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Dissection of the Peripheral Motion Channel in the Visual System of *Drosophila melanogaster*

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SUMMARY

In the eye, visual information is segregated into modalities such as color and motion, these being transferred to the central brain through separate channels. Here, we genetically dissect the achromatic motion channel in the fly *Drosophila melanogaster* at the level of the first relay station in the brain, the lamina, where it is split into four parallel pathways (L1-L3, amc/T1). The functional relevance of this divergence is little understood. We now show that the two most prominent pathways, L1 and L2, together are necessary and largely sufficient for motion-dependent behavior. At high pattern contrast, the two pathways are redundant. At intermediate contrast, they mediate motion stimuli of opposite polarity, L2 front-to-back, L1 back-to-front motion. At low contrast, L1 and L2 depend upon each other for motion processing. Of the two minor pathways, amc/T1 specifically enhances the L1 pathway at intermediate contrast. L3 appears not to contribute to motion but to orientation behavior.

INTRODUCTION

Visual systems process the information from the environment in parallel neuronal subsystems. In higher vertebrates, for instance, the visual modalities of color, form, and motion are segregated at the level of the retina into separate channels (reviewed in Livingstone and Hubel, 1988). Similarly, insects have distinct sets of photoreceptors for motion and color (Kaiser, 1975; Heisenberg and Buchner, 1977; Bausenwein et al., 1992). Investigating the motion channel in the fly *Drosophila melanogaster* we now show that at the next level below the eye, the lamina (Figures 1A and 1B), the motion channel is again split into several functionally distinct parallel pathways.

Directional responses to visual motion have been intensely studied, predominantly in dipteran flies (reviews: Heisenberg and Wolf, 1984; Borst and Egelhaaf, 1989; Borst and Haag, 2002; Douglass and Strausfeld, 2003). They are provided by arrays of elementary movement detectors (EMDs; Figure 1C), the smallest motion-sensitive units that temporally compare the intensity fluctuations in neighboring visual elements (sampling units; for further explanation of EMD see legend to Figure 1C). Their neuronal implementation in flies is still unknown. In the rabbit retina, a candidate interneuron computing directional motion has recently been identified (Euler et al., 2002). The present study is confined to the input side of the movement-detection circuitry.

The compound eye of *Drosophila* is composed of about 750 ommatidia. Each of these contains eight photoreceptors (R1-8) that can be structurally and functionally grouped into two subsystems: six large photoreceptors (R1-6) mediate the detection of motion (Heisenberg and Buchner, 1977), whereas two small ones (R7, R8), together forming one rhabdomere in the center of the ommatidium, are required for color vision (Menne and Spatz, 1977).

The lamina consists of corresponding units called neuro-ommatidia, or cartridges (Figures 1A and 1B; e.g., Boschek, 1971; Strausfeld, 1971; Franceschini, 1975). These are the sampling units of the motion channel, whereas the color channel (R7, R8) bypasses the lamina cartridge to terminate in the second neuropil, the medulla. The lamina is anatomically and ultrastructurally known in exquisite detail (Braitenberg, 1970; Boschek, 1971;

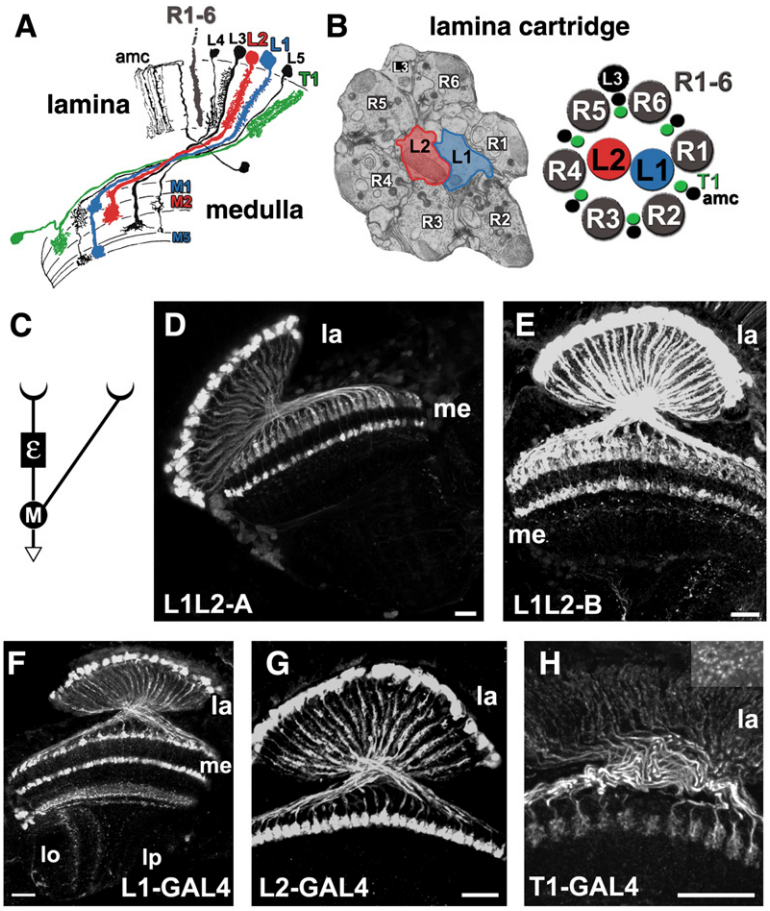

Figure 1. Anatomy of Peripheral Interneurons of the Fly's Visual System

(A) Neurons of the lamina cartridge (modified from Fischbach and Dittrich, 1989). For visibility, only one R1-6 terminal is shown (gray). Interneurons genetically addressed in this study (L1, L2, and T1) are shown in color. Note that L1 and L2 arborize in different layers of the medulla (M1/M5 and M2, respectively), whereas L2 and T1 share the same layer (M2).

(B) Electron microscopical (left, modified from Heisenberg and Wolf, 1984) and schematic cross-section (right) through the lamina cartridge formed by photoreceptors R1-6 (gray) sharing the same optical axis and making synaptic contacts with monopolar cells L1, L2, L3, and amacrine processes (amc). L1 (blue) and L2 (red) are located in the center of the cartridge. L4, L5, and T1 represent second-order interneurons, with T1 being postsynaptic to amc.

(C) Unidirectional elementary motion detector (EMD; Borst and Egelhaaf, 1989). It consists of two input channels that are successively activated when a visual stimulus moves across their receptive fields. The signal of channel 1 is delayed (ε) in order to coincide with the signal of channel 2 at the multiplication stage M, leading to a directionally selective output signal.

(D and E) 10 μm confocal image stacks of a plastic section (left) and an agarose section (right) of two GAL4 lines, L1L2A (D) and L1L2B (E), driving expression of a green fluorescent protein in L1 and L2. Reporter expression was detected in cell bodies and dendritic structures in the lamina (la). In the medulla (me), three rows of arborizations are labeled that correspond to L1 and L2 (compare to Figure 1A).

(F) L1-GAL4 labels L1 neurons in the lamina that have their cell bodies in the lamina (la) cortex and arborize in two medulla (me) layers (compare to Figure 1A). Note that there is additional expression in the proximal medulla (me), as well as in the lobula (lo).

(G and H) 13–20 μm confocal image stacks of drivers expressing GFP in lamina interneurons L2 (L2-GAL4; [G]) and T1 (T1-GAL4; [H]). Inset in (H): horizontal section through optic cartridges reveals the ring of T1's basket processes that enclose R1-6 (compare to schematic in Figure 1B). Note that both interneurons arborize in the same medulla layer (compare to Figure 1A).

Scale bars, 20 μm.

Strausfeld, 1971; Strausfeld and Campos-Ortega, 1977; Shaw, 1984; Fischbach and Dittrich, 1989; Meinertzhagen and O'Neil, 1991). Its functional significance, however, is little understood.

In the lamina, the motion channel is split into four parallel pathways (Figures 1A, 1B, and 2A). In each cartridge, the photoreceptor terminals are connected by tetradic synapses to four neurons, L1, L2, L3, and the amacrine cell α (amc; connecting to the medulla via the basket cell T1). The most prominent of these are the large monopolar cells L1 and L2 (labeled in blue and red in Figures 1A and 1B; Fischbach and Dittrich, 1989; Meinertzhagen and O'Neil, 1991). Their position in the center and their radially distributed dendrites throughout the depth of the cartridge suggest a key role in peripheral processing. This can be visualized by ^{3}H-deoxyglucose activity labeling (Buchner et al., 1984; Bausenwein et al., 1992). Single-unit recordings of L1 and L2 in large flies so far have revealed only subtle differences between them (Hardie and Weckström, 1990; Laughlin and Osorio, 1989). Their specific functional contribution to behavior is largely unknown.

Several hypotheses have been advanced over the last 40 years (reviews: Laughlin, 1981a; Heisenberg and Wolf, 1984; Shaw, 1984; Borst and Haag, 2002; Douglass and Strausfeld, 2003). The loss of L1 and L2 and concomitantly of optomotor responses in the mutant *Vacuolar medulla*^{KS74} had prompted Coombe and Heisenberg (1986) to propose that these cells were involved in motion detection. Later, however, Coombe et al. (1989) claimed that L1 and L2 should be dispensable, because they still measured optomotor responses in flies that they assumed to have complete degeneration of L1 and L2.

If indeed L1 and L2 mediate motion vision, are they functionally specialized or redundant? The latter is unlikely to be the whole answer, considering the differing synaptic relationships of the two neurons. For one, they have their terminals in separate layers of the medulla (Fischbach and Dittrich, 1989; Figures 1A and 1B). Second, L2, but

not L1, has feedback synapses onto R1-6 (Meinertzhagen and O'Neil, 1991). These might play a role in neuronal adaptation and could exert a modulatory influence on the photoreceptor output (Zheng et al., 2006). A functional differentiation had also been proposed by Braitenberg and Hauser-Holschuh (1972), who had suggested that L1 and L2 might be specialized to provide the respective inputs to the two branches of the EMD (see Results).

In *Drosophila*, L2 innervates and reciprocally receives input from a second-order interneuron, L4 (Meinertzhagen and O'Neil, 1991), that has two conspicuous backward oriented collaterals (Figure 1A) connecting its own cartridge to the neighboring ones along the x and y axes of the hexagonal array (see Figure S5A in the Supplemental Data available with this article online; Braitenberg, 1970; Braitenberg and Debbage, 1974; Strausfeld and Braitenberg, 1970). In this network, the L2 neurons are connected to the L4 neurons of two adjacent cartridges, and the L4 neurons are directly connected to all six neighboring L4s (Meinertzhagen and O'Neil, 1991; see also Results). The significance of this circuitry is not yet understood. Braitenberg and Debbage (1974) had speculated that the L4 network might be specialized for front-to-back motion, the prevalent direction in the visual flow-field of fast forward-moving animals. These various hypotheses will be addressed in the present study.

Using the two-component UAS/GAL4 system for targeted transgene expression (Brand and Perrimon, 1993), we manipulated single interneurons or combinations of them, in all lamina cartridges. To study whether a particular pathway was necessary for a given behavioral task, we blocked their synaptic output using the temperature-sensitive allele of *shibire*, shi^{ts1} (Kitamoto, 2001). In addition, we adopted the inverse strategy studying whether single lamina pathways were sufficient for mediating the behavior in the same experimental context. Using a mutant of the histamine receptor gene *outer rhabdomeres transientless* (*ort*; Gengs et al., 2002) that has all lamina pathways impaired, we expressed the wild-type *ort*-cDNA in chosen types of lamina interneurons known to receive histaminergic input from R1-6 (Hardie, 1989). Testing necessity and sufficiency we can now start to relate the structural organization of the lamina to visually guided behavior.

