Speed dependent descending control of innate freezing behavior in *Drosophila melanogaster*

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Summary

The most fundamental choice an animal has to make when it detects a predator, or other threats, is whether to freeze, reducing its chances of being noticed, or to flee to safety. Here we show that *Drosophila melanogaster* exposed to looming stimuli in a confined arena either froze or fled. The probability of freezing versus fleeing was modulated by the fly's walking speed at the time of threat, demonstrating that freeze/flee decisions were context dependent. We describe a pair of descending neurons crucially implicated in freezing. Genetic silencing of DNp09 descending neurons disrupted freezing yet did not prevent fleeing. Optogenetic activation of both DNp09 neurons induced running and freezing in a state-dependent manner. Our findings establish walking speed as a key factor in defensive response choices and reveal a pair of descending neurons as a critical component in the circuitry mediating selection and execution of freezing or fleeing behaviors.

Introduction

Animals rely on similar kinds of cues to detect a predator's rapid approach such as visual looming stimuli (Card, 2012; Carr, 2015; Pereira and Moita, 2016). Although great progress has been made in the study of threat detection mechanisms, much less is known regarding when and how

28 different defensive behaviors are performed. Animals deploy several defensive strategies, grossly
29 categorized into flight, freeze and fight, the display of which is modulated by external factors, such as
30 the presence of offspring or existence of escape, and the animal's internal state (Blanchard et al.,
31 1986; Giaquinto and Volpato, 2001; Llaneza and Frye, 2009; Montgomerie and Weatherhead, 1988;
32 de OCA et al., 2007a; Rickenbacher et al., 2017; Vale et al., 2017; Verma et al., 2016). How each
33 specific defensive response is selected and executed remains unclear.

34 In mammals, mostly from studies using rodents, multiple brain regions have been implicated
35 in the expression of freezing and escape responses including amygdala, hypothalamus and peri-
36 aqueductal grey (Amorapanth et al., 2000; Gross and Canteras, 2012; Kim et al., 2013, 1993; Kunwar
37 et al., 2015; Silva et al., 2013). More recently, microcircuits within these brain regions regulating the
38 expression of flight or freeze behaviors have been characterized (Fadok et al., 2017; Tovote et al.,
39 2016). Much less is known about the mechanisms of expression of defensive behaviors in other
40 vertebrates. In fish, Mauthner cells, a pair of large neurons in the hindbrain, have been implicated in
41 fast escape responses (review in (Korn and Faber, 2005), whereas other spinal chord projecting
42 neurons are involved in slower escapes (Bhattacharyya et al., 2017). Even though the zebrafish
43 habenula has been shown to down regulate freezing, favoring escape responses, how freezing
44 behavior is produced remains unknown. In invertebrates the mechanisms of escape responses have
45 received more attention. In striking similarity with the zebrafish, fruit flies can exhibit either fast or
46 slower escape responses, the first relying on the giant fibers, a pair large descending neurons and the
47 later on other descending neurons (Card, 2012; Fotowat et al., 2009; von Reyn et al., 2014). Although
48 freezing behavior has been reported in fruit flies (Card and Dickinson, 2008b; Gibson et al., 2015), a
49 systematic quantification of this behavior and exploration of its neural underpinnings is lacking.

50 While the neural circuits of defensive behavior have been partially described in multiple
51 organisms, in mammals the focus has been on learned freezing responses, and in other organisms on
52 innate escape responses. In addition, how external or internal factors impinge on these circuits to
53 regulate choice between different responses remains largely unknown. To address this issue we
54 decided to use *Drosophila melanogaster* for its arsenal of genetic tools to dissect neural circuits and
55 the ability to use large sample sizes allowing for detailed quantitation of behavior. We developed a

visual assay to track responses of flies to an expanding shadow mimicking a large object on a collision course - looming stimulus - that triggers defensive behaviors in virtually all visual animals tested, including fruit flies (Card, 2012; Carr, 2015; Pereira and Moita, 2016). Flies were exposed to multiple looming stimuli in an enclosed arena to increase the chances of seeing both escape and freezing responses. In our experimental set-up sustained freezing was the predominant defensive response. When flies did not freeze they displayed escape responses directed away from the looming stimulus. Taking advantage of a close-loop system, which allows the presentation of stimuli dependent on the behavior of flies, we found that the decision to freeze or flee was modulated by the flies' walking speed at the time of threat. Through genetic manipulation of neuronal activity we identified a pair of descending neurons whose activity is required for freezing. Moreover, their ability to drive freezing, through optogenetic activation, depended on the walking speed of flies at the time of stimulation. These results reveal that innate responses to threats can be modulated by the flies' internal state, while identifying an element of the freezing circuit that is modulated by this state.

Results

Flies jumped rarely in response to repeated inescapable looming

Figure 1 shows a schematic representation of the experimental setup. We placed single flies in a covered walking arena and gave them 5 minutes to explore. A computer monitor angled above the arena showed a looming stimulus (black circle expanding on a white background) repeated 20 times over a subsequent 5-min period. As a control, we showed a separate group of flies a sequence of randomly appearing black pixels resulting in a similar change in luminance but with no pattern of expansion (Figure 1B). Notably, flies cannot escape from the arenas.

We found that looming stimuli only occasionally triggered escape jumps, or takeoffs (6.4% of looming stimuli, 384/6000), the most studied defensive response in insects (Card and Dickinson, 2008a, 2008b; Fotowat et al., 2009; Hammond and O'Shea, 2007; Heitler and Burrows, 1977; McKenna et al., 1989; von Reyn et al., 2014; Tauber and Camhi, 1995; de Vries and Clandinin, 2012). The number of jumps per fly was significantly higher for looming than control condition (Wilcoxon Rank Sum test $Z= 14.15$, $p < 0.0001$, Figure 1C) and the large majority of these events

occurred within the window of stimulation, before the circle reached its maximum size (Figure 1D). Still, the efficacy of looming stimuli to elicit jumps was lower in our experimental conditions than that reported previously (Fotowat et al., 2009; de Vries and Clandinin, 2012), where flies were exposed to a single escapable looming stimulus. Furthermore, the probability of jumping decreased over the course of the 20 stimulus presentations (Figure 1E), suggesting that with multiple presentations flies may have habituated to looming. Alternatively, flies could be adopting other defensive strategies.

Most flies responded to looming with sustained freezing

As running is an alternative form of defensive behavior (Lebestky et al., 2009), we analyzed the flies' speed over the course of the experiment. The largest fraction of flies decreased their speed instead (Figure 2A). Visual inspection of the videos led to the observation that flies were not just walking slower, they were completely immobile, i.e. freezing, many times sustaining unbalanced postures for long periods of time (movies S1 and S2). In order to quantify freezing, we created an automated classifier based on pixel changes recorded in a region of interest surrounding the fly (Figure S1).

The fraction of flies freezing increased gradually with each looming presentation, arguing against habituation to the looming stimulus (Figure 2B). By the end of the experiment, 70% of flies were freezing compared to 12% for the control stimulus (Chi-squared test, X^2 =208.599, $p<0.0001$, Figure 2B). Interestingly, there was a sharp decrease in freezing during stimulus presentation. Further examination showed that freezing flies displayed startle responses during looms, but quickly returned to an immobile state (movie S1). These responses were characterized by a spike in pixel motion that peaked at the end of the expansion of the looming stimulus (Figure 2C). Hence flies froze for long periods of time only breaking freezing for the brief moments of startle. Indeed, we observed a bimodal distribution, where most flies either froze for long periods of time (> 3 min) or did not freeze at all, with fewer flies displaying intermediate freezing levels (Figure 2D). To test whether prolonged freezing was due to the repeat stimulation, we exposed flies to five looming stimuli over the course of one minute, and asked whether flies would stop freezing after the stimulation period. We found that a substantial fraction of flies froze after the stimulation period (Figure 2E), and that 50% of the flies

froze more than one minute with many flies freezing up to 5 minutes (Figure 2F). These long periods of time spent freezing are in sharp contrast with previous studies, which have only reported short-lived, occasional freezing in *Drosophila* (Card and Dickinson, 2008b; Gibson et al., 2015; Zabala et al., 2012).

