Cholinergic Circuits Integrate Neighboring Visual Signals in a Drosophila Motion Detection Pathway

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Summary

Detecting motion is a feature of all advanced visual systems [1], nowhere more so than in flying animals, like insects [2, 3]. In flies, an influential autocorrelation model for motion detection, the elementary motion detector circuit (EMD; [4, 5]), compares visual signals from neighboring photoreceptors to derive information on motion direction and velocity. This information is fed by two types of interneuron, L1 and L2, in the first optic neuropile, or lamina, to downstream local motion detectors in columns of the second neuropile, the medulla. Despite receiving carefully matched photoreceptor inputs, L1 and L2 drive distinct, separable pathways responding preferentially to moving “on” and “off” edges, respectively [6, 7]. Our serial electron microscopy (EM) identifies two types of transmedulla (Tm) target neurons, Tm1 and Tm2, that receive apparently matched synaptic inputs from L2. Tm2 neurons also receive inputs from two retinotopically posterior neighboring columns via L4, a third type of lamina neuron. Light microscopy reveals that the connections in these L2/L4/Tm2 circuits are highly determinate. Single-cell transcript profiling suggests that nicotinic acetylcholine receptors mediate transmission within the L2/L4/Tm2 circuits, whereas L1 is apparently glutamatergic. We propose that Tm2 integrates sign-conserving inputs from neighboring columns to mediate the detection of front-to-back motion generated during forward motion.

Results and Discussion

Pairs of L1 and L2 neurons, one for each module or cartridge of lamina neuropile, extend axons down the cartridge axis to terminate in distal strata of the medulla ([8]; Figures 1A, 1B, and 1I). In the lamina they receive input from the outer photoreceptor neurons R1–R6 of the ommatidia [9]. These are rod-like [10], and in Drosophila their terminals each bear ~50 tetrad synapses [11]. The R1–R6 input to L1 and L2 is closely matched because these two cells are invariant postsynaptic partners at the tetrads [12, 13]. In vivo calcium imaging reveals that L1 and L2 both respond positively to light decrements and negatively to light increments [7]. However, behavioral and electrophysiological studies reveal that they nevertheless mediate two separable pathways responding preferentially to moving light and dark edges, respectively [6, 7]. In addition, the L2 pathway is fine-tuned for front-to-back motion detection at low contrasts [14] and differentially modulates translational and rotational walking behaviors [15].

L1 and L2 Provide Independent Pathways to the Medulla

The axons of L1 and L2 traverse the external chiasma, their paired terminals innervating specific strata of each medulla column (Figures 1A and 1B; [16]), L1 in strata M1 and M5 and L2 with a single expansion in M2 [16, 17]. Each horizontal sheet of axons twists en route to the distal medulla so as to invert the retinotopic map by which lamina cartridges project upon the corresponding array of medulla columns [18]. L1 and L2’s terminals align across the array of medulla columns (Figures 1A and 1B), emphasizing the respective strata, and even though the alignment between neighboring columns may not be perfect [16], the strata can be differentiated by immunolabeling with antibodies against synaptic proteins, such as Bruchpilot (or Brp) or Discs large (Dlg; see Figures 1C and 1D). Some strata are differentially labeled by antibodies against various adhesive receptors, such as Capricious, FasciclinIII, and Connectin (Figure 1B; [19]). Thus, the definition of stratum M4 originally revealed from Golgi impregnation [17], although not clear in the single columns from our electron microscopy (EM) series [16] is suggested by the absence of immunolabel using anti-Connectin C1.427 (DSHB) (Figure 1B).

Immunohistochemical evidence reveals a glutamate phenotype in both L1 and L2 [20, 21]. However, genetic reporter studies suggest that these two cells—which define the two motion-sensing channels—might, despite their closely matched R1–R6 input in the lamina [11, 13], actually employ different neurotransmitters [19]. To examine their neurotransmitter and receptor phenotypes, we developed techniques to profile relevant transcripts with single-cell resolution. We manually dissociated laminas and isolated single GFP-labeled L1 and L2 cell somata and designed primers for RT-PCR reactions (for details, see Experimental Procedures and Figure S1 available online). We found that L1 expressed vesicular glutamate transporter (VGlut) and L2 choline acetyltransferase (Cha), but not vice versa, implying that L1 and L2 are glutamatergic and cholinergic, respectively. We quantified VGlut and Cha transcript levels using real-time PCR analyses and found that L1 neurons express 5,980 ± 830 copies of VGlut transcript per cell, whereas L2 neurons express 5,730 ± 710 copies of Cha transcript (see Experimental Procedures, Tables S1 and S2, and Figure S2 for details), indicating that both are highly abundant transcripts comparable to those for Rp49, a ribosomal protein (L1: 8,210 ± 290 copies per cell; L2: 8,529 ± 196 copies per cell). Our finding that L2 lacks VGlut transcript fails to confirm its expression of both glutamate [20, 21] and a glutamate transporter [22]. Previous observations on the
Housefly Musca have revealed that L2 and L4 form a network of reciprocal connections in the lamina mediated by L4’s collaterals that invade two posterior cartridges [23, 24]. This pattern also occurs in Drosophila (Figures 1F–1H; [11, 13]), and a critical role for L4 in motion detection is suggested by a recent behavioral study [25]. L1 and L4 lack direct synaptic connections [13, 26]. To determine the nature of transmission at L2/L4 connections, we extended our profile analyses to all known cholinoceptor transcripts and to L4. We found that, like L2, L4 also expresses Cha but not VGlut. L2 and L4 share expression of the Dα7 and Dβ1 subunits of nicotinic cholinoceptors (nAcR), but each expresses a unique α-subunit (Dα3 in L2 and Dα4/5 in L4; Table S1). In contrast, L1 lacks detectable acetylcholine receptors. Insofar as L2 and L4 express nicotinic but not muscarinic cholinoceptors, we conclude that L2 and L4 probably provide fast reciprocal excitatory inputs to each other.