RESULTS

A Screen for Driver Lines Labeling Interneurons in the First Optic Ganglion

In a database of 3939 enhancer trap GAL4 driver lines (Hayashi et al., 2002), we discovered three that label the lamina monopolar cells L1 and L2. The corresponding P elements were inserted in different regions of the genome. The lines showed additional expression in other parts of the central nervous system that differed between them (Figure S1).

The line L1L2A (NP6298) drove GFP expression in two rows of cell bodies in the lamina cortex. The axonal fibers projecting from these via the outer optic chiasm to the distal medulla revealed radially oriented dendritic structures throughout the lamina neuropil (Figure 1D). In line with the descriptions of lamina cells from Golgi impregnations (Fischbach and Dittrich, 1989), these were identified as the L1 and L2 monopolar cells. The L1 ending from axons crossing the first chiasma was identified by its bistratified specializations in the first and the fifth layer of the medulla (compare to Figure 1A). The L2 terminals were identified by their varicose bilobed endings in the medulla's second layer, immediately beneath the outer swellings of the L1 terminals (compare to Figure 1A). The line L1L2B (NP5214) expressed GFP in a very similar pattern in the lamina (Figure 1E), but additionally labeled L5 neurons characterized by a columnar fiber with only few if any dendritic swellings distally in the lamina (Figure S2A). The stratified terminals of L5 overlap with those of L1 and L2 in the medulla (compare to Figure 1A). Both lines, L1L2A and L1L2B, showed additional, weaker, and apparently nonoverlapping expression in medulla and lobula complex neurons. A third line, L1L2C (NP723), with a similar but weaker expression pattern than L1L2A in the lamina and other medulla and lobula complex cells, was occasionally used in behavioral experiments (Figure S2B).

Moreover, drivers were also identified that labeled only one of the two interneurons. An enhancer trap line (c202a; renamed here L1-GAL4) labeled L1 in the lamina and additional cells of the optic lobe. A driver line labeling L2 (21D-GAL4; renamed here L2-GAL4), had been isolated in our laboratory and was recently described by Gorska-Andrzejak et al. (2005). The GFP staining revealed typical features of L2 neurons. They are characterized by big cell bodies in the lamina cortex, radially arranged dendrites in the lamina neuropil, and axons that project via the outer optic chiasm to the medulla terminating in layer 2 (Figure 1G, compare to Figure 1A). In some flies, occasionally an unknown cell in the distal medulla, presumably a glia cell, was faintly labeled (not seen in Figure 1G).

The screen for neurons labeling lamina interneurons also yielded a driver for the T1 basket cell (T1-GAL4; NP1086) that is well known from Golgi impregnations (Fischbach and Dittrich, 1989). Its cell body is located in the medulla rind, giving rise to a cell body fiber that characteristically branches in a T-shape at the outer surface of the medulla (Figure 1H). One branch ascends outward to the lamina where it provides a basket-like system of processes that embraces the optic cartridge (Figure 1H, inset). The other branch provides a bush-like system of dense processes in the corresponding medullary column, at the level of the L2 ending (Figure 1H, compare to Figure 1G). T1 arborizes together with L2 in layer 2 of the medulla (Campos-Ortega and Strausfeld, 1973; Fischbach and Dittrich, 1989; Figure 1A). In the same layer, L4 has distal arborizations. Together these elements are clearly separated from the bistratified specializations of L1 that are found at a more distal (layer 1) and a more proximal level (layer 5) and those of L3 that terminate in layer 3 (ibid; Figure 1A).

L1 and/or L2 Are Necessary and Sufficient for Directional Responses to Visual Motion

The three L1L2 driver lines (A-C) above allowed us to reassess the question of whether L1 and L2 are required for motion detection. We blocked the synaptic output of L1 and L2 (Figure 2A) by combining the GAL4 drivers with the effector UAS-*shi*^{ts1}. First, we measured head optomotor responses (Figure 2B) that serve to stabilize gaze in walking and flight (Hengstenberg, 1993; Kern et al., 2006). The rotation of a striped drum around the fly leads to syndirectional turning of the head (Hengstenberg, 1993). This turning response around the fly's vertical (yaw, Figure 2B) or horizontal body axis (roll, Figure 2B) can be quantified by measuring the change in head position for clockwise and counterclockwise stimulus rotation.

With L1 and L2 blocked (i.e., under restrictive conditions), neither significant yaw nor roll responses to a moving striped drum ($\lambda = 24°$; $w/\lambda = 3$ Hz) were observed for all three experimental genotypes (orange bars in Figure 2C, right panel). Occasionally, spontaneous (random) yaw and roll movements not consistently in the direction of movement could be seen in all three experimental genotypes, indicating that the motor system was able to perform the respective movements after the shift to the restrictive temperature. At the permissive temperature, no difference was found between experimental and control flies (Figure 2C, left panel).

The driver L1L2A, which in terms of lamina expression is more specific than L1L2B (see above), was also tested in a paradigm measuring optomotor turning responses of walking flies (shown in Figure S2C; Buchner, 1976; Heisenberg and Buchner, 1977). In line with the data found in the head yaw and roll paradigms, exposure of L1L2A/*shi*^{ts1} flies to the restrictive temperature for 15 min abolished the optomotor response even at stimulus conditions known to elicit maximal responses ($\lambda = 45°$, $w/\lambda = 1$ Hz). The effector control flies were not significantly affected by the high temperature (Figure S2C).

The optomotor response in flight (see Figure 3, below) could not be measured, as the experimental flies were reluctant to start flight in a striped drum after the temperature shift (drivers L1L2B and L1L2C) or stopped after brief flight episodes (L1L2A). However, the latter were sufficient for measuring the visually induced landing response. In this paradigm, tethered flying flies respond to an expanding visual stimulus (paradigm shown in Figure S2D) by stretching their forelegs above the head and lowering their middle and hind legs (Fischbach, 1981). This collision-avoidance response could not be elicited in L1L2A/*shi*^{ts1} flies (Figure S2D).

Thus far, these data suggested that L1 and L2 were necessary for motion detection, as their synaptic output was indispensable. To decide whether the two neurons together were also sufficient for this task, we utilized a complementary approach (see Introduction). We started out with a general impairment of all (histaminergic) photoreceptor synapses of the compound eye, due to mutations in the gene *ort* coding for a histamine receptor subunit in the postsynaptic membrane (Gengs et al., 2002). To specifically study the sufficiency of the L1 and L2 pathways, we targeted the *ort*-cDNA to these neurons again using the UAS/GAL4 system and the same driver lines. Note that by restoring the histamine receptor, most likely only the histaminergically innervated neurons (i.e., mainly the first-order lamina interneurons) were affected. Therefore, the effects observed in the rescue experiments could be more specifically attributed to the labeled lamina interneurons than in the *shi*^{ts1} experiments. On the other hand, the severely reduced *ort*$^{+}$ function in several neurons of the lamina and medulla throughout development and adult life might cause additional developmental problems.

With the combination of *ort* alleles used here (see Experimental Procedures) flies were not entirely (motion-) blind. Occasionally, very small responses were observed (see negative controls in Figures 2D and 2E). Restoring the *ort* receptor in L1 and L2 (driver L1L2A) led to flies with head yaw and roll optomotor responses operating at wild-type levels (orange bars in Figures 2D and 2E).

As all three driver lines lost their motion sensitivity if L1 and L2 were blocked and the expression patterns of all three driver lines appeared to overlap only in L1 and L2, and as restoring these pathways was sufficient for mediating optomotor responses, we conclude thus far that at least one of these two neurons is indeed required for the direction-specific responses to large-field motion and for the landing response to expanding stimuli, as had been originally suggested by the mutant *Vam*^{KS74} (see above). The result would be in line with the suggestion of Braitenberg and Hauser-Holschuh (1972) (see Introduction) that L1 and L2 would be the respective inputs to the two branches of the EMD (Figure 1C). The pathways of L3, L5, and amc/T1 are neither necessary nor sufficient to mediate directional responses to motion.

L1 and L2 Mediate Motion Detection Independently of Each Other

The data reported so far do not reveal whether L1 and L2 are both necessary or whether one of them would be sufficient. Blocking or restoring only the L1 or L2 pathway would resolve this issue. We therefore specifically expressed *ort*$^{+}$ cDNA in the *ort* mutant either in L1 (blue bars, Figures 2D and 2E) or L2 (red bars in Figures 2D and 2E) and tested the flies under the same experimental conditions used above. Both pathways were able to independently mediate wild-type optomotor responses (Figures 2D and 2E). This result shows that each of the two major lamina pathways alone can mediate optomotor responses. Under the experimental conditions used so far, L1 and L2 are redundant. The hypothesis of Braitenberg and Hauser-Holschuh (1972) that they need to cooperate being specialized each for one of the branches of the EMD (see Introduction) must be refuted.

L1 and L2 Interact at Low Pattern Contrast

To investigate whether the L1 and L2 pathways were indeed fully redundant, we tested more challenging

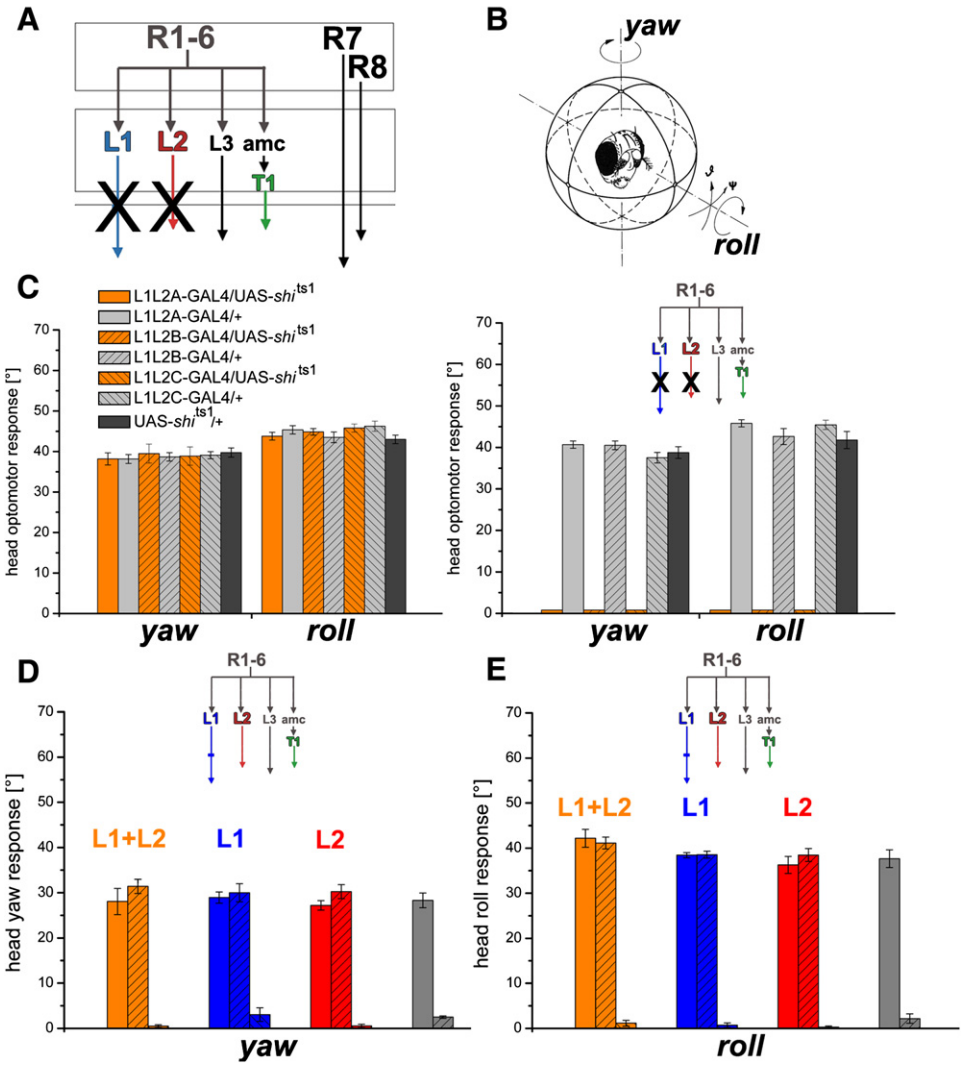

Figure 2. The Functional Role of L1 and L2 in Motion Detection

(A) In the lamina cartridge, photoreceptors R1-6 provide input to four pathways: L1, L2, L3 and amc (that is presynaptic to T1). R7 and R8 bypass this arrangement. *Shi*^{ts1} was expressed in L1 and L2 in order to block the two central pathways. L3 and amc remain functional.