Flies that did not freeze, fled instead

Although a large fraction of flies froze in response to looming, not all flies did so. To determine whether flies displayed an alternate response to the looming stimuli, we next analyzed the flies' behavior excluding all freezing and grooming bouts, hence only periods classified as walking (> 4 mm/s) were analyzed. During the baseline period walking speed gradually decreased, reflecting a common process of habituation to the test arena. However, during the stimulation period, their walking speed increased (one sample Wilcoxon Signed-Rank test, $W=16435$, $p<0.0001$). This was not observed for flies exposed to control stimuli, which further decreased their speed (one sample Wilcoxon Signed-Rank test, $W=-15623$, $p<0.0001$) (see Figure 3A and B). The average difference in walking speed between stimulation and baseline periods was significantly higher for flies exposed to looming relative to control (Wilcoxon Rank-Sum test, $Z=16.33$, $p<0.0001$ Figure 3B). In addition, we observed sharp increases in speed at the time of each looming stimulus (Figure 3A). To examine changes in speed around looming we plotted the average speed of walking flies aligned on looming onset (Figure 3C). Walking speed was relatively constant before the stimulus. Upon looming onset, flies initially paused for about 300ms followed by a rapid burst of locomotion (Figure 3C, marker #2, movie S3). These pauses may correspond to the brief freezing bouts preceding takeoff described previously (Card and Dickinson, 2008b; Zabala et al., 2012). Around the end of the looming stimulus a transient decrease in speed is seen. However, after the offset of the looming stimulus, the flies' speed remained significantly elevated relative to the speed prior to looming (one sample Wilcoxon Signed-Rank test, $W=208460$, $p<0.0001$, Figure 3D). We next asked whether the running bouts reported here correspond to escapes directed away from the threat. We measured the orientation of the paths of walking flies before and after each looming presentation (Figure 3E). Before looming, paths in all orientations could be seen (Figure 3F). Upon looming, we observed a sharp increase in

140 orientation bias toward the side of the chamber furthest away from the threatening stimulus (Figure
141 3G. Wilcoxon Signed-Rank test, W=178808, p<0.0001). Flies heading towards the screen changed
142 direction, while flies heading away from the screen increased their walking speed and kept the same
143 orientation. These findings indicate that running bouts triggered by looming stimuli, are not just a
144 simple increase in locomotion, but constitute directed escape responses.
145
146 **Freezing/fleeing decisions were modulated by the walking speed of the flies at the time of threat**
147 Our data suggest that flies select between two distinct behavioral strategies, freezing or
148 fleeing. A possible factor modulating this selection became apparent when we analyzed the
149 relationship between the flies' movement speed and their response to looming. We sorted looming
150 trials by the speed of flies before looming onset and calculated the probability of freezing at different
151 movement speeds. We observed a sharp decay in freezing probability with increasing speed, such that
152 flies moving slowly or grooming were more likely to freeze upon looming stimulation than flies
153 moving faster (Figure 4A). To further explore the relationship between movement speed at the time of
154 looming and freezing probability, we designed a closed-loop experiment where the speed of the tested
155 fly was tracked online and looming stimuli were delivered at specific speed thresholds. One group of
156 flies received looming at low movement speeds and another at high movement speeds (<1mm/s and
157 >15mm/s respectively. Figure S2). Importantly, there was no difference in the average baseline speed
158 between the two groups, indicating that their overall level of arousal was similar (Student's T-test,
159 t=0.058, p=0.95, Figure 4B). We found that flies walking at low speed when exposed to looming were
160 more likely to freeze than flies exposed to the same stimulus while walking faster (Wilcoxon Rank-
161 Sum test, Z =-6.786, p<0.0001, Figure 4C), thus confirming a modulation of freezing probability by
162 the flies' movement speed. It is possible that the faster the flies walk the more difficult is to come to a
163 fast halt, explaining the sharp decrease of freezing with increased speeds. However, we found that the
164 probability of pausing upon looming was not significantly modulated by the flies walking speed
165 (Figure S2). Indeed at all walking speeds, including the highest, flies paused in response to ~40% of
166 the looming stimuli. Hence, the modulation of freezing probability is unlikely to result from a simple
167 inability to become immobile at higher walking speed.

168

169 **Activity of DNp09 descending neurons is required for freezing but not fleeing in response to**

170 **looming**

171 Next, we searched for the neural mechanisms underlying the defensive behaviors observed.

172 We focused on freezing, as it is conserved across the animal kingdom (Blanchard and Blanchard,

173 1969; Eilam, 2005a; Speedie and Gerlai, 2008) and corresponds to the dominant behavior adopted by

174 flies in our experimental conditions. Given the lack of knowledge regarding the neural mechanisms of

175 freezing in insects, we performed an unbiased screen, testing for looming triggered freezing of fly

176 lines expressing a hyperpolarizing potassium channel, Kir2.1 (Baines et al., 2001), in different subsets

177 of descending neurons (DNs; Namiki et al, in preparation, Janelia Descending Interneuron Project).

178 We focused on DNs, as these convey information from the brain to the ventral nerve cord thereby

179 controlling behavior. From this screen we identified a bilateral pair of DNs, DNp09, with dendrites

180 innervating the posterior protocerebrum and a large axon extending throughout the ventral nerve cord,

181 as well as the posterior slope and gnathal ganglion (Figure 5A. Weak off-target labeling not shown).

182 Silencing these neurons significantly decreased the occurrence of freezing relative to the parental

183 controls (Kruskal-Wallis test, X^2=80.19, p<0.0001. Post-hoc Dunn tests revealed a significant

184 difference between DNp09>Kir2.1 and the parental controls: DNp09>Kir2.1 vs. DNp09/+, Z=-4.98,

185 p<0.0001; DNp09>Kir2.1 vs. UASKir2.1/+, Z=-8.94, p<0.0001. In addition the two parental controls

186 showed a small but significant difference: DNp09/+ vs UASKir2.1, Z=3.96, p=0.0002. Figure 5B and

187 C). This effect was not due to an overall decrease in sensitivity to looming since running was intact

188 (Figure 5D and Figure S4A). Furthermore, the frequency of jumps was increased, raising the

189 possibility that in control flies freezing behavior directly or indirectly inhibits jumps (Figure S4B).

190

191 **DNp09-silenced flies still paused in response to looming**

192 The effect of silencing DNp09 neurons on freezing behavior could reflect a general impairment in the

193 ability to stop. However, DNp09-silenced flies that ran in response to looming still exhibited a pause

194 upon looming onset, which can be seen in the walking speed profiles around looming stimulation

195 (Figure 5E). Indeed, these flies paused in 30% of the looming stimuli (looming stimuli that occurred

while flies were already freezing were excluded from this analysis). This finding indicates that pausing and sustained freezing are mediated by different mechanisms. Interestingly this dissociation is also present in rodents that possess distinct descending neurons for freezing and stopping, and where freezing involves sustained muscle tension whereas stopping does not (Bouvier et al., 2015; Koutsikou et al., 2014; Tovote et al., 2016).

Disruption of freezing was independent of walking speed

Given the negative relationship between speed and freezing probability mentioned above, we investigated whether silencing DNp09 neurons affected the walking speed of the flies. We found that indeed the average baseline speed was increased in silenced flies relative to controls (One-way ANOVA, $F=16.37$, $p<0.0001$. Post-hoc Tukey revealed a significant difference between DNp09>Kir2.1 and the parental controls: DNp09>Kir2.1 vs. DNp09/+, $p<0.0001$; DNp09>Kir2.1 vs. UASKir2.1/+, $p<0.0001$. No difference between parental controls was found, $p=0.6$. Figure 5F). This raises the possibility that the impairment seen in freezing results from the increased walking speed and hence a shift in the probability of freezing behavior. Therefore, we calculated the probability of freezing for looming stimuli occurring at different speeds. We found that despite the upward shift in speed of DNp09-silenced flies relative to controls, freezing probability of these flies was lower for the entire range of speeds (Figure S4C). In addition, we tested DNp09-silenced and control flies in closed loop such that looming stimuli were only presented when flies were at very low speeds (1mm/s) and found that even at these speeds DNp09-silenced flies froze less than controls (Kruskal-Wallis test, $X^2=33.55$, $p<0.0001$. Post-hoc Dunn tests revealed a significant difference between DNp09>Kir2.1 and the parental controls: DNp09>Kir2.1 vs. DNp09/+, $Z=-5.64$, $p<0.0001$; DNp09>Kir2.1 vs. UASKir2.1/+, $Z=-4.16$, $p<0.0001$. No difference between parental controls was found, $p=0.36$. Figure 5G). Together these findings indicate that silencing DNp09 neurons directly disrupts freezing, rather than indirectly affecting freezing behavior by increasing the speed of locomotion.

