LOVIT is a putative vesicular histamine transporter required in *Drosophila* for vision.

**Graphical Abstract**

**Highlights**
- LOVIT is a photoreceptor synaptic-vesicle-enriched transporter
- LOVIT is required for histamine concentration in synaptic vesicles
- Loss of LOVIT in *Drosophila* photoreceptors impairs visual transmission

**Authors**
Ying Xu, Tao Wang

**Correspondence**
wangtao1006@nibs.ac.cn

**In Brief**
Xu et al. identify a member of the SLC45 transporter family, LOVIT, expressed in photoreceptor synaptic vesicles. The authors further demonstrate that LOVIT is required for maintaining the synaptic vesicular concentration of histamine at the *Drosophila* photoreceptor terminal and supports the high rates of histamine release in fly visual neurotransmission.
LOVIT Is a Putative Vesicular Histamine Transporter Required in Drosophila for Vision

Ying Xu1,3 and Tao Wang1,2,4,*
1National Institute of Biological Sciences, Beijing, China
2Tsinghua Institute of Multidisciplinary Biomedical Research, Tsinghua University, Beijing, China
3Department of Surgery, University of Michigan, Ann Arbor, MI, USA
4Lead Contact
*Correspondence: wangtao1006@nibs.ac.cn
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SUMMARY

Classical fast neurotransmitters are loaded into synaptic vesicles and concentrated by the action of a specific vesicular transporter before being released from the presynaptic neuron. In Drosophila, histamine is distributed mainly in photoreceptors, where it serves as the main neurotransmitter for visual input. In a targeted RNAi screen for neurotransmitter transporters involved in concentrating photoreceptor synaptic histamine, we identified an SLC45 transporter protein, LOVIT (loss of visual transmission). LOVIT is prominently expressed in photoreceptor synaptic vesicles and is required for Drosophila visual neurotransmission. Null mutations of lovit severely reduced the concentration of histamine in photoreceptor terminals. These results demonstrate a LOVIT-dependent mechanism, maintaining the synaptic concentration of histamine, and provide evidence for a histamine vesicular transporter besides the vesicular monoamine transporter (VMAT) family.

INTRODUCTION

Histamine was generally accepted as a neurotransmitter in 1984, when histamine was first identified to be located in tuberomamillary nucleus (TM) of the brain (Watanabe et al., 1984). In mammals, histaminergic neurons play an important role in regulating multiple physiological activities, including sleep, synaptic plasticity, and feeding behaviors (Bekkers, 1993; Huang et al., 2001; Parmentier et al., 2002; Vorobjev et al., 1993). Deregulation of histaminergic neurotransmission can lead to several neurologic disorders, such as schizophrenia and multiple sclerosis (Haas and Panula, 2003). In Drosophila, VMAT is absent in photoreceptor cells, which use histamine as their primary neurotransmitter, leading us to speculate that an unknown transporter is responsible for the vesicular transport of histamine (Romero-Caldérón et al., 2008). Here, we demonstrated that a previously uncharacterized Drosophila gene CG45782, called lovit, encodes a photoreceptor-specific vesicular transporter responsible for concentrating histamine at synapses and that its activity is required to transmit visual signals.

RESULTS

Knockdown of CG45782 in Photoreceptors Disrupts Visual Transmission

Drosophila is estimated to have 603 distinct transmembrane transporters (Ren et al., 2007). Because Drosophila’s principle histaminergic neurons are located in the heads, the total mRNA expression profiles from a previous study comparing the heads and torsos of wild-type flies have been used to identify 46 head-enriched transporters (Xu and Wang, 2016). These were considered as candidates involved in the loading of histamine into synaptic vesicles. To examine whether these transporters were involved in the vesicular transport of histamine, the corresponding candidate genes were knocked down individually, using the eye-specific expression of RNAi driven by GMR (glass multiple response element)-Gal4 or using available loss-of-function alleles. In a wild-type paradigm, activity in L1 and L2, two neurons both postsynaptic to photoreceptors, was monitored as ON and OFF transients of the electroretinogram (ERG) at the onset and cessation of light stimulation (Alawi and Pak, 1971; Heisenberg, 1971). Mutant flies with defective synaptic transmission had obvious reductions in their ON and OFF transients. An uncharacterized transporter gene, CG45782, was identified...
The ERG transients were additionally disrupted by expressing CG45782 RNAi in neurons using elav-Gal4 but were not affected by specific knockdown of CG45782 in glia using repo-Gal4, confirming the specific role of CG45782 in neurons rather than glia (Figures 1B and 1C). Phototaxis is a visual behavior that requires the integrity of the visual transmission (Behnia and Desplan, 2015). Consistent with the ERG results, knockdown of CG45782 either in the retina or in neurons impaired phototaxis, whereas wild-type phototaxis was observed in flies with knockdown of CG45782 in glia (Figure 1D). Through protein sequence alignment, we found that CG45782 belonged to the slc45 gene family, which has been reported to consist of proton-associated transporters (Vitavska and Wieczorek, 2013). Among them, SLC45A2 shared the highest protein identity with CG45782, which we named LOVIT (loss of visual transmission) based on the ERG phenotype (Figure S1A).
Photoreceptor-Specific Expression of LOVIT Is Sufficient to Restore Vision

