Walking modulates speed sensitivity in *Drosophila* motion vision

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INVENTORY OF SUPPLEMENTAL INFORMATION

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SUPPLEMENTARY METHODS

Fly Stocks

Flies were reared on standard cornmeal agar under a 12-hr light/12-hr dark cycle at 25°C. All experiments were performed on adult female flies, 2-4 days post-eclosion, and during similar periods of their circadian day. *UAS-GCaMP3.0* flies were generously provided by Loren Looger and Julie Simpson. Line *R27B03-Gal4* was constructed by the methods described in Pfeiffer et al. 2008 [1] and identified as driving expression in the HS-neurons by A. Nern (unpublished).

Fly preparation

Details of the preparation are described in an accompanying publication [2], and many additional experimental details are provided at http://www.flyfizz.org. Briefly, a cold-anesthetized female fly was tethered and mounted on a custom holder under a dissection microscope. The head of the fly was bent forward by about 70-80º to give access to its posterior face. The preparation allowed good optical access to the dorsal part of the head and the LPTCs. The cuticle, some of the fat tissue preventing optical access to the LPTCs, and muscle M16 were removed with fine tweezers, and the proboscis was extended and fixed with wax to prevent motion. The dissected fly was mounted under the microscope where its legs were free to move an air-supported ball.

Two-photon imaging and visual stimulation of the fly

We imaged on a custom-built two-photon microscope (**Fig. 1A**) using ScanImage 3.6 software [3], an Olympus 60×, 0.9 NA LUMPlanFI/IR objective, and a mode-locked Ti:Sapphire Chameleon Ultra II laser (Coherent, Santa Clara, CA) tuned to 920-930 nm. Fluorescence was collected using photomultiplier tubes (Hamamatsu, Hamamatsu City, Japan) after bandpass filtering with a BG22 emission and HQ615/70-2p filter (Chroma Technologies, Brattleboro, VT). Images were acquired in framescan mode (8 Hz).

We used a modular LED arena to present visual stimuli to the fly [4]. Details of visual display are described in an accompanying publication [2]. Briefly, the fly was positioned at the center of an LED arena whose dimensions cover ~157º in azimuth and 45º in elevation of the fly's visual field, with a maximal pixel size of 2.8º. With the fly on the ball, the subtended angle of elevation is reduced to ~40º.

Horizontal moving patterns were generated with vertical bars of constant spatial frequency (λ = 22.4º [5, 6]), close to the optimal spatial frequency for optomotor response [7], moving at different velocities, ranging from 5.6 º/s to 224 º/s, so that temporal frequencies ranged from 0.25 to 10 Hz (velocity/spatial frequency). For temporal frequencies lower than 6 Hz, stripe intensity was adjusted to generate sine wave gratings. For higher temporal frequencies, square gratings were used. Large-field sine or

square gratings of maximal contrast were presented to the fly. Stimuli generally consisted of a 5-second stationary pattern segment after which the pattern moved in the preferred direction (PD) of the neuron for 10-15 seconds. Following PD stimulation, another 5- to 15-second stationary pattern segment preceded motion of the pattern in the anti-preferred or null direction (ND) of the neuron for 10-15 seconds. The trial ended with 5 seconds of the stationary pattern, and lasted 35-55 s.

The same stimulus was repeated at least five times per behavior condition (walking or stationary, i.e. not walking). The inter-trial interval lasted between 60 to 75 seconds. Constructing the temporal frequency tuning curves under the two behavioral conditions (walking and stationary) required experiment durations of 4-5 hr, throughout which the flies showed resting—including grooming—and walking periods (see behavioral analysis).

We only imaged the left half of the fly brain. Furthermore, we restricted imaging to a highly stereotyped branching segment of the dendrite of HS-North (HSN) neuron, which was selected based on ease of optical access across all flies and strength of calcium signal (**Fig. S1** and **Fig. 1B**). Therefore, data presented as HSN always refers to the left HSN neuron, for which PD corresponds to rotations in the counter-clockwise direction.

Data Analysis

Behavioral Analysis: The ball's movements are tracked at high temporal resolution [2] and are the proxy of the fly's walking activity. We used two cameras to track the ball and measure the three axes of rotation: pitch, indicating forward rotation, roll, indicating sideways rotation, and yaw, indicating rotations around the animal's dorsoventral axis. From the ball rotations we obtained displacements along the three axes that were converted into the corresponding forward, side and rotational velocities for the fly.