Recent studies have begun to probe the internal structure of the elementary motion detector circuit (EMD) [7, 27, 28]. Two very different computational models, including a weighted four-quadrant detector and a two-quadrant detector with an additional direct current (DC) component, have been proposed [7, 27]. Despite differences, both computational models, like the original Reichardt EMD, require communication between neighboring visual signals [4, 5]. The topology of the L2/L4 pathway communicates between anteroposterior (AP) rows of columns, and so could provide the substrate for this communication. Missing, however, are the identities

**Figure 1. Strata and Terminals of the Distal Medulla**

(A–E) Expression of specific markers designates six outer strata M1–M6. Scale bar (in A) represents 10 μm.

(A) Relative to photoreceptor-specific anti-Chaoptin (MAb24B10, red), L1’s bistratified terminals (green, arrows) in M1 and M5 are revealed by L1-Gal4 driven GFP, and L2’s terminals in M2 by hemagglutinin (HA)-tagged Ora transientless ORT (HA-ORT; cyan, arrowhead) expression in the 21D enhancer trap pattern.

(B) Relative to the same L1 and L2 markers in (A), anti-Connectin (α-Connectin, red) immunolabels stratum M3 immediately below the terminals of L2, leaving a space, presumably stratum M4, beneath M3 and the deeper terminals of L1 in M5.

(C) Medulla strata revealed by GFP expressed in the ort pattern (green) and anti-Discs Large (Dlg, cyan), L3 (double arrowhead) and the proximal L1 (arrow) terminals are discernable using GFP and anti-Dlg immunostaining.

(D and E) Single-cell flip-out clones of L4 (GFP, green).

(F–H) L4 axon and associated collaterals (green) in the proximal lamina. Scale bar (in F) represents 5 μm.

(F) Wire transformation of (G) with axon marked in cyan. Lamina cartridges are revealed by anti-Chaoptin staining (MAb24B10, red).

(G) View of (F) as seen in a direction looking outwards, from a proximal location toward a distal one. The L4 axon (cyan) is located at the posterior side of its cognate lamina cartridge and extends collaterals (green) to its cognate, posteroventral, and posterodorsal cartridge neighbors.

(I) Diagram of cell types, and the respective tiers of their medulla terminals and dendrites for lamina (R7, orange; R8, red; L1,L4, green; L2, cyan) and medulla (Tm1,Tm2) cells.
of L1 and L2’s target neurons in the medulla. To address these downstream circuits, we chose the circuits of L2 because of their importance for front-to-back motion sensing in a flying insect, their cholinergic neurotransmitter phenotype, and because they engage another lamina cell type, L4.

**L2’s Synapses Incorporate Tm1 and Tm2 Medulla Cell Targets**

L2 has a single subdivided terminal (Figure 2A), and three such completely reconstructed terminals each had between 88 and 98 presynaptic sites in stratum M2 [16]. Like R1–R6 tetrads in the lamina and the synapses of R7 and R8 in the medulla [16], each site was marked by a presynaptic ribbon, T-shaped in cross-section (Figure 2B). All synapses were of the multiple-contact type, with at least three dendrites visiting each presynaptic site. Occasional presynaptic sites and T-bar ribbons seen en face, in the plane of the plasma membrane, had a 4-fold symmetry suggesting that they had four postsynaptic elements, but because these were tiny, we could usually trace at most only three. Although, because of their small size, most dendrites were difficult to trace back to a parent neuron, many received redundant contacts from the same neuron, providing assurance that we had identified individual dendrites accurately. In addition to its presynaptic sites, the L2 terminal in column 2 was also postsynaptic at 26 contacts from other medulla cells, mostly C2 and C3 [16].

Through strata M1–M6 in column 2, we reconstructed two clear columnar neurons with particular morphological features. The axons of both were located in the midposterior position of the column cross-section and ran alongside each other down the length of the distal column. Their identities were confirmed by less complete reconstructions from the two other columns. The first neuron closely resembled transmedulla cell Tm2 previously reported from Golgi impregnation...
profiles of which have already been the subject of detailed light and electron microscopy comparisons [29]. Although we lacked information on its terminal in the next neuropile, the lobula, we could identify this neuron based especially on the defining feature of two or three branched descending dendrites that projected in a proximal direction like walking legs, from their origin at the axon in stratum M2, to a deeper level (Figures 2C, 2D, and 2J). The second neuron was identified as Tm1, based on the presence of a densely branched system of dendrites in stratum M3 (Figures 2C and 2D). Although its morphological identity was less clear, its partnership with Tm2 was suggested by the fact that both axons fasciculated together, whereas the frequent impregnation of Tm1 by the Golgi method [17] suggests that this cell is present in all columns. Tm1 is also one of several previously identified cell types with quantitatively analyzed patterns of coarborization in different medulla strata that belongs to pathway 2, tentatively identified as the L2 pathway [30].

