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A Novel Neuronal Pathway for Visually Guided Escape in *Drosophila melanogaster*

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Fotowat H, Fayyazuddin A, Bellen HJ, Gabbiani F. A novel neuronal pathway for visually guided escape in *Drosophila melanogaster*. *J Neurophysiol* 102: 875–885, 2009. First published May 27, 2009; doi:10.1152/jn.00073.2009. *Drosophila melanogaster* exhibits a robust escape response to objects approaching on a collision course. Although a pair of large command interneurons called the giant fibers (GFs) have been postulated to trigger such behaviors, their role has not been directly demonstrated. Here, we show that escape from visual stimuli like those generated by approaching predators does not rely on the activation of the GFs and consists of a more complex and less stereotyped motor sequence than that evoked by the GFs. Instead, the timing of escape is tightly correlated with the activity of previously undescribed descending interneurons that signal a threshold angular size of the approaching object. The activity pattern of these interneurons shares features with those of visual escape circuits of several species, including pigeons, frogs, and locusts, and may therefore have evolved under similar constraints. These results show that visually evoked escapes in *Drosophila* can rely on at least two descending neuronal pathways: the GFs and the novel pathway we characterize electrophysiologically. These pathways exhibit very different patterns of sensory activity and are associated with two distinct motor programs.

INTRODUCTION

During evolution, many species have developed specialized escape circuits tuned to the sensory signals generated by approaching predators. To be effective, these circuits have to be faster than the predators and yet be accurate and robust enough to elicit reliable escapes. These circuits typically receive visual, olfactory, or mechanosensory inputs that converge on large interneurons that in turn connect to motor centers (Eaton 1984). In the visual domain, ecological manifestations of danger are signified by looming stimuli: the two-dimensional expanding shadows produced by objects approaching on a collision course (Ball and Troninck 1971; Gibson 1979). Such stimuli represent powerful cues to trigger escape behaviors (Fotowat and Gabbiani 2007; Oliva et al. 2007; Preuss et al. 2006; Yamamoto et al. 2003). Neurons that are particularly sensitive to these cues have been characterized in several species (Graziano 1994; Hatsopoulos et al. 1995; Kang and Nakagawa 2006; Oliva et al. 2007; Preuss et al. 2006; Rind and Simmons 1992; Schlotterer 1977; Wang and Frost 1992; Wicklein and Strausfeld 2000).

In the laboratory, a sudden change in luminance (*light off*) triggers a visually mediated escape behavior in white-eyed *Drosophila* mutants. This escape behavior is known to be mediated by the giant fibers (GFs), a pair of command interneurons that convey information from sensory centers in the brain to motor neurons in the thoracic ganglion that control the mesothoracic legs and wings (Fig. 1*A*; Koto et al. 1981). A single spike in the GFs activates a jump and flight motor program in which the fly takes off with its wings folded and tumbles in the air with no apparent directionality (Lima and Miesenbock 2005; Trimarchi and Schneiderman 1995a,b). In wild-type (WT), red-eyed flies, however, the GFs are difficult to activate using light-off stimuli and may require multimodal stimulation (Levine 1974; Thomas and Wyman 1984).

In contrast, an approaching physical object—producing an expanding visual image on the retina as well as mechanosensory cues—elicits a well-coordinated escape behavior in WT flies, in which the fly raises its wings before jumping (Hammond and O'Shea 2007a,b), similar to a voluntary flight initiation (Trimarchi and Schneiderman 1995b). Additionally, during this escape behavior the fly makes preparatory leg movements prior to the wing raise (WR) to generate directional flight after take-off (Card and Dickinson 2008b). Because this escape behavior is quite unlike the GF-evoked light-off response in white-eyed flies described earlier, an important question is whether the GFs play any role in its execution as previously assumed (Card and Dickinson 2008a,b; Hammond and O'Shea 2007a).

We addressed this question using a combination of novel behavioral, electrophysiological, and genetic tools. We simulated the visual component of an approaching object on a computer screen—i.e., a looming stimulus (Ball and Troninck 1971; Gibson 1979). Cues provided by computer-generated looming stimuli were sufficient to generate a well-coordinated escape behavior in both WT and white-eyed flies similar to the behaviors reported previously in studies using real approaching objects. We then directly recorded the activity of the GFs in response to the presentation of looming stimuli. Our results show that the GFs are not activated in response to looming stimuli and that another neuronal pathway mediates the associated escape behaviors in fruit flies.

METHODS

Behavioral experiments

All experiments were performed on adult flies 2–3 days old (*Drosophila melanogaster*). We aspirated single flies into an Eppendorf tube that had its tip sliced off, to leave an opening of about 3 mm. The

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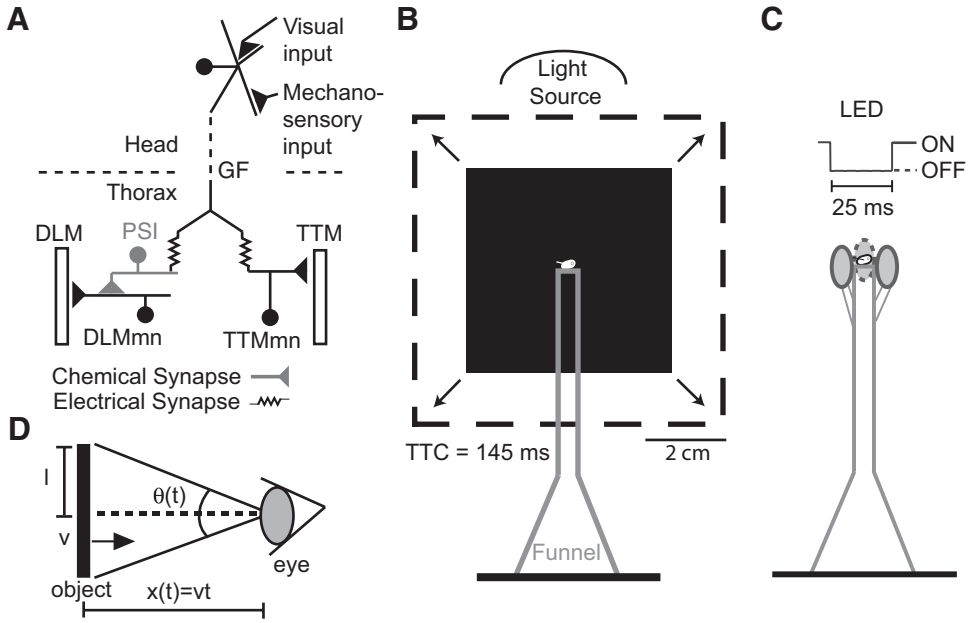

FIG. 1. Experimental arrangement and looming stimuli. *A*: schematic diagram of the giant fiber (GF) system. DLM(mn), dorsolongitudinal muscle (motor neuron); TTM(mn), tergotrochanteral muscle (motor neuron); PSI, peripherally synapsing interneuron. *B*: looming experiments: for these behavioral experiments, flies were placed inside a funnel. Flies then climbed to the top of the funnel opening, which was aligned with the center of the stimulation screen. Once a fly reached the top of the funnel and remained stationary for >3 s, a looming stimulus was presented. The drawing shows the average stimulus size at which flies took off when presented with a looming stimulus with $l/|v| = 60$ ms (see *D*; average time remaining to collision [TTC] = 145 ms; fly length, funnel, and square dimensions drawn to scale). *C*: light-off experiments: for these experiments the flies were placed similarly inside the funnel and once they reached the tip of the funnel and stayed there for ≥3 s, the 4 light-emitting diodes placed on the funnel tip were suddenly turned off for 25 ms. *D*: during monocular stimulation, the time course of the angular size subtended at the eye by a square of half-size l approaching at speed v is a function of the $l/|v|$ ratio: $\theta(t) = 2\tan^{-1}(l/vt)$.

fly was then tapped from the tube through the tip of the funnel (Fig. 1, *B* and *C*). The funnel's tip (top) and its sealed opening (bottom) had diameters of 0.5 and 2 cm, respectively. After exploring the bottom of the funnel for up to a few minutes, the flies climbed along the funnel walls toward a light source placed directly above the setup (negative geotaxis and phototaxis). Once the fly reached the top of the funnel and remained there for ≥3 s, it was presented with either a looming or light-off stimulus. In the looming experiments, the animal had a position parallel (0°) to the video monitor used for visual stimulation in 65% of the trials, resulting in monocular stimulation. In the other 35%, the fly was at an angle with the screen (45–90°) and thus visual stimulation was not monocular. No significant difference was observed in the timing of behavior in these trials and monocular ones. Therefore all trials were pooled in subsequent analyses. The behavior was filmed using a high-speed video camera (see *Video recordings and analysis*). Almost all trials were carried out with different flies. In a few cases the fly could be captured after a trial and was tapped back into the funnel and presented with a second stimulus.