Activation of DNp09 neurons induced freezing

223 If indeed DNp09 neurons are involved in the execution of freezing behavior, activating them
224 artificially, in the absence of looming stimuli, should induce freezing. We expressed the red-shifted
225 channelrhodopsin, CsChrimson (Klapoetke et al., 2014), in DNp09 neurons and exposed single flies
226 to red light using a modified version of our behavioral setup (Figure 6A). CsChrimson requires retinal
227 to function, thus experimental flies were raised in food containing retinal whereas control animals
228 were raised in standard food. Flies were allowed to acclimate to the arena for 2 minutes. We then
229 presented 10 trials of continuous light for 2 seconds, separated by 20-second intervals. We found that
230 CsChrimson activation of DNp09 neurons was sufficient to trigger freezing in ~ 60% of trials (Figure
231 6B and movie S4). Closer inspection of the time course of freezing induction showed that the
232 probability of freezing increased gradually over the course of the 2-second light stimulation (Figure
233 6C). The lag in freezing correlated with an initial increase in walking speed, induced by DNp09
234 activation (Figure 6D). This running bout was much reduced in control flies, showing that running
235 was mostly caused by DNp09 activity (Figure S5). In an unbiased behavioral representation of
236 optogenetic activation of descending neurons it was also observed that activation of DNp09 induced
237 running followed by pausing using different analytical methods (Cande et al., 2017). The finding that
238 flies first ran in response to DNp09 activation contrasts with the observation that, upon looming, flies
239 often first paused and then ran, jumped or froze. Given that looming triggered pauses were
240 independent of DNp09 neurons, it is possible that, with looming, neurons upstream of DNp09 inhibit
241 the initial running bout seen with artificial DNp09 activation. Finally, we observed jumps at light
242 offset in test flies, but not control flies (Chi-squared test, X^2=219.76, p<0.0001, Figure 6E).
243 Moreover, jumps were more likely after stimulations that led to freezing than after stimulations that
244 failed to elicit freezing (Chi-squared test, X^2=72.51, p<0.0001, Figure 6E). One possible
245 explanation for this could be that strong activation of DNp09 neurons inhibits downstream targets
246 involved in jumping behavior, such that when DNp09 activation stops, these neurons are released
247 from inhibition showing rebound excitation, thereby triggering the observed jumps.
248
249 **The probability of freezing upon DNp09 activation was dependent on the walking speed of flies**

250 Given that the probability of freezing in response to looming stimuli was found to depend on
251 the walking speed of flies at the time of threat, we asked whether the ability of DNp09 neurons in
252 driving freezing was also modulated by the flies' walking speed. We found that the probability of
253 freezing upon light activation of DNp09 neurons was negatively correlated with the movement speed
254 of flies (Figure 6F, linear regression, r^2=0.71 p=0.001). To confirm that DNp09-driven freezing is
255 modulated by the movement speed of flies at the time of stimulation, we again tested flies in close
256 loop, such that DNp09 activation would occur when flies were at low, high or very high speeds
257 (1mm/s, 15mm/s and 20mm/s, respectively). The probability of DNp09 activation driving freezing
258 was highest for the flies stimulated at low movement speed (very high vs. high: Chi-squared test, X^2
259 =37.875, p<0.0001; high vs. low: Chi-squared test, X^2 =26.278, p<0.0001. p values Bonferroni
260 corrected. Figure 6G). Together, these findings show that DNp09 neurons are a key element in the
261 circuit mediating the speed modulation of freezing expression, and suggest that this modulation is not
262 a result of locomotion induced changes in visual perception (Chiappe et al., 2010; Maimon et al.,
263 2010).
264
265 **Discussion**
266 Freezing is a defensive response characterized by complete immobility, allowing preys to
267 avoid being detected while remaining attentive to changes in the environment (Eilam, 2005b;
268 Fanselow, 1994). This response has been reported across vertebrates (Blanchard and Blanchard, 1969;
269 Eilam, 2005b; Speedie and Gerlai, 2008). Here we show long lasting freezing in fruit flies similar to
270 that observed in vertebrates and distinct from the brief freezing periods reported for flies (Card and
271 Dickinson, 2008b; Gibson et al., 2015; von Reyn et al., 2014). The pervasiveness of freezing across
272 distant taxa strongly suggests its independent evolution and supports its adaptive value (Schultz and
273 Endler, 1987).
274 Given the prevalence of freezing behavior, it is crucially important to understand whether
275 there are general principles that govern it. For instance, the display of freezing is plastic. Rodents tend
276 to freeze only if there is no escape, and the presence of conspecifics decreases this behavior
277 (Blanchard et al., 1986; Kiyokawa et al., 2007, 2009; de OCA et al., 2007b; Rickenbacher et al., 2017;

278 Vale et al., 2017). Furthermore, it has been previously shown that estrous cycle or feeding state
279 modulate freezing (Llaneza and Frye, 2009; Verma et al., 2016). How animal's surroundings or the
280 animal's internal state regulate this behavior is much less clear. A hint of the conserved nature of the
281 principles governing freezing, is the finding that in other vertebrates, such as fish, the expression of
282 innate defensive behaviors is also plastic (Agetsuma et al., 2010; Bass and Gerlai, 2008; Giaquinto
283 and Volpato, 2001). In this study, we extend this to invertebrate animals by demonstrating the plastic
284 nature of freezing in flies. Flies either ran or froze in response to inescapable looming.

285 The choice between escaping and freezing was strongly modulated by the flies' speed at the
286 time of threat. This effect could be explained by an impact of speed on motor output, sensory
287 processing and/or arousal. An effect on motor output could be simply a consequence of increased
288 difficulty in stopping when walking fast. The findings that pausing in response to looming was
289 independent of the walking speed, and that DNp09 induced freezing was always preceded by running,
290 argues against this possibility. This leaves a possible influence on visual processing (Bennett et al.,
291 2013; Chiappe et al., 2010; Maimon et al., 2010; Vinck et al., 2015) or central motor commands. The
292 finding that DNp09-induced freezing was modulated by movement speed of flies at the time of
293 stimulation argues for the later. Future experiments are required to disambiguate between these
294 scenarios.

295 We next explored the neuronal underpinnings of freezing behavior, contributing to the
296 understanding of how different animals, with different bodies and brains, implement this seemingly
297 simple behavior. We uncovered the key role of a single pair of descending neurons, DNp09, in driving
298 freezing. DNp09 neurons innervate visual input areas in the central brain, thus being in a good
299 position to respond to looming stimuli. The output terminals of DNp09 neurons innervate the
300 posterior slope, which is densely innervated by other descending neurons (Hsu and Bhandawat, 2016),
301 and multiple regions along the ventral nerve chord, allowing the interaction with other motor outputs
302 at different levels.

303 Although freezing is often seen as absence of other behaviors, a passive state of immobility
304 (Fadok et al., 2017; Fanselow, 1994; Gozzi et al., 2010), evidence suggests otherwise. For example in
305 mammals, freezing is accompanied by sustained muscle tension likely involved in postural control

306 (Koutsikou et al., 2014; Watson et al., 2016) and, in response to learned cues, requires sustained
307 activity of several brain regions (Amano et al., 2011; Burgos-Robles et al., 2009; Duvarci et al.,
308 2011). In addition a recent study identified in mice a set of descending neurons that drive stopping
309 behavior that is distinct from those identified for freezing (Bouvier et al., 2015; Tovote et al., 2016).
310 The finding that looming triggered-freezing and pausing could be dissociated supports the idea that
311 freezing is an active defense module pointing to the conserved nature of the distinction between
312 freezing and stopping. Moreover, freezing may require active inhibition of alternate behaviors. An
313 indication that active inhibition of alternate behavior happens in flies comes from our observation that
314 DNp09 silenced flies jump more and that flies jump at the offset of DNp09 neuron activation,
315 presumably resulting from rebound excitation after inhibition of jump. Consistent with an inhibitory
316 effect of freezing on jumping is the observation that jumping steeply decreased with increase number
317 of flies freezing over the course of the repeated looming stimuli. The identification of DNp09
318 descending neurons as central to freezing opens the path to further explore how the active state of
319 freezing is implemented. Activation of DNp09 neurons drove both running and freezing. However,
320 silencing DNp09 neurons left looming triggered escape responses intact suggesting that DNp09
321 triggered running may correspond to a different behavior. Still, it will be very interesting to unravel
322 how a single pair of neurons drives distinct behaviors.

323 Finally, since the flies' speed modulates their response to looming stimuli, we examined
324 whether the ability of DNp09 neurons to drive freezing was also modulated by the flies' speed. We
325 found that the probability of freezing upon DNp09 stimulation was negatively correlated with the
326 flies' movement speed. This finding demonstrated that DNp09 neurons are a key element in the
327 circuit mediating speed dependent defensive decisions. Unraveling how speed impinges on DNp09
328 neurons and possibly other elements of defense circuits will be instrumental for the understanding of
329 the organization of defensive behaviors crucial for survival.

