Glutamate, GABA and Acetylcholine Signaling Components in the Lamina of the Drosophila Visual System

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Abstract

Synaptic connections of neurons in the Drosophila lamina, the most peripheral synaptic region of the visual system, have been comprehensively described. Although the lamina has been used extensively as a model for the development and plasticity of synaptic connections, the neurotransmitters in these circuits are still poorly known. Thus, to unravel possible neurotransmitter circuits in the lamina of Drosophila we combined Gal4 driven green fluorescent protein in specific lamina neurons with antisera to γ-aminobutyric acid (GABA), glutamic acid decarboxylase, a GABAe type of receptor, L-glutamate, a vesicular glutamate transporter (vGluT), ionotropic and metabotropic glutamate receptors, choline acetyltransferase and an acetylcholine transporter. We suggest that acetylcholine may be used as a neurotransmitter in both L4 monopolar neurons and a previously unreported type of wide-field tangential neuron (Cho-Tan). GABA is the likely transmitter of centrifugal neurons C2 and C3 and GABAe receptor immunoreactivity is seen on these neurons as well as the Cha-Tan neurons. Based on an rdl-Gal4 line, the ionotropic GABAe receptor subunit RDL may be expressed by L4 neurons and a type of tangential neuron (rdl-Tan). Strong vGluT immunoreactivity was detected in α-processes of amacrine neurons and possibly in the large monopolar neurons L1 and L2. These neurons also express glutamate-like immunoreactivity. However, antisera to ionotropic and metabotropic glutamate receptors did not produce distinct immunosignals in the lamina. In summary, this paper describes novel features of two distinct types of tangential neurons in the Drosophila lamina and assigns putative neurotransmitters and some receptors to a few identified neuron types.

Introduction

One of the most extensively investigated portions of the insect brain is the first synaptic neuropil in the optic lobe of flies, the lamina. This neuropil corresponds in its processing operations to the outer plexiform layer of the vertebrate retina, and indeed since the seminal work of Cajal and Sánchez [1] insect visual neuropils are known to have a similar structure (see, e.g. [2, 3]). Like the vertebrate retinal ganglion cells, Drosophila lamina interneurons are generally elongated and their processes span the lamina from where they synapse with other lamina interneurons to the medulla [6]. In the lamina the axon terminals of R1–R6 provide synaptic input upon first-order interneurons grouped in cylindrical modules called cartridges [7, 8]. Like the ommatidia that innervate them, these are of determinate composition; each cartridge comprise the six R1–R6 terminals and a fixed set of lamina neurons, one of each type, with the axons of R7 and R8 occupying a position satellite to these, as reported from electron microscopy for Drosophila [9]. The neuron types and their synaptic connections in a cartridge have been described by various techniques in the house fly Musca domestica and other larger fly species [2, 7, 8, 10, 11] as well as in the fruit fly Drosophila melanogaster [9, 12]. For the lamina of Drosophila, the synaptic contacts [9] and their numbers [13], as well as the circuits these constitute, have all been reported for the R1–R6 photoreceptor terminals and 11 major types of interneuron. The neuronal organization of the lamina is characterized by a geometrical precision of the arrangement of its neuronal elements into cartridges. As a result the identification of specific neurons has been greatly facilitated, both at the light and electron microscopical levels. Thus, the Drosophila lamina has become an excellent system for the analysis of the genetic regulation of many aspects of synaptic function, plasticity and synaptogenesis (see [14, 15, 16, 17, 18, 19, 20, 21]).

In parallel with the structural analyses of the lamina’s synaptic circuits, which are most complete for Drosophila, the electrophysiological properties of lamina neurons are reported but mostly from larger fly species (e.g. [22, 23] [24, 25, 26, 27, 28, 29, 30]). Together, these reveal visual phenomena such as spatial summation and amplification of visual signals, lateral inhibition, light adaptation, and even peripheral substrates for movement detection and colour coding (reviewed in [31]). By contrast, only...
limited electrophysiological data are available for lamina neurons in *Drosophila* ([32,33]).

In contrast to the extensive anatomical and electrophysiological investigations, we have little information about the neurotransmitters in the lamina of flies (see [34,35]). It is clear that fly photoreceptors use histamine as their neurotransmitter ([34,36,37,39,40]). When released from photoreceptor synapses, histamine acts as a fast neurotransmitter at ligand-gated chloride channels on postsynaptic lamina interneurons ([36]), which include L1-L3 ([41]). There is also immunocytochemical evidence for GABA in two types of small field centrifugal interneurons, C2 and C3 ([42,43,44,45]). This evidence is based on several antisera to GABA and antisera to the biosynthetic enzyme glutamate decarboxylase (GAD). Some reports indicate the immunolabeling of lamina monopolar cells (first-order interneurons) with antisera to glutamate, in flies ([46,47]) and honeybees ([48]). In *Drosophila*, these cells also label with an antibody against choline acetyltransferase (ChAT), the biosynthetic enzyme of acetylcholine ([49]), which is encoded by the gene *Cha* [50]. *Cha* transcript has also been found by *in situ* hybridization in cell bodies of lamina monopolar neurons [51]. Finally, fly amacrine cells are reported to express glutamate immunoreactivity [47]. Clearly there is some uncertainty in these reports. Some describe tentative identifications of lamina neurons, while in others the antisera used may identify a substance (e.g. glutamate) that is present only as a metabolic intermediate; some studies also do not include *Drosophila*. Thus, for *Drosophila* there is a need to investigate the lamina further with respect to these classical neurotransmitters.

Here we applied immunocytochemistry to the lamina of *Drosophila* to identify neurotransmitters or associated molecules important for neurotransmitter function, including corresponding receptors proteins. Examination of these markers was combined with use of the Gal4-UAS system [52] to drive expression of green fluorescent protein (GFP) in specific neuron populations of the lamina. The focus of our investigation is on neurons expressing markers for acetylcholine, glutamate, GABA, and some of their receptors.

**Materials and Methods**

**Fly strains**

We used adult wild type *Drosophila melanogaster* (Oregon R or w*1118*) strains for basic immunocytochemistry. For correlation with various neuronal phenotypes we performed immunocytochemistry on a variety of Gal4 lines crossed with UAS-GFP, as specified below. L2 monopolar interneurons were visualized by the 21D-Gal4 driver ([53]) (from Tomas Raabe, University Würzburg, Germany). C3 neurons were identified by 5-6-8/Cyo;TM2/TM6B-Gal4 (abbreviated 5-6-8-Gal4; from Larry Zipursky, Howard Hughes Medical Institute at UCLA, Los Angeles, CA). Other Gal4 lines used were: rdl-Gal4 ([4.7 kb upstream rdl-gene; from Julie Simpson, Howard Hughes Medical Institute, Janelia Farm, VA], Cha-Gal4 ([54]) (from Bloomington Stock Center at Indiana University, Bloomington, IN), and OK371-Gal4 ([55], from Hermann Aberle, University of Münster, Germany). These lines were used to visualize expression of the GABA receptor subunit RDL, choline acetyltransferase (Cha), and vesicular glutamate transporter (vGluT) gene products, respectively. To visualize Gal4-expression with GFP, we crossed these lines with flies expressing UAS-mCD8-GFP (from Bloomington Stock Center). Presynaptic sites were visualized by driving a neuronal synaptobrevin-GFP fusion line (w[1];P[w[mC] # UAS-nsyb.eGFP);2; Bloomington stock center) with either the OK371- or 21D-Gal4 lines ([see [56,57]])

**Antisera**

Several antisera were used to detect neurotransmitters and other signaling components in the lamina. The antisera and their corresponding antigens are listed separately (Table 1). Antibody specificities have been carried out for all antisera in earlier publications (listed in Table 1). A comprehensive description of antisera production and specificity tests is given below.

**DmGluRA**. The mouse monoclonal antibody to DmGluRA, 7G11 ([58]; purchased from European Molecular Biology Laboratory, Heidelberg, Germany) was raised against recombinant receptor protein that was purified to homogeneity ([58,59]). Specificity of 7G11 was tested by expressing DmGluRA in a baculovirus-insect cell system and testing cell extract by western blotting ([59]). The 7G11 antibody was also tested on Western blots of head extracts of Drosophila controls (2b) and DmGluRA mutants ([112b] showing loss of staining in mutants [60]).

**DvGluT**. The Rabbit anti-DvGluT (*Drosophila* vesicular glutamate transporter; kind gift from Dr. A. DiAntonio, Washington University School of Medicine, St. Louis, MO; [61]) was raised against a C-terminal peptide (CQMPSYDPSGYQQQ) of the *Drosophila* vGluT, affinity purified and characterized by western blotting and by its detection of transgenically expressed vGluT ([61]). Two other polyclonal rabbit antisera to the *Drosophila* vGluT were raised against the C-terminus (aminic acid 561–632) and N-terminus (aminic acids 21–87) of the transporter protein, respectively. The C-terminus antisera was affinity purified. Both antisera were kindly provided by Dr. H. Aberle (University of Munster, Germany; [55]). In *Drosophila* embryos homozygous for a small deficiency that removes the vGluT gene Mahr and Aberle ([55]) did not observe immunolabeling. They also found a good match between the immunolabeling obtained with the two vGluT antisera (indistinguishable from each other) to in situ hybridization and the OK371 (vGluT-Gal4) expression pattern.

**GABA**. We used two antibodies both raised against L-glutamate conjugated to keyhole limpet hemocyanin (KLH) with glutaraldehyde: a rabbit polyclonal (Cat. no. 1766; Arnel Products, New York, NY) raised by Hepler et al. ([62]; and a mouse monoclonal (Cat. no. G9282; Sigma, St. Louis, MO) raised by Madl et al. ([63]). The specificity of both antibodies in fly tissues is revealed because both gave similar labeling patterns in *Drosophila* to an antibody against vGluT (above), and both immunolabeled the same cells in two other species of fly (*Musca, Calliphora*) that they labeled in *Drosophila*.