Visual stimulation

LOOMING STIMULI. Dark squares of constant half-size (l) and speed (v), approaching on a collision course, were simulated on a computer screen (looming stimuli). The monitor's luminance was calibrated linearly between black (foreground, 7.6 cd/m^2) and white (background, 87.5 cd/m^2) and had a refresh rate of 200 frames/s, which is well above the cutoff frequency for *Drosophila* photoreceptors (Niven et al. 2003). Since the monitor maintained a white background between trials, the flies were in a light-adapted state in these experiments. For monocular stimulation, the time course of the angular size, $\theta(t)$, subtended by the approaching object on the retina fully characterizes the stimulus and is a function of the half-size to speed ratio ($l/|v|$): $\theta(t) = 2\tan^{-1}[l/(vt)]$ (Fig. 1*D*; $v < 0$ for approaching stimuli, $t < 0$ before collision; Gabbiani et al. 1999). Looming stimuli had $l/|v|$ values between 5 and 80 ms, corresponding to approach sequences with total durations between 0.4 and 6 s, respectively. The physical half-size of the stimulus on the screen at each frame was calculated from $r = pd(l/v)/t$ (in pixels; rounded up to the closest integer). In this equation, d is the distance of the eye to the screen, p is the monitor resolution (16.5 pixels/cm), and t is the frame presentation time relative to projected collision. In practice the average distance between the screen and fly eye, equal to the distance between the center of the funnel tip and the screen, was used instead of d. This distance ranged from 6 to 7.5 cm across different experiments. Since the fly could be positioned anywhere on the perimeter of the funnel tip, the distance between its eye and the screen could vary by as much as 0.25 cm around its average (see *Behavioral experiments*). Initial and final half-sizes of the dark squares were preset to 2 and 220 pixels, subtending on average 2–3° and 120–130° on the retina, respectively. Figure 1*B* shows a drawing of the average size of the stimulus at the time of take-off in looming-evoked escape trials (time remaining to collision [TTC] = 145 ms, $l/|v| = 60$ ms).

To test the effect of overall luminance changes on jump responses, black squares approaching on a white background (overall screen luminance decreasing over time) and black and white checkerboard squares approaching on a gray background were presented (4 × 4 checkerboard; fixed overall screen luminance). Luminances of white, gray, and black were 87.5, 47.4, and 7.6 cd/m^2, respectively. Transistor–transistor logic (TTL) pulses were generated at each frame by the stimulation program and were used to synchronize stimulus presentation with data acquisition. All stimulation programs were written in C using the MGL graphics library (SciTech Soft, Chico, CA; http://www.scitechsoft.com) on a personal computer running the QNX4 operating system (QNX, Ontario, Canada; http://www.qnx.com).

LIGHT-OFF STIMULI. Four bright green light-emitting diodes (LEDs) were mounted around the tip of the funnel from which the fly emerged (Fig. 1*D*) and had an estimated average luminance of 95 cd/m^2. This value was calculated by first measuring the illuminance of the four LEDs (600 lx) and dividing it by the solid angle of a half-sphere (2π).

A TTL pulse was used to trigger a custom circuit that transiently turned off the LEDs for 25 ms, evoking a startle-like jump and flight response. Such jump responses could be evoked only in white-eyed flies (Wyman et al. 1984). Since *Drosophila* show little sensitivity to red light (Heisenberg and Buchner 1977), we used a high-power red LED to produce sufficient light for video recordings. During the light-off experiments, the green and red LEDs were the only light sources in the experiment room. In flies, photoreceptor light adaptation is thought to consist of two phases: a fast phase lasting about 100 ms and a slow phase that completes in about 60 s (Hardie and Raghu 2001; Laughlin and Hardie 1978). At the start of the experiment, the flies were exposed to very little light while they were at the bottom of the funnel for a few minutes, since the green LEDs had a small emission angle (30°). As the flies climbed the funnel over tens of seconds, they experienced increasing amounts of light and the LEDs were turned off about 3–5 s after flies remained stationary on the funnel top. Therefore in our experiments the flies were at least partially light adapted and possibly fully light adapted when they were presented with the light-off stimulus. That the state of light adaptation did not play a major role in the behavioral responses we report is in agreement with the fact that they were indistinguishable from those reported earlier for light-adapted flies (Fayyazuddin et al. 2006; Thomas and Wyman 1984; Trimarchi and Schneiderman 1995). In addition, we further confirmed this fact by carrying out a few experiments in fully light adapted flies.

Video recordings and analysis

A high-speed digital video camera (IMPERX, IPX-VGA210), equipped with a zoom lens (LIMZ50M, Kowa), was used to record escape jumps. The video recordings were obtained at 400 frames per second (fps). During looming experiments, we used a broad-spectrum light source (red-eyed flies) or a red LED (white-eyed flies) to illuminate the scene. The first video frame was synchronized with the start of the stimulus and the camera was operated in free-running mode from then on. The video recordings were analyzed frame by frame off-line. The timing of WR was visually identified as the first frame of wing movement. The timing of take-off (TO) was defined as the moment when the fly legs left the funnel (Supplemental Fig. S1).[1]

We investigated the WR in more detail in 50 trials obtained from 50 WT flies in response to looming stimuli with various $l/|v|$ values (10, 40, 60, 70 ms). We categorized these trials into five groups, based on the extent of the WR. Group 1 consisted of trials in which the flies fully raised the wings prior to take-off; group 2 flies did not completely raise their wings, but they did so more than halfway to full extension; group 3 flies extended their wings approximately halfway; group 4 raised their wings less than halfway; and finally, group 5 consisted of flies that did not raise their wings before take-off.

Flies and genetics

To assess the potential impact of GF activation on the flies' responses to looming stimuli, we used a mutant in which GF responses to visual inputs are severely disrupted. The Dα7[PΔEY6] is a null allele of the Dα7 nicotinic acetylcholine receptor that lacks most of the ligand-binding domain generated by imprecise excision of a P-element, EY10801. The Dα7 receptor is abundantly expressed on the ventral lateral dendrites (VLDs) of the GF where the GF receives its visual inputs (Fayyazuddin et al. 2006; Strausfeld and Bassemir 1983). Thus a disruption of Dα7 synaptic receptors will inevitably disrupt a large fraction of the visual input to the GF. In addition, electrical stimulation of the eye fails to activate the GF in *Dα7* mutants, whereas it activates optic lobe neurons presynaptic to the GF and subsequently the GF at latencies <5 ms in WT animals (Fig. 7 of Fayyazuddin et al. 2006). This suggests that the visual inputs to the GF are largely, if not completely, disrupted in *Dα7* mutants.

In addition, white-eyed flies (*bw*; *st*) were used to assess light-off and looming-evoked behaviors and for electrophysiological experiments to further assess the responses of the GF to these stimuli.

