SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: Behavioral responses from the optomotor screen. Related to Figure 1.

(A, C, E, G, I, K, M, O, Q, S): Translation index as a function of time for decrement **(A, E, I, M, Q)** and increment **(C, G, K, O, S)** stimuli.

(B, D, F, H, J, L, N, P, R, T): Rotation index as a function of time for decrement **(B, F, J, N, R)** and increment **(D, H, L, P, T)** stimuli. Genotypes as indicated in plots. Number of tubes of flies per genotype is the same as in Figure 1. Shading denotes +/- 1 SEM.

Figure S2: Characterization of *L3^{0595}-Gal4* and *L4^{0987}-Gal4.* Related to Figure 2.

(A-D) Confocal images of adult brains stained with anti-GFP (green) and mAb 24B10 **(A,B)** or anti-Bruchpilot (nc82) **(C,D)**. **(A)** *0595-Gal4* neurons terminate in the same layer as R8, identifying this as layer M3. *0987-Gal4* neurons terminate distal to layer M3 (M2) and between layers M3 and M6 as identified by 24B10 staining **(B)** identifying this as layer M4. (D) Single cell flp-out clones strongly labeled L3 cells in *0595-Gal4* and L4 cells in *0987-Gal4*. Scale bar: 20 μm.

Figure S3: GRASP and physiological characterization of L4. Related to Figure 3.

(A) GFP reconstitution across synaptic partners (GRASP) between L2 and L4. *L2-Gal4* expressing *UAS-^{myr}tdTOM* and *UAS-CD4::spGFP1-10* as well as *L4^{0980}-LexA* driving *lexAOp-CD4::spGFP11*. Reconstituted Split-GFP signal can be seen in M2 of the medulla and throughout the lamina cartridge, with prominent expression in the proximal lamina. Scale bar: 50 μm.

(B) Model of L4 responses to a gaussian flicker stimulus. The stimulus s(t) is passed through a linear temporal filter F(t), this output is used to predict the actual calcium response, r(t). (R^2=0.77).

Figure S4: Inverted and non-linear responses in L3. Related to Figure 4.

(A,B) Average responses (∆R/R) of L3 to periodic full field flashes chosen by different levels of cross-correlation (corr) of each cell's response with the mean L3 response. The timing of lights-off and lights-on is depicted by the filled and open portions, respectively, of the bar above the traces.

(C) Plot of the measured output against responses predicted by the linear filter. Regression lines were fit to the mean response to contrast increments or contrast decrements, respectively.

(D) Nonlinearity from the two linear regressions shown in (C).

(E) Mean calcium response as a function of stimulus contrast 200 ms earlier, measured from L3 terminals. Error bars denote +/- 1 SEM.

Figure S5: Single fly behavioral assay and responses to rotational stimuli. . Related to Figure 5.

(A) Schematic of the behavioral set-up. The fly is positioned above an air-cushioned ball. Visual stimuli are projected via a DLP projector through a set of fiber optics on to 3 screens that surround the fly. **(B)** Photo of fly-on-ball behavioral set-up. Inset: Close up photo of a fly mounted on the ball. **(C-J)** Each panel shows the experimental *Gal4>shi^{ts}* condition (blue) as well as the corresponding *Gal4/+* (red) and *UAS-shi^{ts}/+* controls. Genotypes and schematics are shown to the left of each panel. Number of flies run per genotype is indicated in parentheses. Shading denotes +/- 1 SEM. The bar plots next to each time trace show integrated responses over a 250 ms window beginning 80 ms after stimulus onset. *p<0.05, ***p<0.001, tested using two-tailed t-tests against both controls. Error bars denote +/- 1 SEM.

(C, D, E, F, J) Turning responses to an opposing edge stimulus. Light and dark edges rotate in opposite directions at 160°/s. **(G)** Turning responses to rotating light edges. Multiple light bars appear on a dark background. One edge of each bar then expands in the same direction at 80°/s. **(H)** Turning responses to rotating dark edges. Multiple dark bars appear on a light background. One edge of each bar then expands in the same direction at 80°/s. **(I)** Turning responses to rotating square wave gratings with 40° spatial period and moving at a contrast frequency of 9 Hz.

Figure S6: Effects of silencing multiple lamina neurons on responses to rotating opposing edge stimuli. Related to Figure 6.

(A-F) Turning responses to an opposing edge stimulus. Light and dark edges rotate in opposite directions at 160°/s. Each panel shows the experimental *Gal4>shi^{ts}* condition (blue) as well as the corresponding *Gal4/+* (red) and *UAS-shi^{ts}/+* (green) controls. Genotypes and schematics are shown to the left of each panel. Number of flies run per genotype is indicated in parentheses. Shading denotes +/- 1 SEM. The bar plots next to each time trace show integrated responses over a 250 ms window beginning 80 ms after stimulus onset. *p<0.05, ***p<0.001, tested using two-tailed t-tests against both controls. Error bars denote +/- 1 SEM.

Figure S7: Full field flicker does not drive strong translational modulation and flies turn to avoid a pole of expansion. Related to Figure 7.

(A) Translational modulation observed in response to a full field flicker presented for 250 ms at 6 Hz (gray area). n=8 flies. Shading denotes +/- 1 SEM. **(B)** Responses to a full field flicker presented at different frequencies, integrated over a 250 ms window beginning 80 ms after stimulus onset. In contrast to responses to motion, slowing responses to full field flicker are not modulated as a function of contrast frequency. n=8 flies. Error bars denote +/- 1 SEM. **(C)** Translational modulation in response to a full field flicker does not become more pronounced when this stimulus is presented at 6 Hz for a longer duration (750 ms, gray area). n=7 flies. Shading denotes +/- 1 SEM. **(D-G)** Average turning **(D, F)** and