To confirm the results above, rescue experiments were conducted in which the CG45782RNAi1-resistant lovit cDNA transgene with six synonymous nucleotide replacements in the short hairpin RNA (shRNA) target region was expressed either in pigment cells or in photoreceptors. Expressing lovit in photoreceptors using the ninaE (neither inactivation nor after potential) promoter restored ON and OFF transients in GMR > CG45782RNAi1 flies, whereas pigment-cell-specific expression of lovit (rhdB-lovit) failed to rescue the loss of ON and OFF transients (Figure 1E). Furthermore, the loss of phototactic behavior in GMR > CG45782RNAi1 was significantly rescued by ninaE-lovit, but not by rhdB-lovit (Figure 1F).

To further confirm lovit is the causal gene, a null allele of lovit was generated through the CRISPR/Cas9 system (Ren et al., 2013; Figures S1B and S1C). lovit1 flies lost ON and OFF transients and exhibited significantly reduced phototaxis (Figures 1G and 1H), consistent with the phenotypes of photoreceptor-specific knockdown of lovit. Homozygous lovit-null mutants were viable and fertile and showed normal morphology of both soma and axon terminal of photoreceptors on transmission electron microscopy (TEM) (Figure S2).

LOVIT Is Enriched in the Fly’s Main Histaminergic Neurons, the Photoreceptors, and Located on Synaptic Vesicles

To determine the spatial expression pattern and subcellular localizations of LOVIT, an antibody against a LOVIT-specific peptide was raised (Figures S3A and S3B). The endogenous LOVIT signal was highly enriched in the lamina, containing the terminals of R1–R6 photoreceptors, and medulla, containing the terminals of R7 and R8 and co-localized with photoreceptor cell marker 24B10 to the medulla (Figures 2A and 2B). Cross-sections of the lamina neuropil, containing terminals of R1–R6, showed that the LOVIT signal was surrounded by the glial marker Ebony and co-localized with synaptic vesicle marker CSP (Figures 2C and 2D). Besides synaptic vesicles, mitochondria, photoreceptor cell membrane, and glial capitate projection were the main membrane structures within
photoreceptor terminals (Rahman et al., 2012). We also checked the co-localization between LOVIT and mitochondria marker CoVI or photoreceptor cell membrane marker Na K-ATPase, and no merged signals were observed (Figures 2D and 2E). These results indicated that LOVIT expressed specifically throughout the R1–R8 photoreceptor neurons and is mainly present in their synaptic vesicles.

The subcellular localization of LOVIT in TEM sections was also determined using horseradish peroxidase-3,3'-diaminobenzidine (HRP-DAB) staining (Rahman et al., 2012). Given the low protein level of LOVIT in vivo, ninaE-lovit-mCherry transgenic flies were used to conduct this experiment. The chimeric protein is functional as endogenous LOVIT protein (Figures S3C–S3E). The electron-dense DAB signal was visualized as small puncta, which filled the terminals, in a typical distribution of synaptic vesicles (Figure S4). Together, these data showed that transporter LOVIT was specifically localized to photoreceptor synaptic vesicles.

**Histamine Fails to Concentrate in lovit Mutant Photoreceptor Terminals**

To further characterize LOVIT function in histamine signaling, a reported antibody was used to label histamine in lovit1 flies (Chaturvedi et al., 2014). In control flies, histamine immunolabeling was exclusively enriched in photoreceptor terminals in both the lamina and distal medulla, co-localized with the
Histamine functions as a key neurotransmitter in multiple circuits to control various behaviors. In *Drosophila* photoreceptor, histamine is produced *de novo* by histidine decarboxylase (Burg et al., 1993); meanwhile, maintaining normal histamine content also depends on the histamine recycling pathway (Borycz et al., 2002; Chaturvedi et al., 2016; Stenesen et al., 2015; Xu et al., 2015). In both pathways, loading histamine into synaptic vesicles is critical for histaminergic neurotransmission (Figure 4). The absence of VMAT in some histaminergic neurons, including fly photoreceptors, suggests the existence of VMAT-independent vesicular transport of histamine. Here, we found a putative vesicular histamine transporter LOVIT in photoreceptor synaptic vesicles, providing the first evidence that a vesicular monoamine transporter other than VMAT protein may exist. It is speculated that other species may use a similar transporter to regulate the location of monoamines.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - *Drosophila* stocks
AUTHOR CONTRIBUTIONS