Electrophysiology

To obtain extracellular recordings from the neck connective (Hengstenberg 1973), flies were anesthetized by brief cooling and fixed ventral side up to a Perspex holder using vacuum grease. The legs were then cut off. We exposed the neck connective and thoracic ganglion by removing the preepisternum of the pro- and mesothoracic segments using a razor scalpel. Ringer solution was used to bathe the thoracic ganglion and was replenished as needed to keep the tissue moist (in mM: 140 NaCl, 5 KCl, 5 $MgCl_2$, 5 $CaCl_2$, 3 $NaHCo_3$, and 6.3 HEPES; pH 7.0). The fly was placed parallel to a computer monitor so that its right eye had a clear view of the screen. Signals from the neck connective were amplified differentially between a reference electrode placed in the thoracic ganglion and a recording electrode in the neck connective. A ground electrode was placed in the abdomen. All electrodes were made of sharpened tungsten insulated to the tip with nail polish.

Extracellular recordings from flight muscles were obtained after anesthetizing the flies by brief cooling, removing their legs, and fixing them dorsal side up on a Perspex holder using beeswax. The holder was mounted parallel to the computer monitor so that the fly's left eye faced the screen. Stimulating electrodes were placed in the eyes and recording electrodes were placed in the left dorsolongitudinal muscle (DLM) 45a and right tergotrochanteral muscle (TTM) according to the map of Levine and Hughes (1973). The flies were first visually stimulated by looming stimuli and the response of the TTM and DLM muscles was subsequently confirmed by electrical stimulation of the GF through the eyes using 200-μs current pulses of 0.75 mA.

Statistical analysis

The Kruskal–Wallis test (KWT) was used to compare the medians of populations across different treatments. The significance level (P values) presented on the figures and in the results are derived from the KWT (P_{KWT}). When no significant difference was detected, we report average values pooled across treatments. Least-squares linear regression was used to fit lines to the timing of TO and WR as a function of $l/|v|$. The variability of linear fit slopes, intercepts, and the angular thresholds was quantified by SEs as described in Moore and McCabe (2006). Variability in the data is otherwise quantified by SD. To compare the slopes and intercepts of the linear fits, an ANCOVA was used (Fotowat and Gabbiani 2007). The corresponding P values are denoted by P_{ANO} (with SD). The Pearson correlation coefficient is denoted by ρ throughout.

RESULTS

Looming stimuli evoke reliable jump and flight after reaching a fixed angular threshold size

An approaching object, such as a predator on a collision course, produces a shadow on the retina that expands as a function of the size and approach speed of the object. In *Drosophila*, jump escape behaviors in response to simulated objects approaching on a collision course have not yet been studied systematically. For this purpose, we designed a behavioral setup similar to that used in a recent quantitative study of locust jump escape behaviors (Fotowat and Gabbiani 2007). The animals were introduced into an inverted funnel positioned close to the stimulation monitor. After the animals had climbed

[1] The online version of this article contains supplemental data.

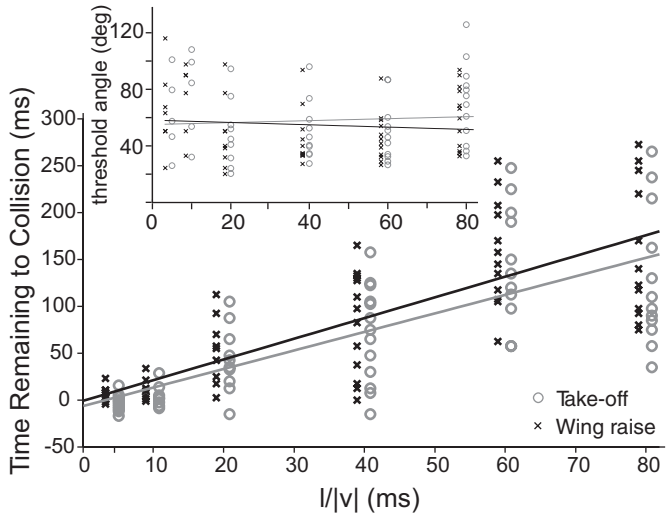

FIG. 2. Fly jump escape behavior in relation to stimulus l/|v|. Timing of wing raise (WR, black × symbols) and take-off (TO, gray circles) as a function of l/|v|. Both timings were positively correlated with stimulus l/|v| (ρ_{WR} = 0.78, ρ_{TO} = 0.73). WR slope = 2.2 (SD = 0.2) and intercept = 0 ms (SD = 10 ms). TO slope = 2.0 (SD = 0.2) and intercept = −6 ms (SD = 11 ms). *Inset* shows the value of the stimulus angular size at the time of WR (black × symbols) and 6 ms before TO (gray circles). The correlation coefficient with l/|v| was −0.09 and 0.07 for WR and TO, respectively, which were not significantly different from zero ($P > 0.5$). See Supplemental Fig. S1 and Movie S1 for a specific example of the data used to generate this figure.

and exited from the tip of the funnel (Fig. 1*B*), they were presented with a two-dimensional expanding dark square simulating an object approaching at constant speed on a collision course with the animal (i.e., a looming stimulus; Ball and Tronick 1971). Although the approach of a square is characterized by two of its physical parameters, the half-size (l) and approach speed (v < 0 for approach), only the ratio of the half-size to the absolute value of the speed, l/|v|, determines the angle θ(t), subtended by the object at the retina, and thus the stimulus experienced by the fly (Fig. 1*D*; Gabbiani et al. 1999). Using this new behavioral assay, we could systematically vary the parameters of the looming stimulus and measure their effect on behavior, which could not be accomplished previously in studies that used real approaching objects (Card and Dickinson 2008a,b; Hammond and O'Shea 2007a,b). Looming stimuli were highly effective at eliciting escape behaviors in WT *Drosophila* regardless of their expansion rate in the tested l/|v| range, producing jump and flight in 82% of the trials. The escape sequence was almost always well coordinated, consisting of an initial WR, followed by a jump triggering take-off and flight (Supplemental Fig. S1; also see movie in Supplementary Material). More specifically, in a subset of 50 trials analyzed in detail, wings were fully raised in 68% of the trials, more than halfway raised in 8%, about halfway raised in 10%, less than halfway raised in 12%, and not raised in only 2% of the trials (groups 1–5; n = 34, 4, 5, 6, and 1, respectively). We found that faster expanding stimuli (i.e., stimuli with smaller l/|v| values) evoked WR and TO closer to the expected collision time (Fig. 2). In addition, both the timing of WR and TO were strongly correlated with l/|v| (ρ_{WR} = 0.78, ρ_{TO} = 0.73). We thus linearly fitted the WR and TO time as a function of l/|v| (Fig. 2*B*; R^2 = 0.6 and 0.5, respectively). The two straight lines obtained in this way were nearly parallel because their slopes were not statistically different (means = 2.2 and 2.0; SDs = 0.2 and 0.2; ANCOVA, $P_{ANO} > 0.05$). This implies that the time interval between WR and TO is constant. Accordingly, the delay between these two events did not significantly change with l/|v| and had a mean value of 11 ms (SD = 11 ms; median = 7.5 ms; Kruskal–Wallis test, P_{KWT} = 0.5). In principle, several distinct stimulus variables could be associated with the behavior (Fotowat and Gabbiani 2007; Sun and Frost 1998). However, it has been shown that linear relationships such as those documented here imply that both WR and TO occur on average at a fixed delay from the moment when the stimulus reaches a fixed angular size on the retina, independent of l/|v|. As explained in detail in earlier work, a reliable method to estimate this average threshold angular size and the corresponding delay is based on computing the slopes and intercepts of the linear fits (Fotowat and Gabbiani 2007; Gabbiani et al. 1999). Specifically, the delay is equal to the intercept and the slope is related to the angular threshold angle through the equation $\theta_{th} = 2 \tan^{-1}$ (1/slope). Thus we find that on average the WR occurred when the stimulus had reached 49° (SD = 4°) on the retina and TO occurred 6 ms (SD = 11 ms) after the angular size reached 54° (SD = 5°). In agreement with this, the correlation between angular sizes at these delays and l/|v| was not significantly different from zero (Fig. 2, *inset*). Although we cannot estimate the variability of the threshold angles and delays across animals since each fly was used only once, earlier estimates based on repeated trials in locusts and pigeons suggest a substantial amount of interanimal variability (Gabbiani et al. 1999; Sun and Frost 1998).