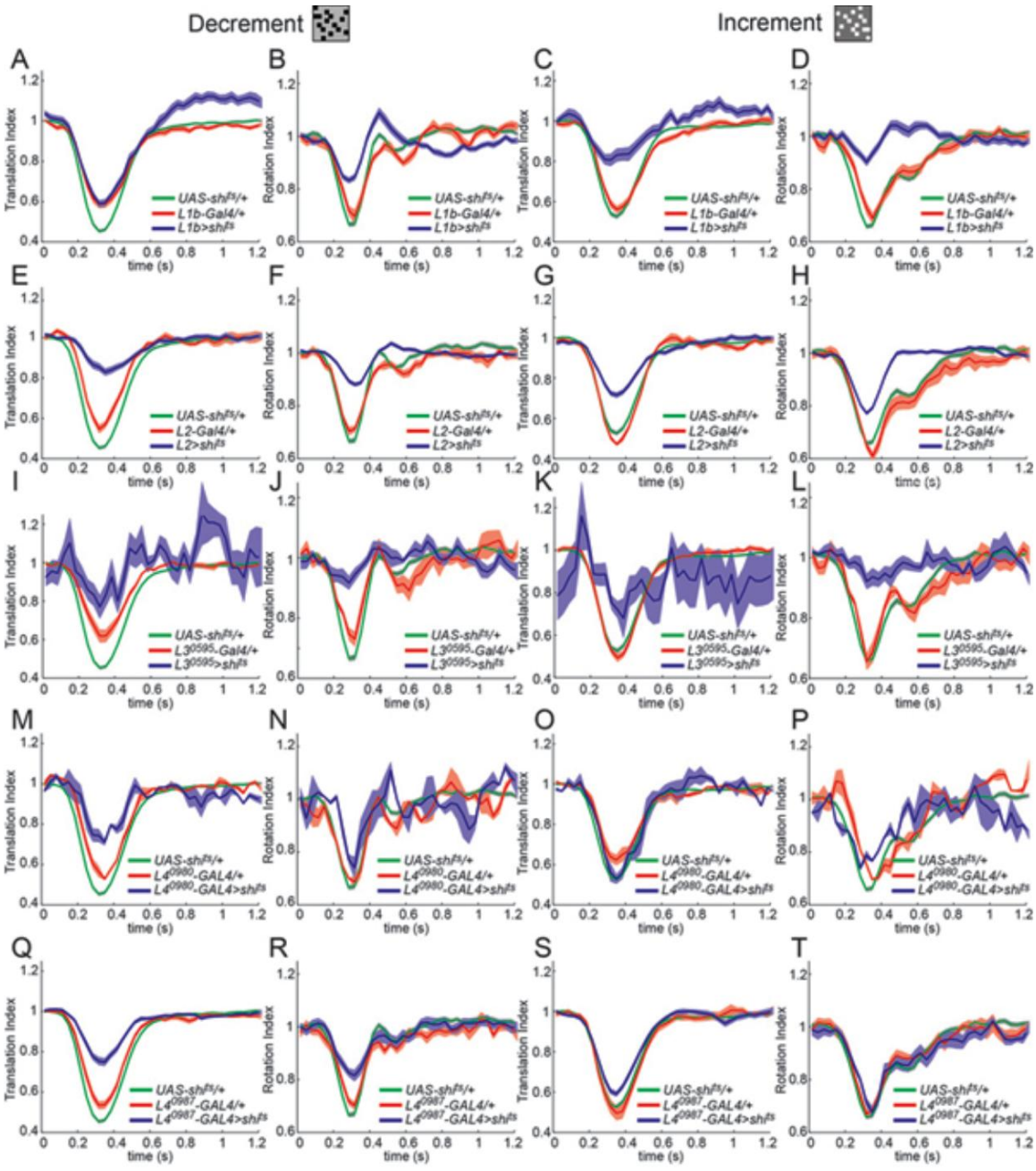
normalized forward walking **(E, G)** responses to front-to-back (**D, E**) and back-to-front **(F, G)** translational motion stimuli moving at the indicated contrast frequencies in the absence of obscured poles of expansion and contraction. The gray filled area denotes the stimulus presentation time (250 ms). Shading denotes +/- 1 SEM. n=8 flies

Figure S8: L2 and L3, but not L1 or L4, are required for modulation of forward walking behavior by translational motion stimuli. Related to Figure 8.

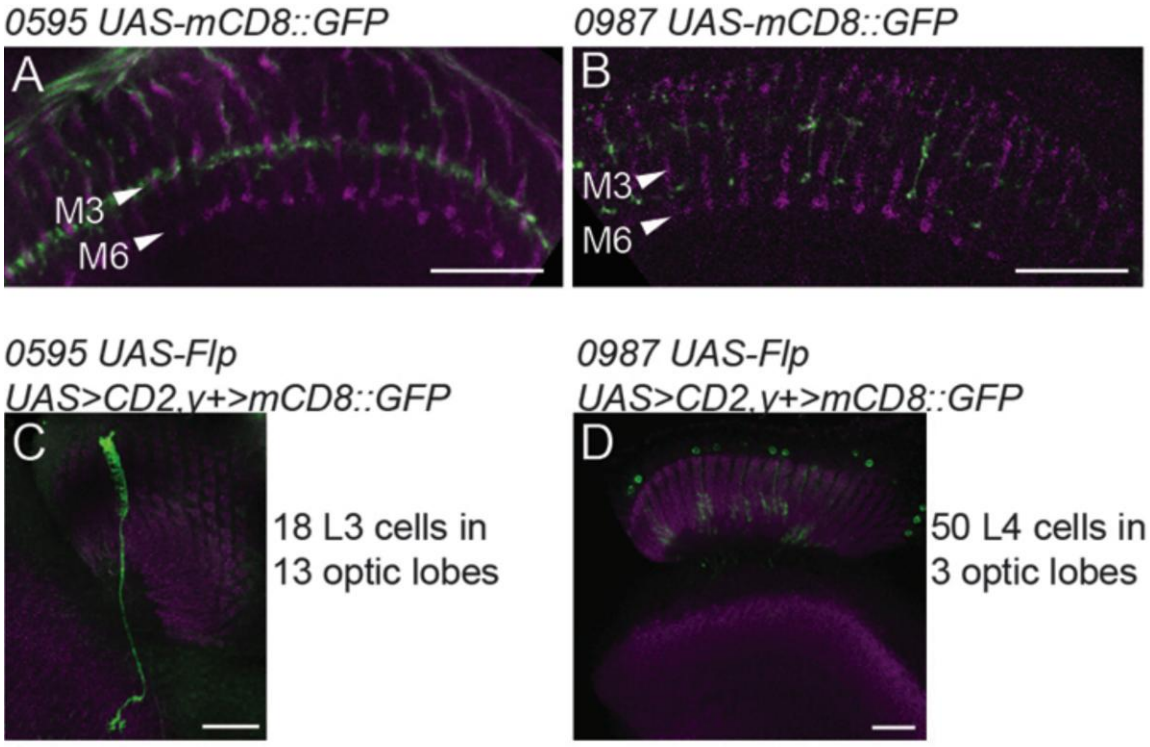
(A-J): Integrated forward modulation in response to a range of contrast frequencies. The integration window is 250 ms and begins 80 ms after stimulus onset. Genotype and schematics are shown to the left of each panel. Number of flies run per genotype is indicated in parentheses. Error bars denote +/- 1 SEM. *p<0.05, two-tailed t-test, tested against the *Gal4*/+ and *UAS-shi*^{ts}/+ controls.
(K) Normalized average forward walking speed as a function of time in response to a back-to-front translational motion stimulus at a contrast frequency of 6 Hz (dark gray box), preceded by a 500ms delay, where the square wave grating is stationary (light gray box). Number of flies run per genotype is indicated in parentheses. Genotype schematics and labeling are given to the left of each panel. Shading around mean traces denotes +/- 1 SEM.