From the 98 presynaptic sites of L2's terminal in column 2, Tm1 and Tm2 were both identified postsynaptic elements at nearly half; at 12 they partnered each other at the same sites. The low probability (12 out of 98, or 0.12) of tracing two postsynaptic elements to the same synapse resulted partly from the combined low probabilities of tracing either Tm1 or Tm2 alone (≈40 out of ~98, or about 0.4). This in turn is because the dendrites are generally very fine and thus hard to follow through consecutive sections. We therefore propose that many more of L2's synapses incorporate both Tm1 and Tm2. Given this low probability, we suggest that Tm1 and Tm2 are the chief targets of L2 at possibly most of its synapses and that both are projection neurons that establish a dichotomous pathway to the lobula. L2 terminals overlapped Tm2 dendrites in every column (Figures 2E–2G), supporting the primacy of this pathway. The terminals of Tm2 expanded in the two outermost strata of the lobula, Lo1–Lo2, where they formed a continuous array across the face of that neuropile (Figures 2H and 2I). Tm1, by contrast, occupies only the superficial stratum Lo1 [17]. To confirm that Tm1 is also present in all columns, we must await a Gal4 driver specific for this cell type.

The L2 Pathway Incorporates Binary Subdivisions

Visual systems process features in a series of parallel pathways in distinct neuropile strata. This requires that light-evoked signals be split to provide input to the circuits of different parallel pathways. Light-evoked signals in R1–R6 are split at the first synapse into two different channels, L1 and L2. As we now see, L2 in turn splits these signals in the medulla into two further channels, Tm1 and Tm2. Other cells also receive the same signal at either synapse. Some, like amacrine cells of the lamina, may provide feedback to the R1–R6 input [13], whereas others may establish an independent pathway, as L3 does in the medulla [16, 19]. The similarity between the matched inputs of L1 and L2 at the first synapse and, at the second, the likely matched inputs of Tm1 and Tm2 in the L2 pathway of the medulla is rather striking, and a candidate mechanism for establishing motion opponent pathways [4]. Further anatomical and functional studies would be needed to determine whether the Tm1 and Tm2 pathways indeed converge antagonistically at downstream targets. Although previous reports (review: [31]) have identified a single type of medulla Tm neuron in the L2 pathway, none has clearly identified two, and thus none has recognized the binary split that occurs in this pathway in the medulla.

Tm2 Also Receives Input from L4

L4's axon moved to one side of its column soon after entering stratum M1 and deeper formed three small terminals that arose from short collaterals and partially overlapped the proximal terminals of L1 and L5 [16], in strata M4/5 (Figures 1D and 1E). The walking leg dendrites of Tm2 descended into the medulla in either a dorsal, ventral, or anterior direction in the column, with possibly some variation. In strata M4/M5, these contacted the terminals of L4 (Figure 1F; Figures 3A and 3B) from which they received synaptic inputs, from the parent column and its two posterior neighbors (Figure 3C). Although synaptic contacts were observed in M4/5, the cells also overlapped each other in M2 (Figures 3D–3F). Thus Tm2 received identified synaptic input from two types of lamina neurons, but in different strata, L2 in stratum M2 and three L4 terminals in strata M4/M5; light microscopy also suggested L4 input in M2 as well. The L4 input helped us to identify a combined L2/L4 pathway in the medulla, which resembles that seen in the lamina, where L4 provides input directly to L2 (Figures 1F–1H) [13, 24, 26]. In the medulla, such input is received at a common target neuron, Tm2, however. Given the inversion of the chiasma between lamina and medulla, L4's synapses in both neuropiles provide input to the columns that neighbor their own in a retinotopically anteroposterior direction (Figure 4). The direction of L4's spread in both neuropiles thus corresponds to an ommatidial sequence from the retina's front to its back and thus to the direction of the fly's forward locomotion.

To determine how reliably the L4-Tm2 connections observed in EM repeat in different columns, we examined approximately 150 pairs of L4/Tm2 neurons by light microscopy. To resolve Tm2's dendritic arbor, which frequently overlaps neighboring Tm2 neurons, we labeled Tm2 neurons stochastically with a membrane-tethered RFP marker, using a pair of split LexA drivers [32] that express only in approximately 40% of Tm2 neurons (Figure S3A). We also randomly labeled L4 axons with a GFP marker using a combined apterous-Gal4 driver and lamina-specific flipase (Dac-Flip), with a UAS-stop>GFP reporter (Table S3). This double-stochastic labeling method allowed us to determine how often Tm2's dendrites overlapped the four potential L4s, one in the cognate and three in neighboring columns (posterior, postero dorsal, and posteroventral). Supporting our EM images in stratum M2, Tm2's dendrites formed apparent contacts with all three of the L4 axons but, despite their being in physical proximity, not with L4 from the posterior column (100%, n = 34, 55, 40, for cognate, postero dorsal, and posteroventral columns, respectively; 0%, n = 29 for posterior columns). Similarly, in strata M4/M5, Tm2's dendrites also formed apparent contacts with the same appropriate L4 neurons. However, these connections were not as robust as those in M2 and failures to overlap were observed. Such failures were not distributed equally for all partners, however, with 3%–4% failures for dorsoventrally aligned pairs, and 9% for the cognate pair (Figure 3M). In addition, we observed a rare posterioroanteriour L4/Tm2 pair (3.4%; Figure 3M).