**GABA**. We used a commercial antisem to GABA (Sigma; Cat. No. A2052) that was raised to GABA-bovine serum albumin (BSA) conjugate and then affinity immunopurified by the manufacturers. The GABA antisem was characterized by dot-blot immunoassay by the manufacturers and was previously applied to *Drosophila* brain ([64,65]).

**GAD-1**. Antisem to full-length gel-purified *Drosophila* GAD1 protein was raised in rabbit (kind gift from Dr. F.R. Jackson; [66,67]). This antisem has been previously characterized by Featherstone et al. ([67]) by Western blotting (recognizes a 57-kDa band, as expected) and by demonstrating the absence of labeling of tissue in a homozygous mutant lacking the *gad1* gene.

**GABAR2**. Production of antisera to GABABR2 was described previously ([63]). In brief, three antisera were raised in rabbits against a sequence (CLNDIVRLSAPPVRREMP) of the C-terminus of the receptor protein conjugated to KLH. These antisera were characterized by ELISA, Western blotting and with standard pre-adsorption tests ([63]). In addition, preimmune sera from the rabbit were collected prior to immunization and used for immunocytochemistry and Western blotting as controls. The best antisem (code B7873/3) was used here.
**Table 1. Antisera used for immunocytochemistry.**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>antigen</th>
<th>fixation</th>
<th>dilution</th>
<th>source (references)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glutamate</strong></td>
<td>L-glutamate conjugated to KLH with glutaraldehyde (GA)</td>
<td>Zamboni’s fixative</td>
<td>1:10,000</td>
<td>Amel. Products, New York, NY Cat. no. 1766 [62]</td>
</tr>
<tr>
<td>Rabbit polyclonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Glutamate</strong></td>
<td>L-glutamate conjugated to KLH with GA</td>
<td>Zamboni</td>
<td>1:5000</td>
<td>Sigma, Cat. no. G9282 [63]</td>
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<tr>
<td>Mouse monoclonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DmGluRA</strong></td>
<td>Drosophila metabotropic glutamate receptor A (recombinant protein)</td>
<td>Zamboni</td>
<td>1:10</td>
<td>European Molecular Biology Laboratory, Heidelberg, Germany [58]</td>
</tr>
<tr>
<td>#7G11 mouse monoclonal</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>DvGluT</strong></td>
<td>Drosophila vesicular glutamate transporter (peptide sequence)</td>
<td>Zamboni, Buin 4% PFA</td>
<td>1:10,000</td>
<td>from Dr. A. D’Antonio, University of California, LA [61]</td>
</tr>
<tr>
<td>C-term rabbit polyclonal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DvGluT</strong></td>
<td>Drosophila vesicular glutamate transporter (amino acids 561–632)</td>
<td>Zamboni, 4% PFA 1:1000</td>
<td></td>
<td>from Dr. H. Aberle, University of Münster, Germany [55]</td>
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<tr>
<td>N-term rabbit polyclonal</td>
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<tr>
<td><strong>DvGluT</strong></td>
<td>Drosophila vesicular glutamate transporter (amino acids 21–87)</td>
<td>Zamboni, 4% PFA 1:1000</td>
<td></td>
<td>from Dr. H. Aberle, Münster University, Germany [55]</td>
</tr>
<tr>
<td>T-term affinity purified rabbit polyclonal</td>
<td></td>
<td></td>
<td>1:500</td>
<td>from Dr. H. Aberle, Münster University, Germany [55]</td>
</tr>
<tr>
<td><strong>DvGluT</strong></td>
<td>Drosophila vesicular glutamate transporter (amino acids 61–632)</td>
<td>Zamboni, 4% PFA 1:1000</td>
<td></td>
<td></td>
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<tr>
<td><strong>DLG</strong></td>
<td>Discs large protein (recombinant protein, PDZ2 domain)</td>
<td>4% PFA 1:2000</td>
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<td>Developmental Study Hybridoma Bank, NICHD, Iowa [104]</td>
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<tr>
<td>mouse monoclonal</td>
<td></td>
<td></td>
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<tr>
<td><strong>GABA</strong></td>
<td>γ-aminobutyric acid (GABA-85A)</td>
<td>4% PFA 1:2000</td>
<td></td>
<td>Sigma-Aldrich [65]</td>
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<td>#A2052 rabbit polyclonal</td>
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<tr>
<td><strong>GAD-1</strong></td>
<td>Glutamic acid decarboxylase-1 (purified protein)</td>
<td>Zamboni, Boiun 1:1000</td>
<td></td>
<td>from Dr. F. R. Jackson [66,67]</td>
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<tr>
<td>Rabbit polyclonal</td>
<td></td>
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</tr>
<tr>
<td><strong>GABA&lt;sub&gt;A&lt;/sub&gt;R2</strong></td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor 2 (peptide sequence)</td>
<td>4% PFA 1:16,000</td>
<td></td>
<td>[65]</td>
</tr>
<tr>
<td><strong>GFP</strong></td>
<td>Green fluorescent protein from <em>Aequorea victoria</em> (purified protein)</td>
<td>4% PFA, Zamboni, Bouin 1:1000</td>
<td>Molecular Probes, Leiden, Netherlands</td>
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<tr>
<td>mAb 3E6 mouse monoclonal</td>
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<tr>
<td><strong>ChAT</strong></td>
<td>Choline acetyltransferase (recombinant protein)</td>
<td>4% PFA 1:1000</td>
<td></td>
<td>Developmental Study Hybridoma Bank [69]</td>
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<tr>
<td>481 mouse monoclonal</td>
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<tr>
<td><strong>NMDA1</strong></td>
<td>Ionotropic glutamate receptor (mammalian) (recombinant protein)</td>
<td>Bouin 1:500</td>
<td>Chemicon, Temecula, CA [47]</td>
<td></td>
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<tr>
<td>subunit mab363 mouse monoclonal</td>
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<tr>
<td><strong>RDL</strong></td>
<td>Drosophila ionotropic GABA receptor (peptide sequence)</td>
<td>Zamboni, Bouin 1:40,000</td>
<td></td>
<td>[68]</td>
</tr>
<tr>
<td>N-term rabbit polyclonal</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><strong>vAChT</strong></td>
<td>Drosophila vesicular acetylcholine transporter (amino acids 441–546)</td>
<td>4% PFA 1:1000</td>
<td></td>
<td>from T. Kitamoto [73]</td>
</tr>
<tr>
<td>C-term Rabbit polyclonal</td>
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**RDL.** For the GABA<sub>A</sub> receptor subunit RDL we synthesized a C-terminal peptide sequence: CLHVSDDVADDLVLGEE, which was coupled to *Lamalus* hemocyanine (LPH) via the N-terminal cysteine. The best RDL antisera (code 7385) produced in rabbit was characterized by Western blotting and immunocytochemistry, by pre-adsorption with peptide used for immunization, and by tests of preimmune serum [68].

**ChAT.** A mouse monoclonal antibody to recombinant ChAT protein (Code 4B1; [69]) was purchased from the Developmental Study Hybridoma Bank. This antibody was characterized by pre-adsorption with crude recombinant ChAT [69,70] and the labeling pattern in *Drosophila* confirmed by in situ hybridization and LacZ expression [see (49)].

**NMDAR1.** Mouse monoclonal antibodies raised against the rat NMDAR1 (mab363) were purchased from Chemicon (Temecula, CA) who performed specificity test of the antibodies (no cross reactivity with other NMDA receptors). The antisera was raised against a recombinant fusion protein containing the amino acids 660–811 of rat NMDAR1 [71]. The NMDAR1 protein displays 46% amino acid identity to a *D. melanogaster* NMDA-like protein [72]. This antibody was previously utilized on the lamina of flies and honeybees by Sinaevitch and Strausfeld [47].

**vAChT.** A rabbit polyclonal antisera to vAChT was raised against amino acids 441–546 of the protein [73]. The antisera was characterized in western blots of extract from wild type (band with Mr of 65 kD) as well as vach- (vesicular acetylcholine transporter) and Cha-mutant flies [73,74].

**vGAT.** Antiserum was raised in rabbits to a peptide sequence (N-terminal amino acids 24–38) of the putative *Drosophila* vesicular GABA transporter (vGAT; CG8394). The peptide was synthesized with a cysteine (QTARQIQPERKDYEQamide for directed conjugation to maleimid-coupled KLH at the N-terminal. The best vGAT antisera (code 1061) was affinity immunopurified. This antisera was characterized by Western blotting, pre-adsorption with the peptide used for immunization, and tests of preimmune serum [68].

**GFP.** A mouse monoclonal antibody to GFP (mAb 3E6; code #A-11120; Molecular Probes, Leiden, Netherlands) was used at 1:1000 for amplifying the GFP signal in some specimens. This antibody was raised against GFP purified from the jellyfish *Aequorea victoria* and characterized by the manufacturer; it produces no immunolabeling in wild type *Drosophila* CNS and thus only amplifies the GFP fluorescence.

**Immunocytochemistry.** For glutamate immunolabeling, brains were dissected out of the head capsule in modified Zamboni’s fixative (4% paraformaldehyde, 1.6% glutaraldehyde, 0.2% saturated picric acid, in 0.1M sodium phosphate buffer, pH 7.4) and left for between 1 h and overnight at 4°C. They were washed in sodium phosphate buffered saline (PBS), and then sectioned at 50–80 µm slices on a Vibratome. The sections were washed in PBS, blocked with normal goat serum (NGS), transferred to 0.5% Triton X-100 in PBS for 30 min prior to primary antibody incubation, and then

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and incubated overnight at 8°C in one of two glutamate antisera (Table 1), a rabbit polyclonal antibody at a dilution of 1:10,000, or a monoclonal antibody at a dilution of 1:5000. After the primary antibody, the tissue was washed several times in PBS, and then incubated with the corresponding secondary antibody (goat anti-rabbit, goat anti-mouse; Jackson ImmunoResearch Labs, West Grove, PA) conjugated to a fluorochrome (Cy3 or FITC).