Figure S9: Responses to translational and rotational motion utilize different input architectures. Related to Figure 9.

(A-N): Integrated forward modulation in response to a range of contrast frequencies. The integration window is 250 ms and begins 80 ms after stimulus onset. Genotype and schematics and shown to the left of each panel. Number of flies run per genotype is indicated in parentheses. Error bars denote +/- 1 SEM. *p<0.05, two-tailed t-test, *Gal4(1)+Gal4(2)>>UAS-shi^{ts}* tested against all other genotypes.



Silies and Gohl et al., Figure S1



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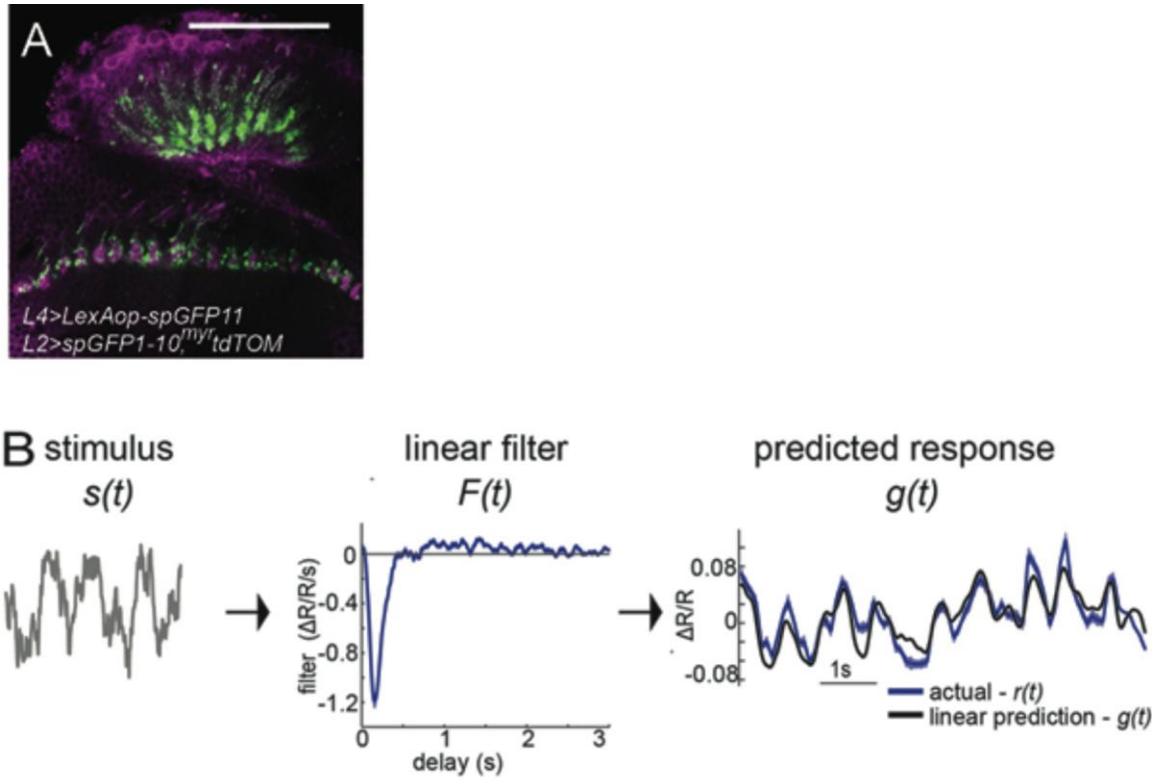

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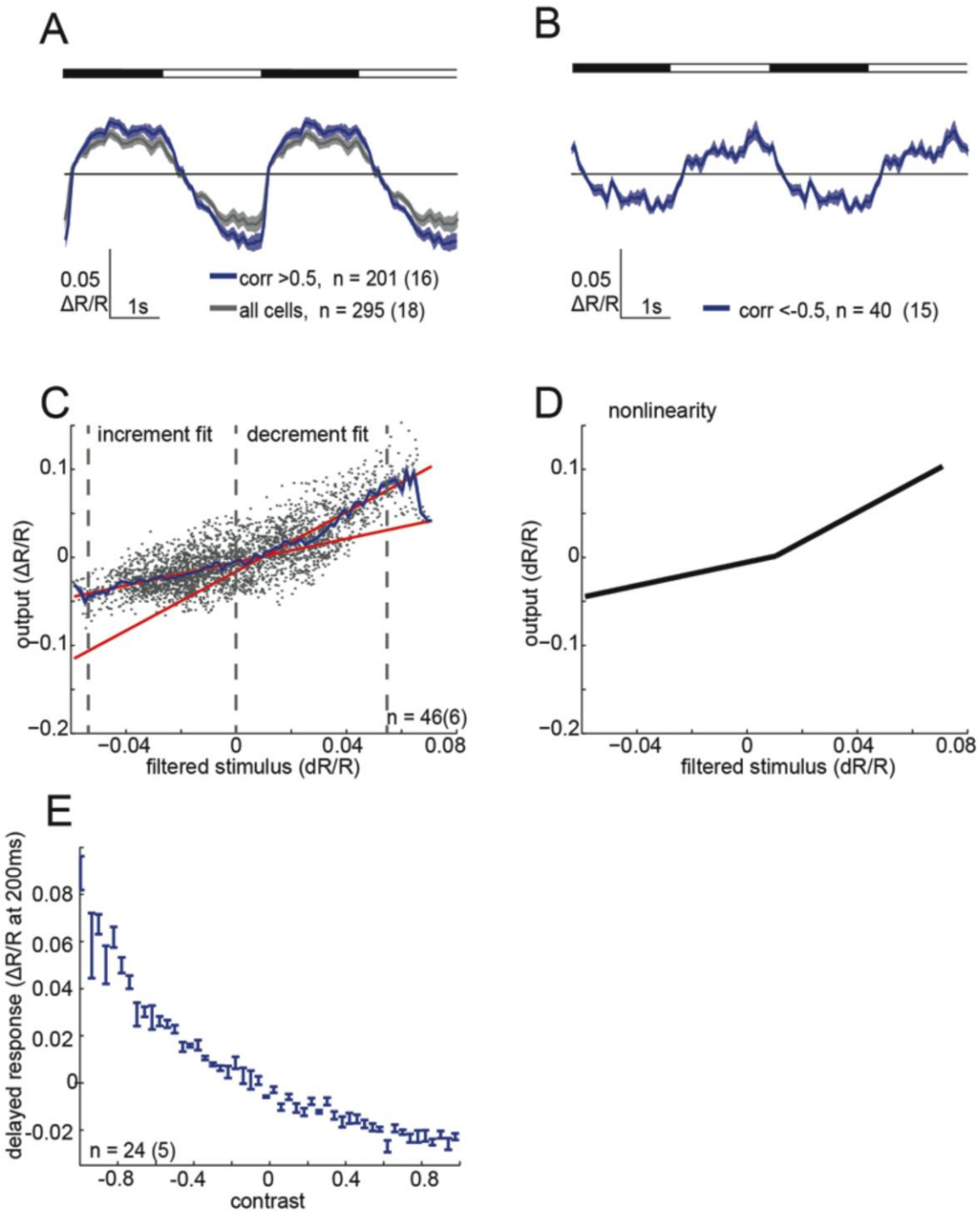