330

331 **References**

332 Agetsuma, M., Aizawa, H., Aoki, T., Nakayama, R., Takahoko, M., Goto, M., Sassa, T., Amo, R.,
333 Shiraki, T., Kawakami, K., et al. (2010). The habenula is crucial for experience-dependent

modification of fear responses in zebrafish. Nat. Neurosci. *13*, 1354–1356.

Amano, T., Duvarci, S., Popa, D., Paré, D., and Pare, D. (2011). The Fear Circuit Revisited: Contributions of the Basal Amygdala Nuclei to Conditioned Fear. J. Neurosci. *31*, 15481–15489.

Amorapanth, P., LeDoux, J.E., and Nader, K. (2000). Different lateral amygdala outputs mediate reactions and actions elicited by a fear-arousing stimulus. Nat. Neurosci. *3*, 74–79.

Baines, R.A., Uhler, J.P., Thompson, A., Sweeney, S.T., and Bate, M. (2001). Altered Electrical Properties in Drosophila neurons developing without synaptic transmission. J. Neurosci. *21*, 1523–1531.

Bass, S.L.S., and Gerlai, R. (2008). Zebrafish (Danio rerio) responds differentially to stimulus fish: The effects of sympatric and allopatric predators and harmless fish. Behav. Brain Res. *186*, 107–117.

Bennett, C., Arroyo, S., and Hestrin, S. (2013). Subthreshold mechanisms underlying state-dependent modulation of visual responses. Neuron *80*, 350–357.

Bhattacharyya, K., McLean, D.L., and MacIver, M.A. (2017). Visual Threat Assessment and Reticulospinal Encoding of Calibrated Responses in Larval Zebrafish. Curr. Biol. *27*, 2751–2762.e6.

Blanchard, R.J., and Blanchard, D.C. (1969). Crouching as an index of fear. J. Comp. Physiol. Psychol. *67*, 370–375.

Blanchard, R.J., Flannelly, K.J., and Blanchard, D.C. (1986). Defensive behavior of laboratory and wild Rattus norvegicus. J. Comp. Psychol. *100*, 101–107.

Bouvier, J., Caggiano, V., Leiras, R., Caldeira, V., Bellardita, C., Balueva, K., Fuchs, A., and Kiehn, O. (2015). Descending Command Neurons in the Brainstem that Halt Locomotion. Cell *163*, 1191–1203.

Burgos-Robles, A., Vidal-Gonzalez, I., and Quirk, G.J. (2009). Sustained Conditioned Responses in Prelimbic Prefrontal Neurons Are Correlated with Fear Expression and Extinction Failure. J. Neurosci. *29*, 8474–8482.

Cande, J., Berman, G.J., Namiki, S., Qiu, J., Korff, W., Card, G., Shaevitz, J.W., and Stern, D.L. (2017). Optogenetic dissection of descending behavioral control in Drosophila. bioRxiv 230128.

Card, G.M. (2012). Escape behaviors in insects. Curr. Opin. Neurobiol. *22*, 180–186.

Card, G., and Dickinson, M. (2008a). Performance trade-offs in the flight initiation of Drosophila. J.

362 Exp. Biol. *211*, 341–353.
363 Card, G., and Dickinson, M.H. (2008b). Visually mediated motor planning in the escape response of
364 Drosophila. Curr. Biol. *18*, 1300–1307.
365 Carr, J.A. (2015). I'll take the low road: the evolutionary underpinnings of visually triggered fear.
366 Front. Neurosci. *9*, 414.
367 Chiappe, M.E., Seelig, J.D., Reiser, M.B., and Jayaraman, V. (2010). Walking modulates speed
368 sensitivity in Drosophila motion vision. Curr. Biol. *20*, 1470–1475.
369 Duvarci, S., Popa, D., Pare, D., and Paré, D. (2011). Central Amygdala Activity during Fear
370 Conditioning. J. Neurosci. *31*, 289–294.
371 Eilam, D. (2005a). Die hard: a blend of freezing and fleeing as a dynamic defense--implications for
372 the control of defensive behavior. Neurosci. Biobehav. Rev. *29*, 1181–1191.
373 Eilam, D. (2005b). Die hard: A blend of freezing and fleeing as a dynamic defense - Implications for
374 the control of defensive behavior. Neurosci. Biobehav. Rev. *29*, 1181–1191.
375 Fadok, J.P., Krabbe, S., Markovic, M., Courtin, J., Xu, C., Massi, L., Botta, P., Bylund, K., Müller,
376 C., Kovacevic, A., et al. (2017). A competitive inhibitory circuit for selection of active and passive
377 fear responses. Nature *542*, 96–100.
378 Fanselow, M.S. (1994). Neural organization of the defensive behavior system responsible for fear.
379 Psychon. Bull. Rev. *1*, 429–438.
380 Fotowat, H., Fayyazuddin, A., Bellen, H.J., and Gabbiani, F. (2009). A novel neuronal pathway for
381 visually guided escape in Drosophila melanogaster. J. Neurophysiol. *102*, 875–885.
382 Giaquinto, P., and Volpato, G. (2001). Hunger suppresses the onset and the freezing component of the
383 antipredator response to conspecific skin extract in pintado catfish. Behaviour *138*, 1205–1214.
384 Gibson, W.T., Gonzalez, C.R., Fernandez, C., Ramasamy, L., Tabachnik, T., Du, R.R., Felsen, P.D.,
385 Maire, M.R., Perona, P., and Anderson, D.J. (2015). Behavioral responses to a repetitive visual threat
386 stimulus express a persistent state of defensive arousal in drosophila. Curr. Biol. *25*, 1401–1415.
387 Gozzi, A., Jain, A., Giovanelli, A., Bertollini, C., Crestan, V., Schwarz, A.J., Tsetsenis, T.,
388 Ragozzino, D., Gross, C.T., and Bifone, A. (2010). A neural switch for active and passive fear.
389 Neuron *67*, 656–666.

390 Gross, C.T., and Canteras, N.S. (2012). The many paths to fear. Nat. Rev. Neurosci. *13*, 651–658.
391 Hammond, S., and O'Shea, M. (2007). Escape flight initiation in the fly. J. Comp. Physiol. A.
392 Neuroethol. Sens. Neural. Behav. Physiol. *193*, 471–476.
393 Heitler, W.J., and Burrows, M. (1977). The locust jump. II. Neural circuits of the motor programme.
394 J. Exp. Biol. *66*, 221–241.
395 Hsu, C.T., and Bhandawat, V. (2016). Organization of descending neurons in Drosophila
396 melanogaster. Sci. Rep. *6*, 20259.
397 Kim, E.J., Horovitz, O., Pellman, B.A., Tan, L.M., Li, Q., Richter-Levin, G., and Kim, J.J. (2013).
398 Dorsal periaqueductal gray-amygdala pathway conveys both innate and learned fear responses in rats.
399 Proc. Natl. Acad. Sci. U. S. A. *110*, 14795–14800.
400 Kim, J.J., Rison, R.A., and Fanselow, M.S. (1993). Effects of amygdala, hippocampus, and
401 periaqueductal gray lesions on short- and long-term contextual fear. Behav. Neurosci. *107*, 1093–
402 1098.
403 Kiyokawa, Y., Takeuchi, Y., and Mori, Y. (2007). Two types of social buffering differentially
404 mitigate conditioned fear responses. Eur. J. Neurosci. *26*, 3606–3613.
405 Kiyokawa, Y., Takeuchi, Y., Nishihara, M., and Mori, Y. (2009). Main olfactory system mediates
406 social buffering of conditioned fear responses in male rats. Eur. J. Neurosci. *29*, 777–785.
407 Klapoetke, N.C., Murata, Y., Kim, S.S., Pulver, S.R., Birdsey-Benson, A., Cho, Y.K., Morimoto,
408 T.K., Chuong, A.S., Carpenter, E.J., Tian, Z., et al. (2014). Independent optical excitation of distinct
409 neural populations. Nat. Methods *11*, 338–346.
410 Korn, H., and Faber, D.S. (2005). The Mauthner cell half a century later: a neurobiological model for
411 decision-making? Neuron *47*, 13–28.
412 Koutsikou, S., Crook, J.J., Earl, E. V., Leith, J.L., Watson, T.C., Lumb, B.M., and Apps, R. (2014).
413 Neural substrates underlying fear-evoked freezing: the periaqueductal grey-cerebellar link. J. Physiol.
414 *592*, 2197–2213.
415 Kunwar, P.S., Zelikowsky, M., Remedios, R., Cai, H., Yilmaz, M., Meister, M., and Anderson, D.J.
416 (2015). Ventromedial hypothalamic neurons control a defensive emotion state. Elife *2015*, 1–30.
417 Lebestky, T., Chang, J.-S.C., Dankert, H., Zelnik, L., Kim, Y.-C., Han, K.-A., Wolf, F.W., Perona, P.,