(B) Schematic view of two degrees of freedom of head movements that serve the stabilization of gaze (modified from Heisenberg and Wolf, 1984). Flies respond to the movement of a surrounding striped drum with syndirectional turning of the head. Such optomotor responses elicited by a large-field stimulus moving around the vertical axis are called yaw responses and around the animal's long body axis roll responses.

(C) Studying the necessity of L1 and L2 in motion detection. The effect of *shi*^{ts1} driven in L1 and L2 by three different GAL4 lines on head yaw and roll responses at the permissive (left panel) and restrictive (right panel) temperature. Responses were invariably zero in all three experimental groups L1L2A-C/UAS- *shi*^{ts1} (orange bars) after the temperature shift (right), in contrast to the GAL4 driver (gray) and UAS-*shi*^{ts1} effector (dark gray) controls that were unaffected by the elevated temperature. N = 16–28 trials per genotype; $\lambda = 24°$, $w/\lambda = 3$ Hz, m = 100%. Error bars indicate SEM; ***$p < 0.001$ compared to both controls (ANOVA test).

(D and E) Studying the sufficiency of L1 and L2 in motion detection. Restoring both L1 and L2 (orange bar), or either L1 (blue bar) or L2 (red bar) in an *ort* mutant background (for details, see Results). Color coding: the first bar is always the rescue genotype, the second (hatched right up) is the positive (heterozygous) GAL4 control, and the third (hatched left up) is the negative GAL4 control in the *ort* mutant background. Grey bar, positive (heterozygous) UAS-*ort* effector control; hatched bar, negative UAS-*ort* effector control in the mutant background. At 100% pattern contrast, the rescue of both L1 and L2, as well as rescue of the single pathways, is sufficient for mediating full-sized head optomotor yaw (D) and roll (E) responses. Panels above bars show the four lamina pathways (compare to [A]). N = 4–6 animals per genotype.
Error bars indicate SEM.

stimulus conditions. As under critical conditions head roll and yaw measurements are compromised by the fly's head movements, we switched to optomotor yaw torque responses (paradigm shown in Figure 3A). In this experiment, the tethered fly has its head glued to the thorax and is kept in the center of a striped drum. Its yaw torque

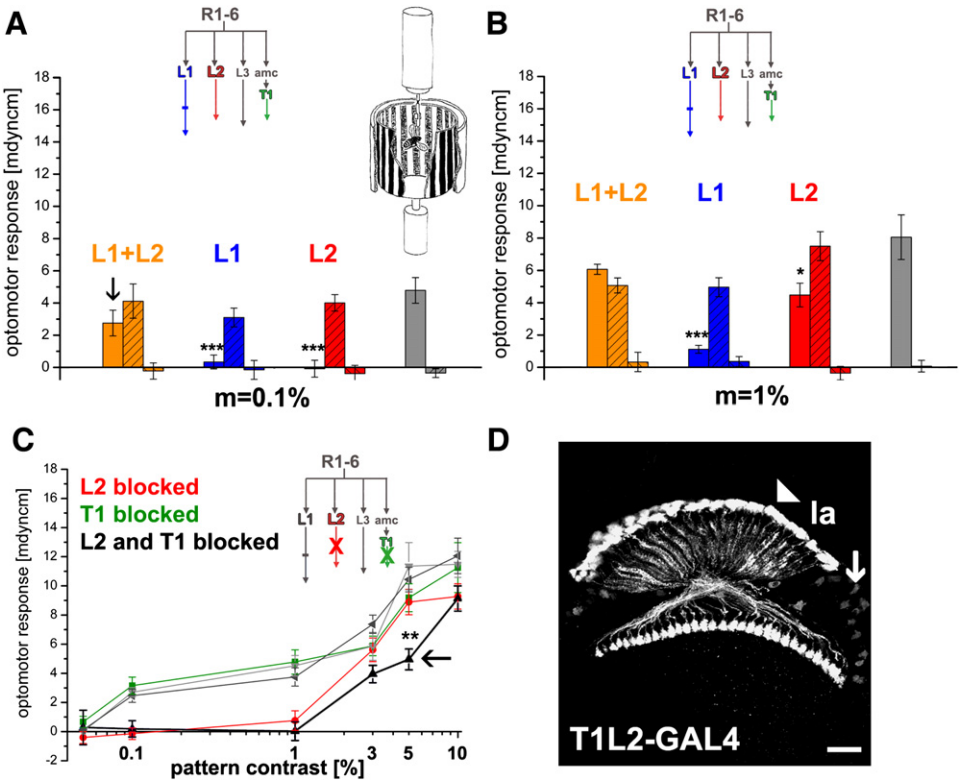

Figure 3. Differences between the L1, L2, and T1 Lamina Pathways at Low Pattern Contrast

Color coding and genotypes in (A) and (B) as in Figures 2D and 2E. (A) Upper right inset: schematic drawing of the setup used to measure optomotor yaw torque responses in tethered flight. Flies try to follow the motion of the striped drum by producing syndirectional yaw torque. At very low pattern contrast (m = 0.1%), both the L1 and L2 pathways (orange bar, arrowed) have to be functional for motion vision. Rescue of either pathway alone is not sufficient (blue and red bar; $p < 0.001$ in comparison to positive controls, ANOVA test). (B) At pattern contrast m = 1%, again the rescue of both pathways leads to wild-type optomotor responses. The rescue of L1 is not sufficient ($p < 0.001$ in comparison with the positive control, and no significant difference in comparison with the negative control; ANOVA test). In contrast, restoring L2 leads to highly significant responses that are close to positive controls (hatched red bar; $p < 0.05$; ANOVA test). This shows that the L2 pathway has a higher sensitivity under these conditions. N = 5–11 per genotype. (C) Optomotor responses at different pattern contrasts of flies that had the output of L2 (red), T1 (green), and both interneurons (black; expression pattern shown in [D]) blocked. Pattern wavelength and contrast frequency were kept constant ($\lambda = 18°$; $w/\lambda = 1$ Hz). Light gray curve, heterozygous T1L2-GAL4/+ driver control; dark gray curve, heterozygous UAS-*shi*^{ts1} effector control. Blocking L2 prevented the response to contrasts of 1% and below. Note the significant reduction (**$p < 0.01$ in comparison to both control genotypes; ANOVA test) after the block of T1 and L2 at 5% contrast (arrow). N = 3–15 animals per data point. Error bars indicate SEM. (D) T1L2-GAL4 labels both interneurons. Note that only one medulla layer is labeled and that T1's cell bodies are located in the medulla rind (arrow), whereas those of L2 are found in the lamina cortex (arrowhead). Scale bar, 20 μm. Error bars indicate SEM.

is recorded in real-time while it tries to turn with the rotating stripes (Götz, 1964; Heisenberg and Wolf, 1984).

At the lowest pattern contrast tested (m = 0.1%), responses of wild-type and positive controls were already reduced to about 25% of maximal responses (compare to pattern contrast m = 10%; Figure 3C). Under these conditions, *ort* mutant flies were entirely motion blind. We compared the responses of flies that had the L1 and L2 pathways or only L1 or L2 restored. Expression of *ort*$^{+}$ cDNA in L1 and L2 rescued optomotor yaw responses at m = 0.1% pattern contrast (arrowed, Figure 3A). In contrast, optomotor responses were not rescued if the L1 and L2 pathways (blue and red bars in Figure 3A, respectively) were separately restored, indicating that both L1 and L2 were required under these conditions.

At m = 1% pattern contrast, mutant *ort* flies with a restored L2 pathway showed optomotor responses that were only slightly reduced compared to the positive controls (red bars, Figure 3B). In contrast, the L1 pathway alone was not sufficient to mediate optomotor responses under these conditions. Its rescue did not cause a significant difference in comparison to the negative control (blue bars, Figure 3B). At pattern contrast m = 3%, both rescue groups showed wild-type responses (data not shown).

In the inverse approach, we expressed *shi*^{ts1} in L2 leaving only L1 of the L1L2 pair functional. At the restrictive temperature, optomotor responses were abolished at pattern contrast m = 0.1% and m = 1% (red curve, Figure 3C), confirming that the L1 pathway alone was not sufficient under these conditions. At m = 3% pattern contrast and

above, no significant difference from the positive control genotypes was detected (Figure 3C). Taken together, all these data suggest that the L1 and L2 pathways can mediate optomotor responses independently of each other. The L2 pathway is more sensitive to low pattern contrast than L1. At very low pattern contrast, L1 and L2 need to interact. Qualitatively similar results were found in the head yaw paradigm (Figures S3A and S3B). No *shi*^{ts1} experiments were performed with the L1-GAL4 driver line, as it showed considerable expression in other parts of the optic lobes. This would not have allowed an unambiguous interpretation of the results.

Blocking L2 also abolished the head roll optomotor response to a vertically rotating drum at pattern contrast m = 1% (Figure S4A). A significant reduction of the response was also observed at 3% (data not shown) and 5% pattern contrast. This demonstrates that the L2 pathway also plays a role in the detection of vertical flow fields at low pattern contrasts. For values equal to or bigger than 10%, no difference was detected between the experimental group and the controls. Note that in control flies, head roll responses, like body roll responses (Blondeau and Heisenberg, 1982), saturate at much higher pattern contrast than yaw torque responses (compare to Figure S3). The latter are known to saturate at low stimulus strengths, suggesting a large open loop gain (Heisenberg and Wolf, 1984). Restoring L2 function led to head roll responses that were at the level of the positive controls at all pattern contrasts tested (Figure S4B).

To summarize, at the low end of the contrast range, the L1 and L2 pathways interact to provide directional motion sensitivity. At higher pattern contrast, the two pathways mediate motion vision independently of each other. The L2 pathway is more sensitive to low pattern contrast than the L1 pathway.

The amc/T1 Pathway Supports the L1 Pathway at Intermediate Pattern Contrast

Blocking synaptic output of T1 (T1-GAL4/*shi*^{ts1}) had remarkably little effect on optomotor yaw torque as well as head yaw and roll responses at any of the pattern contrasts tested (Figure 3C and data not shown). This was also true for flies that had the inwardly rectifying potassium channel (Kir$^{2.1}$) expressed in T1 for 5 days or the attenuated diphtheria toxin DTI for 7 days (Figure S4C), while 4 days were enough to block photoreceptors R1-6 using *rh1*-GAL4 as driver (data not shown). As T1 does not receive direct photoreceptor input (Meinertzhagen and O'Neil, 1991), we were not able to selectively reconstitute the amc/T1 pathway by rescuing *ort* function.