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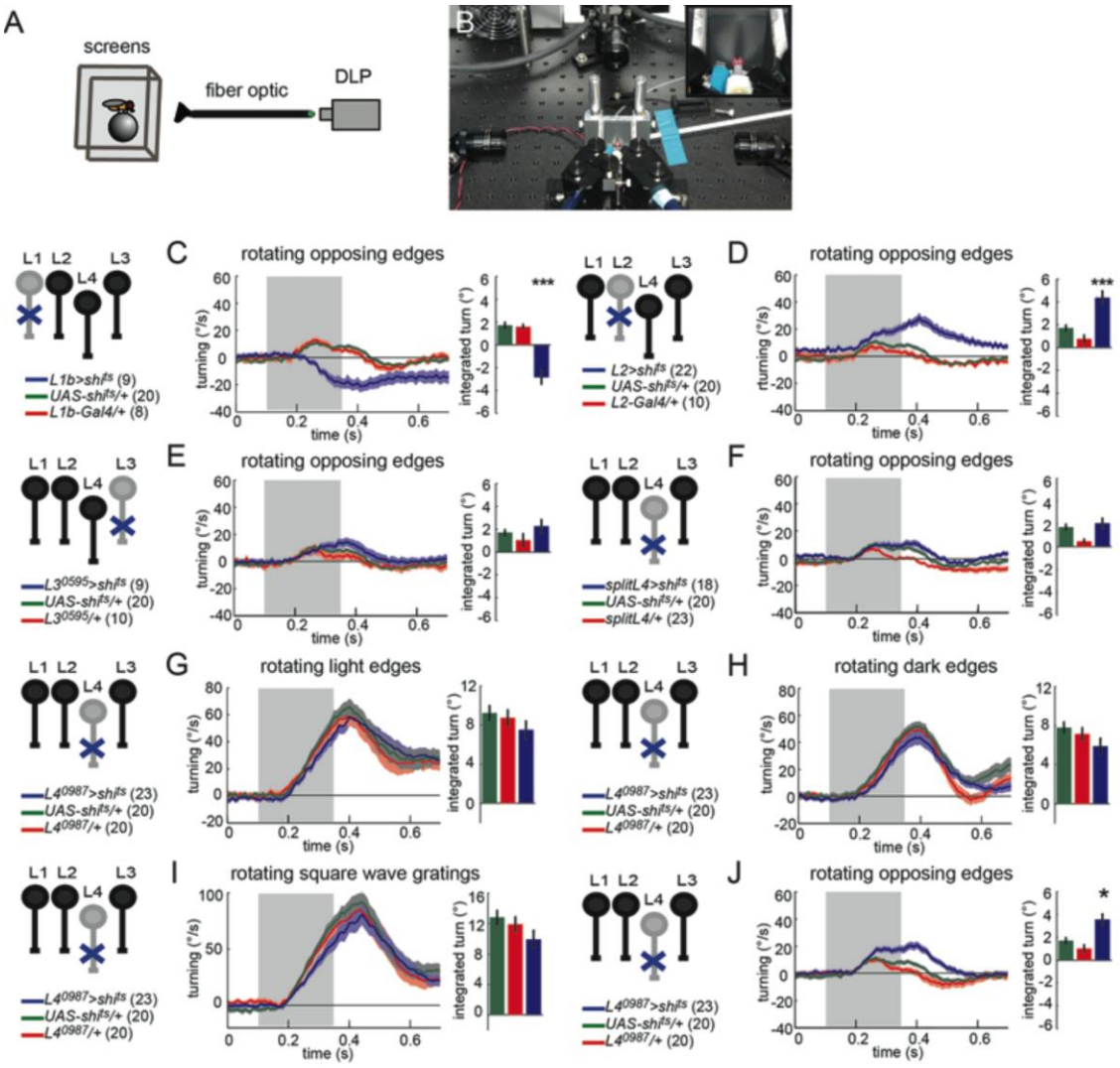

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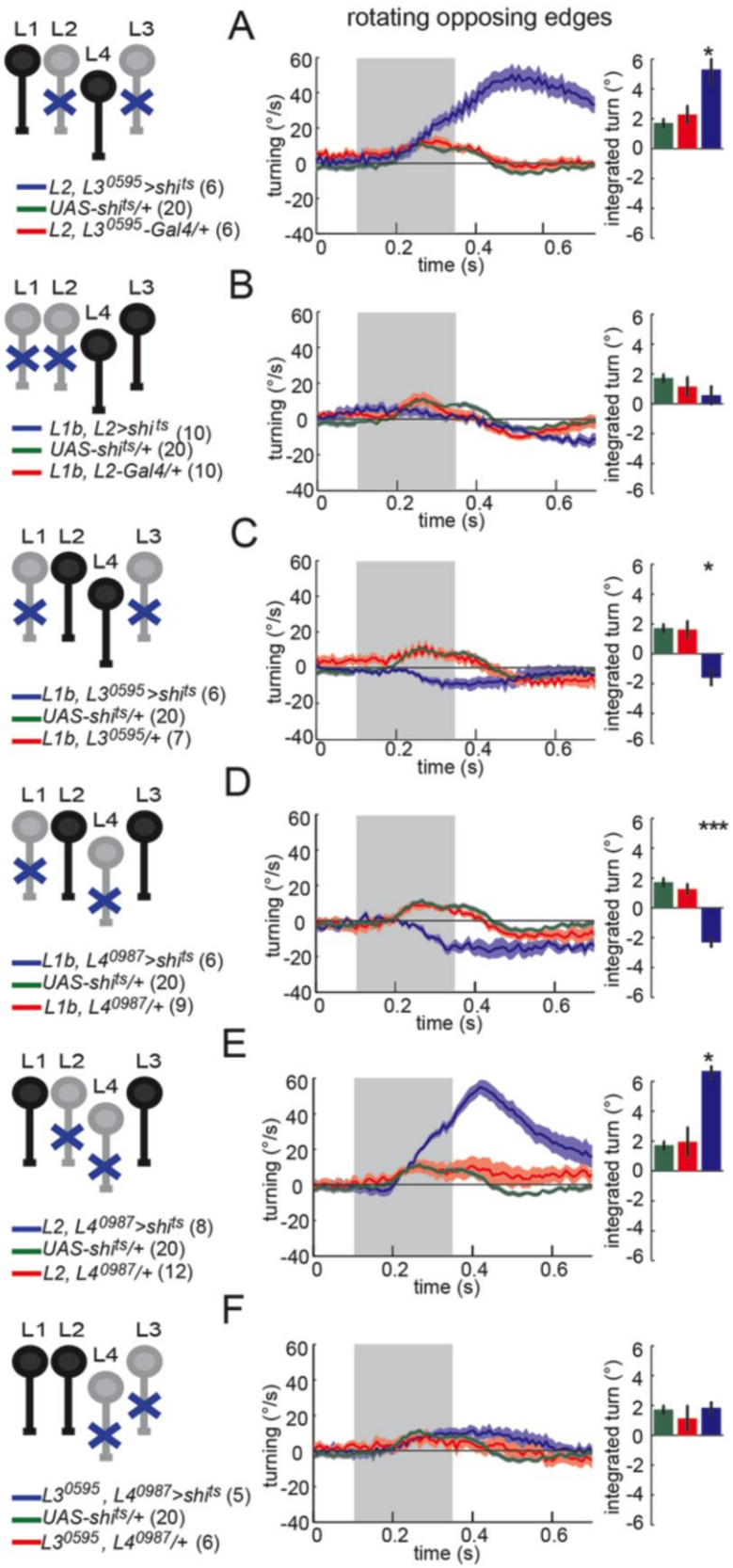