Y.X. and T.W. designed the experiments. Y.X. performed the experiments. Y.X. and T.W. analyzed and interpreted the data. Y.X. and T.W. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

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EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila stocks
The following Drosophila stocks were obtained from the Bloomington Stock Center: (1) 61340, y[1] v[1]; P(y[+t7.7] v[+t1.8] = TRiP.HMJ23232)attP40; (2) 38367, y[1] sc[*] v[1]; P(y[+t7.7] v[+t1.8] = TRiP.HMS01836)attP2; (3) 40876, y[1] sc[*] v[1]; P(y[+t7.7] v[+t1.8] = TRiP.HMS02043)attP40; (4) 3605, w1118; and (5) 24749, M(vas-int.Dm)ZH-2A;M(3xP3-RFP.attP)ZH-86Fb. The (nos-Cas9)attP2 flies were obtained from the lab of Dr. J. Ni at Tsinghua University, Beijing, China. The carT1, ebonyKO, GMR-Gal4, elav-Gal4 and repo-Gal4 flies were maintained in the lab of Dr. T. Wang at the National Institute of Biological Sciences, Beijing, China. Flies were maintained in 12-h-light–12-h-dark cycles with 2000 lux illumination at 21°C, except when mentioned differently in the text.

METHOD DETAILS

Generation of plasmid constructs and transgenic flies
The lovit cDNA sequence was amplified from cDNA clone GH10292 obtained from DGRC. To construct pninaE-lovit and prdhB-lovit, the entire coding region of lovit was amplified from cDNA clones, and the CG45782RNAi1 shRNA target region (50-TGGCTAGGAGATGATAATTTA-30) was replaced with six synonymous mutations (50-TGGCTTGGTGACGACAACTTG-30). Finally, CG45782RNAi1-resistant lovit was cloned into the pninaE-attB and prdhB vectors (Xu et al., 2015). To construct pninaE-lovit-mCherry-attB, the entire CDS sequence excluding the stop codon from ninaE-lovit, was cloned into the pIB-cmCherry vector, and the lovit-mCherry sequence was subcloned into the pninaE-attB vector. These constructs were injected into M(vas-int.Dm)ZH-2A;M(3xP3-RFP.attP)ZH-86Fb or w1118 embryos, and transformants were identified based on eye color. The (3xP3-RFP.attP) locus was removed by crossing with P(Cre) flies.

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The lovit1 flies were generated using the Cas9/sgRNA system. The recognition sequence of guiding RNA (sgRNA-sense: 50-TTCGTGAAGCTTATCCATTGCTGT-30 and sgRNA-antisense: 50-AAACACAGCAATGGATAAGCTTCA-30) was designed with tools available at https://www.flyrnai.org/crispr/. The recognition sequence was cloned into the U6b-sgRNA-short vector, and then the U6b-sgRNA sequence was amplified and cloned into paust-attB using HindIII and EcoRI, which removed the UAS sequence. The pU6b-gRNA-attB vector was injected into M(vas-int.Dm)ZH-2A;M(3xP3-RFP.attP)ZH-86Fb embryos. The pU6b-gRNA-attB transgenic fly was crossed with (nos-Cas9)attP2, and then a single cross between the F1 flies and the TM6/MKRS flies was undertaken. PCR sequencing of the lovit locus of F2 flies was used to identify flies with lovit deletions.

Generation of anti-LOVIT antibody
A LOVIT peptide VFKGDPKATLGSLPQKRYEEGVC was synthesized by ChinaPeptides (Soochow, China), linked with BSA and injected into rats by the Antibody Center at NIBS to generate anti-LOVIT antibodies. The animal work for generating the antisera was conducted following the National Guidelines for Housing and Care of Laboratory Animals in China, and performed in accordance with institutional regulations after approval by the IACUC at NIBS (Reference# NIBS2016R0001).