Given that both L2 and L4 express Cha and are thus genotypically qualified to synthesize acetylcholine and provide cholinergic input to Tm2, we next profiled the expression of acetylcholine receptors in Tm2. This proved more complex than for L2 and L4. In addition to D:7 and D:1 aNaCr shared with L2 and L4, Tm2 also expressed D:o1/2 and D:1 nAcR (Table S1; Figure 4C). The exclusive expression of nicotinic rather than muscarinic receptors (nAcR not mAcR) in Tm2
suggests that both L2 and L4 provide fast excitatory inputs to Tm2. We also found that Tm2 expressed Cha but not VGlut, indicating that, like L2 and L4, Tm2 is also genotypically cholinergic. In summary, these data predict that both synaptic connections in the L2/L4/Tm2 network are mediated by excitatory acetylcholine systems, and therefore sign conserving.

Asymmetrical Connections in the Motion Detection Pathway

Although either the L1 or L2 channel alone can mediate rudimentary motion detection, each also responds differentially in walking flies [15], and in head-yaw assays, the L2 pathway is preferentially tuned to front-to-back motion [14]. Although the connections between L4 and L2 along the anteroposterior direction might account for this front-to-back preference [24], these connections are reciprocal [13, 24, 26] so that targets, only Tm2 receives two additional L4 inputs from neighboring posterior columns; Tm1 does not. These L2/L4/Tm2 connections are highly determinate, underscoring a critical role in connecting neighboring L2 channels along the AP direction, in what is arguably the most important motion direction for flies because it occurs during forward flight. Interestingly, other flies have a Tm neuron closely resembling Drosophila’s Tm2 morphologically, for example Tm1 in the calliphorid Phaenicia [34]. This is proposed to receive L2 inputs, suggesting that an L2/L4/Tm2 network might be conserved in higher Diptera.

Tm2 could conceivably serve as half of the EMD’s multiplier stage, comparing the temporally delayed input from collateral L4s with the cognate signal from L2. However, electrophysiological investigations on calliphorid “Tm1” neurons, which resemble morphologically Drosophila’s Tm2 [17], have yet to

Figure 3. L4 Provides Input to Tm2

(A and B) Corresponding electron microscopy (EM) reconstructions of L2 terminal (blue), L4 (red) and Tm2 (green) seen from anterior (A) and ventral (B) views. Three sets of L4 terminals (arrow [posteroventral], arrowhead [cognate], and double arrows [posterodorsal]) contact descending walking leg dendrites from Tm2. Scale bar represents 5 μm.

(C) L4 synapses (arrowhead) onto a dendrite of Tm2. L4 terminal contains vesicle profiles with a mean diameter of 37.85 ± 2.21 (SD) nm, predicted to contain acetylcholine. Other postsynaptic elements at this synapse are unclear. Scale bar represents 0.5 μm.

(D–F) Tm2 dendrites (pseudocolored in green) form apparent contacts (yellow, arrows) with L4 axons (pseudocolored in red) from cognate (D and G), posteroventral (E and H), and posterodorsal (F and I) columns at two neuropile levels, in M2 and M4/M5. For clarity, the neighboring L4s and Tm2s are not shown (see Figure S3 for details). Scale bar represents 10 μm.

(G–I) Wire transformations of the corresponding panels (D–F) (above) shown relative to an array of 24B10-immunolabeled R7/R8 pairs at the center of each column at stratum M3.

(J–L) A Tm2 neuron (green) and two L4 axons from the posterior and posteroventral columns. For clarity, the neighboring processes have been blocked from view.

(J) The blue channel has been removed from (K). The Tm2 dendrites form apparent contacts (arrows) with L4 from the posteroventral column, but not with L4 from the posterior column (double arrow).

(K) Wire representation of (J), viewed from the same direction.

(L) View of (K) as seen in a direction looking outwards, from a proximal location toward a distal one.

(M) A schematic representation of Tm2 (green) and four neighboring L4s (red) from the cognate (C), posterior (P), posterodorsal (PD), and posteroventral (PV) columns. Photoreceptor axons (blue) mark the column array. Probabilities of overlap between the L4 terminals of four surrounding columns and the dendrites of Tm2 in the central column are shown as percentage values, first in M2 then in M4, of the total numbers of such pairs examined, shown in parentheses. Despite the proximity between Tm2 and the L4 terminals of all four columns, L4 in the posterior column almost never (only 1 in 29) overlapped Tm2, whereas the three others did so in at least 91% of pairs. All errors occurred in stratum M4.
provide strong evidence for this role [35, 36]. An alternative interpretation is that the L2/L4/Tm2 network serves instead as a prefilter in the preprocessing stage, whereas Tm2’s output feeds into the multiplier stage [37]. The topology and sign-conserving nature of L4/Tm2 connections suggest the spatial summation of neighboring visual signals, which could increase light sensitivity at the expense of spatial acuity. It has been suggested that under low luminance conditions, neighboring visual signals are indeed pooled prior to their interaction at the multiplier stage, whereas at higher luminance levels, nearest-neighbor interactions dominate motion detection [38–40]. Alternatively, the L4/Tm2 connections could convert visual signals sampled from the hexagonal ommatidial array into an orthogonal coordinate upon which motion signals can be derived [41, 42]. Differentiating between these possibilities must await future investigations that combine genetic and electrophysiological approaches.