418 and Anderson, D.J. (2009). Two different forms of arousal in Drosophila are oppositely regulated by
419 the dopamine D1 receptor ortholog DopR via distinct neural circuits. Neuron *64*, 522–536.
420 Llaneza, D.C., and Frye, C.A. (2009). Progestogens and estrogen influence impulsive burying and
421 avoidant freezing behavior of naturally cycling and ovariectomized rats. Pharmacol. Biochem. Behav.
422 *93*, 337–342.
423 Lopes, G., Bonacchi, N., Frazão, J., Neto, J.P., Atallah, B. V, Soares, S., Moreira, L., Matias, S.,
424 Itskov, P.M., Correia, P.A., et al. (2015). Bonsai : an event-based framework for processing and
425 controlling data streams. *9*, 1–14.
426 Maimon, G., Straw, A.D., and Dickinson, M.H. (2010). Active flight increases the gain of visual
427 motion processing in Drosophila. Nat. Neurosci. *13*, 393–399.
428 McKenna, M., Monte, P., Helfand, S.L., Woodard, C., and Carlson, J. (1989). A simple chemosensory
429 response in Drosophila and the isolation of acj mutants in which it is affected. Proc. Natl. Acad. Sci.
430 *86*, 8118–8122.
431 Montgomerie, R.D., and Weatherhead, P.J. (1988). Risks and Rewards of Nest Defence by Parent
432 Birds. Q. Rev. Biol. *63*, 167–187.
433 de OCA, B., Minor, T.R., and Fanselow, M.S. (2007a). Brief flight to a familiar enclosure in response
434 to a conditional stimulus in rats. J. Gen. … *134*, 153–172.
435 de OCA, B.M.B., Minor, T.R.T.R., and Fanselow, M.S.M.S. (2007b). Brief flight to a familiar
436 enclosure in response to a conditional stimulus in rats. J. Gen. … *134*, 153–172.
437 Peirce, J.W. (2007). PsychoPy--Psychophysics software in Python. J. Neurosci. Methods *162*, 8–13.
438 Pereira, A.G., and Moita, M.A. (2016). Is there anybody out there? Neural circuits of threat detection
439 in vertebrates. Curr. Opin. Neurobiol. *41*, 179–187.
440 von Reyn, C.R., Breads, P., Peek, M.Y., Zheng, G.Z., Williamson, W.R., Yee, A.L., Leonardo, A.,
441 and Card, G.M. (2014). A spike-timing mechanism for action selection. Nat. Neurosci. *17*, 962–970.
442 Rickenbacher, E., Perry, R.E., Sullivan, R.M., and Moita, M.A. (2017). Freezing suppression by
443 oxytocin in central amygdala allows alternate defensive behaviours and mother-pup interactions. Elife
444 *(accepted*, 1–17.
445 Schultz, D.L., and Endler, J.A. (1987). Natural Selection in the Wild. Syst. Zool. *36*, 87.

Silva, B.A., Mattucci, C., Krzywkowski, P., Murana, E., Illarionova, A., Grinevich, V., Canteras, N.S., Ragozzino, D., and Gross, C.T. (2013). Independent hypothalamic circuits for social and predator fear. Nat. Neurosci. *16*, 1731–1733.

Speedie, N., and Gerlai, R. (2008). Alarm substance induced behavioral responses in zebrafish (Danio rerio). Behav. Brain Res. *188*, 168–177.

Tauber, and Camhi (1995). The wind-evoked escape behavior of the cricket Gryllus bimaculatus: integration of behavioral elements. J. Exp. Biol. *198*, 1895–1907.

Tovote, P., Esposito, M.S., Botta, P., Chaudun, F., Fadok, J.P., Markovic, M., Wolff, S.B.E., Ramakrishnan, C., Fenno, L., Deisseroth, K., et al. (2016). Midbrain circuits for defensive behaviour. Nature *534*, 206–212.

Vale, R., Evans, D.A., and Branco, T. (2017). Rapid Spatial Learning Controls Instinctive Defensive Behavior in Mice. Curr. Biol. *27*, 1342–1349.

Verma, D., Wood, J., Lach, G., Herzog, H., Sperk, G., and Tasan, R. (2016). Hunger Promotes Fear Extinction by Activation of an Amygdala Microcircuit. Neuropsychopharmacology *41*, 431–439.

Vinck, M., Batista-Brito, R., Knoblich, U., and Cardin, J.A. (2015). Arousal and Locomotion Make Distinct Contributions to Cortical Activity Patterns and Visual Encoding. Neuron *86*, 740–754.

de Vries, S.E.J., and Clandinin, T.R. (2012). Loom-sensitive neurons link computation to action in the Drosophila visual system. Curr. Biol. *22*, 353–362.

Watson, T.C., Cerminara, N.L., Lumb, B.M., and Apps, X.R. (2016). Neural Correlates of Fear in the Periaqueductal Gray. *36*, 12707–12719.

Zabala, F., Polidoro, P., Robie, A., Branson, K., Perona, P., and Dickinson, M.H. (2012). A simple strategy for detecting moving objects during locomotion revealed by animal-robot interactions. Curr. Biol. *22*, 1344–1350.

Acknowledgements

We would like to thank the Scientific Software Platform of Champalimaud Centre for the Unknown (CCU) for developing the fly tracking software; the Scientific Hardware Platform of the

CCU, for building LED array for optogenetic activation experiments; the Fly facilities of the CCU and Grace Zheng from the fly facility of Janelia Research Campus; Eugenia Chiappe and Joseph Paton for comments on the manuscript. This work was funded by the Champalimaud Foundation, the visiting scientist program of Janelia Research Campus and the ERC Starting Grant CoCO 337747.

Author Contributions

RZ performed all experiments and analyzed data. RZ, MLV and MAM designed all experiments, discussed results and wrote manuscript. SN and GC created the split-gal4 lines of descending neurons and provided the image of DNp09 neuron labeling. GC, MLV and MAM supervised the unbiased DN-silencing screen performed at Janelia Research Campus; GC commented on the manuscript. Fly lines should be requested to GC. All data are reported in the main text and supplementary materials, stored at Champalimaud Research and available upon reasonable request.

Conflict of interest

Authors declare no conflict of interests.

Experimental Procedures

Animal husbandry and fly strains

All animals used in experiments were 4-6 day old mated female *Drosophila melanogaster*. Flies were raised at 25ºC and 70% humidity in a 12h:12h dark:light cycle. Strains and sources:Canton S used in in figures 1, 2, 3 and 4; DL used to cross with parental strains in figure 5 and 6: 10XUAS-IVS-eGFPKir2.1 (attP2) (DL) (DL denotes chromosomes derived from the DL fly strain)(von Reyn et al., 2014); 20xUAS-CsChrimson.mVenus in attp2(Klapoetke et al., 2014); DNp09 line (Namiki et al, in preparation; https://www.janelia.org/project-team/fly-descending-interneuron).

Behavior

All behavioral experiments were performed in the 4-hour period preceding lights off and under the same conditions as rearing.

Arenas

Behavioral arenas were custom built from opaque white and transparent acrylic sheets. Chambers were 30 mm in diameter and 4 mm in height.

Apparatus

We recorded behavior of unrestrained flies while presenting visual stimulation (Figure 1a). A monitor tilted at 45 degrees over the stage delivered visual stimulation. To image fly locomotion, a custom-built infrared (850 nm) LED array was placed under the stage (backlight). A diffuser (2 mm white opaque acrylic sheet) was placed on top of the LED array to produce homogeneous illumination. Fly behavior was recorded using a USB3 camera (PointGrey Flea3) with a 850 nm long pass filter.

Each fly was aspirated into a chamber and placed on the stage. Flies were observed for 20 seconds to ensure that no gross motor defects were present before video acquisition started.

Video acquisition and tracking

Videos were acquired using Bonsai(Lopes et al., 2015) at 60 Hz and width 1104 x height 1040 resolution. Image segmentation was performed by custom software in python using OpenCV. Fly positions were calculated from centroid, and motion quantified by pixel change in an 100x100px region of interest surrounding the centroid.

Visual stimulation

Visual stimuli were presented on a 24-inch monitor running at 144 Hz (ASUS VG248QE). All stimuli were generated in a custom python script using PsychoPy(Peirce, 2007).