Electroretinogram recordings
Two glass microelectrodes filled with Ringer’s saline were inserted into small drops of electrode cream and placed on the surfaces of the compound eye and thorax. A Newport light projector (model 765) was used for stimulation. The source light intensity was

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~0.3 mW/cm², and the wavelength was ~550nm (the source light was filtered by FSR-OG550 filter). ERG signals were amplified with a Warner electrometer IE-210 and recorded with a MacLab/4 s A/D converter and the Clampex 10.2 program (Warner Instruments, Hamden, USA). All recordings were carried out at 21°C.

**Immunohistochemistry**

Whole-mount immunolabeling was performed to locate endogenous LOVIT in adult flies. Fly heads were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 2h at 4°C, and the heads labeled with the following primary antibodies: mouse anti-24B10 (1:100, DSHB), rat anti-RFP (1:200, Chromotek, Martinsried, Germany), rabbit anti-Ebony (1:200, lab of Dr. S. Carroll, University of Wisconsin, Madison, USA), rat anti-LOVIT (1:100), or anti-CSP (1:100, DSHB). Goat anti-rabbit IgG conjugated to Alexa 647 (1:500, Invitrogen, USA), goat anti-rat IgG conjugated to Alexa 488 (1:500, Invitrogen, USA), and goat anti-mouse IgG conjugated to Alexa 568 (1:500, Invitrogen, USA) were used as secondary antibodies.

Section staining was performed as previously described. Briefly, fly heads were fixed with 4% EDAC in PB for 1h, and then fixed with 4% paraformaldehyde for 1h. Fixed heads were immersed in 12% glucose overnight at 4°C, embedded in OCT, and 10 μm thick cryosections were immunolabeled with mouse anti-24B10 and rabbit anti-histamine (1:100, ImmunoStar, USA) as primary antibodies. For histamine immunolabeling, the antibody was preadsorbed with carcinine, as previously reported (Xu et al., 2015). Sections were incubated with secondary antibodies, goat anti-rabbit IgG conjugated to Alexa 568 (1:500, Invitrogen, USA) or goat anti-mouse IgG conjugated to Alexa 488 (1:500, Invitrogen, USA) at 25°C for 1 h, and the images recorded with a Nikon A1-R confocal microscope.

**Transmission Electron Microscopy**

To visualize the LOVIT-mCherry chimera by TEM, the heads of four-day-old *pninaE-lovit-mCherry* flies were dissected in 4% paraformaldehyde, and the retinas were removed. The following whole-mount labeling was conducted as described above. To amplify the signal, heads were incubated with anti-rat biotinylated secondary antibody, washed five times with PBST, incubated with ABC-HRP kit (Vector labs, CA, USA) for 1h, washed five times with PBST, and developed in the DAB peroxidase substrate kit (Vector labs) for 2 min. Stained heads were washed in PBS, treated with 0.2% osmium tetroxide for 30 s, and then processed to the normal TEM procedure as described previously (Chaturvedi et al., 2014). To maintain the contrast of DAB staining, thin sections were not stained with uranyl acetate or lead citrate, and were directly examined under a transmission electron microscope FEI Tecnai Spirit Twin 120kV.

**Phototaxis assay**

Flies were dark adapted for ~15 min and tapped into the end of 20 cm transparent glass tube, as described (Xu and Wang, 2016). A white light source (with an intensity of ~6,000 lux) was put at the other end of the tube, and the number of flies that walked past an 11-cm mark on the tube within 70 s was counted. A phototaxis index was calculated as total number of flies / number of flies that crossed the mark.

**Liquid chromatography–mass spectrometry (LC-MS)**

LC-MS was performed as previous reported (Xu et al., 2015). The Dionex Ultimate 3000 UPLC system was coupled to a TSQ Quantiva Ultra triple-quadrupole mass spectrometer (Thermo Fisher, CA, USA), equipped with a heated electrospray ionization (HESI) probe operated in negative ion mode.Extracts were separated by a Fusion-RP C18 column (2 × 100 mm, 2.5 μm, Phenomenex, CA, USA). Data were acquired in selected reaction monitoring (SRM) for histamine with a transition of 112/95.2. Both precursor and fragment ions were collected with a resolution of 0.7 FWHM. The source parameters are as follows: spray voltage: 3000 V; Ion transfer tube temperature: 350°C; vaporizer temperature: 300°C; sheath gas flow rate: 40 Arb; auxiliary gas flow rate: 20 Arb; CID gas: 2.0 mTorr. Data analysis and quantification were performed using software Xcalibur 3.0.63 (Thermo Fisher, CA, USA).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All data in bar and line graphs are expressed as Means ± SDs. Significant differences between different groups were determined using unpaired two-tailed t tests in Graphpad Prism 6 (Graphpad software Inc.). (***p < 0.001; **p < 0.01; ns, not significant).