For all other immunocytochemistry the adult fly heads were dissected in PBS-TX (0.01M phosphate-buffered saline with 0.5% Triton X-100, pH 7.2) before fixation. For DmGluRA, DvGluT and DLG immunolabeling, opened heads were fixed 2 h in ice-cold 4% paraformaldehyde (pH 7.4) or 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB) at pH 7.4. For GABA and GAD immunolabeling, the heads were fixed for 2 h in ice-cold 4% paraformaldehyde in 0.1M PB (pH 7.2). For GABA and GAD immunolabeling, the heads were fixed in freshly prepared ice-cold Bouin’s fixative for 30 min (tissues were also fixed in Zamboni’s fixative to obtain better GFP preservation). For ChAT and vAChT immunolabeling, adult fly heads were fixed for 2 h in ice-cold 4% paraformaldehyde (pH 7.4) or in 0.1M sodium phosphate buffer at room temperature (about 22°C). For GABABR2 immunolabeling, fly heads were fixed for 2 h in ice-cold 4% paraformaldehyde (pH 7.4) or in Zamboni’s fixative. Additional labeling with anti-GFP was necessary to amplify GFP fluorescence partly quenched after Bouin fixation.

Tissues were thoroughly washed with 0.1M PB and incubated overnight at 4°C in 20% sucrose in 0.1M PB. 20µm thick sections of the head were cut on a Leitz 1720 Cryostat at −23°C and collected on chromalum-gelatin-covered microscope slides. After washing in PBS-TX, tissues were incubated with primary antibodies in 4°C overnight or for 48 h. Brains were washed in PBS-TX at room temperature (about 22°C) and incubated with fluorophore-tagged secondary antibodies (Cy2- or Cy3-tagged IgGs, raised in goat; Jackson ImmunoResearch) diluted 1:1000, either overnight at 4°C or 2h at room temperature (around 22°C). After washing in PBS-TX and rinsing in 0.01M PBS, tissue was mounted under a coverslip in 20% glycerol in 0.01M PBS.

Pre-embedding immuno-electron microscopy

We also used both wild-type and white eye mutants for electron microscopy of preparations immunolabeled for glutamate by the pre-embedding method using the polyclonal rabbit anti-glutamate [62]. Tissue incubated as above in this primary antibody was next incubated in a biotinylated goat anti-rabbit antibody (Vector Labs) and then in a solution of peroxidase conjugated Avidin Biotin Complex (ABC complex, Vector Labs). Labeling was detected with 3,3’-diaminobenzidine (DAB) as the substrate. The sections were then osmicated, dehydrated in graded ethanol series, changed into propylene oxide and then flat-embedded between Aclar sheets (Ted Pella Inc, Redding, CA) in Poly/Bed 812 resin. The tissue was sliced at 80µm using a Vibratome and selected slices subsequently resectioned at 60nm for electron microscopy. Ultrathin sections were then viewed at 60kV with a Philips 201 C electron microscope, photographed on 35mm film at primary magnifications of between 5,000 and 20,000, and the prints then labeled and scanned.

Imaging

For glutamate-immunolabeled specimens, Vibratome slices were mounted in Vectashield (Vector Labs, Burlingame, CA) and viewed with a Zeiss LSM 410 confocal microscope (Zeiss, Jena, Germany). All other specimens were imaged with a Zeiss LSM 510 confocal microscope. Confocal images were obtained at an optical section thickness from 0.1–0.35µm and were processed with Zeiss LSM software and edited for contrast and brightness in Adobe Photoshop CS3 Extended version 10.0.

Results

The optic lobe of Drosophila consists of four neuropil regions located beneath the retina: the lamina, medulla, lobula and lobula plate (Fig. 1A). Each of these neuropils exhibits a columnar organization that derives from the pattern of photoreceptor innervation from the ommatidia of the overlying retina. R1–R6, the six outer of eight photoreceptor neurons in each ommatidium, terminate in the lamina in columnar modules termed cartridges [5,7], while the two inner neurons, R7 and R8, penetrate the lamina and innervate the distal strata of columns in the medulla (see Fig. 2B). The optic lobe neuropils are also stratified (Figs. 1A,D, and 2B), the result of overlap between stratum-specific terminals of (1) columnar centripetal neurons (those running from periphery to center), (2) columnar centrifugal neurons (those running in the opposite direction); and lateral arborizations (dendrites or collaterals) of (3) various columnar neuronal elements and (4) tangentially oriented wide-field branches of non-columnar neurons (see [12,75]). Some neurons do not display a pronounced stratified organization within the lamina. For example the L2 monopolar neurons form uniform arrangements of short, radially-directed dendritic spines throughout the depth of the lamina neuropil (Figs. 1B,C, and 2B). In contrast to their processing counterparts in the vertebrate retina, a distinctive feature of insect neurons is that they have their cell bodies located in a cortex surrounding the synaptic neuropil (Fig. 1C). Thus all interneurons referred to in this report have cell bodies in the lamina cortex, or in a cortex of the deeper optic lobe.

To facilitate interpretation of the immunolabeling and GFP expression patterns in the lamina and distal medulla we first briefly present the neuron types of the Drosophila lamina. The neuronal morphologies depicted in Fig. 2 are based on analyses of a large number of Golgi impregnations of Drosophila [12]. There are 3 types of photoreceptor axon and 11 types of interneuron associated with the lamina. Most of these are columnar, with an axon oriented parallel to the main axis of the visual columns, thus establishing the retinotopic organization of the optic lobe. All the interneurons, except the wide-field elements (amacrines and tangential neurons), are readily distinguished and morphological counterparts have been identified in other fly species [2,12,76] that have been reasoned to be evolutionary homologues [77]. Together, the columnar elements form a bundle of invariant pattern and composition, the axon of each contributing a distinct profile to the cartridge cross section (Fig. 5A, 4A,B).

There is, however, some ambiguity with respect to amacrine and tangential neurons. This is important to point out in order to accurately interpret our immunolabeling and Gal4-GFP expression patterns (see later sections). Fig. 2A depicts one type of amacrine (Am) and one of two types of tangential neurons (5-HT-IR Tan). The other (Tan; designated La w f1 by Fischbach and Dittrich [12]) has arborizations in the distal synaptic layer of the lamina (Fig. 2B), while 5-HT-IR Tan (designed Lat by [12]), has all its varicose processes in a layer distal to the lamina neuropil, and is known in Drosophila and larger flies to react with antisera to serotonin (see [35]). In the paper by Fischbach and Dittrich [12] a possible third type of tangential neuron (La w f2) is depicted (in their Fig. 24F). This also has processes reaching into the distal lamina, but its morphology differs from that of Tan (their La w f1). La w f2 has tangential branches with large boutons hanging down into the lamina neuropil. Only one type of amacrine (Am; designated Lai by Fischbach and Dittrich [12]) was described in Drosophila, with tangential processes sprouting characteristic axo-axonic processes running between the R1-R6 terminals in the cartridges. These make many synapses [13]. However, in other flies a second
Fig. 2. Neuron types in the lamina of Drosophila melanogaster. The neurons were revealed by Golgi silver impregnation in Drosophila melanogaster (The figure was modified from Meinertzhagen and O’Neil [9], after Fischbach and Dittrich [12]). A wide-field amacrine neuron (Am, designated Lai by Fischbach and Dittrich [12]) and wide-field serotonin-immunoreactive tangential neuron (5-HT-IR Tan). B The different types of narrow-field neurons of the lamina (and one wide-field neuron: Tan, designated Lat by Fischbach and Dittrich [12]) and their relationships in the 10 medulla strata comprise: R1–R6, terminate in the lamina; R7 and R8, in the medulla; L1–L5 lamina monopolar neurons; C2 and C3 narrow-field centrifugal neurons; T1, a narrow-field centripetal neuron with input in the lamina; and Tan (originally called La wf1), a wide-field tangential neuron. A second type of tangential neuron, La wf 2, illustrated by Fischbach and Dittrich [12], is not incorporated in this figure. doi:10.1371/journal.pone.0002110.g002
exhibits a range of labeling patterns. A  

Acetylcholine is a major excitatory neurotransmitter in  

Acetylcholine signaling components in the lamina  

has yet employed vAChT antiserum to this part of the brain. We examined the Drosophila lamina with antisera to both proteins. ChAT-immunolabeling reveals several types of lamina neuron (Fig. 5A). The cell bodies of large and small monopolar neurons are ChAT-immunolabeled (Fig. 5A,E), and what appear to be the axons and tripartite lamina collaterals of L4 monopolar neurons also react with ChAT antiserum (Fig. 5A, see also 5B). The axons of other monopolar neurons were not seen. Using 21D-Gal4 to drive GFP in L2 cells we could also show that anti-ChAT labeled L2 cell bodies, but no immunolabeling was visible in their dendritic processes in the lamina (Fig. 5E).