Looming: The visual angle of the expanding circle was determined by the equation

$\theta(t) = 2\tan^{-1}(l/vt)$

where l is half of the length of the object and v the speed of the object towards the fly. Virtual object length was 1 cm and speed 25 cm/s (l/v value of 40 ms). Each looming presentation lasted for 500 ms. Object expanded during 450 ms until it reached maximum size of 78 degrees where it stayed for 50 ms before disappearing.

Random dots: an array of approximately 3 degree dots was added each frame in random positions to generate similar change in luminance as the looming but without any expanding pattern.

Behavioral classifiers

528 Jumping: a fly was classified as having jumped if frame by frame speed exceeded a threshold (75
529 mm/s) identified by a discontinuity in the speed distribution.
530 Freezing: a fly was considered to be freezing when average pixel change in a region of interest around
531 the fly was lower than 25 pixels (approx.< 5% of fly area) over 500 ms.
532 Walking: a fly was considered to be walking if its average speed over a 500 ms period exceeded 4
533 mm/s.

534 **Closed-loop looming stimulation**

535 Fly positions were tracked in real time and used to trigger looming stimuli using Bonsai(Lopes et al.,
536 2015). Threshold for looming stimuli were defined based on the path length in 500 ms windows. Low
537 speed loomings were triggered when path length was < 1 mm in 500 ms. High speed loomings were
538 triggered when path length was > 7.5 mm in 500 ms. A refractory period of 15 seconds was imposed
539 after each triggered stimulation.

540 **Optogenetic activation**

541 Responses of freely moving flies to CsChrimson(Klapoetke et al., 2014) activation were captured
542 using a modified version the behavioral apparatus described in figure 6a. High-powered 627 nm LEDs
543 were interspersed between the infrared LEDs on the backlight board. Each arena was irradiated with
544 four LEDs for total radiance of $0.025 mW.mm^{-2}$. Experimental flies were raised on standard fly food
545 with 0.2 mM all trans-retinal (Sigma, R2500) and control flies were raised on standard fly food
546 without retinal. Flies were allowed to explore for 2 minutes and then were stimulated with 10
547 repetitions of 2 seconds light on, 20 seconds light off.

548 **Closed-loop optogenetic activation**

549 Fly positions were tracked in real time and used to trigger red light activation using Bonsai(Lopes et
550 al., 2015). Speed thresholds used were the same as in the looming stimulation. Threshold for looming
551 stimuli were defined based on the path length in 500 ms windows. Low speed loomings were
552 triggered when path length was < 1 mm in 500 ms. High speed loomings were triggered when path
553 length was > 7.5 mm in 500 ms. A refractory period of 15 seconds was imposed after each triggered
554 stimulation.

555 **Staining and imaging**

556 Imaging of DN morphology was performed as part of the Janelia Descending Interneuron project.
557 The DNp09DN split-GAL4 driver line, SS1540, was crossed to 5XUAS-IVS-Syt::smGFP-HA and -
558 5xUAS-IVS-myr::smGFP-FLAG and the central nervous systems of the progeny were dissected and
559 stained for anti-GFP according to the standard Janelia FlyLight protocol. Brains were subsequently
560 mounted in DPX and imaged with a confocal microscope. Detailed immunohistochemistry staining
561 and DPX mounting protocols are avilable online at https://www.janelia.org/project-
562 team/flylight/protocols. To best illustrate DNp09 morphology, off-target expression was removed
563 from the image using Photoshop.

564 **Data analysis and statistics**

565 Data analysis was performed using custom Python scripts. All data, except those from animals
566 excluded due to tracking errors, were analyzed. Prior to statistical testing, data were tested for
567 normality with a Shapiro-Wilk test and the appropriate non-parametric test was chosen if data were
568 not normally distributed. All statistical tests are specified in the results section of the text or figure
569 captions and are two-sided.

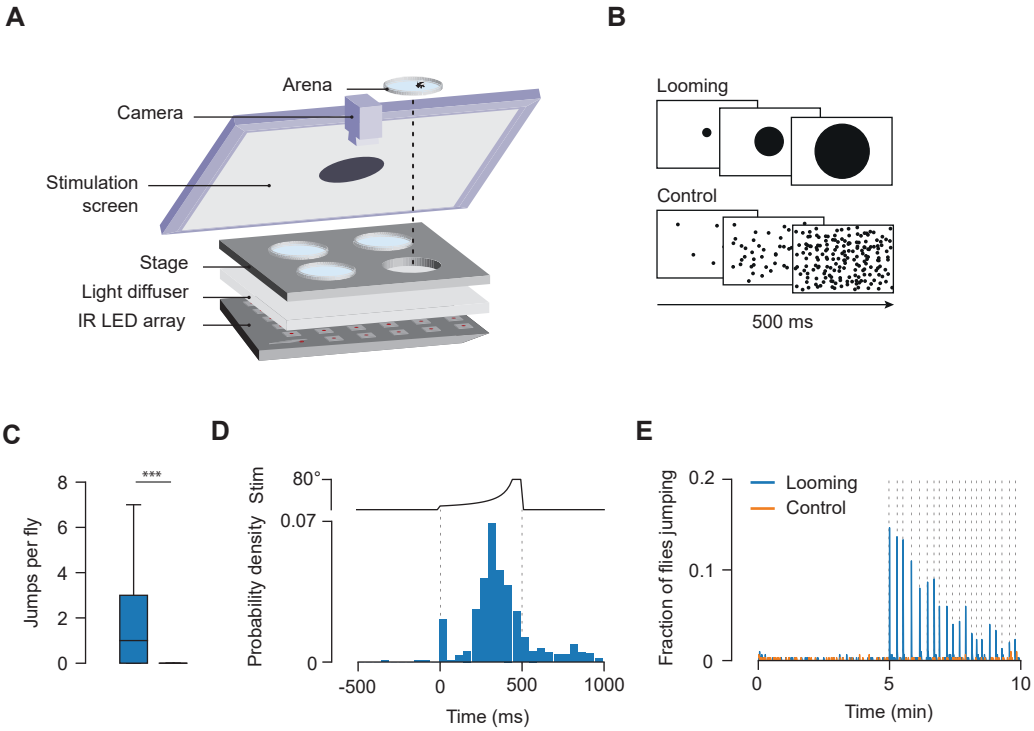

Figure 1 – *Flies jump in response to repeated looming.*
(A) Schematic of behavioral assay. (B) Schematic of visual stimuli, 300 flies tested in each condition. (C) Number of jumps per fly during stimulation period. *** denotes, $p<0.0001$. (D) Probability density of jumps around the looming stimulus. Dotted lines indicate beginning and end of looming. Top, visual angle of looming disk. (E) Fraction of flies jumping. Orange corresponds to flies exposed to control stimulus and blue to flies exposed to looming stimulus. Dotted lines represent stimulus presentations.