Looming is sufficient to trigger escape

The neural pathway thought to trigger visually guided escape behaviors in higher Diptera is the GF pathway. In white-eyed mutants, this pathway is readily activated by a sudden

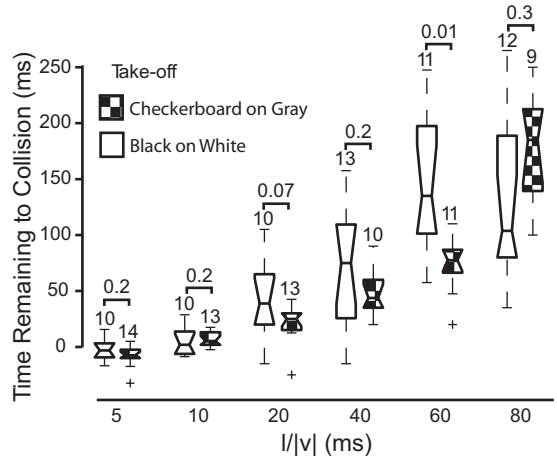

FIG. 3. Effect of an overall luminance decrease on looming evoked escapes. Timing of TO relative to the expected collision time in wild-type (WT) flies in response to black squares looming on a white background (white symbols) and checkerboard black and white squares looming on a gray background (checkerboard symbols) at different l/|v| values. For both stimuli, TO occurred earlier relative to collision for larger l/|v| values. The notched box plots show the lower quartile, the median, and the upper quartile values. The whiskers (i.e., the lines extending from the end of the boxes) show the extent of the rest of the data. Outliers are shown with a +. The number of trials (n) is given immediately above each notched box and Kruskal–Wallis test P values (p_{KWT}) are shown above the square brackets indicating the compared values.

decrease in luminance (light-off stimuli). Since expanding black squares produce a large decrease in luminance, particularly toward the end of the approach, we reasoned that this component of the stimulus might trigger the GF and elicit the escape behavior. To test whether flies jump in response to a decrease in luminance or whether the looming component of the stimulus is sufficient to trigger the escape response, we presented flies with checkerboard squares looming on a gray background. For these stimuli the overall screen luminance is constant during approach (see METHODS, *Visual stimulation*). Surprisingly, flies raised their wings and took off just as reliably in response to checkerboard stimuli as black ones, with 87% of successful trials. Therefore looming alone is sufficient to trigger escape responses and a decrease in luminance had no impact on escape rate. Figure 3 shows the timing of TO for WT flies in response to black on white (BW; white symbols, replotted from Fig. 2) and checkerboard on gray stimuli (CB; checkerboard symbols). The timing of WR in response to BW and CB stimuli was similar (Supplemental Fig. S2). Both timings were slightly less variable and better correlated with $l/|v|$ for the CB stimuli ($\rho_{WR} = \rho_{TO} = 0.9$). Except for one $l/|v|$ value (60 ms) no significant difference was observed between the timing of behavior in response to BW and CB stimuli. With respect to the BW stimuli, the slopes and intercepts of the linear fits of WR and TO in response to CB stimuli were not significantly different (WR slope = 2.1, SD = 0.1; intercept = −14 ms, SD = 6 ms; TO slope = 2.1, SD = 0.1; intercept = −22 ms, SD = 5 ms; $P_{ANO} > 0.05$). Moreover, the slopes and intercepts of WR and TO in response to BW and CB stimuli were not significantly different ($P_{ANO} > 0.05$). On a trial-by-trial basis, the timing of WR was highly correlated with that of TO ($\rho_{BW} = 0.7$, $\rho_{CB} = 0.8$). The delay (D) between WR and TO did not significantly change with $l/|v|$ ($P_{KWT} = 0.65$), but was significantly shorter ($P_{KWT} = 0.006$) and less variable for the CB compared with BW stimuli (D_{BW} = 11 ms, SD = 11 ms; D_{CB} = 8 ms, SD = 5 ms). We thus conclude that looming is a powerful visual cue, sufficient to control the escape behavior.

Light-off and looming stimuli evoke distinct behaviors in white-eyed flies

Both looming stimuli and abrupt changes in luminance trigger escape responses in *Drosophila*. Since the characteristics of these two types of escape behaviors, such as escape probabilities and reaction times, had not yet been compared quantitatively, we carried out experiments that addressed these points. As expected, a light-off stimulus did not evoke an escape in red-eyed flies (10 flies; see also Wyman et al. 1984). Therefore we used white-eyed flies to compare escape responses to light off and looming. The looming experiments were carried out in the same manner as described in the previous sections. For the light-off experiments, the animals were introduced into an inverted funnel whose tip was illuminated by four bright green LEDs (Fig. 1*C*). After the animals climbed and exited from the tip of the funnel, the LEDs were suddenly turned off for 25 ms and the video recording was

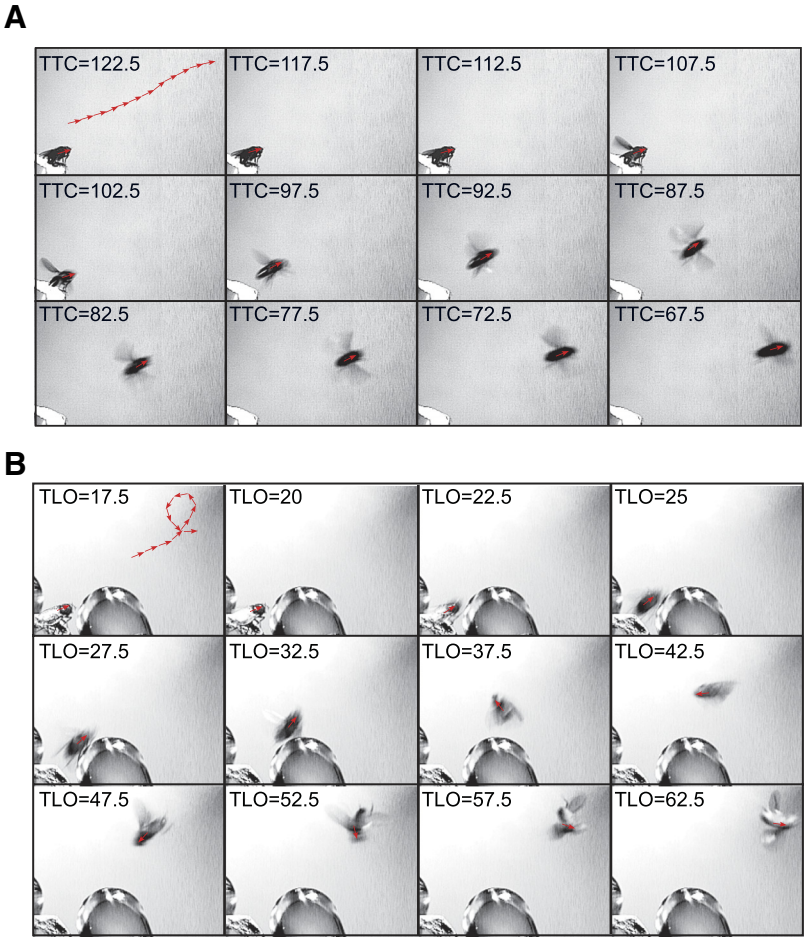

FIG. 4. Comparison of looming and light off evoked escapes in white-eyed flies. *A* and *B*: example frames from the escape response to looming ($l/|v|$ = 40 ms) and light-off stimuli, respectively, in 2 different white-eyed flies. Jump and flight trajectories are marked with red arrows. *Inset* in the first frame of *A* and *B* shows the complete escape trajectory obtained from all depicted frames. TLO, time after light off (ms); TTC, time remaining to collision (ms).