Relating Synaptic Connections to Circuit Functions

Our study begins the difficult task of using three-dimensional reconstructions to identify the many different types of medulla cell, especially transmedulla cells with subtly different arbors and, in *Drosophila*, very fine caliber neurites [17]. We adopted a sparse, cell-by-cell reconstruction strategy to identify relevant circuit elements in the motion pathway. Our results have reinforced the hope that even in densely packed neuropiles, such as the medulla, it is technically feasible, though laborious, to identify connections and assign them to identified neurons, a process also open to validation against genetic reporters [29]. Annotating synaptic contacts must proceed in the face of axon terminals that are not exclusively presynaptic and dendrites that are not exclusively postsynaptic [16]. Indeed, in addition to the L2 inputs they receive in stratum M2, Tm1 and Tm2 dendrites were both also presynaptic, having T-bar ribbons in strata M2 and M3 (Figure S4) that provide inputs to neurons yet to be identified. Axon terminals, such as those of L2, can also be postsynaptic [16]. The power of EM reconstructions is that these alone reveal such important details.

Of course, a complete map of synaptic circuits does not easily relate synaptic connections to neuronal functions. EM reconstruction is moreover low-throughput, making it difficult to determine whether an identified connection occurs reliably in different regions and/or different animals. Here we confront that difficulty by combining a light-microscopic approach to assess the robustness of connections identified from EM. For example, despite their paucity, L4 to Tm2 contacts are highly determinate, suggesting that synapse number need not accurately predict functional significance in any simple synaptic democracy. Although connections revealed by EM provide information on the direction of transmission, they fail to reveal its polarity or dynamics, which requires knowledge of receptor expression, as we now provide for the cholinergic genotype of the L2/L4/Tm2 pathways. Unlike promoter constructs, which even when available may suffer from positional effects, single-cell transcript profiling demonstrated in this study directly probes the expression of neurotransmitter receptor genes. Determining not only the anatomical connections but also their robustness and neurotransmitter components will all be crucial both to understand how information is transmitted and to manipulate it to achieve particular behavioral outcomes.

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**Figure 4. Network of Connections between L2 and L4 and Their Common Medulla Target Tm2**

(A) Plan view. Top shows connections between L2 and L4 in the lamina. L4 reciprocally contacts its own L2 and through collaterals the L2 of the two posterior cartridges [13, 24, 26], shown for a patch of seven cartridges with orientations abbreviated as follows: A, P, anterior, posterior; D, dorsal; eq, equator. The chiasma between lamina and medulla inverts this array. Bottom shows that in the medulla, Tm2 receives input from its own L2 and L4 and from the L4 of two posterior columns. See text for details.

(B) The same connections as in (A), in side view. Note that the chiasma has been inverted to emphasize that connections between L4 and L2, in the lamina, or between L4 and L2’s target in the medulla, Tm2, are both directed in a retinotopically posterior direction. Note the binary split from R1–R6 to L1/L2 in the lamina and between L2 and Tm1,Tm2 in the medulla.

(C) Neurotransmitter and receptor profiles for L1, L2, L4, and Tm2. The L2/L4/Tm2 network is mediated by cholinergic connections (ACh) and each neuron expresses a shared pair of nicotinic receptor subunits (nAcRα7/β1) as well as type-specific nAcR receptor subunits. L1 is genotypically glutamatergic (Glu) and expresses no detectable acetylcholine receptors. In addition, L1 and L2 are connected via gap junction (resistor symbol).
is transformed within identified synaptic circuits and to attain a major goal of functional connectomics.

Experimental Procedures

Fly Stocks
Fly stocks were maintained on standard fruit fly medium at 23°C–25°C. Fly stocks used in this study are listed in Table S3 and described in the Supplemental Experimental Procedures.

Single-Cell Transcript Profiling
Single L1, L2, L4, and Tm2 neurons labeled with GFP were dissociated from adult lamina or medulla and collected using a custom-made capillary aspiration system. Total RNA from lysed single cells was reverse-transcribed to complementary DNA. PCR analyses were carried out to determine the presence of specific transcripts for VGluT, Cha, and nicotinic and muscarinic acetylcholine receptors. Real-time PCR was carried out to quantify the transcript levels of VGluT and Cha. Rp49, which encodes a ribosomal protein, was used as an internal reference. Detailed procedures for cell dissociation, single-cell isolation, PCR primer design, single-cell PCR assay, and real-time PCR assay are provided in the Supplemental Experimental Procedures.

Immunohistochemistry
Immunohistochemistry, confocal imaging, image deconvolution, and 3D image rendering were performed as described previously [43]. Neuronal processes were traced using the FilamentTracer module in Imaris (Bitplane). Images shown in figures are maximal projections of multiple optical sections (0.2 µm). The following concentrations of primary antibodies were used: 24B10 (DSHB), 1:100 dilution; Connectin C1.427 (DSHB), 1:100 dilution; Cha, 1:100 dilution; mouse anti-GFP Mab (IgG2a, Invitrogen), 1:200 dilution; rat anti-C2D2 (Serotec), rabbit anti-GFP, 1: 500 dilution (Torrey Pines Biotabs). The secondary antibodies including goat anti-rabbit, rat, or mouse IgG coupled to Alexa 488, Alexa 568, or Alexa 647 (Invitrogen) were used at 1:400.

