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

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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 primary sensory neurons. Subsequently, the released histamine is taken up by perisynaptic epithelial glia and converted into inactive carcine through conjugation with β-alanine for transport. In contrast to a previous assumption that epithelial glia deliver carcine directly back to photoreceptor axons for histamine regeneration within the lamina, we detected both carcine and β-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 β-alanine accumulation in retinal pigment cells and impairs carcine 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 to 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.

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 glial 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-β-alanyl-biogenic amine synthetase Ebony conjugates histamine to β-alanine to form inactive carcine for storage and transport (15). Carcinine is then transported back to photoreceptors via an unknown mechanism, where it is hydrolyzed into histamine and β-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

I n 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.

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.

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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 β-alanine in both wild-type and ge-
etically 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
β-Alanine. Previous work has shown that carcinine and β-alanine
are present in both the retina and lamina (20). By immunostaining,
we confirmed the presence of all three molecules (i.e., his-
tamine, carcinine, and β-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>jk210</sup>) (16) and diminished in a tan<sup>1</sup> mutant fly. Car-
carine expression was dramatically reduced in both hdc<sup>jk210</sup>
and ebony<sup>1</sup> mutants, whereas β-alanine levels were significantly
lower in tan<sup>1</sup> and a Stu<sup>r</sup>,<sup>t</sup>;<sup>b</sup> triple mutant (Fig. S1 B and C),
which is deficient in two pathways of endogenous β-alanine
synthesis (21, 22). Moreover, carcinine expression was increased
in the retinal and distal laminar regions of tan<sup>1</sup> mutants, whereas
β-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 pho-
toreceptors (the rhabdomere). When costained with chaoptin,
the majority of histamine, carcinine, and β-alanine signals in the
retina were visualized between chaoptin-labeled rhabdomere
bundles (Fig. L4). 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 basolateral part of photoreceptor cell bodies and/or in
second-order pigment cells (Fig. L4), 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 α subunit of Na<sub>a</sub>+/K<sup>+</sup>
ATPase (ATPα). ATPα resides in the pigment cell membrane and
the basolateral membrane of photoreceptors, and thus can be used
how the boundary of ommatidia. Any histamine/carcinine/β-alanine
signal detected within the staining region of ATPα would be in
pigment cells, whereas signals on the border or outside of the
ATPα staining region are considered to be in photoreceptors.
We first examined that β-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. 1 B
and Fig. S2). Next, we performed immunogold labeling of carci-
nine 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. 1 C and D), confirming the lo-
calization of carcinine in both cell types.

Photopigment Cells Accumulate Carcinine and β-Alanine in tan<sup>1</sup>
and ebony<sup>1</sup> Mutants, Respectively. To test whether the presence of histamine,
carcinine, and β-alanine in pigment cells is relevant to the
recycling of histamine, we costained these molecules with ATPα
in the retina of ebony<sup>1</sup> and tan<sup>1</sup> mutants (Fig. 1 B). In the retina of
tan<sup>1</sup>, which is defective in the hydrolysis of carcinine, both hist-
amine and β-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. 1 B and Fig.
S1 B). In contrast, carcinine was only slightly increased in the
lamina of this mutant (Fig. S1 B), 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
photoreceptors (Fig. 1 B). Both cell types also exhibited a re-
duction in histamine level (Fig. 1 B). In contrast, the level of
β-alanine increased significantly in pigment cells of ebony<sup>1</sup> (Fig.
1 B and Fig. S2). Thus, pigment cells serve as a repository for
both β-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, pseudocartridge, 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-glial 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 electoretinogram (ERG) recordings (Fig. S4 A 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. S4 C 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-Cas9::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 Gial 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 Inx2RNAi 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. 3 A and B). In Inx2RNAi 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. 3 A and B and Fig. S6D). 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, Inx2RNAi, and flies defective in histamine metabolic enzymes, i.e., hdcRNAi, ebony1, and tan1 mutants (Fig. 3B). The result indicated that, in all layers of glia, the reduction of carcinine in Inx2RNAi flies was at least as severe as in the ebony1 mutant.