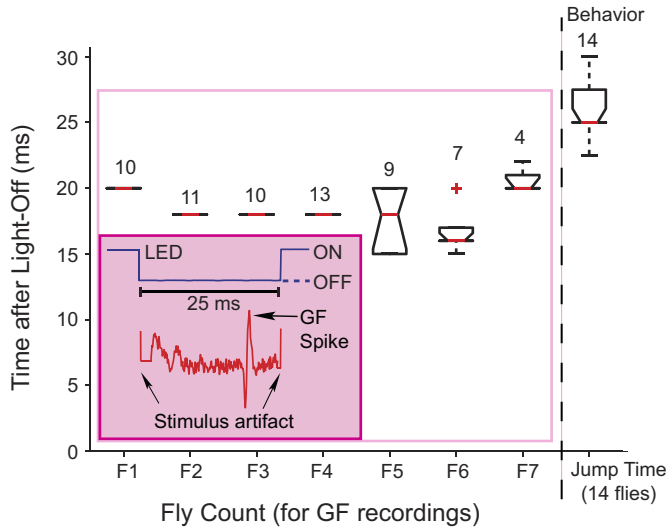

FIG. 5. Timing of the light-off–evoked GF spike and its relation to the timing of jump. In white-eyed flies a single spike is triggered in the GF in response to a light-off stimulus (*inset*: stimulus artifact has been partially removed for clarity). The timing of the GF spike was usually very reliable, as illustrated for 7 flies (F1–F7; timing is relative to light off). The number of trials for each fly is shown on the plot. In flies F1–F4 the GF spike occurred at the same time in all trials; thus there was no error in timing. The red cross depicts an outlier data point for the corresponding fly. The escape jump evoked by light-off stimuli occurred within a few milliseconds of the GF spike (Behavior, one trial per fly in 14 flies).

triggered. Flies responded to both light off and looming by jump and flight with similar probabilities (Pr) ($Pr_{light\text{-}off}$ = 45%, Pr_{loom} = 45%). In these experiments, TO occurred on average 25 ms (SD = 2 ms) after the light-off stimulus (Fig. 5; Behavior) and 22 ms (SD = 21 ms) after the stimulus reached a threshold of 47° (SD = 7.5°) in response to looming (Supplemental Fig. S3, red circles). Therefore the average reaction times following the cues were similar in response to light-off and looming stimuli, although the onset of the looming-evoked response was more variable. On the other hand, there was a clear difference between the two behaviors in the evoked flight trajectory and the duration of WR prior to TO. In response to looming stimuli, flies jumped and flew in a controlled directional manner (Fig. 4*A*), whereas light off evoked a jump followed by tumbling, resulting in a flight trajectory similar to an open spiral (Fig. 4*B*; Supplemental Videos S2 and S3). Only in half of the trials did the flies slightly raise their wings before take-off in response to light off. In those trials, TO occurred on average 5 ms after WR (SD = 0.9 ms). In response to looming on the other hand, in all trials, the wings were raised on average 15 ms (SD = 12 ms) before TO. Looming and light off therefore evoke different behavioral sequences before and after TO, suggesting that they are mediated via separate pathways.

GF responds to light-off and not looming stimuli

To find whether light-off and looming-evoked escapes are mediated by the GF or an alternate sensory pathway, we recorded extracellularly the activity evoked in the neck connective, which contains among others, the axons of the GFs. Recordings from the nerve cord of white-eyed flies consistently revealed the occurrence of a single spike on average 18 ms (SD = 1.5 ms) after the lights went off (seven flies, Fig. 5). The timing of this single spike is in agreement with previous reports of the GF spike recorded intracellularly in response to light off in white-eyed flies (Thomas and Wyman 1984), as well as the timing of the evoked jump (Fig. 5). We therefore conclude that it belongs to the GF.

The GF responded consistently to light off in all seven white-eyed flies, but not to looming stimuli (Fig. 6). In WT flies, the GF did not spike in response to light off, consistent with their lack of behavioral response to the same stimuli. To confirm that the GF does not respond to looming stimuli, we also used an alternate strategy for measuring its activity. It is known that a single spike in the GF results in activation of the TTM and DLM muscles through their respective motor neurons in a precise sequence determined by the synaptic connectivity of these elements. The TTM is activated first, followed by the DLM <1 ms later (Tanouye and Wyman 1980). We thus used the evoked pattern of activation of TTM and DLM muscles as a proxy for GF activation (Trimarchi and Schneiderman 1995c). We recorded extracellular potentials from the TTM and DLM muscles while presenting looming stimuli in 13 WT flies. Eleven of 13 flies did not show activation of the TTM and DLM in response to these stimuli but we could detect activity in the TTM or DLM in the remaining 2 flies. One of these flies responded to the stimulus with a single spike in both the TTM and DLM muscles in four of six trials (Fig. 7*A*). In

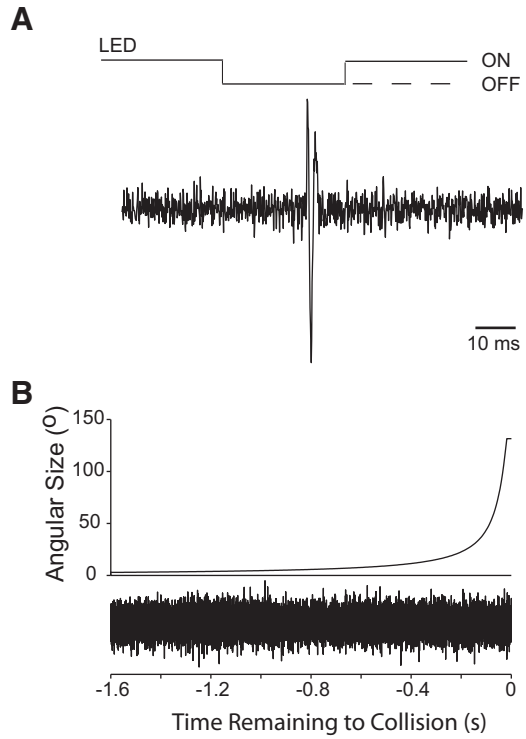

FIG. 6. The GF responded to light-off and not looming stimuli in white-eyed flies. Example of a GF spike in response to light-off (*A*) and looming (*B*) stimuli. In this fly, the GF responded to light off in all 11 trials but did not respond to looming when presented for 11 trials. In 4 of the 7 tested flies, no looming response could be detected despite consistent light-off responses. In 2 of the remaining 3 flies, looming response could be detected only after the electrode was moved to a new position on the nerve cord. In one fly we could detect the GF spike in response to light-off and the non-GF spikes in response to looming, although the GF spike amplitude was larger than that of the non-GF spikes.