Electron Microscopy
Specimens were prepared for electron microscopy and reconstructions made from the same series of 60 nm sections, both as previously reported [16]. The procedures are summarized in the Supplemental Experimental Procedures.

Supplemental Information
Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.10.053.

Acknowledgments
This work was supported by National Institutes of Health (NIH) grant EY-03592 (to I.A.M.) and the Intramural Research Program of the NIH, Eunice Kennedy Shriver National Institute of Child Health and Human Development (grant HD008776-06 to C.-H. L.). We are grateful to Matthew Murphey and Satoko Takemura for assistance in making the EM reconstructions; Tara Edwards for executing Figure 4; Christina Dollar for carrying out the PCR assay, and real-time PCR assay are provided in the Supplemental Experimental Procedures.

References
Supplemental Information

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Supplemental Inventory

1. Supplemental Figures and Tables
   - Figure S1
   - Figure S2
   - Figure S3, related to Figure 3
   - Figure S4
   - Table S1
   - Table S2
   - Table S3

2. Supplemental Experimental Procedures

3. Supplemental References
Figure S1. Dissociated Single Cells
Representative inverted microscope (A-A'); and confocal (B-B') image of dissociated single L2 cells. Scale bar: 150 μm in A and A'; and 7 μm in B and B'.
Figure S2. PCR Amplification of Specific Transcripts and Standard Curves for Real-Time PCR Analyses of Cha, VGlut and Rp49

(A) PCR-amplified products of specific transcripts using optic lobe cDNA as templates. Lane M: 100bp DNA ladder marker; (C) negative control (without reverse transcription); 1: VGlut; 2: Cha; 3-9: nAcR α1-7; 10-12: nAcR β1-3; 13: mAChR-60C; 14: mAChR-CG7918.

(B,D,F) Standard curves were generated by plotting CT values against the known initial DNA copy number. The x axis represents the logarithm of the input cDNA; the y axis represents the number of PCR cycles required to reach a given fluorescence signal level (Ct). CT values (y-axis) were obtained using a controlled amount of input cDNA (VGlut [B]; Cha [D]; Rp49 [F]). The final PCR product was analyzed by DNA gel electrophoresis (C,E,G). An inverse linear relationship between Ct and the logarithm of the input cDNA was observed over a large concentration range. Standard linear regression analysis was performed to calculate the standard curve, the amplification factor (AmF), PCR efficiency (E), and the coefficient of determination ($R^2$) (as indicated). The standard curves were used to calculate the VGlut, Cha and Rp49 experimental data by interpolation. Data points are averages of three independent dilution series.

Figure S3: Tm2 Received Highly Stereotyped Inputs from Three L4s

(A-D) Tm2 and L4 neurons were labeled stochastically with RFP (pseudo-colored in green) and GFP (pseudo-colored in red), respectively (see main text for details) and the contacts (arrows) between Tm2 dendrites and L4 axons were examined by confocal microscopy. Photoreceptor axons labeled with mAb24B10 (blue) were used as landmarks.

(B) The blue channel was removed from (A) for clarity. Note the apparent contacts (arrows) between Tm2 dendrites and L4 processes in strata M2 and M4.

(C) The processes of Tm2 (green) and its cognate L4 (red) were manually traced and skeletonized. The locations of neighboring photoreceptor axons at the R8 level were marked with white spheres. Tm2’s axon lies posterior to the photoreceptor axon in the same columns and extends dendrites anteriorly to surround the photoreceptor axon. The L4 axon lies anterior to the photoreceptor axon in its cognate column.

(D) A proximal-distal view of (C).
Figure S4: Tm1 and Tm2 Are Both Presynaptic in the Medulla Neuropile

(A) Tm1 forms a presynaptic T-bar ribbon (arrowhead) onto three small dendrites with postsynaptic densities.

(B) Tm2 forms a presynaptic T-bar ribbon (arrowhead) onto two clear postsynaptic dendrites in a dyad configuration. Note that neither presynaptic site has a clear cumulus of synaptic vesicles. Scale bar: 0.5 µm.
Table S1. The mRNA Expression Pattern for Neurotransmitter Genes in Identified Lamina and Medulla Neurons

Nicotinic (nAcRs) and muscarinic acetylcholine receptor subunits (mAcRs) from lamina neurons (L1, L2, and L4) and transmedulla neuron Tm2.

(+) detectable, (-) non-detectable.

<table>
<thead>
<tr>
<th>Neuron Type</th>
<th>Gene Name</th>
<th>Alternative name &amp; CG number</th>
<th>L1</th>
<th>L2</th>
<th>L4</th>
<th>Tm2</th>
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<td></td>
<td>vglut</td>
<td>DvGlut - CG9887</td>
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<tr>
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Table S2. List of Genes and Their Sequences Used for Single-Cell Analysis

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<th>Gene Name</th>
<th>Alternative Name &amp; CG number</th>
<th>First-round PCR Primer Sequence (5' - 3')</th>
<th>Nested PCR Primer Sequence (5' - 3')</th>
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<td>VGlut</td>
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<td>Da3 - CG2302</td>
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<td>F-GGCGATACGCTGCAAGCTGCA</td>
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<td>Rp49</td>
<td>RpL32-CG7939</td>
<td>F-TCAGAGGACGGAAGAGGAAGAG</td>
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Table S3. Summary of Experimental Genotypes

