (University of Bonn, Bonn, Germany). The two *UAS-Inx2* RNAi lines were from the Vienna Drosophila RNAi Center and the Transgenic RNAi Project.

**VAR Behavior Assay.** VAR assay was performed by challenging a fly with a moving block (0.4 cm) in a vertical chamber. As the fly reached the middle of the chamber, the block was moved from left to right across the fly visual field and its response was recorded with a microscope digital camera for analysis (Movie S1). See *SI Methods* for details.

**ACKNOWLEDGMENTS.** We are grateful to Peiyi Guo, Zhuo Luan, and Caitlin Quigley for critical comments on the manuscript. We thank the Bloomington Drosophila Stock Centre, the Vienna Drosophila RNAi Center, the Transgenic RNAi Project, and the Developmental Studies Hybridoma Bank for fly stocks and antibodies, and we are especially thankful to Drs. R. Bauer and M. Freeman for sharing flies and reagents. This work was supported by National Institutes of Health Grants R01AG022508 and R01EY021796.

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