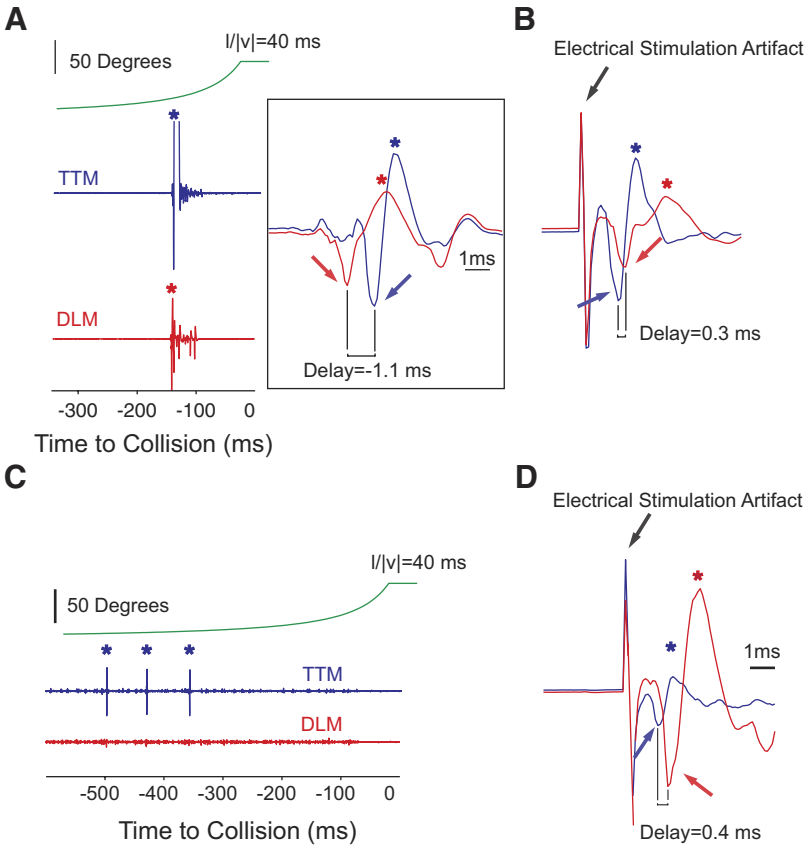

FIG. 7. Looming-evoked activity in the TTM and DLM muscles. *A*: activity evoked by a looming stimulus in the TTM (*blue trace*) and DLM (*red trace*) muscles in one animal. The TTM and DLM spikes (asterisks) were identified based on their amplitudes by comparison with the activity evoked by electrical stimulation of the eye (see *B*; spikes in *A* and *B* are shown with identical vertical scale). The box shows the same spikes on an expanded timescale (1-ms scale bar applies to *B* as well). The looming stimulus evoked a spike in the DLM 1 ms before the TTM. *B*: electrical stimulation evoked a spike in the TTM 0.3 ms before the DLM. *C*: looming-evoked activity in another fly. Three spikes were evoked in the TTM but none in the DLM. *D*: activity evoked in the TTM and DLM by electrical stimulation in the same animal as shown in *C*. In *A*, *B*, and *D* delays were measured from the start of potential rise (indicated by black vertical lines).

these four trials, the looming-evoked spike in the TTM followed that in the DLM with a delay of 1 ms, which is the opposite of the pattern expected from GF activation of these muscles. In the second fly, we recorded spikes in only one of six trials and these were limited to the TTM, with the DLM not responding at all (Fig. 7*C*). At the end of each experiment, we activated the GF by electrical stimulation through the eyes to record the shape, size, and timing of the TTM and DLM muscle potentials and thus confirm the identity of those recorded during visual stimulation. Under these conditions, all 13 flies invariably showed TTM and DLM spikes in the temporal sequence characteristic of GF activation, with the TTM spike occurring on average 0.24 ms before the DLM spike (SD = 0.05 ms; Fig. 7, *B* and *D*). This confirmed that even when TTM and DLM spikes were evoked by looming stimuli, they did not match the pattern expected of GF activation. Taken together, these results show that the GF is not activated by looming stimuli, indicating that other units in the neck connective must convey looming-evoked activity to motor centers.

Looming stimuli evoke activity in a novel descending pathway correlated with behavior

Next, we recorded neural activity in response to looming stimuli using three $l/|v|$ values in WT flies (10, 40, and 70 ms, five flies per stimulus $l/|v|$). In all trials, activity in the connective increased and was always clearly distinguishable on the audio monitor connected to the extracellular amplifier. In the majority of trials, we could reliably isolate spikes with amplitudes larger than the baseline (Fig. 8*A*; percentages of such trials were 90, 50, and 70 for $l/|v| = 10$, 40, and 70 ms, respectively). Examples of spike rasters obtained by thresholding nerve cord activity according to this criterion are shown in Fig. 8*A*. We used these spike rasters to calculate the instantaneous firing rate for each trial. In each case, the firing rate increased during the approach and then decayed after reaching a maximum. The average instantaneous firing rate across these animals and trials is shown in Fig. 8*A* (solid black lines). We observed that the peak firing occurred earlier relative to collision for larger $l/|v|$ values. The peak time for each trial in each animal is plotted in Fig. 8*B* along with the TO times obtained from behavioral experiments (replotted from Fig. 2). Remarkably, the timing of the peak firing rate showed a positive correlation with stimulus $l/|v|$ ($\rho_{peak} = 0.6$) entirely analogous to that of TO. This result suggests that the timing of the peak, or a threshold in the firing rate, determines the timing of behavior. Moreover, the slopes and intercepts of the linear fits to the timings of peak firing and take-off were not significantly different, suggesting that the peak occurs around take-off (slope = 1.35, SD = 0.18; intercept = −14 ms, SD = 8 ms, $P_{ANO} > 0.05$). Consistent with this, we found no significant difference between the median peak and TO times (peak times for $l/|v| = 10$ and 40 ms: 7 and −37 ms; TO times: −2 and −75 ms, $P_{KWT} = 0.1$ and 0.4, respectively). Thus the activity evoked in the nerve cord corresponds to one or more neurons different from the GF likely involved in mediating looming-evoked escape responses.

To test whether these neurons make electrical connections with the TTM motorneuron like the GFs do, we attempted to fill them transynaptically through gap junctions from the TTM

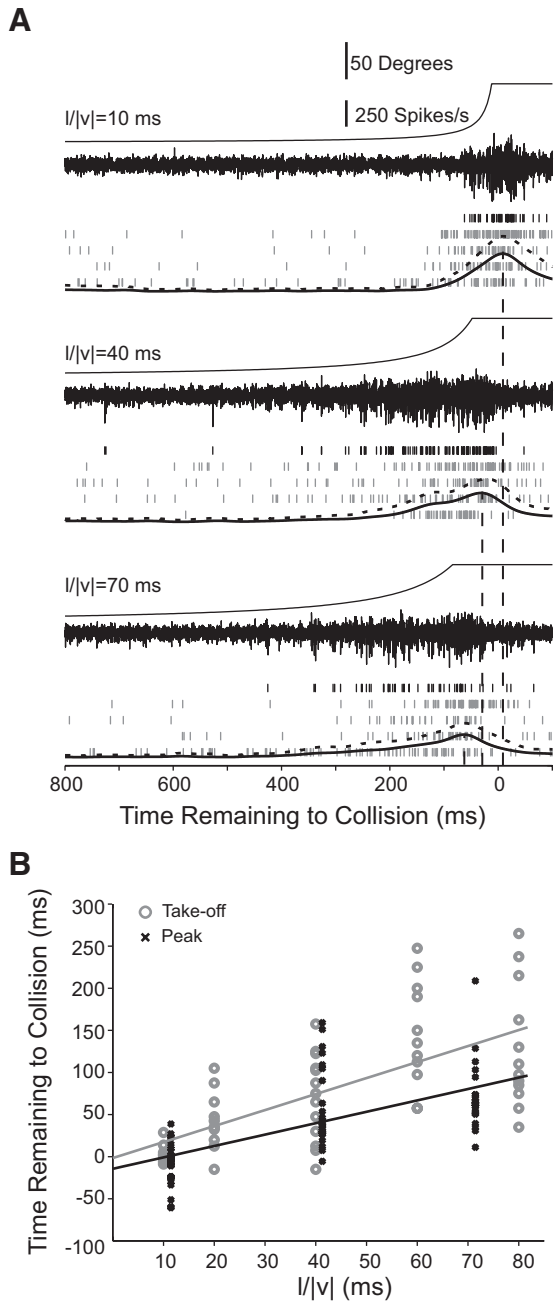

FIG. 8. Looming-evoked activity in the nerve cord of WT flies. *A*: activity evoked in the nerve cord in response to looming stimuli with $l/|v| = 10$, 40, and 70 ms. Representative examples of extracellular recordings obtained from one animal are shown for each speed. The looming-evoked activity was detected by thresholding the spikes above baseline (activity level before stimulus onset). The black raster plots show the spikes detected in this way from the example traces. The other 4 rasters (gray) correspond to spikes detected from representative trials taken from the other 4 tested flies. The solid (dashed) *black traces* superimposed on rasters show the average (+SD) instantaneous firing rates across these trials for each $l/|v|$ value. The timing of the peak for each $l/|v|$ value is mapped to the time axis via the dashed lines. The peak occurs earlier relative to collision for larger $l/|v|$ values. *B*: timing of the peak for each trial/animal (black asterisks) and that of the TO (gray circles, replotted from Fig. 2, obtained in a set of flies different from those used for electrophysiology).

using Neurobiotin injections (Fayyazuddin and Dickinson 1996; Trimarchi and Murphey 1997). However, the only descending neuron that was filled from the TTM in these experiments was the GF (data not shown). It is therefore likely that descending neurons belonging to the looming-sensitive pathway make chemical synapses with the TTM motorneuron or indirect connections through local interneurons.