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<tr>
<th>Figures</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>1A-B</td>
<td>w; c202a y'-Gal4/+; 21D-2HAORT/20XUAS-IVS-mCD8GFP,attP2</td>
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<tr>
<td>1C</td>
<td>w; ort-LexAVP16/+; LexAop-rCD2GFP GMR-mRFP/+</td>
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<tr>
<td>1D-H</td>
<td>DacFLP/w; ap&lt;sup&gt;mcd4&lt;/sup&gt;-Gal4/+; UAS-FRT-stop-FRT-mCD8GFP/+</td>
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<tr>
<td>2E-G</td>
<td>w; otD-Gal4/+; 21D-2HAORT/20XUAS-IVS-mCD8GFP,attP2</td>
</tr>
<tr>
<td>2H-J</td>
<td>hsFLP122/w; otD-Gal4/+; UAS-FRT-CD2 stop y-FRT-mCD8GFP/+</td>
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<tr>
<td>3D-L, S3A-D</td>
<td>DacFLP/w; ET77BVP16AD, ort-LexADBD/ ap&lt;sup&gt;mcd4&lt;/sup&gt;-Gal4; UAS-FRT-stop-FRT-mCD8GFP/LexAop-rCD2mRFP</td>
</tr>
</tbody>
</table>
Supplemental Experimental Procedures

Fly Stocks
The following fly stocks were used in this study. (1) yw; c202a-Gal4/CyO [15]; (2) yw++; 21D-Gal4/Tm3 [15]; (3) yw; ap-mds44-Gal4/CyO; (Bloomington Stock Center); (4) yw; otd-Gal4/CyO [S1]; (5) w;; 20XUAS-IVS-mCD8GFP,attP2 [S2]; (6) w; ET77B-VP16AD, ort-LexADBD; [S3]; (7) Dac-FLP++; [S4]; (8) UAS-FRT-stop-FRT-mCD8GFP (Bloomington Stock Center); (9) LexAop-rCD2mRFP [S5]; (10) UAS-D2y-mCD8GFP; (11) 21D-2xHA-ORT/Tm6B; (12) hsFLP 122++; (Bloomington Stock Center).

The Generation of 21D-2xHA-ORT Flies
The p{y+, 2xHA-ORT} enhancer trap vector was constructed using regular cloning techniques and further confirmed by DNA sequencing. Cloning procedures were as below:
2xHA DNA fragment (which encodes two copies of a HA tag) was generated by primer self-annealing and then subsequently cloned into the Msc I site of pBS2-ORT to generate pBS2-2xHA-ORT. A 2xHA-ORT fragment isolated from an EcoR I and Kpn I-digested pBS2-2xHA-ORT construct was subcloned to pUAST to generate pUAST-2xHA-ORT. The 2xHA-ORT-SV40trem fragment isolated from EcoR I and the Stu I-digested pUAST-2xHA-ORT construct were used to replace the Gal80-hs70term fragment in the enhancer trap vector p{y+, Gal80} (gift from Christopher Potter) to generate a p{y+, 2xHA-ORT} enhancer trap vector.

To generate 21D-2xHA-ORT flies, the p{y+, 2xHA-ORT} construct was inserted into the genomic location of the 21D-Gal4 enhancer trap by P-element replacement (S6). The 2xHA-ORT transgene recapitulated the expression pattern of the 21D-Gal4 enhancer trap and HA staining was readily detected in the lamina L2 cells.

Cell Dissociation and Single-Cell Isolation
Cell dissociation was carried out as described previously (S7,S8) with the following modifications. Transgenic flies were decapitated under CO₂ anesthesia. Optic lobes were manually dissected and transferred to a 35 mm Petri dish in Shields and Sang M3 insect medium (Sigma) with antibiotics (Penicillin-streptomycin-kanamycin, 100 μg/ml). Laminas (containing the somata of L1, L2, and L4) or medullas (Tm2 somata) were further dissected and transferred into a 0.5 ml microcentrifuge tube and incubated in a protease mixture (Collagenase/Dispase [1 mg/ml] and liberase I [0.1 Wünsch units/ml], Roche) for 30 min at 23°C. To facilitate cell dissociation, tissue was gently triturated 10 times or until most tissue was dissociated to single cells. Cells were pelleted by brief centrifugation at 150g and then washed three times with M3 medium including 2% Fetal Bovine Serum (HyClone). The resulting cell suspension was filtered using a Pre-Separation filter (30-μm nylon mesh) (Miltenyi Biotech Inc) to remove large tissue debris and fragments. The final cell suspension was diluted in M3 medium and transferred to a custom-made coverslip chamber with multiple agarose wells 1 mm in diameter to limit cell movement. After the cells settled to the bottom of the wells, GFP-positive somata were identified using an inverted fluorescence microscope (Carl Zeiss). A custom-made microcapillary system was used to aspirate single GFP-positive cells and transfer these to the lid of a 200 μl PCR tube in a volume of approximately 2 μl. After adding 50 ng/μl random hexamers or 2 μM of gene-specific primer (to quantify PCR analysis), 10 mM dNTP mix and DEPC-treated water, cells were quickly frozen on dry ice and stored at -80°C. An additional 2 μl of the medium surrounding the cells was collected for a negative control.
Primer Design, Validation, and Determination of Sensitivity Of Transcript Detection

Nested PCR primers were designed to amplify specific transcript regions that spanned two exon-intron boundaries to minimize the amplification of genomic DNA (Table S2). The first run of PCR amplified transcript regions of 100-600 bp (Figure S2A) and the second run amplified transcript regions of 80-300 bp. All primers were checked against genomic and annotated gene sequences for off-target hybridization using the BLAST search program (http://flybase.org/). The efficiency and specificity of these primers were tested using cDNA generated from total RNA of wild-type adult flies.