Additional to the cell bodies and presumed L4 processes, the ChAT antiserum also labeled enlarged boutons at the level of the C2 terminals (Fig. 5A, 6A,C). These structures seem to be associated with tangential neuronal elements having boutons in the distal lamina neuropil. Using Cha-Gal4 to drive GFP we obtained strong fluorescence in tangential neurons with similar boutons (Fig. 6A), but no labeling of any monopolar neurons. It is not clear whether these tangential processes seen with Cha-Gal4 are derived from the Tan tangential neurons (see Fig. 2B) or a novel type of tangential neurons (or even new amacrine neurons, like Am2 of other flies), both with more pronounced varicosities distal to the lamina neuropil than Tan. Arguing against the amacrine neuron possibility, the Cha-Gal4 expressing neurons appear to derive from neurons with axons projecting towards or even connecting to the medulla (Fig. 6A). Thus they are most likely to be a form of wide-field tangential neuron. For simplicity we will...
Fig. 5. Monopolar neurons in the lamina labeled with different antisera or Gal4 lines. A ChAT immunoreactive lamina neurons. Asterisk: layer with large monopolar cells (L1–L3); triangle: layer with small monopolar cells (L4 and L5); square: layer with processes of Cha-Tan neurons (see Fig. 8 and 9). Arrow labels level of branching of L4 neurons in the proximal lamina. Scale bar = 10 μm (for all images, except panel B). B GFP expression in lamina driven by the rdl-Gal4 reveals L4 neurons. Triangle: L4 cell body; arrow: characteristic branching of L4 collaterals in the proximal lamina. GFP is also seen in branches of a wide-field tangential neuron, in the distal lamina. Scale bar = 10 μm. C Weak immunolabeling in cell bodies of large monopolar neurons with antiserum to vGluT. Strong immunolabeling in the lamina neuropil is seen in processes of amacrine neurons. D1–3 Distributions of vesicular acetylcholine transporter (vAChT) immunoreactivity and rdl-Gal4 driven GFP expression co-localize to arborizations of the L4 neurons (arrow) in the proximal lamina, but not in their cell bodies (cb) and not in processes of rdl-Tan neurons (asterisk in D1) in the distal lamina. However, vAChT immunoreactivity is seen in enlarged boutons of another tangential neuron in this dorsal layer (large arrow). Scale bar = 5 μm. E1–3 Cell bodies of L2 monopolar neurons are ChAT immunoreactive, revealed by 21D-Gal4 driven GFP (green) in L2 neurons labeled with anti-ChAT (α-Cha; magenta). Co-localization of label is seen in cell bodies, but not clearly in their neurites. Scale bar = 10 μm. F1–3 Anti-vAChT labeling (α-vAChT; magenta) is not co-localized in L2 monopolar cells displayed by GFP driven by 21D-Gal4. Scale bar = 10 μm.

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Fig 6. Tangential and amacrine neurons in lamina, and markers for GABA, glutamate and acetylcholine signaling. A GFP expression driven by Cha-Gal4 reveals putative choline acetyltransferase containing lamina neurons. GFP expression in cell bodies near the medulla (arrow), and lamina morphology together suggests that Cha-Gal4 reports Tan neurons (La wf1) in the lamina. Scale bar = 20 \mu m. A1 Magnification of the putative Tan varicosities in the distal lamina. Scale bar = 10 \mu m. B GFP expression in Rdl-Gal4 reveals tangential neurons in lamina with cell bodies localized above medulla (arrow). Scale bar 10 \mu m. B1 Magnified view of the Rdl-Gal4-expressing lamina neurons. Scale as in A1. C(1–3) Distribution of ChAT immunoreactivity (C1, \alpha-Cha; magenta), in relation to Cha-Gal4 driven GFP (C2, green) in lamina cross section. Co-localization (C3) is seen in the distal rosette-like structures (see magnifications in insets). Scale bar = 10 \mu m. D (1–3) Cross section of lamina showing co-localization of GFP in Cha-Gal4 and anti-vAChT (D1, \alpha-vAChT; magenta) in distal boutons of Cha-Tan neurons. Scale bar = 10 \mu m. E (1–3) GABABR2 immunoreactive neurons (E1, \alpha-GBR2; magenta) in relation to Cha-Gal4 driven GFP in tangential neurons (E2, green). Close contacts and some co-localization (E3) between labels are seen (insets show magnified views), suggesting localization of GABABR2 on these tangential neurons. Scale bar = 5 \mu m.

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therefore refer to these neurons henceforth as Cha-Tan neurons. We found co-localization of Cha-Gal4 driven GFP and anti-ChAT immunolabeling in the tangential processes and enlarged boutons of these cells (Fig. 6C), but ChAT-immunolabeling was detected mainly in the GFP-labeled processes in the distal lamina, not in their cell bodies or axons (data not shown). Our immunocytochemistry thus confirms that the lamina neurons seen in the Cha-Gal4 reporter line actually express ChAT-immunoreactivity. We could exclude that the ChAT-immunolabeling in this distal layer is derived from C2 neurons, although, as we show later, C2 neurons may contact the Cha-expressing tangential neurons on these enlarged boutons. Seen in cross section, it appears that the overlapping Cha-Tan neurons form aggregates of boutons, each aggregate associated with an underlying cartridge (see section on GABA receptors).

The antiserum to the vAChT confirmed most of the ChAT-immunolabeling in the lamina. We could detect vAChT-immunolabeling in basal processes likely to represent collaterals of L4 neurons (Fig. 5D) and in the dilated boutons of the Cha-Tan neurons (Fig. 6D). Double-labeling with rdl-Gal4 and vAChT antiserum showed the close match between the two in the morphology of the L4-like profiles (Fig. 5D). This double-labeling also clearly showed the distinction between the rdl-Gal4 (described below) and Cha-Gal4 expressing tangential processes in the distal lamina. Whereas the Cha-Gal4 tangential profiles co-localize another acetylcholine marker, vAChT (Fig. 6D), the rdl-Gal4 processes did not (see Fig. 5D). Furthermore, the vAChT antiserum did not label neuronal cell bodies in the lamina cortex (Fig. 5D), and as a result we could not match the rdl-Gal4 signal with ChAT-immunolabeling in monopolar cell bodies, even those of L4 cells.

**Glutamate signaling components in the lamina**

Immunocytochemistry has previously suggested the presence of glutamate in the large lamina monopolar neurons, L1 and L2, in the flies *Drosophila*, *Musca*, *Calliphora* and *Phaenicia sericata* [46,47] as well as in type 1 amacrine neurons of the latter fly [47]. Here, we examined the *Drosophila* lamina for evidence of glutamate neurotransmission by applying antisera to two essential molecules, the neurotransmitter glutamate and the vesicular glutamate transporter (vGluT). The presence of glutamate is a requirement for its candidacy as a neurotransmitter, but given the widespread availability of glutamate as an intermediary metabolite, this evidence alone is unacceptably weak. On the other hand, vGluT is required to load synaptic vesicles with glutamate and is a highly specific marker for sites of glutamate neurotransmission so that, for example, vGluT antiserum label motoneuron varicosities [55,61] that are known to utilize glutamate in the lamina, we examined the lamina from two other fly species, the housefly *Musca domestica*, and the blowfly *Phaenicia sericata*, which use glutamate as a neurotransmitter [81].

**Glutamate immunolabeling.** To seek the presence of glutamate in the lamina, we examined the lamina from preparations sectioned in either a tangential (Fig. 4A,B) or frontal plane (Fig. 4C,D) or, in immuno-EM preparations, in a plane cut at a tangent to the lamina’s surface (Fig. 3A), to reveal cross-sections of individual cartridges (Fig. 3B). Strong glutamate immunolabeling of monopolar cell profiles was apparent in all cartridges, and the corresponding terminals in the medulla.

The labeling pattern in *Drosophila* visible by confocal microscopy was substantially similar to, but varied in details from, that seen in two other fly species, the housefly *Musca domestica*, and the blowfly *Calliphora erythrocephala* (Figures S1, S2, and S3). A number of immunoreactive profiles were visible in single cross-sections of the cartridge, but the slender axon size *Drosophila* lamina cells gave some uncertainty in the exact determination of which profiles were axons and which dendrites. The small cartridge diameter relative to the somata of monopolar cells in the lamina cortex, and the short axon path between cortex and neuropil, made it particularly easy to identify the cell body fiber of immunoreactive monopolar cells (Fig. 4B). There were two rows of such somata above the cartridge (Fig. 4A). Similarly, it was easy to see the axons of monopolar cells extending into the chiasma (Fig. 4C). The deeper neuropiles showed qualitatively similar labeling patterns to those in the larger flies, but were not examined further.

For immuno-EM studies, we used a pre-embedding method with the polyclonal antiserum [62] This revealed a clear pattern of labeling that confirmed at higher resolution much of what was seen by confocal microscopy, and resolving the pattern of labeling of tiny profiles in *Drosophila*. From the enhanced resolution of the preparations we could also demonstrate that there was no difference in the labeling patterns in the lamina between preparations from wild-type flies, with red eyes, and mutant with white eyes. The consensus pattern was also highly consistent in all three fly species examined (Figures S1A-E).

The pattern of immuno-EM labeling in individual preparations varied somewhat. In some only a single monopolar cell axon profile, probably of L2 (Fig. 3A), was labeled. The basis for this identification was twofold. First, it was generally the larger of the small amacrine cell fibers near R4 [9]. Such profiles did not accompany all cartridges however and were sometimes ambiguous, leaving some residual doubt about the identity of the labeled profile. Other preparations had the profiles of both L1 and L2 labeled (Fig. 3B), as was also seen in *Musca* (Figures S2, S3). Unlike the two other fly species, L3 was apparently not labeled in *Drosophila*. Cartridge profiles in the same preparation had the same immunolabeling patterns, so that variation was mostly between specimens.