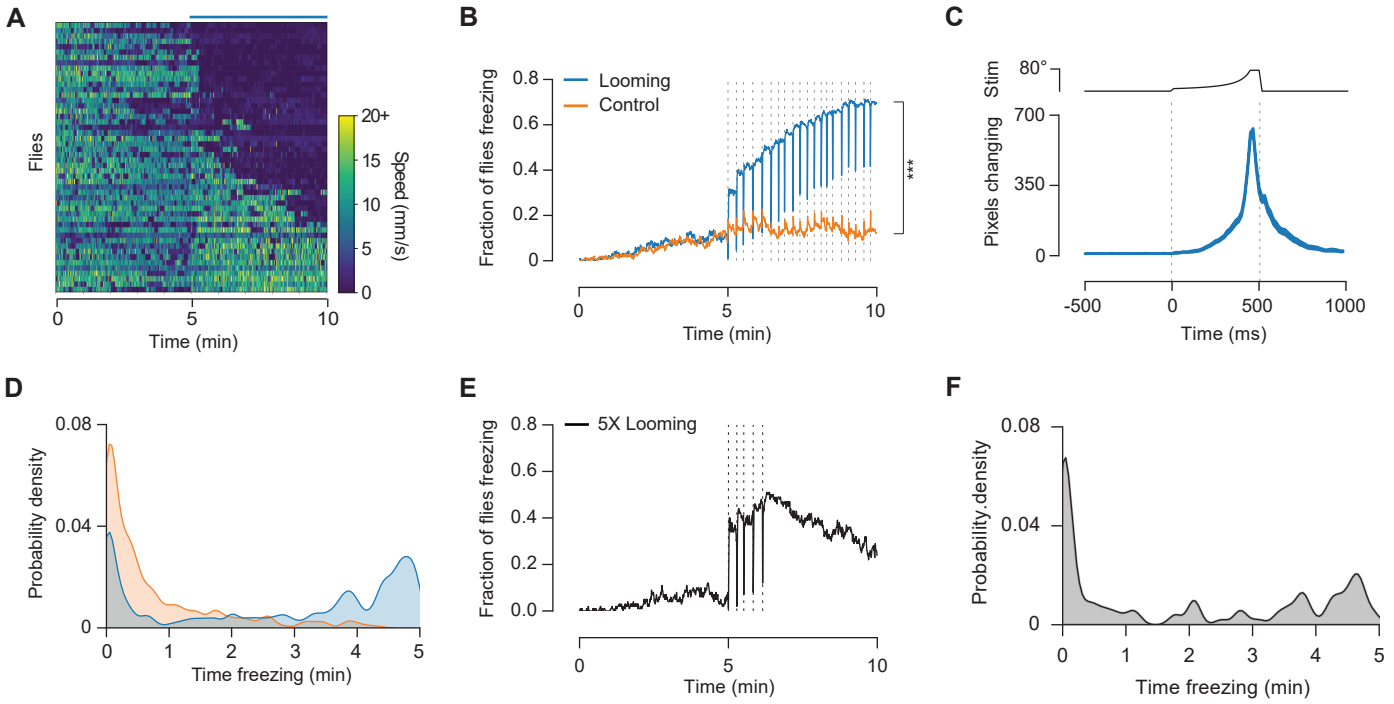

Figure 2 - *Flies freeze upon looming stimulation.*
(A) Speed in 500 ms bins for a subset of 50 flies. Each row corresponds to one fly, rank-ordered by time spent immobile. Blue bar on top indicates the 5 min stimulation period. In B-D orange corresponds to flies exposed to control stimulus and blue to flies exposed to looming stimulus. (B) Fraction of flies freezing. Dotted lines represent stimulus presentations. (C) Average (±sem) number of pixels changing around the fly, aligned on looming events where flies were freezing before and after the stimulus (n=1434). Dotted lines indicate beginning and end of looming. Top, visual angle of looming disk. (D) Probability density of time spent freezing by individual flies. (E) Fraction of flies freezing to a shorter stimulation (5 looming stimuli), n=100. Dotted lines represent looming presentations. (F) Probability density of percentage of time freezing during stimulation period for flies that received only 5 looming stimuli.

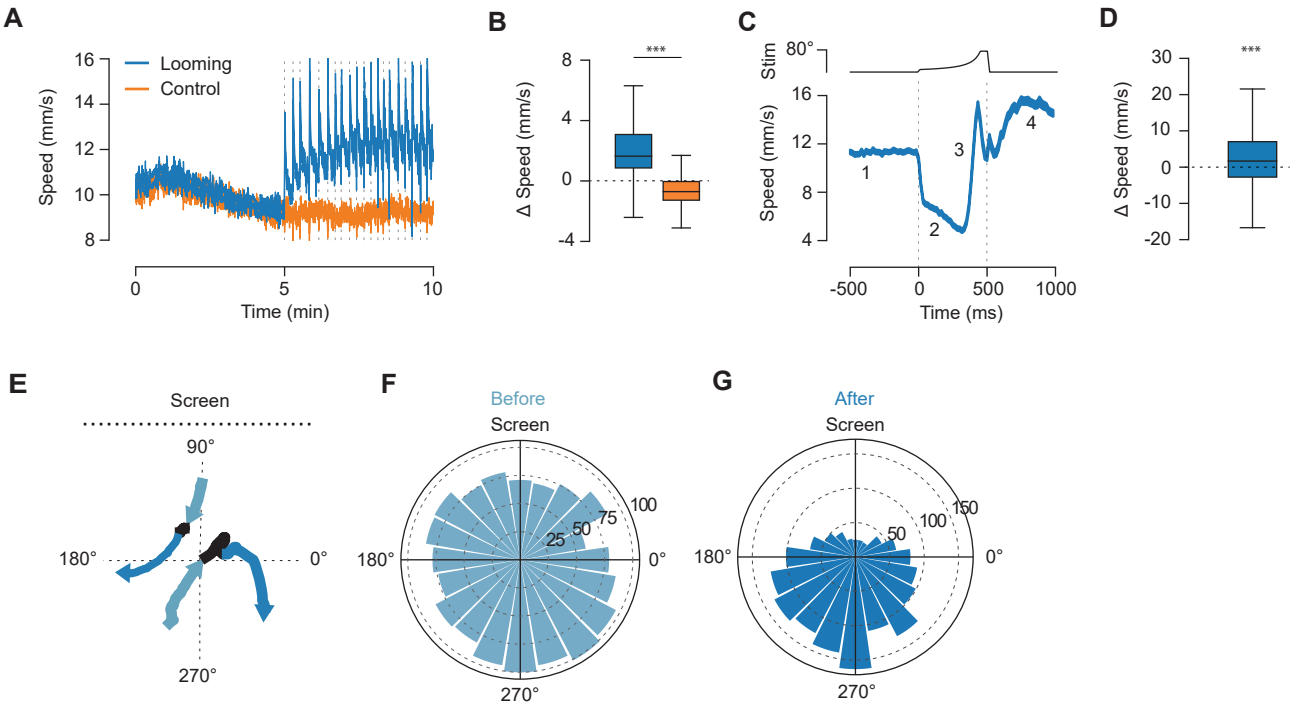

Figure 3 - *Flies run upon looming stimulation.*
A-G include walking bouts only (looming, n=280, and control, n=298). (A) Average walking speed (±sem). Dotted lines represent stimulus presentations. (B) Change in walking speed (baseline subtracted from stimulation period). In C-G n=1574 looming events. (C) Average speed (mean ± s.e.m.) around looming of walking flies. Numbers represent four stages of the response: 1- pre-looming, 2 - pause, 3 - run, 4 - post-looming. Top, visual angle of looming disk. (D) Change in walking speed (pre-stimulus baseline subtracted from post-stimulus period). (E) Two example paths during looming presentation within the chamber. Path before, during and after looming in light blue, black and dark blue, respectively. (F) Distribution of path orientation before looming. Bar heights indicate looming counts. (G) Distribution of path orientation after looming. *** denotes, p<0.0001.

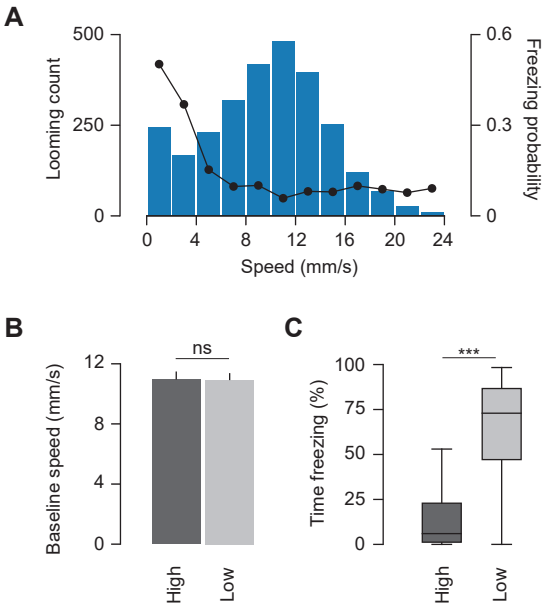

Figure 4 – *Response to looming is modulated by walking speed.*
(A) Distribution of speeds 500 ms before looming where flies were not freezing (blue bars, n=2740 loomings) overlaid with the probability of freezing after looming for each movement speed interval (black line with black dots). In a closed loop experiment flies were exposed to looming when moving slowly (low threshold, n=60) or fast (high threshold, n=56). (B) Average speed (mean ± s.e.m) during baseline period for high and low movement speed groups. (C) Percent time spent freezing during stimulation period for both groups. *** denotes, $p<0.0001$.