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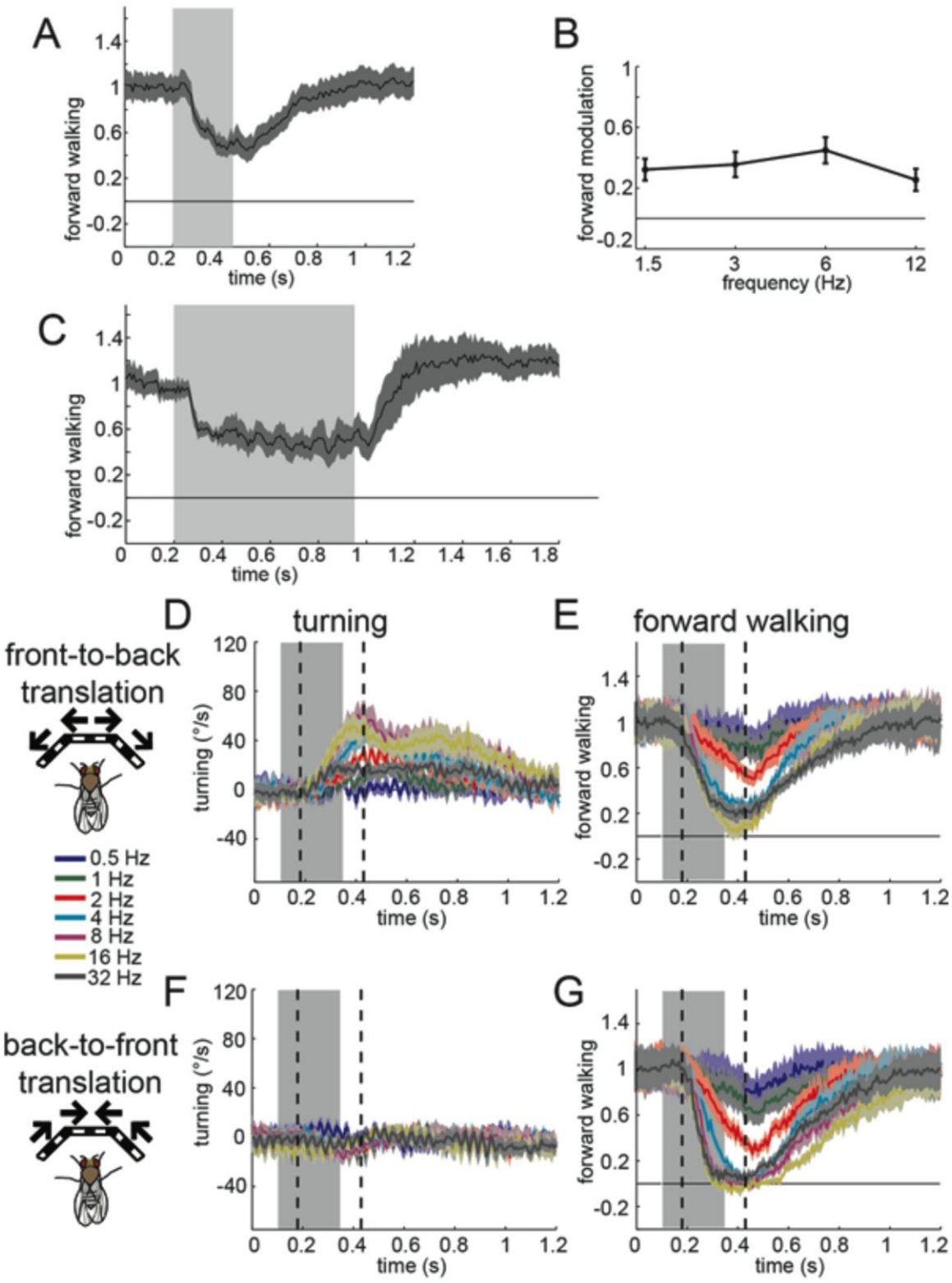