In addition to axon profiles of L-cells, small immunoreactive profiles were visible between profiles of the R1–R6 terminals. These were especially clear in the cartridges of *Drosophila* (Fig. 3C,D) when labeled heavily with the pre-embedding method, compared with those of *Musca* (e.g. Figure S3B). Such locations are occupied by dendrites of both L1–L3 and amacrine cell alpha-processes that approach tetrad photoreceptor synapses [9]. In some preparations it was clear that immunolabel in the L-cell axon profiles disappeared at the base of the dendrites that arose from these (e.g. Fig. 3B: *Drosophila*; Figure S2A: *Musca*). The small labeled profiles between R1–R6 also never connected with the axon profiles of L1 and L2 (Fig. 3C,D). Both observations provide strong evidence that the immunolabeled profiles were instead those of the alpha-processes from amacrine cells. Corresponding somata of the amacrine cells were not examined.

**Drosophila vGluT immunolabeling.** To confirm that immunoreactivity to glutamate signified a capacity for glutamatergic transmission in the monopolar cells, we also applied four different antisera to the *Drosophila* vGluT and obtained identical labeling with each (Fig. 7). Strong vGluT immunolabeling was detected in profiles similar to α-processes of the amacrine neurons or possibly like β-processes of T1 neurons (Fig. 7; Figure S4). Weak vGluT immunolabeling of cell bodies was seen in the chiasma between the lamina and medulla, in a position corresponding to those of amacrine cells (Fig. 2A), but it was not possible to connect these to lamina processes (Figure S4A). The vGluT immunosignal in the lamina was mostly distinct from that seen with the OK371-Gal4 [55], representing vGluT promoter expression (Fig. 7A,B). The OK371-Gal4 drove GFP
Fig. 7. Distribution of vesicular glutamate transporter (vGluT) immunolabeling. Different antisera were combined with OK371-Gal4 driven GFP expression, which reports vGluT-expressing neurons. A1–3 Frontal sections of the lamina (vGluT antiserum) revealing lack of co-localization between vGluT immunolabeling (magenta) and GFP expression (A3 merged) in L2 monopolar cells (A1, green). Scale bar = 5 μm. B1–3 Cross-section of the same lamina region with enlargements in insets. B1 GFP in L2 neurons, B2 vGluT immunolabeling, B3 merged. Note that the vGluT immunolabels six structures surrounding the margin of the cartridge and at the extensions of L2 dendrites. C–E Similar structures labeled with three other antisera to vGluT. All vGluT antisera display the same immunolabeling in the lamina and medulla. C1–3 Affinity purified antiserum to vGluT (magenta) applied to OK371-Gal4 driven GFP (green). C1 Frontal section of lamina. Scale bar = 10 μm. C2 Cross-section of lamina (same magnification). C3 Frontal section of medulla showing that vGluT immunolabeling is not in GFP-labeled terminals of L2 cells in stratum M2 (arrow). Scale bar = 10 μm. D1–3 Similar structures labeled with antiserum to N-terminus of vGluT. Same scales as C. E1–3 Similar images using antiserum to the C-terminus of vGluT. Scales as in C. doi:10.1371/journal.pone.0002110.g007
in smaller or larger populations of large monopolar neurons (Fig. 7A–E). Thus, instead of a complete co-localization of OK371 driven GFP and anti-vGluT expression, we saw neurons expressing vGluT lying adjacent to the GFP-labeled large monopolar neurons (Fig. 6A3, B3). In cross-section, six profiles in each cartridge expressed vGluT immunolabeling and surrounded the GFP labeled monopolar neurons (Fig. 7B, C2, D2, E2). To confirm the failure of vGluT immunolabeling to localize to processes of monopolar neurons, we investigated the relation between this label and OK371-driven GFP, in neurons of monopolar neurons in the medulla (Fig. 7C3, D3 and E3). No clear co-localization was detectable. However, some vGluT immunolabeling can be seen in cell bodies of large monopolar neurons (Fig. 5C) and we could not rule out low levels of vGluT immunolabeling in dendrites of monopolar cells that also express OK371-Gal4 (see Fig. 7A3, B3). Thus there is a lack of correspondence between data from the antisera and data from the Gal4 driver. OK371 expression indicates that at least the large monopolar cell expresses vGluT [vglut-promoter], just as they also contain glutamate, whereas at best the vGluT antisera only weakly label the corresponding cell bodies and tips of dendrites. On the other hand the vGluT immunolabeling seen probably in amacrine cell processes is not matched by a similar pattern of GFP-labeling for the vGluT promoter. Part of this discrepancy may reflect the different intraneuronal distribution of the markers: vGluT antibodies label predominantly presynaptic sites while GFP (cd8-GFP) expression is distributed in the plasma membrane throughout the neuron. There may also be very small amounts of highly localized vGluT protein in L1 and L2 compared with the surrounding amacrine cell processes. To investigate this possibility we used a neuronal synaptobrevin-GFP fusion (nsyb-gfp) to target GFP primarily to presynaptic sites (Figure S5). Using the 21D-Gal4 to drive nssb-GFP resulted in fluorescence localized predominantly or exclusively to the medulla terminals of the L2 neurons (Figure S5A), but still no co-localization was seen with vGluT immunolabeling (Figure S5 B–D). Moreover with OK371-driven nssb-GFP there was no co-localization to vGluT immunolabeling (Figure S5 E1–3). Finally, we cannot exclude that the OK371-Gal4 expression in neurons is incomplete because it lacks promoter/enhancer elements in the construct.

To reveal more clearly the relationship between vGluT-immunolabeled amacrine cell processes and the terminals of photoreceptors R1–6, we used antibodies to Discs large (DLG) as a marker. The DLG protein is a membrane associated guanylate kinase (MAGUK) family protein located at the pre and postsynaptic area of functional glutamatergic synapses, at least in the Drosophila neuromuscular junction [84]. The vGluT immunolabeled structures are likely to be amacrine α-processes that seem to make contacts with DLG immunolabeled photoreceptors (Figure S4B1).

Drosophila GluR immunolabeling. As a further step, we also tried to localize glutamate receptors to lamina neurons using antisera to the Drosophila metabotropic glutamate receptor DmGluRA and one of the subunits of a mammalian ionotropic NMDA1 receptor. The DmGluRA antisera is highly specific and has been used for analysis of both Drosophila neuromuscular junctions [60] and in the clock neuron circuits [85]. When applied to the Drosophila optic lobes distinct and strong immunolabeling was seen in the medulla and lobula complex, but not in the lamina (Figure S4C). In the lamina, the DmGluRA antisera produced diffuse labeling that was hard to distinguish from background labeling. Several fixation protocols yielded the same result. The most likely site for glutamate release, the medulla terminals of L2, in particular, did not express presynaptic receptor immunolabeling.

The antiserum to the NMDA1 subunit was raised to a sequence of the protein that is quite well conserved between invertebrates and mammals, but has not been properly characterized on fly tissue. In a report on the lamina of another fly, P. sericata, the same antisera was reported to label T1 processes in the lamina [47]. In spite of using the same protocol as these authors, and as well as testing several modifications (and different fixatives), we failed to obtain any proper immunolabeling in the lamina or medulla (Figure S4D1). We did, however, obtain strong immunolabeling with this NMDA1 antisera in the mushroom body lobes (Figure S4D2), indicating that the antisera recognized a Drosophila epitope. Possibly the lack of immunolabeling in the optic lobe reflected levels of receptor expression in Drosophila that were too low, or an inconvenient species difference.

GABA signaling components in the lamina

GABA and GAD immunolabeling. GABA is a major inhibitory neurotransmitter in Drosophila and other insects and distributed in large numbers of neurons [68,86,87]. Proven markers for GABAergic neurons are antisera to GABA, vesicular GABA transporter (vGAT) and the biosynthetic enzyme GAD. Here we employed GABA, vGAT and GAD (GAD-1) antisera to label lamina neurons. To identify C3 neurons we employed the 5-6-8-Gal4 line (Fig. 8A). Previous studies have shown that the centrifugal neurons C2 and C3 in different fly species display GABA immunoreactivity [42,43,44,45]. Our study confirmed GABA and GAD immunoreactivity in C2 and C3 neurons in Drosophila (Fig. 8B–D, J). In a recent report from our laboratory [68] vGAT immunolabeling was also detected in C2 and C3 neurons. This suggests that the C2 and C3 neurons indeed both contain and utilize GABA as a neurotransmitter in the lamina: the C2 and C3 neurons indeed both contain and utilize GABA as a neurotransmitter in the lamina. C2 and C3 neurons probably releasing the transmitter from presynaptic sites that localized to enlarged boutons in a distal layer and C3 from similar sites at varicosities along their length in the lamina [9].

We analyzed the relations between the GFP-labeled C3 neurons (5-6-8-Gal4) and ChAT-immunolabeling and found no co-localization of markers (Fig. 8I). However, the C3 neurons were seen close to the ChAT-immunolabeled monopolar axons (which are most likely L4 neurons) and terminated close to the enlarged boutons of Cha-expressing tangential neurons, Cha-Tan.

GABA receptors. The localization of the metabotropic GABAB receptor 2 (GABAB2R2) has previously been demonstrated in the brain of Drosophila by means of a specific antisera [65,68]. Here we show the distribution of GABA2R2 immunoreactivity (GBR-IR) in relation to the different lamina neurons visualized by GFP driven by specific Gal4-lines (Fig. 8E–H). The major expression of GBR-IR was seen on the distal varicosities of C2 neurons (Fig. 8F–H) and in boutons of C3 neurons (Fig. 8E–H), as well as on enlarged boutons of Cha-Gal4-expressing tangential neurons, Cha-Tan (Fig. 6E). GBR-IR expression in the lamina is thus likely to be localized presynaptically in C2 and C3 boutons and postsynaptically on the tangential neuron boutons. This would explain why the distribution of GBR-IR signal in this region appears in coherent aggregates larger than the C2 terminals and larger than the Cha-Gal4-expressing boutons (Fig. 6E). To investigate the relationship between C2 neurons and Cha-expressing neurons further, we also double-labeled tissues with anti-GABAB2R2 antibodies and anti-ChAT (Fig. 9D). Again we saw that the immunolabeled Cha-Tan neuron boutons co-expressed GBR-IR material.