In order to detect possible interactions between the T1 and L2 pathways, we combined the drivers labeling T1 and L2 and obtained a stock (T1L2-GAL4) that labeled both cell types. As expected, it displayed a superposition of the two expression patterns (compare Figure 3D to Figures 1G and 1H). The cell bodies of both cell types were clearly resolved in the lamina rind (arrowhead in Figure 3D) as well as in the distal medulla rind (arrow).

The block of both T1 and L2 paralleled the results of blocking only L2 at low contrasts (Figure 3C). For m < 3%, the response was not significantly different from zero, as had been observed with the L2 driver. At 3% contrast, the effect of blocking T1 and L2 just failed to reach significance compared to control flies ($p = 0.055$; Figure 3C). At 5% contrast, however, the block of both T1 and L2 led to a significant reduction of the optomotor response in comparison to the L2 block alone and the controls (arrowed in Figure 3C), suggesting a contribution of T1 to the L1-dependent motion circuitry. At 10% contrast, however, the L1 motion circuitry was fully sufficient without T1. No significant difference was found in comparison to the other genotypes. Again, these results were paralleled by those of head yaw experiments (Figure S3A). In the head roll paradigm, blocking both interneurons led to similar results at low contrasts as those obtained by blocking L2 alone (Figures S4A and S4B). Remarkably, for m = 10%–30%, again a significant reduction of the response was observed in the T1L2/*shi*^{ts1} flies (arrowed in Figure S4A).

Taken together, the data suggest that in the absence of L2, T1 contributes to motion detection at an intermediate pattern contrast at which the response of the remaining system (L1 pathway) reaches its maximal output.

The L2 Pathway Is More Sensitive than L1 at Low Light Intensity

As shown above, the L2 pathway was more sensitive to low pattern contrast than the L1 pathway. In order to determine whether this also applied to low light intensities, the optomotor yaw torque response of tethered flying flies was tested in the same setup (pattern contrast m = 100%, pattern wavelength $\lambda = 18°$, contrast frequency $w/\lambda = 1$ Hz) at low light intensities that were several orders of magnitude below usual room illumination (close to the low-intensity threshold of humans). Under these conditions, photon noise contaminates the photoreceptor responses.

A reduction of the background illumination to $I = 5 \times 10^{-4}$ cd/m^2 abolished the response of the L2/*shi*^{ts} flies, whereas control flies still showed significant responses (Figure 4A, left). Extensive dark adaptation of experimental flies before the test did not improve the score, and also, responses did not improve during the 3 minute recording time. This excludes that a putative effect on the dynamics of dark adaptation in the photoreceptors caused the mutant phenotype, for example due to the loss of feedback from L2 or L4. Flies without L2 output significantly responded at a background luminance of $I = 10^{-3}$ cd/m^2, but the response was reduced to about half of the average control level (Figure 4A, right). Interfering with synaptic output from T1 at low luminance had no significant effect, and also the block of T1 and L2 did not enhance the deficit of L2/*shi*^{ts1} flies at low luminance.

The expression of the *ort*-cDNA in L2 (red bar in Figure 4B), but not in L1 (blue bar in Figure 4B), fully restored the optomotor response at low light intensities. This demonstrates that flies with a restored L2 pathway

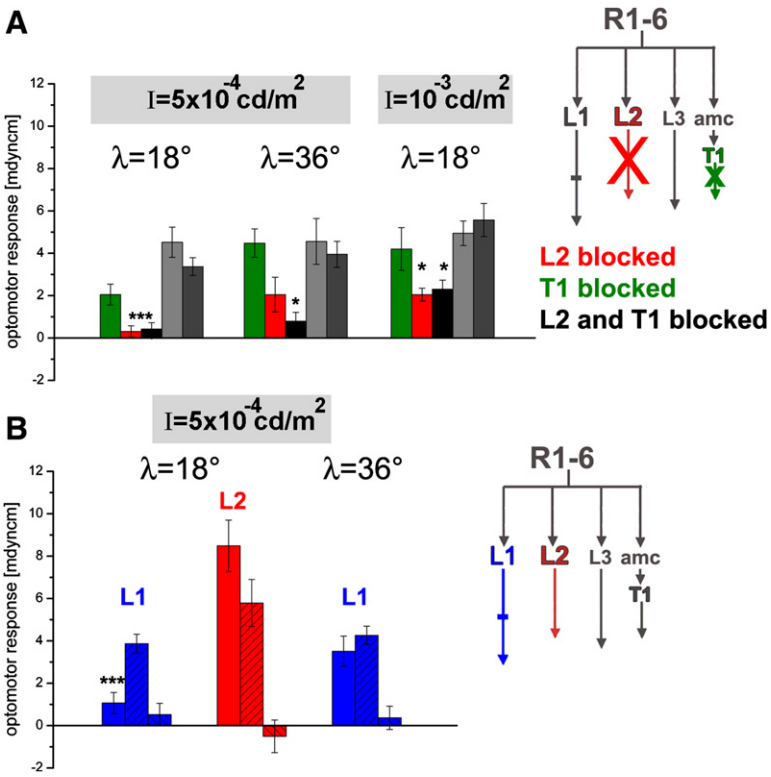

Figure 4. The Role of the L1, L2, and T1 Pathways at Low Light Intensities

(A) Blocking the L2 and T1 pathways at low light intensity reduces optomotor responses in tethered flight (contrast frequency w/λ = 1 Hz); pattern contrast m = 100%). Color coding and genotypes are as in Figure 3C. L2 output was necessary for yaw turning responses at $I = 5 \times 10^{-4} cd/m^2$ and pattern wavelength of λ = 18°. ***$p < 0.001$ compared to both controls; ANOVA test. Doubling the pattern wavelength (λ = 36°) to allow for spatial pooling led to significant responses without L2 output (red bar). A significant reduction (*$p < 0.05$; ANOVA test) was found after blocking both interneurons L2 and T1 (black bar). N = 9–18 flies per genotype. At an intensity two times higher, $I = 10^{-3} cd/m^2$ (λ = 18°; right panel), responses were significantly reduced (*$p < 0.05$; ANOVA test) after blocking L2 (red bar), but well above zero (t test). The additional block of T1 did not further decrease the score (black bar). Error bars indicate SEMs. (B) Studying the sufficiency of the L1 and L2 pathway at low light intensity. Color coding and genotypes are as in Figure 3A. The rescue of the L1 pathway alone was not sufficient to mediate significant responses at the lowest intensity (blue bar; $p < 0.001$ in comparison to positive driver control; response was not significant in comparison to negative driver control; ANOVA test). N = 7–17 animals per genotype. In contrast, restoring L2 function (red bar) led to a wild-type optomotor response at low light intensity ($I = 5 \times 10^{-4} cd/m^2$; λ = 18°; w/λ = 1 Hz). N = 8–14 animals per genotype. At double pattern wavelength λ = 36°, flies with a restored L1 pathway showed wild-type responses (right). N = 5–10 animals per genotype. Error bars indicate SEM.

are able to cope with low luminance levels in mediating optomotor responses, whereas L1 is not sufficient under these conditions. In summary, the L2 pathway has a lower low-intensity threshold of the optomotor response than the L1 pathway.

The L1 Pathway Mediates Spatial Pooling

Like humans, flies have been shown to have a special adaptation mechanism that enhances sensitivity at low light intensity at the expense of visual acuity (*Drosophila*: Heisenberg and Buchner, 1977; *Musca*: Pick and Buchner, 1979). It improves motion sensitivity by pooling the visual input over many visual elements and acts at spatial wavelengths λ > 18°. In order to determine whether the L2 pathway is also necessary for this pooling mechanism, L2-GAL4/UAS-*shi*^{ts1} flies were again tested at the intensity of $I = 5 \times 10^{-4}$ cd/m^2, but at the double spatial wavelength of λ = 36°. If the response would still be abolished, then the L2 pathway would also be involved in the adaptation mechanism. Interestingly, under these conditions, the experimental flies showed a significant optomotor response (Figure 4A, middle). Hence, L2 output seems to be dispensable for this mechanism trading sensitivity for acuity. This also implies that among the remaining pathways one or more must provide the necessary input for this special low-intensity adaptation in the optomotor network. One candidate is the amc/T1 pathway that might again interact with the L1 pathway, as the block in L2 and T1 caused a significant reduction (Figure 4A). However, the rescue of L1 function demonstrates that L1 alone is able to mediate spatial pooling, as the response was wild-type after doubling the spatial wavelength (Figure 4B, right). The L1 pathway is therefore connected to an unknown neuronal substrate that mediates lateral interactions required for spatial pooling.

No Difference in Contrast Frequency Dependence of the L1 and L2 Pathways

To test whether the motion circuitry consists of different components specialized for different parts of the contrast frequency range, L2 rescue flies and flies with L2 neurons blocked by *shi*^{ts} were compared at various contrast frequencies ranging from w/λ = 0.2 Hz to w/λ = 40 Hz. The spatial wavelength (λ = 18°) and pattern contrast (m = 10%) were kept constant. Under these conditions, no difference, neither in the contrast frequency optimum nor in the high or low frequency range, was detected between these two kinds of experimental animals (data not shown).

At Low Pattern Contrast the L1 and L2 Pathways Mediate Unidirectional Optomotor Responses

In the optomotor experiments performed thus far, the rotatory large-field stimuli consisted of both front-to-back

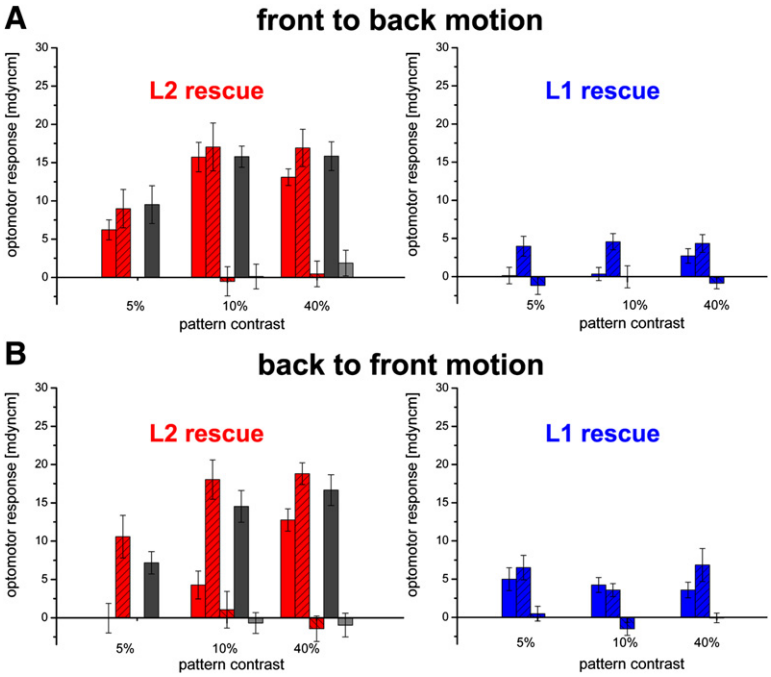

Figure 5. Unidirectional Optomotor Responses at Intermediate Pattern Contrast

Color coding and genotypes are as in Figure 4B. Dark gray bar, positive (heterozygous) UAS-*ort* effector control; light gray bar, negative UAS-*ort* effector control in *ort* mutant background. (A) At low pattern contrast (m = 5%), the L2 pathway mediates optomotor responses in tethered flight only to front-to-back (ftb) motion ($p < 0.001$; t test). The back-to-front (btf) response is not different from zero. At 10% contrast, back-to-front response deviated significantly from chance level ($p < 0.05$; t test), but was clearly reduced (**$p < 0.01$; ANOVA test) in comparison to the positive control (driver control; light gray bars). At m = 40%, no difference was detected between rescue and positive control flies. N = 6–21 animals per genotype. Error bars indicate SEM. The L1 pathway mediates no responses to front-to-back motion at m = 5% and m = 10%. Responses to back-to-front motion are at the level of positive controls (compare right panel in [A] to right panel in [B]). Responses to front-to-back and back-to-front motion are not significantly different at m = 40% pattern contrast.

motion on one eye and back-to-front motion on the other one. As mentioned in the Introduction, from its connections in the lamina, the second-order interneuron L4 might specifically support front-to-back motion detection. In *Drosophila*, it receives chief input from L2 (Figure S5A; Meinertzhagen and O'Neil, 1991). Thus, the L4 network is restored together with the L2 pathway in our rescue experiments. To test whether L1 or L2 specifically provide input to unidirectional motion detectors (see Introduction), these directional stimulus components were separated using a screen that restricted the motion stimulus to a $\Delta = 45^\circ$ window in the fronto-lateral visual field either on the right or the left side of the animal (see Experimental Procedures).