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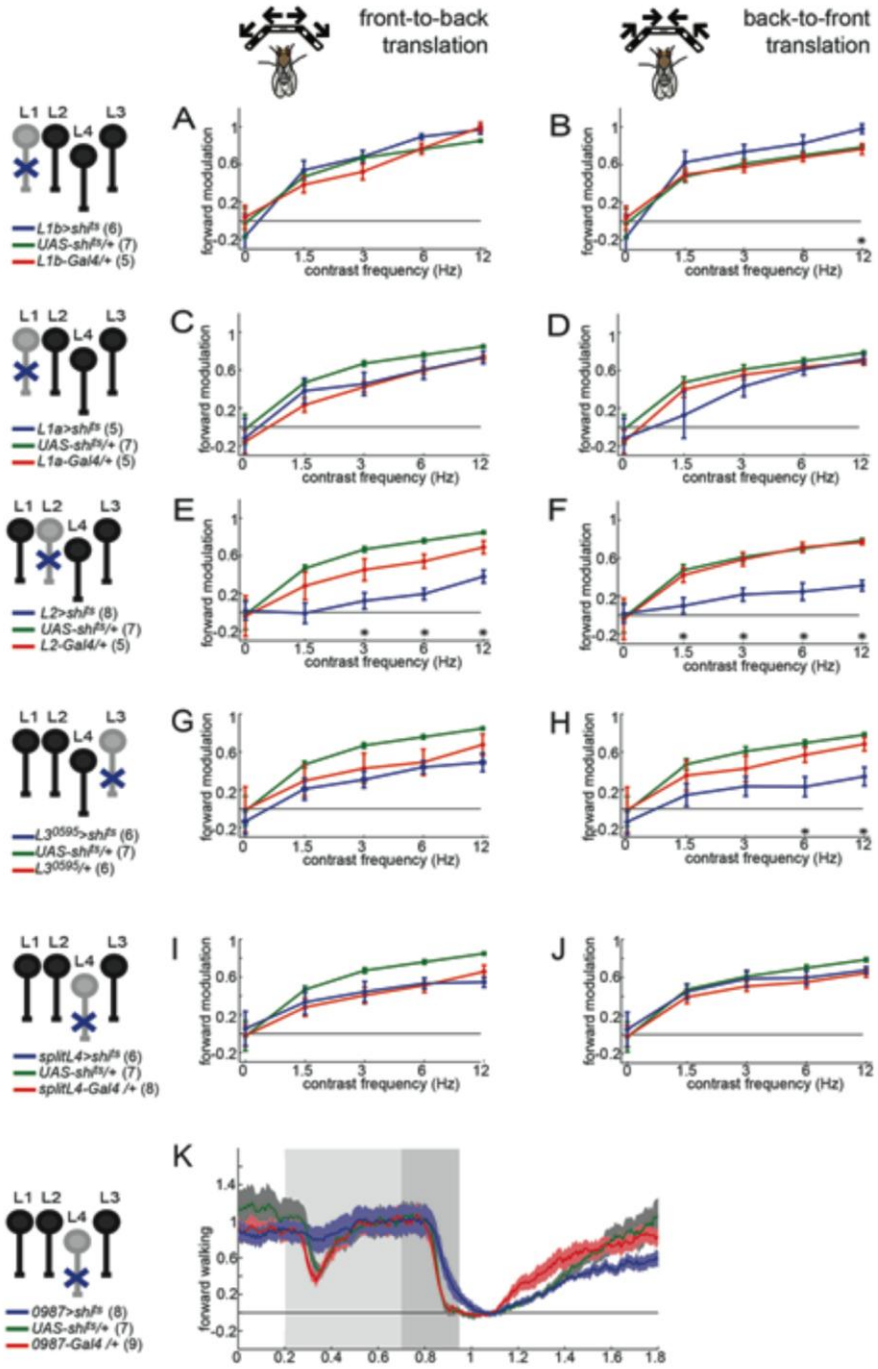

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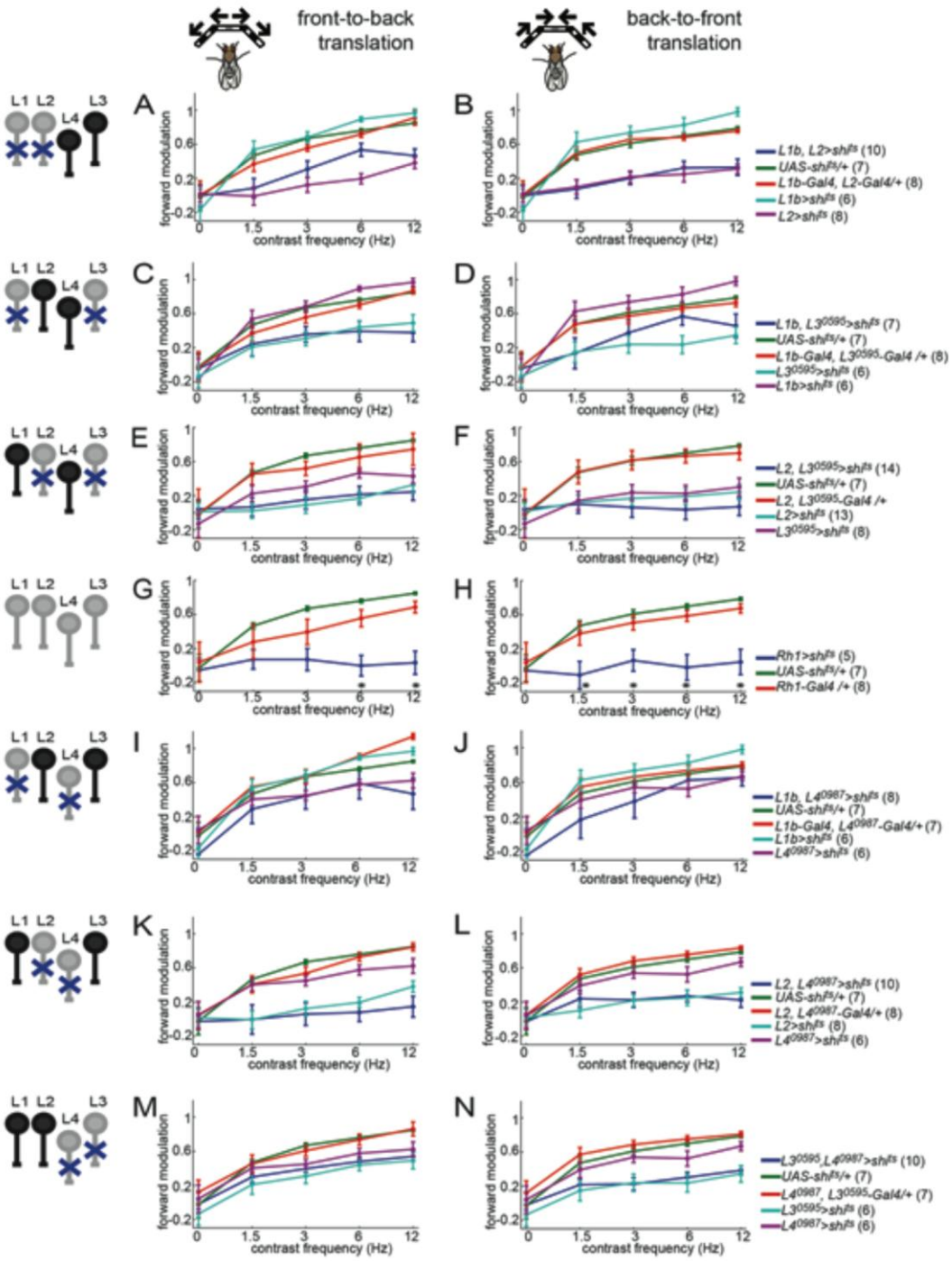

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

FLY HUSBANDRY

Behavioral experiments

For behavioral experiments, flies were grown on molasses-based food on a 12:12 hour light:dark cycle, at a temperature varying daily between 18° and 21°C. Female flies of all genotypes were shifted to 34°C right before the experiment and tested at 34°C, a restrictive temperature for Shi^{ts} activity. For population behavioral assays, flies were tested during their high-activity periods (3 hours after lights-on or 3 hours prior to lights-off). All genotypes were collected 1-2 days after eclosion using CO_2 for sedation, transferred onto fresh food the following day and tested 2 days after collection. Each tube of flies tested contained 33 female flies of the same genotype. For single fly behavior, flies were collected 1 day after eclosion using CO_2 for sedation and run two days later. Chilling was used to immobilize flies for mounting.