The GBR-IR material associated with the C3 neurons appear to be predominantly co-localized within the membranes of the C3 boutons throughout the depth of the lamina (Fig. 8F, G). This we interpret to represent presynaptic GABA2R2 in GABAergic C3s.

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Transmitters in Fly Lamina

GABA and GAD immunolabeling. GABA is a major inhibitory neurotransmitter in Drosophila and other insects and distributed in large numbers of neurons [68,86,87]. Proven markers for GABAergic neurons are antisera to GABA, vesicular GABA transporter (vGAT) and the biosynthetic enzyme GAD. Here we employed GABA, vGAT and GAD (GAD-1) antisera to label lamina neurons. To identify C3 neurons we employed the 5-6-8-Gal4 line (Fig. 8A). Previous studies have shown that the centrifugal neurons C2 and C3 in different fly species display GABA immunoreactivity [42,43,44,45]. Our study confirmed GABA and GAD immunoreactivity in C2 and C3 neurons in Drosophila (Fig. 8B–D, J). In a recent report from our laboratory [68] vGAT immunolabeling was also detected in C2 and C3 neurons. This suggests that the C2 and C3 neurons indeed both contain and utilize GABA as a neurotransmitter in the lamina; the C2 and C3 neurons indeed both contain and utilize GABA as a neurotransmitter in the lamina. C2 and C3 neurons probably releasing the transmitter from presynaptic sites that localized to enlarged boutons in a distal layer and C3 from similar sites at varicosities along their length in the lamina [9].

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The GBR-IR material associated with the C3 neurons appear to be predominantly co-localized within the membranes of the C3 boutons throughout the depth of the lamina (Fig. 8F, G). This we interpret to represent presynaptic GABA2R2 in GABAergic C3s.
Fig. 8. Centrifugal neurons in the lamina and markers for GABA signaling. A GFP expression in columnar C3 neuron terminals in the lamina (La) and medulla (Me) driven by the 5-6-8-Gal4. The C3 cell bodies (cb) proximal to the medulla are also visible. Ch: optic chiasma. Scale bar = 10 μm. B Distribution of glutamic acid decarboxylase-1 (GAD) immunoreactivity in C2 and C3 neurons (overview of lamina and part of medulla). Scale as in A. C Higher magnification of C2 and C3 terminals in lamina revealed by anti-GAD antiserum; note C3 varicosities along the axons. Scale bar = 10 μm. D Both C3 and C2 (labeled by arrow) terminals can be visualised in the lamina by anti-GABA antiserum. Same magnification as in C. E GFP in C3 neurons.
(in green) driven by 5-6-8-Gal4 with anti-GABABR2 immunolabeling (magenta) in horizontal section of lamina. The C3 axons traverse the optic chiasma. Note that much of the receptor immunolabeling is in neurons other than C3, but that some appears co-localized (white). Scale bar = 10 μm.

F–H Details of double-labeling with GFP expression in C3 neurons and antisera to GABA<sub>B</sub>R2. Some GABA<sub>B</sub>R receptor expression is in C3 neurons (arrows in F, G and H). At other sites the receptor is expressed on neuronal structures that appear to be closely adjacent to C3 neurons (long arrowheads in G and H) or in C2 terminals indicated by short arrowhead in F. Scale bar for F–H (F1): 5 μm. I C3 neurons (5-6-8-Gal4 driven GFP, green) do not co-localize ChAT immunoreactivity (magenta) but appear to be located close to ChAT immunolabeled profiles. Scale bar = 5 μm. J1–3 GFP-labeled C3 cell bodies (J1, green) express GABA-immunoreactivity (J3, magenta) as seen in merged image (J2). Scale as in F.

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Discussion

By combining immunocytochemistry with Gal4-directed GFP expression, we have mapped some components of the acetylcholine, glutamate and GABA signaling pathways in the peripheral visual system underlying the compound eyes of Drosophila [summarized in Table 2]. We confirmed some previous reports for Drosophila: for example, the presence of GABA in the centrifugal neurons C2 and C3 [15] and the cholinergic phenotype of some lamina monopolar neurons [49,51]. As discussed below, data to support a neurotransmitter function for glutamate in monopolar neurons L1–L2 are less decisive. We also have some new findings such as evidence for expression of choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (vAChT) protein in the monopolar neuron L4, and expression of ChAT-immunolabeling and Cha-Gal4 driven GFP in what appears to be a previously unreported wide-field lamina tangential neuron, which we designate Cha-Tan. ChAT expression in Drosophila had previously been reported for somata of lamina monopolar cells in general [49,51] and in Calliphora for amacrine neurons [88]. Another new finding is the presence of vesicular glutamate transporter (vGlut) immunoreactivity in what are probably the α-processes of lamina amacrine neurons. This finding confirms with a more reliable phenotypic marker earlier indications of glutamate immunoreactivity in amacrine cells of another fly species [47], which we extend with observations made here from electron microscopical immunocytochemistry. Attempts to map the distribution of GABA<sub>A</sub> and GABA<sub>B</sub> receptors, as well as ionotropic and metabotropic glutamate receptors in lamina circuits met with variable success. Only GABA<sub>B</sub> receptors were clearly identifiable by immunocytochemistry in the lamina, although GABA<sub>A</sub> receptors were expressed in the medulla. However, rd1-Gal4 driven GFP indicated possible expression of the GABA<sub>A</sub> receptor subunit RDL in a wide-field tangential neuron (rdl-Tan), similar to a variant type of tangential neurons previously designated La w2 [12], and in L4 monopolar neurons.

Acetylcholine signaling components

The best evidence for neurons qualified to use acetylcholine for signaling was obtained for the wide-field Cha-Tan neurons. Both Cha-Gal4 expression and the ChaAT- and vAChT-antisera identify these neurons. The Cha-Tan neurons give rise to enlarged boutons, most probably associated with distal C2 neuron terminals. Thus, we believe they were mistaken for C2 neurons in earlier reports on ChAT-immunolabeling in flies [89]. Especially with Gal4-driven GFP expression it is clear that these large boutons are parts of the Cha-Tan neurons, and may thus be partly regions receiving input from centrifugal neurons. The Cha-Tan neurons also produce varicose processes that run between the boutons and that have short branches hanging down into the lamina synaptic neuropil. It therefore seems that a portion of the cholinergic neurotransmission from Cha-Tan neurons is confined to a shallow layer in the distal lamina. The wide spread of these processes was the reason that the synaptic connections of wide-field tangential neurons were not investigated by Meinertzhagen and O’Neil [9], so the synaptic targets or inputs of these neurons are still unknown in Drosophila.
Fig. 9. Comparison between *rdl*-Tan and *Cha*-Tan neurons in distal part of lamina. Arrows indicate differences in structures between the two types of tangential neurons. *rdl*-Tan have thin varicose processes hanging down into the lamina, whereas the *Cha*-Tan have enlarged boutons in the same layer. A (1–3) *rdl*-Tan neurons (A2, green), visualised by GFP expression driven by *rdl*-Gal4, contact GABA<sub>R</sub>-immunolabeled cells at arrows, but do not coexpress the receptor (A1, magenta). B (1–3) *Cha*-Tan cells visualised by GFP expression driven by *Cha*-Gal4 (B2, green) co-express GABA<sub>R</sub> immunoreactivity (B1, magenta) in their boutons (arrow). C (1–3) *rdl*-Tan neurons in cross-section are organized in widely branched network with arborizations in each cartridge (C2, green). Close contacts with GABA<sub>R</sub>-immunopositive cells (C1, magenta) are visible. D (1–3) *Cha*-Tan neurons also are organized in a network but they have more distinct aggregates of boutons in each cartridge and these boutons co-express GABA<sub>R</sub>-immunolabeling (shown in Fig. 8E) and co-localize anti-ChAT (D1, magenta, D3 – merged). Scale bar for images A to D = 10 µm. E (1–2) Higher magnification of the *rdl*-Tan processes distally in the lamina in cross section (E1) and contacts with GABA<sub>R</sub>-immunolabeled neurons (E2). Scale bar = 10 µm.

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Another layer of cholinergic neurotransmission may occur in the proximal portion of the lamina, by means of collaterals of the L4 monopolar neurons.

Published reports on the immunocytochemical localization of acetylcholine receptors in the CNS of Drosophila have also shed some light on the lamina circuitry. Two nicotinic receptor subunits and the muscarinic receptor have previously been detected in the lamina [90,91,92]. Whereas the muscarinic receptor [92] and the vGluT immunolabeling seen only in L1 and L2 cell bodies, not processes. The immunolabeling pattern resembles that of the boutons of the ARD distribution closely matches that of the boutons of the Cha-Tan neurons, but it is not clear what neuron type(s) expresses the receptor.

L4 monopolar neurons have three collateral processes in the basal portion of the lamina [12]. These interconnect the L4 neurons in adjacent cartridges, as well as the L2 cell and photoreceptor terminals within the neighboring and native cartridges, and appear to be the major outputs from the L4s within the lamina [9]. At intermediate pattern contrasts, L2 in Drosophila recruits L4 as the substrate for detection of front-to-back motion [93]. We find that the collateral branches of L4s strongly express ChAT and vAChT immunoreactivities, suggesting that a cholinergic pathway may be responsible for this recruitment in the lamina. In two earlier reports on ChAT-immunoreactivity in Drosophila [49,89] the L4 collaterals are visible in the figures, but did not receive specific comment. Interestingly the rd1-Gal4 drives GFP in what appears to be L4 neurons (and a set of tangential neurons, rd1-Tan). The failure of our antiserum to provide matching immunocytochemical evidence for RDL expression in these neurons, means that it is not clear whether the neurons express this GABA receptor subunit, or – more likely – whether they may have a capacity to synthesize it, or whether the vesicular transporter is expressed at too low levels to detect.