Under these conditions, positive control flies showed no reliable responses for pattern contrasts m < 5%. At 5% pattern contrast, reliable responses were measured that did not differ between front-to-back and back-to-front motion in control genotypes (compare hatched bars in Figure 5A to Figure 5B). Interestingly, in flies with restored L1 function (blue bars) in the *ort* mutant background, the response to front-to-back motion was absent (Figure 5A, right), while flies with a restored L2 pathway (red bars) were wild-type (Figure 5A, left). In contrast, rescuing the L1 pathway allowed responses to back-to-front motion that were at the positive control level (Figure 5B, right), whereas the L2 pathway alone reached significant responses only at 40% pattern contrast (Figure 5B, left). Interestingly, the L1 pathway also showed no asymmetry under these conditions (Figure 5B, right). Note that the response level of the L1-GAL4 driver control flies did not increase with increasing higher pattern contrast between 5% and 40%, suggesting a limiting effect of the P element insertion on the motor output.

When L2 output was blocked, also no significant responses were detected to front-to-back motion at the lowest pattern contrast (Figure S5B), confirming the findings reported above. However, at 10% contrast, no asymmetry was found (Figure S5B). Blocking T1 in addition did not lead to reduced front-to-back sensitivity. The reason for this discrepancy is not known (see Discussion).

To summarize, at low pattern contrast the L1 and L2 pathways are specialized to mediate responses to back-to-front and front-to-back motion, respectively.

Involvement of Lamina Pathways in Landmark Orientation

Because flies lacking output of L1 and L2 failed in all tests of motion-driven behaviors, we investigated orientation toward stationary landmarks by freely walking animals (paradigm shown in Figure 6A). This can be measured by releasing flies with clipped wings in the center of an illuminated arena with two opposing black stripes of a given width. Flies are scored when reaching one of 12 segments of a measuring circle. After each run (12 in total), flies are caught and released in the center of the arena again.

In a pilot experiment, the entire motion channel was blocked using the driver *rh1*-GAL4 (Mollereau et al., 2000) expressing *shi*^{ts1} exclusively in photoreceptor terminals R1-6 (Rister and Heisenberg, 2006). As expected, these flies were motion blind and did not fixate narrow landmarks (width $\delta < 30^\circ$; upper panel in Figure 6A), indicating that subsystems R7 and R8 without R1-6 mediate

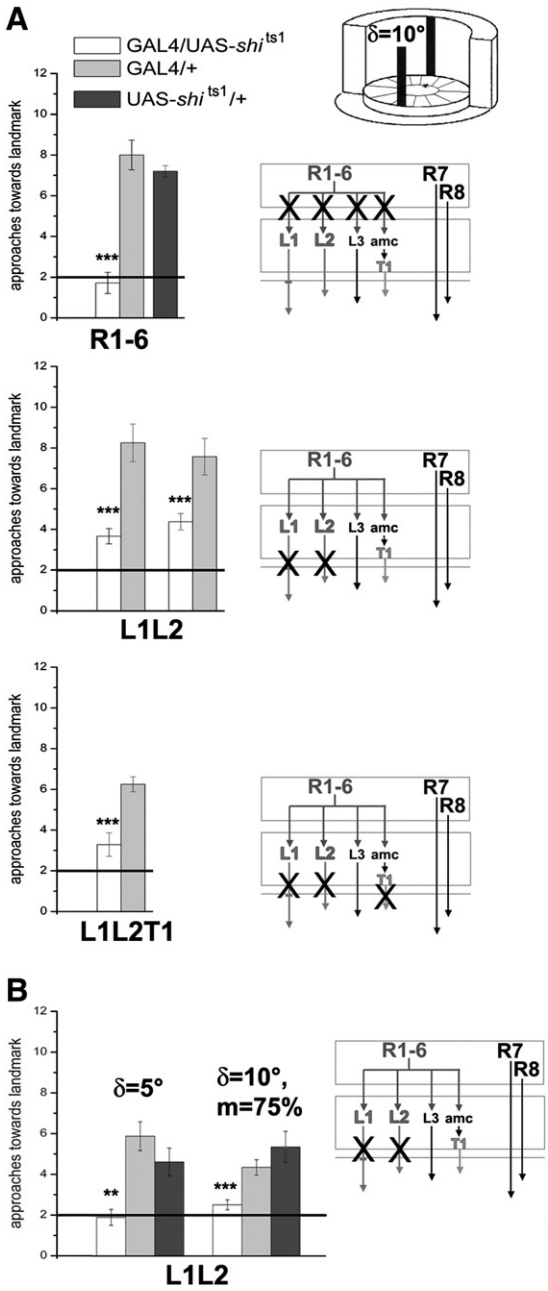

Figure 6. Role of Lamina Pathways in Landmark Orientation

Single flies with clipped wings are released in the center of an illuminated arena (paradigm shown in upper right panel) containing two vertical stripes (width δ = 10°). Each fly performed 12 runs. As a measure of fixation efficiency, it was scored which of the 12 sectors the fly entered. After each run, the fly was caught and released again in the center of the arena. Control flies (gray, heterozygous GAL4 driver controls; dark gray, UAS-*shi*^{ts1}effector control; pooled from all experiments in [A]) fixate these with high efficiency. Controls are compared to flies with blocked lamina interneurons (experimental group, white bar) that are listed below the x axis. (A) Blocking the synaptic output of photoreceptors R1-6 (labeled by *rh1*-GAL4) abolished orientation responses of walking flies toward δ = 10° wide objects. Blocking L1 and L2 (labeled in driver lines L1L2A and L1L2B) significantly reduced orientation (***p < 0.001 for both drivers, ANOVA test) in comparison to the controls (gray and dark gray bars), but did not abolish it. The additional block of L5 (driver L1L2B) or T1 (driver L1L2T1-GAL4) did not further reduce the fixation efficiency toward δ = 10° stripes. N = 7–16 flies per genotype. Horizontal line: chance level. Error bars indicate SEM. (B) Output from L1 and L2 was required for the fixation of objects when the latter were narrow (δ = 5°, left) or reduced in contrast (right: δ = 10°, m = 75%). In both cases, responses of experimental flies (white bars) were significantly different from control genotypes (**p < 0.01 and ***p < 0.001, respectively; ANOVA test) and not significantly different from chance level (indicated by horizontal line; p > 0.05; t test). N = 6–16 flies per genotype. Error bars indicate SEM.

orientation toward large landmarks of δ = 30° or wider (data not shown).

For all three L1L2/*shi*^{ts1} combinations, fixation of a 10° stripe was significantly reduced ($p < 0.001$; ANOVA test) compared to control flies (Figure 6A, middle panel and data not shown). Walking activity was lower, and walking trajectories were less straight. However, the flies were clearly able to target the sectors containing the landmarks. These data indicate that motion detection might improve orientation in freely walking flies. Yet, L1L2/*shi*^{ts1} flies could still fixate 10° stripes, in contrast to *rh1*-GAL4/*shi*^{ts1} flies that had the R1-6 channel blocked (compare middle to upper panel in Figure 6A). This shows that it is not only motion detection that is contributed by the lamina to orientation behavior. Rather, position information is improved (compared to R7 and R8) by some of the lamina pathways that still remained functional (L3, L5, amc/T1). However, as in the driver line L1L2B also L5 was blocked in the *shi*^{ts1} experiment, this neuron can be excluded. To address the role of amc/T1, we combined L1L2A (labeling L1 and L2) with a driver line labeling T1 (T1-GAL4, see below) to generate flies expressing *shi*^{ts1} in the three cell populations: L1, L2, and T1. In these animals, fixation of the 10° stripes was not further reduced in comparison to the block of L1 and L2 (compare lower to middle panel in Figure 6A), suggesting that when L1, L2, and T1 are blocked, the remaining L3 pathway contributes to orientation toward small landmarks.

If stripe width was further diminished to δ = 5°, or if 10° stripes were presented at 75% pattern contrast, L1L2/*shi*^{ts1} flies no longer showed any preferred orientation, while orientation responses of control flies were only slightly reduced (Figure 6B, compare to Figure 6A, middle panel). These data corroborate the above conclusion that L1 and/or L2 do indeed contribute to the orientation toward narrow stripes. In short, the motion-detection circuitry supports orientation toward small objects via L1 and/or L2. Independent of visual motion, the lamina contributes to landmark orientation, most likely via the L3 pathway.

DISCUSSION

The analysis of neuronal networks underlying complex behaviors is a major challenge in neuroscience. In this study,

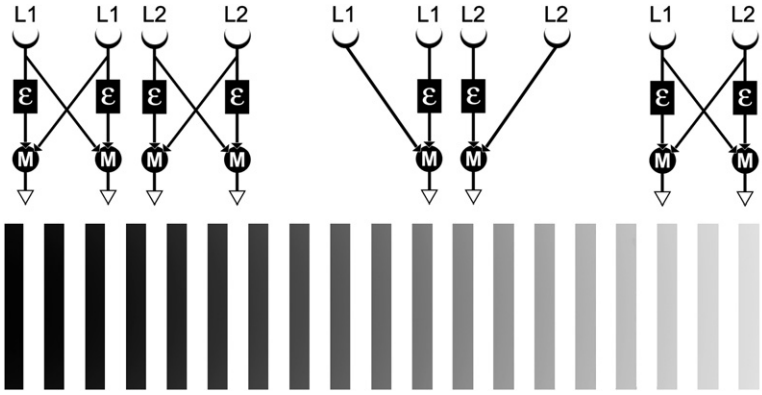

Figure 7. The L1 and L2 Pathways Both Mediate Motion Responses
The two pathways are redundant at high pattern contrast, they are specialized for front-to-back (L2) and back-to-front (L1) motion in the intermediate contrast range, and they cooperate at low contrast. (Note that at low pattern contrast, motion detection might alternatively be unidirectional.)

we report the first steps into the genetic dissection of the neuronal circuitry mediating motion and position detection, the main perceptual processes of visual orientation behavior and gaze control. Some basic properties emerge: we identified two subsystems, the L1 and L2 pathways, that both mediate directional motion independently of each other. A third subsystem, the L3 pathway, may provide position information for orientation. The two motion pathways were remarkably redundant under a broad range of visual conditions, in line with the general observation that motion detection is a very robust phenomenon. To detect an impairment with only one of the pathways remaining intact, one had to drive the system to its operational limits.