Imaging experiments

For imaging experiments, flies were grown on molasses-based food at 25°C and tested at room-temperature (20°C). For imaging, female flies were collected on CO_2 within 1 day of eclosion, and tested within 1-2 days for L4, or within 1 day for L3. Flies were anaesthetized on ice for mounting into the microscope holder. For genetic silencing experiments, *UAS-shi^{ts}* expressing or control flies were incubated in a 37°C waterbath for 1 hour (Joesch et al., 2010). Mounting and dissection was performed at room temperature within 10 min. and all data were acquired within 45 minutes after temperature shift.

CONFOCAL IMAGING

To image expression patterns, *Gal4* driver lines were crossed to *UAS-mCD8::GFP*, and dissected, fixed, and stained for GFP (chicken anti-GFP, abcam, 1:2000) and Bruchpilot (nc82) (DSHB, 1:30) using standard methods (Lee et al., 2001). For GRASP experiments, mouse anti-GFP (Invitrogen, 1:100) and rabbit anti-RFP (abcam, 1:200) were used. Brains were imaged using a Leica TCS SP2 AOBS confocal microscope (Bensheim, Germany) with either a 20x (numerical aperture (NA) = 0.7), 40x (NA = 1.25), or 100x (NA = 1.4) lens. Confocal images were rendered in three dimensions using Imaris (Bitplane), adjusted using cropping and thresholding tools in Photoshop (Adobe), and assembled into figures using Illustrator (Adobe).

POPULATION BEHAVIOR EXPERIMENTS / SCREEN

The behavioral assay, tracking, and analysis were performed described as in Katsov and Clandinin (2008). Visual stimuli were displayed on a CRT monitor at 200 Hz refresh rate and had a dot density of 20%, a dot size of 14 pixels (screen resolution 480x640), and moved at 6 pixels/frame (759 mm/s) with 98% coherence in motion trials, and 0% coherence in noise trials. The fly's mean distance from the monitor was 38.2 mm. From the fly perspective, this resulted in a similar stimulus velocity range to that used in Katsov and Clandinin (2008), with a maximum angular velocity of 1131°/s. Contrast values for increments and decrements were 0.47 and -0.47, respectively. Contrast was calculated as the

Michelson contrast of luminance values: dot-background/dot+background. Translation indices (TI) and rotation indices (RI) were calculated as in Katsov and Clandinin (2008). In brief, TI and RI were calculated relative to the prestimulus period as follows:

$$TI(t) = \left(\frac{1}{\overline{TI_0}} \left(\frac{\#fast}{\#fast + \#slow} \right) \right) - 1$$

and

$$RI(t) = \left(\frac{1}{\overline{RI_0}} \left(\frac{\#turning}{\#turning + \#not\ turning} \right) \right) - 1$$

where $\overline{TI_0}$ and $\overline{RI_0}$ represented metric averages over the 1 sec before motion onset and where “fast” and “slow” walking flies displayed translational velocities of more or less than 1.9cm/s respectively. Stopped flies were excluded from the analysis. Similarly, “turning” and “not turning” flies displayed angular velocities of more or less than 100°/s (Katsov and Clandinin, 2008). Longer integration windows were used for the metrics shown in Figure 1 (200 ms starting at stimulus onset for rotation on contrast decrement stimuli, 433 ms starting at stimulus onset for translation on contrast decrement and translation and rotation on contrast increment stimuli). The values shown represent the mean integrated translation or rotation index for each tube.

SINGLE FLY BEHAVIORAL EXPERIMENTS

Behavioral set-up

The fly-on-a-ball assay was largely as described previously (Clark et al., 2011). Visual stimuli were provided using a digital light projector (DLP) focused

onto coherent optic fiber bundles as described in detail in Clark et al (2011). However, the screens around the fly were modified from the previous set up: in the experiments presented here, the stimulus was projected by camera lenses from the ends of the fiber bundles onto rear projection screens arrayed around the fly-on-the-ball, abutting each other in a similar configuration to the screens in Clark et al (2011), but further away from the fly. Each screen was 3x3 cm, arranged at 90° angles around the fly to form 3 sides of a cube. The screens extended for +135° and -135° azimuthally from the fly heading, and from -45° to +45° in elevation. As in the previous set-up, stimuli were presented at 240 Hz, and the stimulus was projected appropriately to correct for oblique viewing angles, so that flies were situated in a virtual cylinder.

Single fly behavior stimuli

For rotational stimuli, the luminance of the stimulus was 16 cd/m^2, while for translation experiments it was 3x brighter. 100% contrast was used throughout unless otherwise indicated. Fly behavior was examined by monitoring the position of a near-frictionless ball rotating under each suspended fly as described by Clark et al. (2011). All rotational stimuli (rotating square wave gratings, dark and light edges and opposing edges) are described in detail in Clark et al (2011).

Translational motion stimuli consisted of a 100% contrast (unless otherwise indicated) 40° period square wave moving past each eye in the azimuthal plane, either from front-to-back or back-to-front on both sides. The pole

of expansion or contraction, situated directly in front of the fly, was obscured by a 10° wide bar that flickered at 30 Hz. Compared to white, gray or black bars, the flickering stripe was the smallest manipulation necessary to suppress turning evoked by the singularity, and does not introduce net luminance changes. Individual motion stimuli were interleaved randomly, separated by periods of 1 s of gray. Stimuli appeared and remained stationary for 500 ms before a 250 ms period of motion, during which the bars moved at the rates noted. The non-delayed translational stimulus used in Figure 8I,J lacks the 500ms stationary period and interleaves 1 s of gray with 250 ms motion epochs

The translational stimulus was usually run back to back with the rotational stimulus, or flies were otherwise pre-heated at 34°C for 15-20 min for the translational stimulus. Flies walking at a baseline speed (pre-stimulus) of less than 2 mm/s were considered non-cooperative and were excluded from analysis. The forward walking speed of all flies of one genotype was normalized by their mean forward walking speed in the 100 ms period preceding the stimulus.

The full field flicker stimuli were presented at 100% contrast and different frequencies for 250 ms or 750 ms, respectively, interleaved with 1s of gray.