Is the GF necessary for looming evoked jumps?

The results of the previous sections indicate that the GF is not required for generating escape responses to looming stimuli. To further corroborate this finding, we used null mutants for the Dα7 nicotinic acetylcholine receptor that have a significantly lower jump probability in response to light-off stimuli (Fig. 2*B* of Fayyazuddin et al. 2006). The mutation in this receptor disrupts the visual sensory input to the GFs as well as the synapse between peripherally synapsing interneurons (PSI) and DLM neurons (Fig. 1*A*), resulting in both sensory and motor defects in the GF pathway (Fayyazuddin et al. 2006; METHODS). We tested the *Dα7* mutants with BW and CB looming stimuli. In contrast to previous results with light-off stimuli, the mutants jumped with a probability similar to that of WT flies in response to looming stimuli ($Pr_{BW,D\alpha7} = 81\%$, $Pr_{CB,D\alpha7} = 89\%$ vs. $Pr_{BW,WT} = 82\%$, $Pr_{CB,WT} = 87\%$). This result is consistent with the electrophysiological data in white-eyed flies, showing that the GF does not respond to looming (Fig. 6*B*). Therefore the disruption of the GF pathway in *Dα7* mutants affects the light-off evoked, but not the looming-evoked escape behaviors. Figure 9, *A* and *B* shows the timing of TO for WT and mutant flies in response to BW and CB stimuli, respectively. The overall results were very similar in both types of flies, with slight differences at some speeds (see Supplemental RESULTS). The timing of their WR and TO were again highly correlated with stimulus $l/|v|$ (BW: $\rho_{WR} = 0.7$, $\rho_{TO} = 0.8$; CB: $\rho_{WR} = 0.6$, $\rho_{TO} = 0.8$). Similar to the WT flies, the slopes and intercepts of the linear fits to the timing of WR and TO were not significantly different for the BW and CB stimuli in mutants ($P_{ANO} > 0.05$). Moreover, recordings from the nerve cord of the *Dα7* flies revealed a looming response distinct from the GF and similar to that found in the WT flies (Fig. 10, *A* and *B*, seven flies). These results provide strong evidence that the GF is not necessary for looming-evoked escapes and support the idea that the alternate pathway, which is present in both WT and *Dα7* flies, plays an important role in generating those behaviors.

DISCUSSION

We used a new behavioral assay in combination with genetic and electrophysiological methods to analyze visually mediated escape responses in *Drosophila*. Our results show that the animals react to looming stimuli by raising their wings before jumping to take off. These behavioral stages occur at a fixed delay after the looming stimulus reaches a fixed angular threshold size, independent of stimulus parameters. In addition, we ruled out a significant role of the GF pathway in this behavior using two independent pieces of evidence: *1*) looming stimuli fail to activate the GF, as well as the jump and flight muscles in the stereotyped sequence that is a hallmark of GF activation; and *2*) mutant flies in which visual activation of the GF and the GF-evoked light-off escape is severely disrupted (Fayyazuddin et al. 2006) still escape in response to looming stimuli. We propose that one or more descending neurons different from

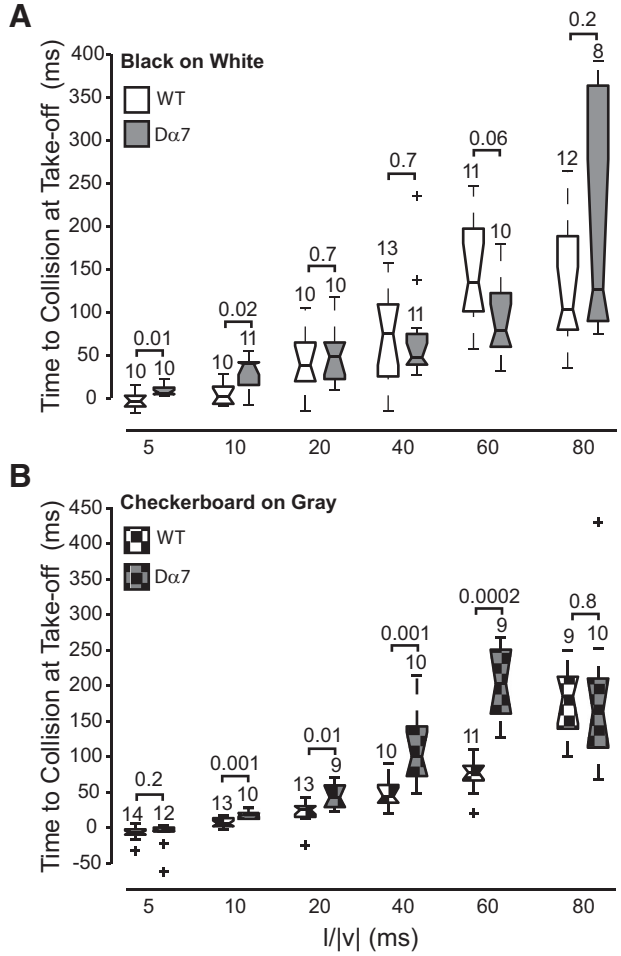

FIG. 9. Effect of *Dα7* mutation on jump timing. *A*: timing of TO in response to black squares looming on a white background in WT (white symbols) and *Dα7* mutant flies (gray symbols). Timings of responses at each l/|v| value were not significantly different except at l/|v| = 5 and 10 ms, where mutant flies jumped significantly earlier than WT flies. *B*: timing of TO in response to checkerboard squares looming on a gray background for WT (black and white checkerboard symbols) and *Dα7* mutant flies (black and gray checkerboard symbols). Jumps occurred significantly earlier for mutant flies in 4 of 6 stimulus l/|v| values (10, 20, 40, and 60 ms).

the GF mediate the behavior because electrophysiological recordings in vivo from the neck connective revealed activity whose peak firing rate was tightly correlated with the timing of the escape jump in both WT and mutants with a disrupted GF pathway.

In *Drosophila*, the canonical pathway mediating visual escape behaviors has been postulated to be the GF system (Allen et al. 2006; Wyman et al. 1984). However, in WT animals the GF pathway is rarely activated by visual stimulation alone, in contrast to white-eyed mutants in which this can be readily achieved using abrupt light-off stimuli (Levine 1974; Nakashima-Tanaka and Matsubara 1979; Thomas and Wyman 1984). Our analysis of neural responses to looming stimuli reveals that, although they are powerful at generating escape behaviors, by themselves they may only rarely, if ever, activate the GF. Instead, multimodal visual and mechanosensory stimuli are likely required to reliably activate the GF in WT animals (Levine 1974; Milde and Strausfeld 1990). This conclusion is consistent with the anatomy of the GF since it receives visual input on one prominent dendrite and mechanosensory input on another (Bacon and Strausfeld 1986; Strausfeld and Bassemir 1983). It is possible that the looming-sensitive activity we recorded in the *Drosophila* cervical connective corresponds to