The Role of the L1 and L2 Pathways in Motion Detection

Clearly, the L1 and L2 pathways play the principal role in motion detection. Flies without the L3 and amc/T1 pathways are fully motion competent, as far as the present analysis can reveal. In contrast, flies with both L1 and L2 blocked are motion blind using optomotor yaw torque responses, motion-driven head movements, and landing response as criteria. This result is based on three independent driver lines and is in line with the findings of Coombe and Heisenberg (1986) on the mutant *Vam*^{KS74}. We suggest that the discrepant results of Coombe et al. (1989) are due to incomplete degeneration of L1 and/or L2 in the *Vam*^{KS74} mutant. As the L2 pathway mediates optomotor responses at very low stimulus strengths, it would not be surprising if few functional L2 neurons were sufficient to have mediated the response, like when there are few residual ommatidia in *sine oculis* mutant flies (Götz, 1983).

The relation between the L1 and L2 pathways is of particular interest. Throughout most of the pattern contrast range either pathway alone provides full-sized motion responses. At high pattern contrast, the two pathways are redundant, while in the intermediate contrast range they are specialized for front-to-back and back-to-front motion, respectively. Only at the low end of the contrast range do the two pathways depend upon each other (Figure 7).

L1 and L2 Mediate Unidirectional Motion Detection

In natural habitats of insects, intermediate pattern contrasts prevail (Laughlin, 1981b). It is in this contrast range where the L1 and L2 pathways show unidirectional sensitivity for back-to-front and, respectively, front-to-back movement. As mentioned in the Introduction, a specialization of L1 and L2 for these two directions of motion had been proposed a long time ago (Braitenberg and Hauser-Holschuh, 1972). Different strengths of the respective optomotor responses in large flies (Reichardt, 1970) and reduced responses for only one of the two directions in *Drosophila* mutants (Heisenberg, 1972; Bausenwein et al., 1986) had suggested separate arrays of EMDs for the two directions. The new data are compatible with at least two models. In the first one, which is the sparser one, either neuron would serve its array of unidirectional EMDs: L1 an array for back-to-front, L2 one for front-to-back motion. The model would entail crosstalk between the two pathways at high pattern contrast, most likely in the medulla, and a more complex interaction between them at the low end of the pattern contrast range. The second model envisages EMDs for both directions to be served by either pathway. In this case, no crosstalk would be required at high pattern contrast, but one would be in need of additional explanations for the unidirectional responses in the intermediate contrast range.

The asymmetry of the L4 collaterals (Figure S5A) and the close interaction between L4 and L2 described in the Introduction are an intriguing structural correlate of the unidirectional contrast sensitivity of the L2 pathway. No equivalent network with opposite polarity has been detected in the lamina for the L1 pathway, but might still be found in the medulla. As long as no physiological data exist of L4 in *Drosophila*, it is not possible to tell whether the L4 network provides lateral inhibition, lateral pooling, or the second input pathway for an array of front-to-back EMDs.

The L2 pathway is more sensitive to pattern contrast and low light intensity than the L1 pathway. As this distinction was observed with three independent genetic variants, an artifact due to the genetic methods is unlikely. The enhanced contrast sensitivity of L2 might be

attributed to the feedback synapses of L2 onto photoreceptors R1-6, possibly providing some kind of gain control (Zheng et al., 2006), or also to the L4 network. Enhancing sensitivity for front-to-back motion could be useful for fast flying animals, as this type of flow field prevails during fast forward flight. How these differences between the two pathways at low light intensity and pattern contrast show in flight behavior when both pathways are operating remains to be investigated.

Somewhat surprisingly, the two lamina pathways seem not to be differentiated for speed or contrast frequency. Possibly, only one array of EMDs might exist for each direction (sparse model) and the two may have to be tuned the same. Genetic intervention in the lamina as studied here obviously does not affect the tuning of EMDs. This supports the view that motion processing is located proximal to the lamina.

Redundancy of the L1 and L2 Pathways at High Pattern Contrast

At high pattern contrast, the L1 and L2 pathways are redundant. L1 and L2 both mediate motion sensitivity in both directions. As pointed out above, bidirectionality at high contrast can be interpreted as crosstalk between two unidirectional pathways. This could be a property of the regular circuitry or due to wiring defects in the absence of neural activity in one of the pathways during development. The latter explanation is rather unlikely. In the L2-GAL4/*shi*^{ts1} flies, we observe about equal back-to-front and front-to-back responses at m = 10% pattern contrast, whereas the L1-GAL4 *ort*$^{+}$ rescue flies at this pattern contrast respond only to back-to-front motion. Why should the permanently low neural activity in the L2 pathway during development (caused by the mutated histamine receptor) render an originally bidirectional L1 pathway more unidirectional?

The L1 and L2 Pathways Interact at Low Pattern Contrast

The anatomical differences between the L1 and L2 pathways had prompted Braitenberg and Hauser-Holschuh (1972) to speculate that the splitting of the signal from R1-6 into two pathways could correspond to the delayed and nondelayed input channels of the EMD (Figure 1C; Hassenstein and Reichardt, 1956; Borst and Egelhaaf, 1989; Borst and Haag, 2002). The present analysis refutes this idea as an overall explanation of the duplicity of the large lamina monopolar neurons. As discussed above, either pathway alone mediates motion stimuli at high and intermediate pattern contrast. Hence, both neurons can serve the delayed as well as the nondelayed branch of the EMD. Yet, at the low end of the pattern contrast range of wild-type (m = 0.1%; λ = 18°) this is different. Neither L1 nor L2 alone mediate optomotor responses. The two pathways need to interact to provide motion sensitivity. Conceivably, by combining two unidirectional EMDs of opposite polarity one can more than additively improve their signal-to-noise ratio. Indeed, the original motion-detector model of Hassenstein and Reichardt (1956) contains a subtraction of the signals of the two antidirectional EMDs to eliminate the dependency of the output upon light intensity. Alternatively, at this very low pattern contrast L1 and L2 might, after all, specialize to serve the delayed and respectively nondelayed branch of the EMD.

Finally, it is not yet clear whether the motion response based on the interaction of the L1 and L2 pathways operating at low pattern contrast is uni- or bidirectional. At the lowest contrast we were able to measure in the window experiments (m = 5%), no directional preference was found in the control flies, although the overall response was already reduced to less than 50%.

The Sensitivity-for-Acuity Trade-Off Mechanism Does Not Require L2

The high sensitivity for pattern contrast of the L2 pathway is paralleled by a low threshold for light intensity. At the lowest intensity measured at which wild-type is still responsive ($I = 5 \times 10^{-4}$ cd/m^2), the L2 pathway is not only necessary but also fully sufficient, implying again that under these conditions the L2 neurons serve both input channels to the EMD. It remains open whether at even lower intensities an interaction between L1 and L2 might be found as is the case with low pattern contrast.

Our data indicate that the special trade-off at low light intensity, whereby sensitivity is gained at the expense of acuity, can use the L1 pathway as input (Figures 4A and 4B). The mechanism is supposed to pool the signals of many visual elements for the delayed as well as the nondelayed channels of an array of EMDs with large sampling base (Pick and Buchner, 1979). In our experiments, the L1 pathway at the broad pattern wavelength (λ = 36°) is about as sensitive as the L2 pathway at λ = 18°. This shows that the role of the L1 and L2 pathways in pooling is not yet understood well. Lower light intensities may reveal an involvement of also L2 in pooling.

The amc/T1 Pathway

Recently, it has been shown that the T1 neuron has no conventional chemical synaptic output sites in the medulla as judged by its ultrastructure (S.Y. Takemura and I.A. Meinertzhagen, personal communication). Hence, it is an open question whether and how *shi*^{ts1} expression in T1 might block a presumed *non*synaptic output from T1. Expressing *shi*^{ts1} at the restrictive temperature has, on the other hand, been found to perturb the organization of microtubules in the expressing photoreceptor cells (X.T. Sun and I.A. Meinertzhagen, personal communication). Moreover, it is likely that the processing of other membrane vesicles (Di et al., 2003) and hormone secretion at the Golgi apparatus (Yang et al., 2001) are affected as well. Our data consistently show an effect of *shi*^{ts1} expression in T1 neurons at the restrictive temperature (Figures 3C and 4A and Figures S3A and S4A). Optomotor responses are reduced at intermediate pattern contrast, if

L2 is blocked as well. The mechanism mediating this effect is not known.

Assuming *shi*^{ts1} to block T1 output, we conclude that the amc/T1 pathway supports the L1 pathway at intermediate pattern contrast, at which the response of the L1 pathway just reaches saturation. Under these conditions, disturbance of T1 reduces the gain of the system and shifts the saturation range to higher contrast levels. The finding that saturation is eventually reached could be explained by the assumption that neurons like L5 with a presumed higher response threshold might be added to the system at still higher pattern contrast. In line with this hypothesis is the finding that the on-off units recorded by Arnett (1972) in the outer chiasm of large flies, which might correspond to L5 (Shaw, 1984), did not respond to contrasts smaller than 10% in electrophysiological recordings (Jansonius and van Hateren, 1991). We take the rather subtle effect of blocking T1 to indicate that the stimulus conditions for T1 function have not yet been properly defined. It is unlikely that we failed to observe T1 functions because *shi*^{ts1} did not block T1 output. Expression of DTI and Kir$^{2.1}$ in T1 neurons did not show a more substantial effect.

Separating Subsystems for Motion and Position Processing

In contrast to earlier assumptions (Reichardt and Poggio, 1976), evidence has been accumulating that orientation toward landmarks does not necessarily require motion. In *Musca*, position-sensitive torque responses could be elicited in stationary flight, if the luminance of a stationary vertical stripe was sinusoidally modulated (local flicker; Pick, 1974). In *Drosophila*, torque responses toward stationary dark objects ($\delta = 5°$) have directly been documented by Bausenwein et al. (1986).

In the present study, we have genetically separated neuronal pathways mediating motion and position detection. We have shown that motion-blind animals are still able to approach landmarks, corroborating the notion that motion vision is not essential for the detection and fixation of a stationary object. On the other hand, our data also suggest that motion detection improves the fixation of landmarks, especially when these are narrow or have a reduced contrast. Note, that in our paradigm testing freely walking flies we do not exclude motion vision experimentally. Obviously, in visual orientation both neuronal subsystems are at work, and genetic dissection will help to unravel their interaction.

In flies having the entire motion channel (R1-6) blocked, the color channel (R7/R8) alone provides basic position information. With only L1 and L2 blocked, flies are still completely motion blind in all paradigms tested, but their orientation behavior is distinctly superior to that of flies with the entire motion channel blocked. Apparently, elements among the remaining lamina pathways improve landmark orientation as mediated by R7 and R8. Given that L5 was blocked in one of the driver lines without an additional impairment of orientation behavior, we assume that at the conditions of the paradigm L5 did not substantially contribute to orientation behavior.

Blocking T1 in addition to L1 and L2 caused no further reduction of the orientation response. Hence, the amc/T1 pathway seems not to contribute significantly to this behavior either (but see the caveat regarding the unconventional output of T1 discussed above). This means that the L3 pathway, possibly interacting with the R7 and R8 pathways in color vision, may mediate orientation behavior, since flies without functional L1, L2, and amc/T1 still show better orientation behavior than flies with the entire R1-6 channel blocked. The residual orientation behavior in flies without functional L1 and L2 is very sensitive to a reduction in object contrast. This suggests that the underlying phototactic or tropotactic orientation mechanism might integrate the visual input over large parts of the visual field, reducing the apparent pattern contrast of small targets below threshold. This spatial integration might occur at any level in the system.

In summary, genetic dissection indicates that position detection might be as robust and redundant as motion vision. The color channel (R7/R8), L1, L2, and L3 all contribute to position detection. Presumably, single pathways are sufficient for this task. Detecting a singularity in space may require a less sophisticated neural mechanism than motion detection based on a temporal comparison of signals from neighboring visual elements.