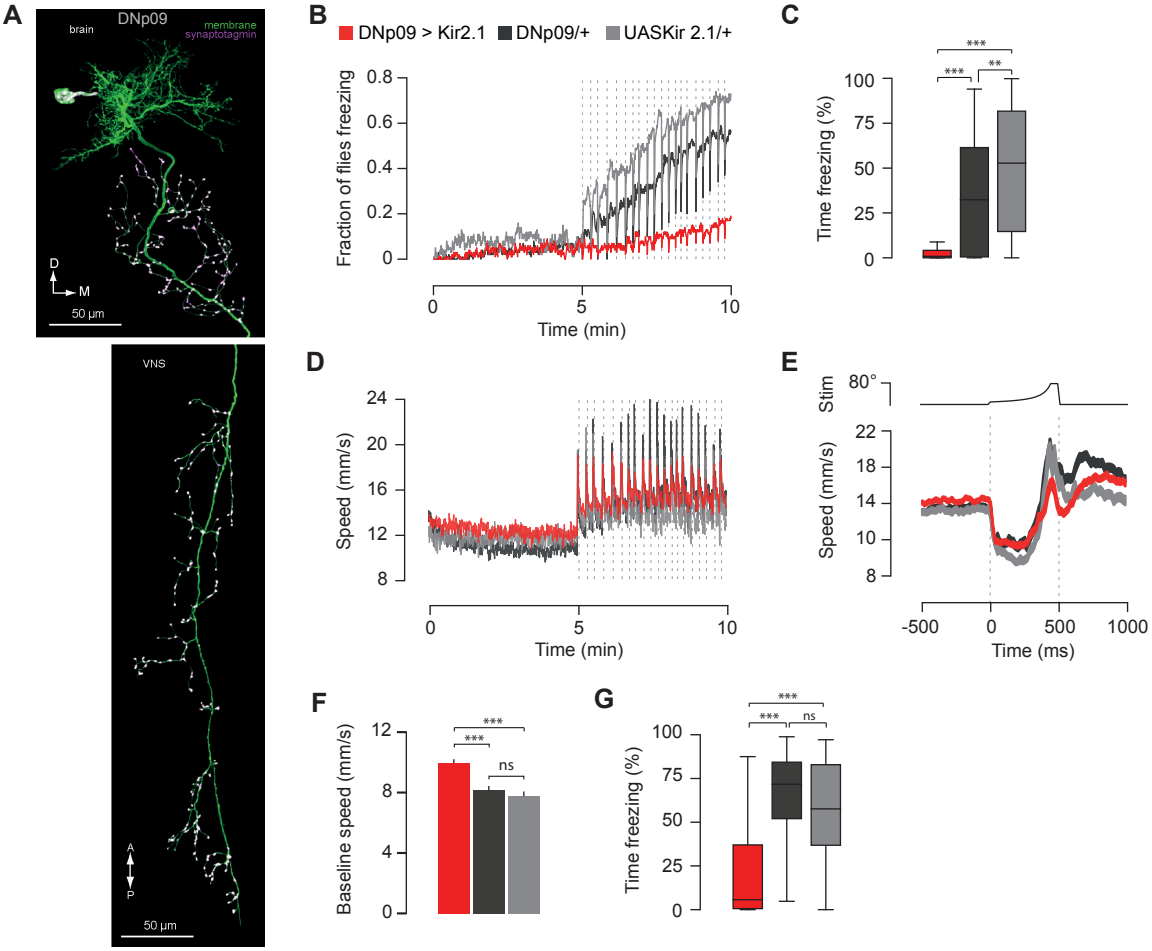

Figure 5 – *Silencing DNp09 descending neurons affects freezing but not running.*
(A) We used a split-GAL4 driver line specific for DNp09 neurons (SS1540). DNp09 morphology is depicted in this confocal image of the driver line crossed to a GFP marker with anti-synaptotagmin-HA staining. B-F include n=100 flies per genotype. (B) Fraction of flies freezing for flies with DNp09 neurons silenced (red line) or not manipulated (gray lines correspond to parental controls). Dotted lines represent stimulus presentations. (C) Percent time spent freezing during stimulation period for each genotype. D-E shows speed for periods classified as walking for flies with DNp09 neurons silenced. (D) Average walking speed (±sem). Dotted lines represent stimulus presentations. (E) Average speed (mean ± s.e.m.) around looming. Dotted lines indicate beginning and end of stimulus. (F) Average baseline speed (mean ± s.e.m.) for each genotype. (G) Percent time spent freezing in closed-loop when looming stimulation was restricted to low speeds. DNp09>Kir2.1, n=42; DNp09/+, n=49; UASKir2.1/+, n=50. ** denotes $p<0.001$ and *** $p<0.0001$.

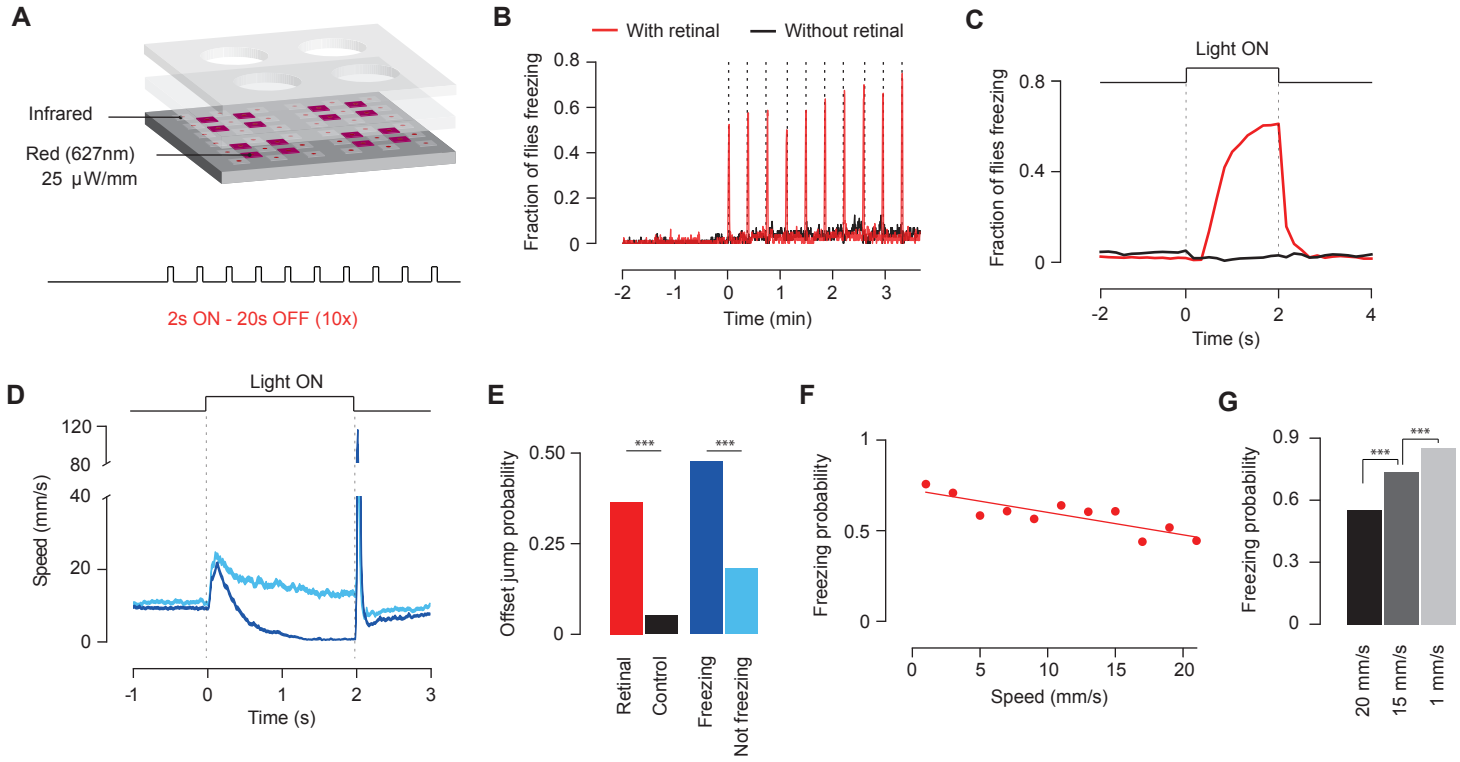

Figure 6 – *Activation of DNp09 descending neurons leads to freezing*.
(A) Schematic of experimental set-up for chrimson stimulation and stimulation protocol. Test flies supplemented with retinal (n=80) and control flies without (n=72). (B) Fraction of flies freezing. Vertical doted lines indicate light stimulation. (C) Fraction of flies freezing aligned on light activation. In e-g n=800 stimulation events with retinal, and n=720 stimulation events without. (D) Average speed (mean ± s.e.m.) aligned on light presentation for events that induced freezing (dark blue) and events that did not (light blue). (E) Probability of jumping at light offset for control and test flies (black and red bars). Probability of jumping at light offset for stimulation events of test flies that induced freezing and events that did not (dark, n=492, and light blue, n=308, bars). (F) Linear regression of probability of freezing by test flies upon red light stimulation at different pre-looming speeds. (G) Probability of freezing for DNp09>CsChrimson tested at different speeds (number of stimulation events for very high, high and low n=403, 674 and 593, respectively). *** denotes p<0.0001