We binned the velocity traces into 50-ms (20 Hz) bins and set threshold criteria to identify movements from walking activity. Based on Ronald Strauss's [8] work on the analysis of walking in *Drosophila*, we considered a minimal step time of 200 ms and a minimal step size of 1 mm for translation and 1º for rotational motion. If motion accumulation within a bin was lower than our threshold, we set the original velocity trace to zero for those time points. The velocity traces processed in this way had the same time resolution as the original traces but with putative noise removed. For display only (**Fig. 2A** and **Fig. 3A**), we calculated velocity at each point as a change in position over 0.5 s periods. In all figures showing behavioral data, we only show rotational velocity—the component of the behavioral response that is most sensitive to the horizontally-motion stimulus we use [2].

Trials in which the fly began walking before or during PD stimulation were considered walking trials. On the other hand, trials in which the fly did not walk during the entire PD were considered non-walking trials, here referred to as stationary trials (but see below for another definition of the stationary condition).

We noticed that the HS response amplitude was also slightly modulated by grooming (data not shown), although the effect was less evident and less robust than when the fly started walking. For that reason, we used two other cameras to record side and posterior views of the fly to differentiate stillness, which we refer to as the "still-legs" condition, from grooming in stationary trials, i.e., those with no detectable rotations of the ball.

However, for some experiments, flies displayed long periods of walking interrupted only by grooming. In these cases, we achieved the stationary condition by waxing the legs, (the "fixed-legs" condition). Because the irreversible fixed-legs condition was usually implemented during the last 1-2 hrs of the experiment, we checked to see if the manipulation resulted in HSN responses and tuning curves different from the more natural stationary condition (i.e., still-legs) (**Fig. 2D,E**). When available, we used the fixed-legs as the stationary condition since this also prevented the fly from grooming.

Two-photon Imaging: Image processing was performed using custom code written and run in MATLAB (The MathWorks, Inc., Natick, MA). Additionally, to correct motion artifacts due to behavior, we implemented image registration by translational compensation based on discrete Fourier analysis (efficient subpixel registration, available from http://www.mathworks.com/matlabcentral/ [9]). We perform experiments using GFP-expressing neurons to estimate artificial changes in fluorescence related to motion [2].

For all trials, we computed the HSN response as the percentage of the ratio between the change in fluorescence (with respect to baseline fluorescence) and the baseline fluorescence. Because of residual bleed-through of the arena [2], raw fluorescent signals were background-subtracted and the baseline was calculated during the first 5 s of stationary pattern. Peak responses were calculated as the mean of a 0.5-s window centered at the maximum value within the PD segment. We choose this definition of peak response instead of the mean response in the last 0.5 s of PD stimulation because the response of the neuron decreased if the fly stopped walking before PD termination. This was particularly relevant during higher temporal frequency stimulation. Thus, we always compared peak responses of walking trials with stationary trials at the corresponding time segment. We used the peak responses to obtain the temporal frequencies tuning curves of HSN by plotting the mean peak response of the neuron to a particular temporal frequency of stimulation across walking and stationary trials (**Fig. S2**).

We calculated the increase of the HSN neuron response during walking (response gain) as the ratio between the peak walking-trial neuron's response and the peak response of stationary trials per temporal frequency. We computed the peak response gain as the mean of a 0.5s window centered at the maximum value of the response gain curve.

Statistical Analysis

For comparison of two independent groups, significance of differences was tested with the Mann-Whitney test using the Statistics Toolbox (The MathWorks, Inc.). Differences were considered significant when $p < 0.05$. Throughout the paper, data from a single fly is given in mean ± standard deviation, whereas data across different flies is given in mean ± s.e.m. In all figures *, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Figure 1. Anatomy of dendrites of HSN neurons in *Drosophila*; related to Fig. 1.

We recorded the activity of HSN neurons from a highly stereotyped dendritic branch that could be identified across all animals. Shown in red are examples of this dendritic region from 4 different flies (different from the fly shown in **Fig. 2B**). HSE = HS-Equatorial neuron.

Supplementary Figure 2. HSN temporal tuning curves during walking and stationary trials; related to Fig. 4.