Concluding Remarks

Applying circuit genetics, we find the peripheral neuronal network of the fly optic lobe to be functionally more complex than what previous behavioral, anatomical, and electrophysiological studies on wild-type animals had revealed and, maybe, what the early pioneers of the 1950s and 1960s (Hassenstein and Reichardt, 1956; Götz, 1964; Braitenberg, 1967; Kirschfeld, 1967) had envisaged. Still, with this new approach, the fly optic lobe once again proves to be a uniquely suited case study for gaining basic insights into the neuronal mechanisms of visual information processing and, more generally, for the comparison of structure and function in neural networks.

EXPERIMENTAL PROCEDURES

Ort Rescue Constructs

HisCl-a1 cDNA (Gisselmann et al., 2002) was cloned into pUAST to generate pUAST-HisCl-a1 (a generous gift from Benjamin White, Bethesda). Detailed cloning procedures are available upon request. Transgenic flies were made in house using standard methods (Spradling and Rubin, 1982).

Fly Stocks

Fly strains were reared on standard *Drosophila* medium at 25°C (or 18°C for induction experiments) and a 14/10 hr light/dark cycle at 60% relative humidity. Only 2- to 4-day-old female flies were investigated.

A strain with insertions of UAS-*shi*^{ts1} on the X- and third chromosomes was used (Kitamoto, 2002). Heterozygous control genotypes were obtained by crossing the GAL4-driver and UAS-effector strains to wild-type Canton S. A third chromosomal insertion of UAS-GFP obtained from Bloomington Stock Center was used for visualizing

expression patterns. The *ort* alleles (Gengs et al., 2002) used for rescue experiments were ort^1 (recombined with 21D-GAL4 and combined with NP6298), ort^{US2515} (combined with a second chromosome insertion of UAS-*ort*). Heteroallelic combinations were used to generate the mutant background. Positive controls were the respective driver or effector strain in a heterozygous mutant background (i.e., crossed to Canton S); negative controls were driver or effector in a homozygous mutant background.

Immunohistochemistry

Agarose Sections

Agarose sections were obtained as described by Rister and Heisenberg (2006). Sections were incubated overnight at 4°C with polyclonal rabbit anti-GFP antiserum (1:1000, Molecular Probes, Eugene, OR). As secondary antibody, either goat anti-rabbit IgG Alexa Fluor 488 conjugated (Fab′) fragment of IgG (1:100, Molecular Probes) or goat anti-rabbit Cy5-conjugated (Fab′) fragment of IgG (Molecular Probes) was used. Three-dimensional image stacks were captured with a 40× or a 63× oil objective at 0.8 μm steps with a Leica confocal microscope and further processed using the software Amira (Mercury Computer Systems, Berlin).

Plastic Sections

Fly brains were fixed in 4% paraformaldehyde using a special microwave (Ted Pella, Pelco 3450, Redding, CA). Brains were microwaved for three 2 min bursts, each separated by 2 min hold, and were left in the same fixative overnight at 4°C. Next, brains were washed in PBST. Then they were blocked with swine serum (Dako, Carpinteria, CA) for 1 hr at room temperature. The primary antibody (anti-GFP, see above) was added. The microwave treatment was repeated. The samples were placed on a shaker and then left at room temperature overnight. Next, they were washed in PBST. The secondary antibody was added (Cy5, see above). A third microwave treatment followed, and the samples were left overnight at room temperature on a shaker. Next, the brains were washed in PBST three times for 20 min, then with PBS six times for 20 min. Brains were permeabilized by dehydration in an increasing series of ethanol (10 min in 70% EtOH, 10 min in 90% EtOH) and eventually washed two times for 10 min in pure EtOH. Next, they were placed first for 15 min in pure acetone, then for 1 hr in a 1:1 mixture of acetone and Spurr's plastic embedding medium (Spurr, 1969) and finally two times for 1 hr in pure Spurr's medium. After this, brains were embedded in pure Spurr's medium and were polymerized at 60°C for 12 hr. Serial 15 μm horizontal sections were cut with a sliding microtome. Eventually, sections were mounted with Fluoromount (Serva, Heidelberg, Germany) and viewed with a confocal epifluorescence microscope (LSM 5 Pascal, Carl Zeiss, Thornwood, NY).

Behavioral Assays

Optomotor Responses in Flight

The torque compensator (Figure 3A) and preparation of flies has been described (Götz, 1964; Heisenberg and Wolf, 1984). Briefly, more than 12 hr before the experiment, flies were anesthetized by cooling to 4°C–7°C. Small hooks made of copper wire were attached between head and thorax with a UV-sensitive glue that was hardened by illumination with a UV lamp (Megadent/Megalux CS). Flies were kept isolated in small plastic vials with a few grains of sucrose and access to water.

In standard optomotor experiments, a striped drum pattern of defined pattern wavelength, contrast, and background intensity (I = 19 cd/m, see also Visual Stimuli) was rotated at constant speed around the animal for 3 min. The first minute of the recording was discarded. The rotatory direction was changed every 30 s. The optomotor response [mdyncm] was calculated by a software program developed by Reinhard Wolf (University of Würzburg) from the remaining 2 × 30 s responses to clock- and counterclockwise rotation. For each fly, an averaged trace of these 2 min recordings was obtained and an integral value of the torque in the direction of the moving pattern was calculated.

For stimulating only with front-to-back or back-to-front motion, we placed a stationary concentric 315° cylinder out of white plastic into the striped drum. The remaining 45° window was positioned at 15°–60° to the frontal right or left of the fly. Each fly was first tested for 30 s with both rotatory directions on one eye and then on the other. Torque responses to front-to-back and back-to-front motion were calculated separately. Before the screen was moved to a new position between two consecutive recordings, the illumination of the arena was switched off in order to avoid responses of the fly to the moving screen.

Visually Induced Landing Response

Flies were prepared the same as for flight experiments. They were exposed to a spiral pattern (kindly provided by Roland Strauss, University of Würzburg) generating the illusion of an image expansion when rotating in one direction. This visual flow field elicited the landing response (Fischbach, 1981), i.e., the fly lowered the second and third pairs of legs and stretched its forelegs above the head. Leg extension was visually recorded under a microscope. Each fly was tested ten times.

Head Roll and Yaw Optomotor Responses

Flies were briefly anesthetized by cooling, and a small pin was glued to their thorax with nail polish. To prevent leg movements, the latter were either glued together or to the body by using nail polish. Flies were allowed to recover for at least 10 min. Visually induced head yaw and roll responses were measured as described by Heimbeck et al. (2001). The steady-state angle of head yaw or roll in response to a moving vertical stripe pattern ($\lambda = 24°$, $w/\lambda = 3$ Hz, I = 240 cd/m) was recorded for clockwise and counterclockwise rotation. Each fly was tested four times.

Optomotor Response of Walking Flies

A grating of vertical stripes ($\lambda = 45°$; $w/\lambda = 1$ Hz; I = 1.3 cd/m) rotated around a tethered fly that walked on an air-supported styrofoam ball. Its rotations were optoelectronically recorded and served as a quantitative measure of the optomotor turning response (Buchner, 1976). A response value of zero corresponds to purely forward walking, whereas a positive (negative) value indicates rotations with (against) the moving pattern.

Orientation Behavior toward Stationary Objects

Object fixation behavior of walking flies was measured as described earlier (Heimbeck et al., 2001). At least one day prior to the experiment, flies were immobilized by cooling to 4°C, and their wings were shortened to about one third of normal length. More than 3 hr before the experiment, flies were starved, but had access to water. Individual flies were placed in the center of a circular arena (I = 3200 cd/m) that contained two opposing vertical black stripes (of different angular width, height: 64°) and was divided into 12 sectors. It was recorded in which of these sectors the flies crossed the line of a measuring circle (Figure 6A; diameter: 10 cm). Each fly performed 12 runs, and the runs toward one of the stripes were counted.

Visual Stimuli

Patterns

Square-wave patterns of desired spatial wavelength were designed with Adobe Photoshop, and the respective contrast was adjusted. Patterns were printed on transparencies (Avery, Holzkirchen, Germany) with a HP4600 printer.

Illumination

The background light intensity of the arena of the respective paradigms was measured with a photometer (Minolta Luminance Meter 1°, Minolta, Ahrensberg, Germany). The intensity was reduced by using "Neutralglas" filters (NG, Schott, Germany). In order to observe the animals in these experiments, they were illuminated by infrared LEDs and recorded by an IR-sensitive camera.

Induction of the shi^{ts1} Effector

Prior to testing, flies were attached via their hook (flight experiments, see Behavioral Assays) or pin (head optomotor experiments) to a metal

clamp or were individually placed with clipped wings in plastic vials (orientation experiments) that were fixed in a holder and placed above the water in an illuminated water bath (air temperature T = 36°C–37°C). Flies were kept under these conditions for 15 min (optomotor experiments) or 15–20 min (orientation experiments). During experiments, the arena was heated to the restrictive temperature.

Statistical Methods

For comparison between genotypes, a one-way ANOVA was performed, followed by the Student-Newman-Keuls multiple comparisons test. Single genotypes were tested against chance level with a one-sample t test. The significance level between experimental flies and controls or chance level indicated in the figures refers to the highest p value obtained for the comparisons. Data are represented as means ± SEM.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/56/1/155/DC1/.

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Supplemental Data

Dissection of the Peripheral Motion Channel in the Visual System of *Drosophila melanogaster*