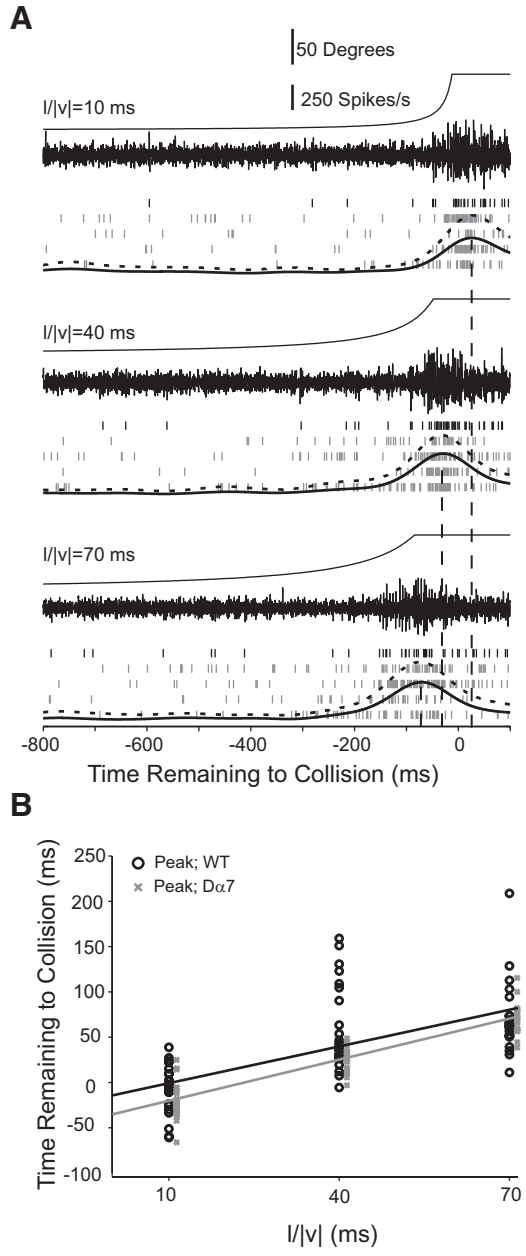

FIG. 10. Looming-evoked activity in the nerve cord of *Dα7* mutant flies. *A*: activity evoked in the nerve cord in response to looming stimuli with l/|v| = 10, 40, and 70 ms. Representative examples of extracellular recordings obtained from one animal are shown for each speed. The raster plots show the spikes detected from the example traces. The other 4 rasters (gray) correspond to spikes detected from representative trials taken from the other 4 tested flies. The solid (dashed) *black traces* show the average (+SD) instantaneous firing rates across all trials and flies for each l/|v| value. The timing of the peak for each l/|v| value is mapped to the time axis via the dashed lines. The peak occurs earlier relative to collision for larger l/|v| values. *B*: comparison of the timing of peak firing rate of the non-GF pathway in WT (black circles) and *Dα7* mutant flies (gray × symbols). The high positive correlation with stimulus l/|v| is conserved in the mutants ($\rho_{\text{peak-WT}} = 0.6$, $\rho_{\text{peak-D}\alpha7} = 0.9$). Slopes and intercepts of the linear fits were as follows. For WT: slope = 1.3 (SD = 0.2), intercept = −14 ms (SD = 8 ms); for *Dα7* mutants: slope = 1.5 (SD = 0.08), intercept = −35 ms (SD = 4 ms).

one or more descending neurons belonging to a homologous cluster. Similarly, in blowflies, looming-evoked escape behaviors do not appear to rely on the GF (Holmqvist 1994; Holmqvist and Srinivasan 1991) and the GFs belong to a cluster of descending neurons that receive visual and mechanosensory inputs and send their axonal terminals to the motor centers (Milde and Strausfeld 1990). Moreover, comparative anatomical studies suggest that a non-GF pathway might be the primary circuit mediating escape in Diptera, since the GF pathway is absent in primitive flies (Jablonski and Strausfeld 2001). Our experiments are the first to quantitatively investigate the visual cues that trigger escape responses to looming stimuli in Diptera and conclusively demonstrate the presence of a looming-sensitive descending pathway distinct from the GF in *Drosophila*. Whether escape in other flies is mediated by homologous descending pathways activated by angular threshold size remains to be seen.

Classically, escape behaviors associated with GF activation have been characterized as consisting of a jump without a preceding WR, as opposed to voluntary flight initiation, which consists of an initial raising of the wings followed by a jump (Trimarchi and Schneiderman 1995a,b). We observed a slight WR in response to light off in some trials as well, but the escape response to looming stimuli always consisted of a WR preceding the jump, with a full WR in half of the trials. Moreover, the flight trajectory after take-off in response to light-off stimuli consisted of nondirectional tumbling in the air, whereas looming stimuli evoked a coordinated, planned escape sequence once a threshold angular size was reached by the stimulus. Our measurements of the reaction times in response to looming and light off show that escape responses to both stimuli occur at similar delays after the triggering cue (on average 25 ms after light off and 22 ms after the looming stimulus reaches a threshold angular size of 47°). Moreover, since the novel neural pathway we describe here is active throughout the expansion of a looming stimulus, it could contribute to different stages of the escape sequence in response to approaching objects (Card and Dickinson 2008b; Hammond and O'Shea 2007a).

Interestingly, this pathway shares many common features with looming-activated escape pathways recently documented in other species. The timing of the peak activity in response to looming stimuli recorded from the nerve cord and its dependence on $l/|v|$ are similar to those documented in the LGMD/DCMD neurons of locusts (Gabbiani et al. 1999; Matheson et al. 2004), neurons of the optic tectum and nucleus rotundus of pigeons and frogs, and the Mauthner cell of gold fish (Kang and Nakagawa 2006; Preuss et al. 2006; Sun and Frost 1998; Wu et al. 2005). Remarkably, in frogs and locusts take-off also occurs at a fixed delay after the looming stimulus reaches a fixed angular threshold size (Fotowat and Gabbiani 2007; Yamamoto et al. 2003).

Since *Drosphila* has retained through evolution two distinct pathways that can mediate escape behaviors to purely visual and combined visual/mechanosensory stimuli, we may speculate on their functional role in relation to natural stimuli. Typically, the onset time of the mechanical air disturbance associated with an approaching object will depend on its texture, size, and speed. A large object may, for example, generate a detectable disturbance only after it is too late to escape. In such a situation, a pathway devoted to processing exclusively looming information, such as the new pathway described here, would be advantageous to generate well-coordinated escape responses. Retaining a second pathway activated by combined visual/mechanosensory cues could prove advantageous by being more broadly tuned to visual stimuli, since the generation of escape responses would still be gated by conjunctive mechanosensory inputs to minimize inappropriate activation.

Escape behaviors can be divided into two broad categories: those that are driven by "command" neurons (Kupfermann and Weiss 1978, 2001) and those that are not. A single spike in a command neuron can activate an entire escape program, as exemplified by the giant fibers mediating the tail-flip of crayfish (Edwards 1999; Krasne and Wine 1984), the Mauthner cell–mediated C-start of teleost fish (Korn and Faber 2005), or the GF-mediated escape of *Drosophila* (Wyman et al. 1984). Escape behaviors that appear not to be command neuron mediated—such as those associated with the LGMD/DCMD in locusts (Fotowat and Gabbiani 2007) or the nongiant escape reactions of crayfish (Kramer and Krasne 1984; Wine and Krasne 1972) and zebrafish (Kohashi and Oda 2008)—are typically less stereotyped, exhibit increased complexity and directionality, and occur with longer and more variable latencies. Such behaviors are associated with more persistent, time-varying activity of descending neurons (Fotowat and Gabbiani 2007; Kramer and Krasne 1984; Oliva et al. 2007). The escape pathway we demonstrate in *Drosophila* in response to looming stimuli appears to be of the latter sort, whereas the GF may be the pathway of last resort, usually activated to generate the fastest escape behaviors in response to abrupt stimuli and only when both visual and mechanosensory cues signal imminent collision. Thus *Drosophila* is endowed with at least two parallel neural pathways for escape in response to visually threatening stimuli and offers a genetic model to study how such pathways interact to generate escape behaviors of varying degrees of complexity.

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