To generate test cDNA, total RNA was isolated using TRIZOL (Invitrogen) and purified using the RNeasy Mini Kit (Qiagen) according to the Manufacturer's instructions. The RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies) and its quality determined by agarose gel electrophoresis. First-strand cDNA synthesis was carried out using the SuperScript III First-Strand Synthesis System with random hexamer primers. Multiple runs of PCR amplification were carried out to optimize both the primer concentrations and annealing temperature for each assay. PCR-amplified products were cloned into pCR4-TOPO vector using the TOPO cloning kit (Invitrogen) and confirmed by DNA sequencing. These cDNA clones were used to determine the sensitivity of single-cell PCR assays and construct standard curves for quantitative real-time PCR analysis.

Single-Cell RT-PCR Assay

Frozen single cells were briefly spun down, heated at 65°C for 5 min and rapidly transferred to ice for 1 min. Reverse transcription (RT) was carried out in a 20 µl reaction according to the Manufacturer's instructions (Invitrogen) using the SuperScript III First-Strand Synthesis System. To multiplex assays, random hexamers were used as primers for RT. The amplification program for the RT step was as follows: 25°C for 10 min, 50°C for 50 min, 85°C for 5 min and followed by RNase H treatment at 37°C for 20 min, and stored at -20°C for subsequent analysis. 1-2 µl (equivalent to 1/10-1/20 of a single cell) of the RT products was used as a template for the subsequent PCR reaction. The first round of PCR amplification comprised 5 min of initial denaturation at 95°C followed by 40 cycles consisting of 96°C for 5 s, 60°C for 5 s, 68°C for 20 s, and a final extension step at 72°C for 1 min using fast-cycling PCR master mix containing HotStarTaq Plus DNA Polymerase (Qiagen). A second round of PCR amplification (nested PCR) was performed using 1 µl of the first-round reaction and appropriate inner primers for 30 PCR cycles. Both RT and PCR reactions were carried out on a T3000 thermocycler (Biometra). The following controls were included: (1) buffer collected from surrounding cells; and 2) cell extract without the addition of reverse transcriptase. In addition, the glia-specific gene repo (reversed polarity) was used as the negative control and mCD8 for the positive control (mCD8). The sizes of the PCR products were examined by DNA gel electrophoresis (1% agarose) and gels were imaged using a Gel Logic 2200 PRO Imaging System (Carestream Health, Inc.)

The sensitivity of the PCR assays was estimated using plasmids containing relevant cDNA. Controlled amounts of plasmid DNA (1 to 300 copies per reaction) were used in PCR reactions under the same condition as the experimental groups. We found that amplified product can be reliably detected after the first round of PCR (40 cycles) for over 30 copies of target DNA while nested PCR readily detects the presence of >3 copies of target DNA. Because 1-2 µl of RT product (equivalent to 1/10-1/20 of a single cell) was used for each assay, we estimated that our PCR assays are sensitive to detect 60 copies of target DNA per cell.

Real-Time RT-PCR Assay

Real-time PCR analysis was carried out as previously described (S7) except that the dsDNA dye SYBR Green (Fast SYBR Green Master Mix, Applied Biosystems) was used to detect PCR
products. To increase specificity, cDNA was synthesized from total RNA samples using gene-specific primers (VGlut: CAGGATACCATTCTTTCGCAG; Cha: GAGTGGACGCACTTTTCGTAGG; Rp49: ACAAATGTGTATTCCGACCACG). Real-time PCR amplification was carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems) according to the Manufacturer’s specifications. The following thermocycling program was used for PCR amplification: 95°C for 20 s and 40 cycles of 95°C for 3 s and 60°C for 30 s. For each assay, three biological samples, each in triplicate, were tested. The rhodamine derivation ROX dye present in the PCR master mix was used as a passive reference to normalize the signal. After the reactions, PCR products were examined with melting curve analysis and DNA gel electrophoresis for product specificity and primer-dimer formation.

Controlled amounts of VGlut, Cha and Rp49 cDNA were used as a template to derive standard curves and PCR efficiency (Figure S2). Real-time PCR data were analyzed using StepOne software version 2.1 (Applied Biosystems). The apparent linearity in the standard curves so derived indicated that the assays were accurate over a wide range (10-100,000 fg/µl). To obtain sufficient concentrated cDNA for quantification, for each assay three cells were pooled together for the reverse transcription reaction. The threshold cycles (C_T) measured for the experimental groups were within the linear range of the standard curves. The amounts of VGlut, Cha and Rp49 transcript were calculated by interpolation. Rp49, which encodes for a ribosomal protein, was used as an internal reference.

Electron Microscopy
Briefly, we used software [sEM Align; S9] to register three manually selected fiducial marks on each consecutive EM image, and traced manually outlined profiles of individual cells to reconstruct each cell in three dimensions, using additional software [Reconstruct: S10]. Medulla cell dendrites are tiny (>100nm), scarcely more than section thickness, their profiles consequently often changing and untraceable between sections.
Supplemental References