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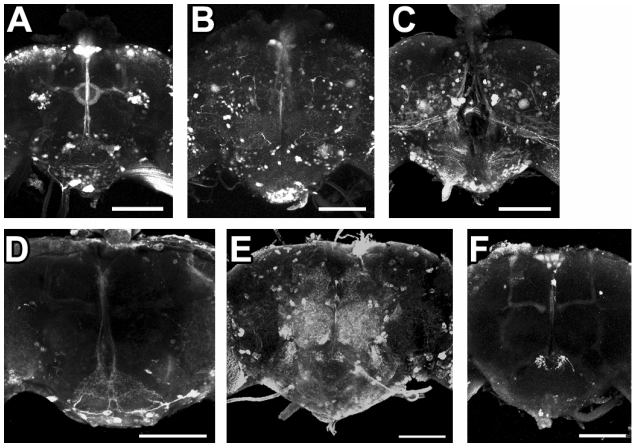
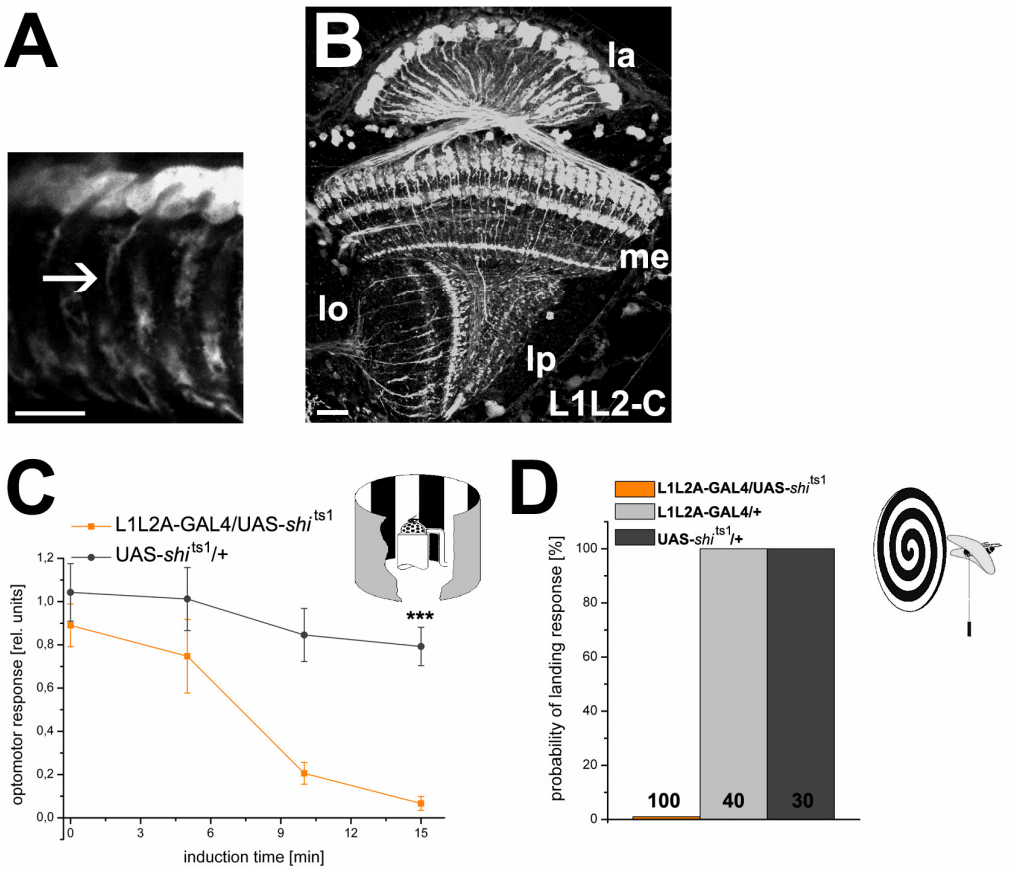
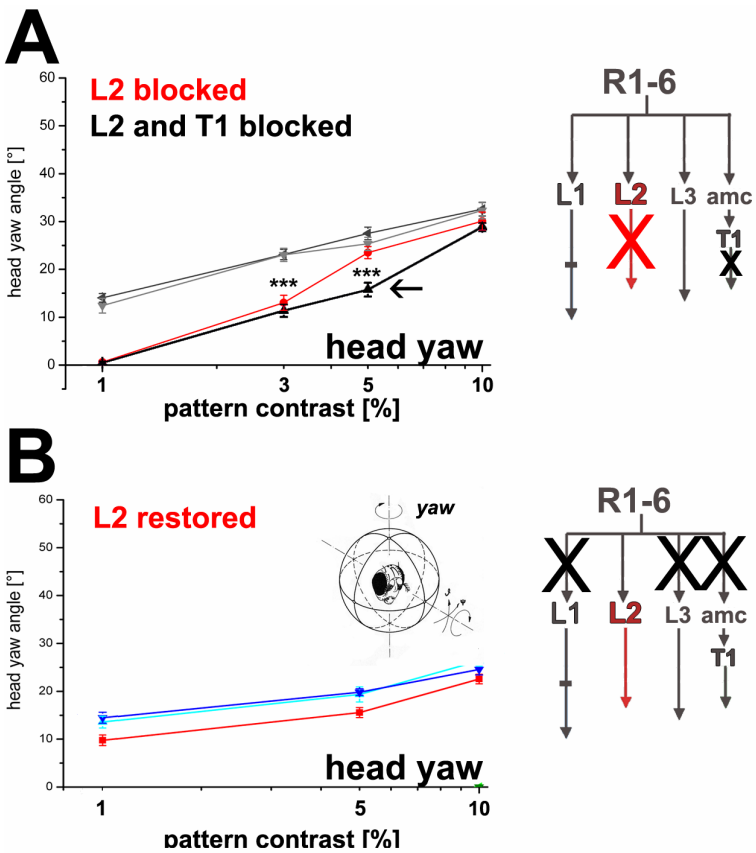
Supplementary Fig. 1: Whole mount projection views of the central brain expression patterns of the GAL4 drivers shown in Figs. 1D-H. (A-C): The three L1L2 drivers L1L2-A (A), L1L2-B (B) and L1L2-C (C) show different central brain GFP expression. (D) L2-GAL4. (E) L1-GAL4. (F) T1-GAL4. Scale bars, 100µm.



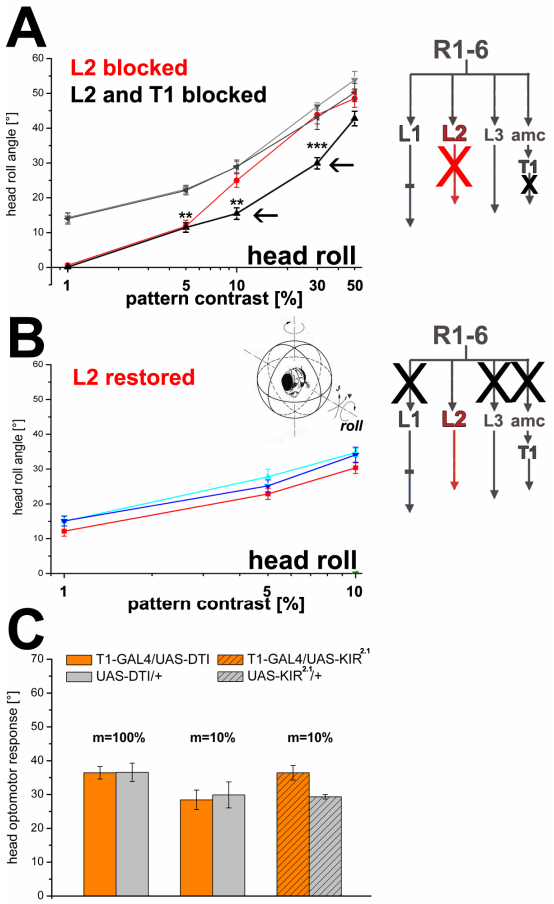
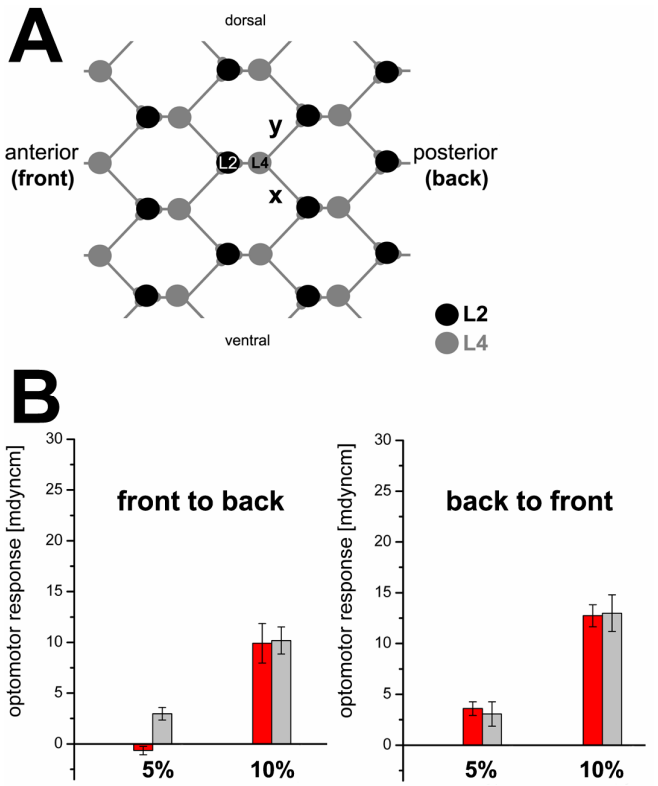
Supplementary Fig. 2: (A) Line L1L2B drove GFP expression also in a columnar neuron (arrow) with only few dendritic arborizations and smaller cell bodies in the lamina cortex, most likely corresponding to the L5 neuron (compare to Fig. 1A). Scale bar, 10µm. (B) GAL4 line L1L2-C drives GFP expression in L1 and L2 in the lamina (la), but also in other unidentified cell types of the medulla (me) and the lobula complex. Scale bar, 20µm. (C) Shi^{ts1} driven by driver line L1L2A also abolished optomotor responses ($p>0.05$; t-test) of tethered walking experimental flies (N=4, orange curve) after 15 minutes at the restrictive temperature, in contrast to the effector control animals (grey, N=6; $\lambda=45°$; $w/\lambda=1$ Hz). ***$p<0.001$, t-test. (D) The visually induced landing response to an expanding stimulus (see Methods; schematic drawing kindly provided by Roland Strauss, Würzburg) was also absent ($p>0.05$; t-test) at restrictive conditions when Shi^{ts1} was driven by L1L2A (orange bar). Both GAL4 driver and Shi^{ts1} effector control genotypes (light and dark gray bars) were unaffected by the elevated temperature. Numbers indicate performed trials. Error bars indicate SEM.



Supplementary Fig. 3: The involvement of lamina pathways T1 and L2 in head yaw responses at low pattern contrasts. (A) Head yaw optomotor responses (λ=24°, w/λ=3 Hz) were affected at 1-3% contrast when L2 was blocked (red curve). Blocking T1 and L2 (black curve) paralleled this effect, but additionally reduced the turning response level (**p<0.01 and ***p<0.001; ANOVA) at intermediate contrast (5%, arrow). Genotypes and color coding as in Fig. 3C. (B) Restoring the L2 pathway (red) rescued head yaw responses at m=1%. Negative controls (driver and effector in *ort* mutant background, green; ***p<0.001 in comparison to all other genotypes; ANOVA test) showed no significant responses (p>0.05; t-test) at pattern contrast as high as m=10% and were therefore not measured at lower pattern contrasts (λ=24°; w/λ=3 Hz). Light and dark blue: Positive (heterozygous) L2-GAL4 driver and UAS-*ort* effector controls. Error bars indicate SEM.



Supplementary Fig. 4: The involvement of lamina pathways T1 and L2 in head roll responses (A, B) at low pattern contrasts. Color coding and genotypes as in Supplementary Fig. 3. (A) Head roll responses were affected at 1-5% contrast when L2 was blocked (red curve). At intermediate contrast (10-30%, arrows) the response was significantly reduced (**p<0.01 and ***p<0.001; ANOVA test) after the block of T1 and L2 (black curve). Note that saturation of the head yaw response (Supplementary Fig. 3A) was reached at much lower contrasts. N=4-18 animals per data point. (B) Restoring L2 (red curve) fully restored head roll responses at all pattern contrasts tested (λ=24°; w/λ=3 Hz). Negative controls (green; ***p<0.001 in comparison to all other genotypes; ANOVA test) showed no significant responses (t-test) at 10% pattern contrast and were not measured at lower pattern contrasts. (C) Induced expression of diphtheria toxin (DTI) for seven days or the human inwardly recifying potassium channel $KIR^{2.1}$ for five days in T1 (orange bars and orange hatched bar, respectively) did not abolish head roll optomotor responses (λ=18°, w/λ=5 Hz, pattern contrast m as indicated). This is in line with the experiments using UAS-*shi*^{ts1} as effector (see Results). Grey bars: UAS-DTI control; hatched grey bar: UAS- $KIR^{2.1}$ effector control. N=3-4 animals per genotype. Error bars indicate SEM.



Supplementary Fig. 5: (A) The L4 network in the lamina. The two collaterals (grey lines) of each L4 neuron (grey circle) connect the L2 neurons of the home cartridge (adjacent black circle) to the neighbouring dorso-posterior and ventro-posterior one. (B) Blocking the output from L2 abolishes the response to front-to-back motion at 5% pattern contrast in tethered flight (red bar, left), but not to back-to-front motion. The response at 10% contrast was unaffected for both stimulus directions in comparison to the heterozygous UAS-*shi*^{ts1} effector controls (grey bar). Compare to L1 rescue data in Fig. 5. N=4-9 animals per genotype. Error bars indicate SEM.