CALCIUM IMAGING

Fly preparation and imaging set-up

Mounting of the flies was as described in Clark et al. (2011). Axonal terminals in the medulla were imaged using a Leica TSC SP5 two-photon

microscope, with a Leica HCX APO 20X/1.0 NA water immersion objective (Leica, Bensheim, Germany) and a pre-compensated Chameleon femtosecond laser (Coherent, Inc., Santa Clara, CA). The excitation wavelength was 830 nm and between ~10 and 20 mW of power was applied at the sample. CFP- and citrine-emitted photons were collected using two emission filters and a 495-LP beam splitter (Semrock, Rochester, NY). The chosen emission filters (514/30 for citrine and 447/60 for CFP) allowed us to present visual stimuli through a 562/40 filter without it being detected by the microscope PMTs. Synchronization between imaging and stimulus presentation was established using triggering functions provided by the LAS AF Live Data Mode software from Leica. A DAQ (NI USB-6211) connected to the computer used for stimulus generation was used to generate a trigger at the beginning of stimulus presentation, this trigger was read by the imaging software and used to initialize imaging at the same time. In addition, a trigger was produced by the imaging software to indicate the beginning of the acquisition of each frame and was acquired via the same DAQ on the stimulus computer such that stimulus presentation details were saved together with imaging timing and used in subsequent processing. All data was acquired at a constant frame rate of 10.6 Hz using frame sizes of 200*50 pixels and a line rate of 700 Hz in unidirectional scanning mode. Visual stimuli were generated using open-gl and C++ code and provided using a digital light projector (DLP) as described in Clark et al (2011). Like the screen modification to the single fly behavioral apparatus described above, after passing through the filter and the coherent fiber optic bundle, the visual stimulus from the DLP was

re-projected on a 8 cm x 8 cm rear-projection screen positioned anterior to the fly spanning 55° of the fly visual field horizontally and 58° vertically. The stimulus was updated at 240 Hz, and had a luminance of approximately 76.4 cd/m^2. Given the stimulus spectrum and its cross-section with the Rh1 absorption spectra, this corresponds to 1.2 times the luminance used for translational behavioral stimuli (Salcedo et al., 2003).

Visual stimuli

Periodic 2s light 2s dark full-field flash presentation

Periodic presentation of light flashes lasting 2 s were interleaved with 2 s of darkness. The stimulus was typically presented for approximately 40 s or 400 imaging frames, such that each cell observed at least 8 flashes of light.

Moving bright bar on a dark background

An approximately 2.5° wide bar moving at approximately 10°/s in one of four possible orientations was presented in a randomized order (a distinct order was chosen every time the stimulus was applied). The four orientations included either a vertical bar moving left or right across the horizontal extent of the screen or a horizontal bar moving up or down across the vertical extent of the screen. There was a 9 s gap between bar presentations to allow responses to fully decay. The stimulus was presented long enough to allow the bar to pass the screen at least 5 times. Response traces of different cells were aligned by shifting different time-traces by the delay that brought the cross-correlation between the specific trace and a sample trace (L2 mean trace) to a maximal value.

Gaussian flicker stimulus

A gaussian noise stimulus was used to examine the cellular responses to a dynamic stimulus. The screen intensity was updated according to a near-gaussian distribution with a standard deviation of 50% fractional contrast about a mean luminance of 50% of maximum. Values of greater than 100% or less than 0% luminance were set to 100% and 0% luminance, respectively. The sequence of samples had an exponential correlation function with a time-constant of 200 ms.

Analysis of calcium imaging data

Raw images in each time series were aligned using an ImageJ macro based on Turboreg (http://bigwww.epfl.ch/thevenaz/turboreg/) and regions of interest around medulla projections of lamina cells were identified in average time-series images as previously described (Clark et al., 2011). The average intensity within each region of interest was computed for each frame to generate a time-trace of the response of each cell as a function of time. All responses and time-traces of presented stimuli were interpolated to 100 Hz prior to averaging. In order to remove slow fluctuations of the baseline ratio a 4^{th} order polynomial was fit to each response trace and subtracted from it. First, mean responses of all cells imaged are calculated for each fly. These mean responses are then averaged across all flies to generate the response traces shown in figures. All cells imaged for all responsive flies of a given genotype are included in the analysis, unless otherwise stated. For each cell, the mean response to each stimulus epoch is

computed whenever multiple stimulus presentations occur within a time trace.

We used a Linear-Nonlinear (LN) model to capture the cell's response to the gaussian full field flicker stimulus. For each case, the model consists of two functions, a linear temporal filter and a static nonlinearity. The model predicts the response of the cell, *r'(t)*, by convolving the stimulus, *s(t)*, with the linear filter, *F(t)*

$$g(t) = \int F(\tau)s(t-\tau)d\tau \qquad \text{(eq 1)}$$

and passing the result, *g(t)*, through the nonlinearity *N(g)*:

$$r'(t) = Ng((t)) = N(\int F(\tau)s(t-\tau)d\tau) \qquad \text{(eq 2)}$$

The filter was computed in Fourier space as described in Clark et al (2011) and then transformed back to temporal space. The nonlinearity was computed by plotting the response, *r(t)*, against the linear prediction, *g(t)*. Values of *g* were ranked by order of magnitude, and the corresponding *r* values were then averaged over evenly spaced bins and the curve was fit with two linear regression lines (Figure S4). Bins on the edges that contained few data points were not considered for the fitting. The linear filter represents the temporal sensitivity and polarity of the cell, while the nonlinearity conveys the gain or rectification of the cells' mean response. Similar methods have been used previously (Chichilnisky, 2001; Sakai et al., 1988). Cells included in the analysis for the LN model were selected based on the predictivity of the filter, calculated by a cross correlation of the predicted output with the output of a cell (cells with a cross correlation coefficient >0.4 were included). Using this criterion, 24% (L3)

and 77% (L4, layer M2) of the imaged cells were used to calculate the linear filters and nonlinearities. To display the delayed responses to contrast, we analyzed the response of cells that were significantly anti-correlated with the input contrast. Cells were included in the analysis when this cross correlation coefficient was less than -0.27. This criterion included between 13% (L3) and 77% (L4, layer M2) of all imaged cells.

MOLECULAR BIOLOGY

To generate *QUAS-TN-XXL*, the *TN-XXL* CDS was amplified from genomic DNA from the *UAS-TN-XXL* transgenic flies, using the oligonucelotides gen UASFOR and genUASREVsv40 as described in Potter et al (2010):
genUASFOR (GCTTCGTCTACGGAGCGACAATTCAATTCAAAC)
genUASREVsv40 (GCAGTAGCCTCATCATCACTAGATGGCATTTCTTC)
The resulting PCR product was then sublconed into pQUAST using *Eco*RI and *Xho*I restriction sites. Subsequent sequencing showed that the *TN-XXL* sequence in *UAS-TN-XXL* transgenic flies (published in Mank et al. 2008, Reiff et al. 2010) and subsequent constructs contained only one domain of chicken TroponinC and did not contain any of the described superfolder mutations in ECFP or Citrine cp174. All EF hand mutations described by Mank et al. 2008 were present. Transgenesis was performed using standard procedures by Rainbow Transgenic Flies, Inc (Camarillo, CA).