Glutamate signaling components

We validated previously published data, including our own [46] on glutamate signaling components by using different antisera to the Drosophila vesicular glutamate transporter (vGluT), as well as analyzing vGluT-Gal4 expression. Glutamate signaling seems to be performed at two main candidate sites in the lamina, large monopolar cells and amacrine neurons.

We obtained clear evidence for glutamate-like immunoreactivity in the large monopolar cells L1–L3 in the lamina and medulla of two fly species (Musca and Calliphora), whereas only two of these neurons, L1 and L2, were detected in Drosophila. There was, however, some variation in the latter species, L2 alone being

### Table 2. Distribution of signaling components in fly lamina indicated by various markers.

<table>
<thead>
<tr>
<th>Neuron type</th>
<th>Marker</th>
<th>Tentative marker</th>
<th>Reference</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1-R6</td>
<td>α-Histamine</td>
<td>α-GABA /GAD</td>
<td>8,9</td>
<td>-</td>
</tr>
<tr>
<td>R7/R8</td>
<td>α-Histamine</td>
<td>α-GABA /GAD</td>
<td>8,9, 3</td>
<td>-</td>
</tr>
<tr>
<td>L1</td>
<td>α-Glutamate</td>
<td>α-ChAT, cha in situ</td>
<td>11, 12</td>
<td>α-ChAT, α-vGluT, α-Glutamate</td>
</tr>
<tr>
<td>L2</td>
<td>α-Glutamate α-RDL</td>
<td>α-ChAT, cha in situ</td>
<td>6,11, 12</td>
<td>α-ChAT, α-vGluT, α-Glutamate, vGluT-Gal4</td>
</tr>
<tr>
<td>L3</td>
<td>α-Glutamate</td>
<td>11</td>
<td>not detectable in Drosophila</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>-</td>
<td>α-ChAT, α-vAChT Rd1-Gal4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>α-GABA, α-GAD α-vGAT</td>
<td>α-ChAT</td>
<td>3,4,6,7,10, 2</td>
<td>α-GABA R, α-GABA, α-vGAT</td>
</tr>
<tr>
<td>C3</td>
<td>α-GABA, α-vGAT</td>
<td>10,11</td>
<td>GABA, GAD α-vGAT</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>α-“NMDA-R1”</td>
<td>11</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Am</td>
<td>α-Glutamate α-ChAT</td>
<td>11, 5</td>
<td>α-vGluT</td>
<td></td>
</tr>
<tr>
<td>Tan 1 α Cha-Tan</td>
<td>α-GABA R</td>
<td>α-ChAT, cha-Gal4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tan 2 α rd1-Tan</td>
<td>rd1-Gal4</td>
<td>rd1-Gal4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Notes.**

1. Immunocytochemical identification of putative neurotransmitter/substrate, protein, biosynthetic enzyme or receptor in a specific neuron type. Evidence is more complete for underlined neuron types.
2. Tentative identification of neuron type with marker (no clear statement/commitment was made in papers).
4. Including Gal4 driven GFP.
5. Only cell bodies labeled with CHAT antiserum.
6. The vGluT immunolabeling seen only in L1 and L2 cell bodies, not processes.
7. The tangentially arranged processes detected with these markers do not completely match tangential neurons (La wf1) or amacrine neurons described from Golgi impregnations [12]. Thus we refer to them as Cha-Tan and rd1-Tan neurons. The rd1-Tan resemble the La wf2 neurons, a possible variant of La wf1 neurons [12].

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invariably labeled. It most plausible to attribute this variation to different levels of cytoplasmic glutamate that could have existed under different functional states prior to preparation for immunolabeling. Compatible with these neurons having the capacity to store vesicular glutamate, the OK371-Gal4 line, specific for vglut expression, also drives GFP in the large monopolar neurons, but we did not detect clear vGluT immunolabeling in monopolar neurons. However, low levels of vGluT immunolabeling were seen in cell bodies of the large monopolar neurons and immunolabeling in dendrites of these neurons may be masked by the stronger immunolabeling seen in amacrine processes. Another more likely possibility is that the amount of vesicle-bound glutamate (and vGluT) is simply too low to detect. We thus do not have conclusive evidence that L1 and L2 have the capacity to store vesicular glutamate, and consequently that they are glutamatergic in Drosophila. These neurons have most of their synaptic output in the medulla and either no (L1) or a limited number (L2) of output synapses in the lamina [9,13]. Insofar as L1 sometimes clearly expresses a glutamate phenotype but lacks output synapses in the lamina, we would predict the absence of glutamate containing vesicles and corresponding vGluT in the lamina, at least in L1. On the other hand, as revealed by the 21D-Gal4 line, we did not detect vGluT immunolabeling in the L2 medulla terminals either, again possibly because there was insufficient protein to yield a clear immuno signal. Thus it cannot be entirely excluded that the glutamate immunoreactivity seen previously [46,47] may represent non-vesicular amino acid stored as a metabolic intermediate. On the other hand, the possibility that these monopolar neurons, the major output neurons of the fly’s lamina, might use two fast neurotransmitters, glutamate and acetylcholine, may not be unprecedented [94]. However, our evidence provides no support for the possibility that the cells might release these at different sites, or even in different neuropils (L1 in the medulla, and L2 also in the lamina).

While this paper was in revision an elegant study appeared on the distribution of a vesicular glutamate transporter in Drosophila [95]. The authors of that report used a different vglut promoter Gal4, but one of the vGluT antisera [61] also used in our investigation. Although the paper did not report on vGluT distribution in the lamina, the authors report expression in the medulla, where, as in our study, they found no conclusive evidence for vglut or vGluT expression in the terminals of L1 to L3.

In addition to the monopolar neurons, strong vGluT immunolabeling was seen in structures resembling the α-processes of amacrine neurons, and this could be correlated with immunoreactivity to glutamate seen by electron microscopical analysis. Sinakevitch and Strausfeld [47] also detected such immunoreactivity in the fly’s Phaenicia sericata, thus providing some measure of support for a glutamatergic phenotype in the lamina amacrine cells.

Overall, there are some incongruencies in the data for glutamate signaling: the processes of monopolar neurons express immunoreactivity for glutamate but not the vesicular transporter, while the amacrine cells express immunolabeling for the transporter but not the expected Gal4 expression. To resolve some of these issues, we had hoped to see DmGluRA expression in lamina circuits, but very weak labeling was seen and this could not readily be assigned to any specific neuron type. Since we detected very strong DmGluRA immunolabeling in neurons of the medulla, we presume the expression level is just very low in the lamina neurons. It was therefore surprising that the antisera to the NMDA1 receptor subunit used in a previous study [47] labeled neither neuron in the lamina nor elsewhere in the visual system. The antisera was raised against a sequence of a mammalian NMDA1 receptor protein with limited similarities to that in Drosophila and thus not likely to display much cross-reactivity in Drosophila. However, we could show rather strong immunolabeling of neurons in the mushroom body lobes, suggesting that again the lack of signal could be a matter of low levels of expression in the visual system of Drosophila.

Adopting cautious criteria, we can summarize the positive findings on glutamate signaling components in the lamina as follows. We find evidence that the α-processes of lamina amacrine neurons express vGluT, and glutamate. These neurons, which we might therefore predict to be glutamatergic, have many outputs onto β-profiles of T1 neurons, and onto R1, R6 and L1–L3 neurons [9]. Compatible with this suggestion, Sinakevitch and Strausfeld [47] reported NMDA1 receptor-like immunoreactivity on T1 neurons in P. sericata. Glutamate may thus be used as a transmitter in amacrine neurons for signaling within the lamina at some of its minority classes of synapses, but that neither L1 nor L2 shows clear evidence of doing so at their chief output terminals in the medulla.

**GABA signaling**

Our immunocytochemical data show that C2 and C3 neurons (identified by Gal4-driven GFP) express both GABA and GAD. Neither of these neurons was detected using a GAD1-Gal4 line [96] tested here, and no other lamina neuron clearly expressed GAD1 or GABA immunoreactivity. An exclusive GABA phenotype among centrifugal neurons is confirmed earlier reports on Drosophila and other flies [42,43,44,45]. Previously we have also shown that the C2 and C3 neurons express the *Drosophila* vesicular GABA transporter [68], further suggesting that these neurons signal by means of GABA.

We localized GABA<sub>B</sub>R immunoreactivity in relation to various identified neurons. For this we used an antisera to the GABA<sub>B</sub>R2, a G-protein coupled receptor known to dimerize with the GABA<sub>A</sub>R1, to form a functional receptor complex [97,98]. Thus our observations are likely to reveal functional GABA<sub>B</sub> receptor sites [see [68]]. At least three neuron types seem to express GABA<sub>B</sub>Rs: C2, C3, and the tangential neuron Cha-Tan. Possibly there is an additional neuron type not identified that may be postsynaptic to the C3 neurons that express GABA<sub>B</sub>Rs, since we also see immunoreactivity adjacent to C3’s boutons. The likely contacts between GABAergic C2 neurons and large boutons of Cha-Tan neurons are quite distinct and express high levels of GABA<sub>B</sub> receptor immunoreactivity. The presence of GABA<sub>B</sub>R on C2 terminals in the distal lamina indicates the presence of presynaptic GABA receptors at a GABA output site of these neurons. Similarly GABA<sub>B</sub>R immunoreactivity is associated with the varicosities of the GABA<sub>A</sub>C3 neurons. These varicosities can be assumed to be GABA release sites, and are known to provide input to L1–L3 and amacrine cell processes and to receive no inputs themselves [9,13]. Thus the GABA<sub>B</sub> receptor may be presynaptic in both the C2 and C3 neurons. Both pre- and postsynaptic locations of GABA<sub>B</sub>Rs have in fact been identified in mammals (see [99]). There, presynaptic GABA<sub>B</sub>R activation inhibits transmitter release by inhibiting voltage-gated Ca<sup>2+</sup> channels via the β<sub>2</sub>/γ subunit of the G-protein, or by inhibiting adenylate cyclase via G<sub>11</sub> proteins [99]. In this way, GABA release from C2 or C3 may be negatively regulated.