Inx2 KD in Satellite Glia Caused β-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 β-alanine from the retinal pool to laminar epithelial glia, the site of carcinine synthesis. We examined the levels and distribution of β-alanine in Inx2RNAi flies and found that the β-alanine level was significantly increased in the retina (Fig. 3C and Fig. S6A). Comparison of staining intensities of β-alanine in (29) and Inx2RNAi mutant was partially rescued by hdcRNAi, which indicates a significant reduction of β-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 β-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 Inx2RNAi 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. 3 D and E and Fig. S6B). However, considerable accumulation of histamine was observed in regions of epithelial glia in ∼55% of Inx2RNAi 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 Gial 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 lamina using an antibody against the synaptic vesicle marker cysteine string protein (CSP). In Inx2RNAi flies, CSP staining was significantly lower than that in wild type (Fig. 4 A and B and Fig. S7A). A similar reduction in CSP signal was observed in tan1 and hdcRNAi mutants (Fig. 4B and Fig. S7A), whereas ebony1 mutant 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, Inx2RNAi flies had normal arrays of ommatidia (Fig. S8) and cuticles (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 Inx2RNAi, wild type, and histamine metabolism enzyme mutants. The result indicated that the density of synaptic vesicles in Inx2RNAi flies was ∼50% of wild type (Fig. 4D), a larger reduction than that in ebony1 mutants, which have previously been found deficient in vesicle number (30). The maximum loss of vesicles is observed in hdcRNAi, tan1, Inx2RNAi, and ebony1 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
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, ebony, and hdc (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 of Inx2KD flies was as severe as in the enzyme mutants and as in or1, a null mutant of the LMC histamine receptor (Fig. 4F). In contrast, normal ON and OFF transients were detected in black and Su(r),r,b; b 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 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 hdcRNAi mutants showed no response; 25% exhibited a delayed response, and a brief-pause response was observed in 15% of flies. Some 65% of tan mutants displayed no response, and a delayed response 25% of the time, whereas 40% of ebony 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),r;b mutants showed wild-type 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 carcine/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), carcine 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 β-alanine by Tan. Though the regenerated histamine diffuses to the photoreceptor axon for vesicle reloading, β-alanine is sent through pigment cells and distal laminar glia back to epithelial glia for new carcine 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 carcine. Second, pigment cells, but not epithelial glia, accumulate carcine upon mutation of the photoreceptor enzyme Tan, suggesting an obstructed transport of carcine 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, β-alanine is concentrated in pigment cells (20). This pool of β-alanine is further increased when Ebony activity is impaired and thus may represent a major source of β-alanine for carcine synthesis. Fifth, when the gap-junction protein Inx2 is knocked down in satellite glia, β-alanine is accumulated in the retina and the level of carcine is reduced drastically, which suggests that β-alanine trafficking across distal laminar glia is essential for...
carcinine synthesis in epithelial glia. Finally, although histamine appears to accumulate in epithelial glia of Inx2RNAi 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 β-alanine, carcinine, and histamine within the laminar glial network. KD of Inx2 in satellite glia causes β-alanine to accumulate in retinal and distal laminar glia, suggesting that Inx2 is required to transport β-alanine across satellite glia. Because the low level of carcinine in Inx2RNAi flies can be explained by limited carcinine synthesis in the absence of laminar β-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 Inx2RNAi flies, but not in ebony 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 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 shunting 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

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**Fig. 4.** Satellite glial Inx2 is essential for fly visual synaptic transmission. (A) CSP staining signal is reduced in the lamina of Inx2RNAi 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) Inx2RNAi 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, Inx2RNAi, 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).

**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 flies freeze 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-Inx2RNAi/+), Inx2 RNAi 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.) β-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), 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 pyramid 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+, ebony+, hdc<sup>+</sup>, Su(var)<sup>+</sup>, brk+, orchid+, and norpA were obtained from the Bloomington Drosophila Stock Centre. The m20709-Gal4 flies were obtained from the Freeman laboratory at the University of Massachusetts Medical School, and UAS-SNAT-QFP 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.

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