Temporal frequency tuning curves of HSN neurons in walking and stationary conditions. In all 8 flies, the tuning curves increased in amplitude for temporal frequencies > 2Hz. Furthermore, the peak of the tuning curves shifted from 1 Hz during stationary conditions to 2 or 3 Hz during walking for 6 out of 8 flies. Number of trials for different flies varied.
Fly 1: 20 trials per frequency, varying numbers of walking/non walking trials for each
Fly 2: 10 walking trials and 5 non-walking trials per frequency condition
Fly 3: 10 walking trials and 5 non-walking trials per frequency condition
Fly 4: 5 trials each for walking and non-walking conditions per frequency
Fly 5: 5 trials each for walking and non-walking conditions per frequency
Fly 6: 5 trials each for walking and non-walking conditions per frequency
Fly 7: 5 trials each for walking and non-walking conditions per frequency
Fly 8: 5 trials each for walking and non-walking conditions per frequency

Supplementary Figure 3. Stimulus temporal frequency effect on fly walking behavior; related to Fig. 4.

A. Box-plots of median rotational velocities of each fly when the animals were presented with different speeds of horizontally moving vertical gratings. Trial numbers for each fly are indicated within each panel. **B**. Mean ± s.e.m fraction of time spent walking across all 8 flies during different temporal frequency of visual stimulation. **C**. Mean ± s.e.m of median rotational velocity across all flies (for 0.25 Hz and 0.5 Hz n = 6, all other temporal frequencies, n = 8).

Supplementary References

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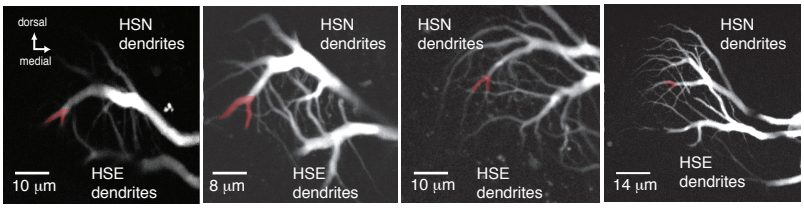
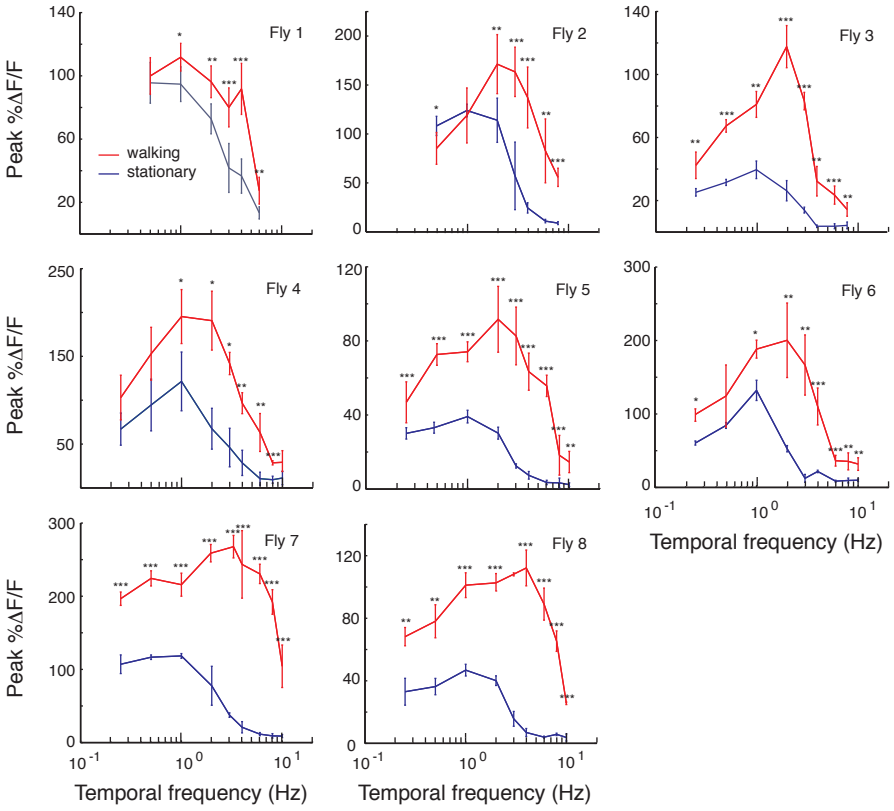
Supplemental Figure 1

Supplementary Figure 1. Anatomy of dendrites of HS-North neurons in *Drosophila*



Supplemental Figure 2

Supplementary Figure 2. HSN temporal frequency tuning curves during walking and stationary trials



Supplemental Figure 3

Supplementary Figure 3. Effect of stimulus temporal frequency on fly walking behavior