The distribution of GABA<sub>A</sub> type receptors in the lamina is still not clear, because the antisera to the *Drosophila* GABA<sub>A</sub> receptor subunit RDL failed to produce distinct lamina immunolabeling. An earlier study suggested that at least part of the RDL-immunolabeling may be localized to L2 monopolar cells [68]. Here we utilized an rdl-Gal4 line to drive GFP, and although we

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**Transmitters in Fly Lamina**

- In Drosophila, the authors of a report used a different *vglut* expression to drive GFP in large monopolar neurons, but did not detect clear *vGluT* immunolabeling in monopolar neurons. Low levels of *vGluT* immunolabeling were seen in cell bodies of large monopolar neurons and dendrites of these neurons may be masked by stronger immunolabeling seen in amacrine processes. Another possibility is that the amount of vesicle-bound glutamate (and *vGluT*) is too low to detect.

- The authors detected *vGluT* immunolabeling in the L2 medulla terminals of flies, again possibly because there was insufficient protein to yield a clear immuno signal. They could not completely exclude the presence of glutamate immunoreactivity seen previously in other studies.

- In the monopolar neurons, strong *vGluT* immunolabeling was seen in structures resembling the α-processes of amacrine neurons, which could be correlated with immunoreactivity to glutamate seen by electron microscopy. Sinakevitch and Strausfeld detected such immunoreactivity in *Phaenicia sericata*.

- Overall, there are incongruencies in the data for glutamate signaling. The processes of monopolar neurons express immunoreactivity for glutamate but not the vesicular transporter, while amacrine cells express immunolabeling for the transporter but not the expected Gal4 expression.

- The distribution of GABA<sub>B</sub> and GABA<sub>A</sub> receptors in the lamina is still not clear due to limitations in the antisera. GABA<sub>B</sub> receptors are likely to reveal functional receptor sites.

- GABA<sub>B</sub>R immunoreactivity is associated with varicosities of GABA<sub>A</sub>C3 neurons. GABA<sub>B</sub>Rs can be assumed to be GABA release sites, providing input to L1–L3 and amacrine cell processes.

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**GABA signaling**

- Our immunocytochemical data show C2 and C3 neurons (identified by Gal4-driven GFP) express both GABA and GAD.

- Neither of these neurons was detected using a GAD1-Gal4 line tested here, and no other lamina neuron clearly expressed GAD1 or GABA immunoreactivity.

- GABA<sub>B</sub> signaling is confirmed by earlier reports on Drosophila and other flies. Previous studies have shown that the C2 and C3 neurons express the *Drosophila* vesicular GABA transporter, further suggesting these neurons signal by means of GABA.

- We localized GABA<sub>B</sub>R immunoreactivity in relation to various identified neurons. For this, we used an antisera to the GABA<sub>B</sub>R2, a G-protein coupled receptor. The presence of GABA<sub>B</sub>Rs in C2 terminals in the distal lamina indicates the presence of presynaptic GABA receptors at a GABA output site of these neurons.

- Similarly, GABA<sub>B</sub>R immunoreactivity is associated with varicosities of the GABA<sub>A</sub>C3 neurons. These varicosities can be assumed to be GABA release sites, and are known to provide input to L1–L3 and amacrine cell processes and to receive no inputs themselves.

- The GABA<sub>B</sub> receptor may be presynaptic in both the C2 and C3 neurons. Both pre- and postsynaptic locations of GABA<sub>B</sub>Rs have been identified in mammals. There, presynaptic GABA<sub>B</sub>R activation inhibits transmitter release.
were unable to demonstrate that lamina neurons revealed by rdl-Gal4 actually produce RDL, a good match between the markers has been seen in many parts of the larval CNS (Enell and Näsbl, unpublished). The rdl-Gal4 labels L4 monopolar neurons and rdl-Tan neurons. At least rdl-Tan neurons may be targets of GABAergic C2 neurons as seen in our study, whereas the L4 neurons are not known to be postsynaptic to either C2 or C3 neurons [9], so that receptor expression on these monopolar cells is unexplained and may be targeted to the medulla terminals.

In summary, GABA seems to be primarily (or exclusively) used by centrifugal neurons from the medulla with outputs in the lamina, one of which (C2) may signal to wide-field tangential neurons of the lamina.

Conclusions
This study has increased the number of lamina neurons for which a putative neurotransmitter has now been identified and has also localized GABA\(_B\) receptors to identified neurons (see Table 2). There are, of course, still many neuron types for which transmitters remain unknown. Perhaps the greatest mystery of all remains whether the large monopolar neurons utilize glutamate or acetylcholine as neurotransmitters, or whether they may possibly release both. They appear qualified to use either, but it is neither clear which they actually use, nor whether release is the same at sites in the lamina and medulla, or in different strata of these neuropils. It is also evident that glutamate receptors and RDL subunits of GABA\(_A\) receptors are expressed at levels too low to be reliably detected in the lamina. Our study now prompts the complete morphological characterization of the possibly novel types of tangential neurons Cha-Tan and rdl-Tan. These are perhaps variants of the La w1 and 2 neurons already described. It is also urgent to determine the neurotransmitter of the L1 and L2 neurons and to localize ionotropic receptors for acetylcholine, GABA and glutamate in the lamina circuits.

Since, in contrast to the lamina of locusts, cockroaches or other non-dipteran insects [100,101,102], it appears that lamina interneurons in flies express neither monoamines such as histamine, dopamine, octopamine or serotonin (see [35,103]) nor identified neuropeptides, further work will be required to screen for small-molecule neurotransmitters in those neurons not yet assigned a signal molecule. Co-expression of yet unidentified neuromodulators clearly remains an additional possibility, revealed for example by dense-core vesicles in C2 [9]: their Fig. 36A), alongside the clear vesicles which we may now presume to contain GABA. Thus, the complete neurotransmitter repertoire of the medulla reveals not only immunoreactive chiasmal fibers, as seen in Musca (B,D) but also their axon profiles and terminals in the array of medulla columns, and tangential fibers. Scale bar: 1 µm. Profiles and terminals in the array of medulla columns, and tangential fibers. Scale bar: 1 µm.

Figure S2 Immuno-EM labeling of lamina cartridges in Musca to be reliably detected in the lamina. Our study now prompts the complete morphological characterization of the possibly novel types of tangential neurons Cha-Tan and rdl-Tan. These are perhaps variants of the La w1 and 2 neurons already described. It is also urgent to determine the neurotransmitter of the L1 and L2 neurons and to localize ionotropic receptors for acetylcholine, GABA and glutamate in the lamina circuits.

Supporting Information
Figure S1 Confocal examination of glutamate-like immunoreactivity in the optic lobes of Musca and Calliphora. A-D: Musca. A: Tangential section of the lamina, revealing the array of cartridges, and the repeated pattern of immunoreactive profiles. B: Horizontal section, showing longitudinally sectioned axon profiles in the lamina, and medulla, and the heavy labeling in the external chiasma between the two neuropils. C: At higher magnification, each cartridge is revealed by large immunoreactive profiles at its core (small circle) circumscribed by a ring of small profiles (within the large circle) contributed by α-processes of amacrine cells. The perikarya of some monopolar cell somata in the lamina cortex also exhibit faint immunoreactivity. D: Paired axon profiles (circles) are especially clear deep in the proximal lamina, in a section plane that cuts the adjacent chiasma. E: Calliphora. Tangential section

In summary, GABA seems to be primarily (or exclusively) used by centrifugal neurons from the medulla with outputs in the lamina, one of which (C2) may signal to wide-field tangential neurons of the lamina.
Attempts to correlate the distribution of vesicular glutamate transporter (vGluT) immunolabeling with structures revealed by Gal4-driven GFP. Here we used an n-synaptobrevin-GFP fusion (nSyb-GFP) to direct GFP mainly to synaptic terminals (green). A, The 21D-Gal4 driver nSybGFP primarily in the medulla (Me) terminals of the L2 neurons. Commensurate with the 10-fold fewer presynaptic sites in the lamina (L), B Details of nSyb-eGFP expressing L2 terminals in the medulla in oblique cross section. C The same terminals seen with vGluT immunolabeling. The two labels do not co-localize, indicating that the L2 neurons do not express vGluT in the medulla. D1–D3 Frontal sections of the medulla showing L2 terminals displayed by 21D-Gal4 crossed to UAS-nSyb-GFP and vGluT immunolabeling (magenta). Again there is no co-localization of labels. E1–E3 Frontal sections of medulla comparing the distribution of OK371-Gal4-driven nSyb-eGFP and vGluT immunolabeling. In contrast to many other parts of the brain the two markers do not co-localize in most structures, except partly in the inner medulla layers (IL). In particular, clear-cut labeling was seen neither in the L1 nor L2 terminals.

References


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Author Contributions

Conceived and designed the experiments: IM DN AK. Performed the experiments: AK XS. Analyzed the data: IM DN AK. Contributed reagents/materials/analysis tools: DN. Wrote the paper: IM DN.


