Safety in numbers is mediated by motion cues and depends on lobula columnar neurons in *Drosophila melanogaster*

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Abstract

Being in a group can increase the chances of survival upon an encounter with a predator. This safety in numbers effect allows animals to decrease their defenses when in groups. Despite its wide prevalence, the mechanisms by which group size regulates defensive behaviors remains largely unknown. Here we show that fruit flies displayed a graded decrease in freezing behavior, triggered by an inescapable threat, with increasing group sizes. Furthermore, flies used the cessation of movement of other flies as a cue of threat and its resumption as a cue of safety. Finally, we found that lobula columnar neurons, LC11, mediate the propensity for freezing flies to resume moving in response to the movement of others. Taken together our results suggest that flies rely on motion cues of others to infer danger, allowing a decrease in defensive behaviors when in groups. By identifying neurons implicated in this process this study sets the stage for the search of the neuronal basis of safety in numbers.

Main body

Predation is thought to be a key factor driving group formation and social behavior (reviewed in *1*). It has long been established that being in a group can constitute an anti-predatory strategy (*2*, *3*), as it affords the use of social cues to detect predators (*4*, *5*), enables coordinated defensive responses (*6*) or simply dilutes the probability of each individual to be predated(*3*). A major consequence of the safety in numbers effect, reported in taxa throughout the animal kingdom, is that animals tend to decrease their individual vigilance (reviewed in *4*), stress levels or defensive behaviors when in a social setting (*8*). Despite its wide prevalence the mechanisms that lead to a decrease in defensive behaviors are largely unknown. Hence, in order to gain mechanistic insight into how increasing group size impacts defense behaviors, we decided to use *Drosophila melanogaster* since it allows the use of groups of varying size, the large number of replicates required for detailed behavioral analysis and genetic access to specific neuronal subtypes. Importantly, fruit flies display social behaviors in different contexts (*9*–*14*), namely social regulation of anti-predation strategies, such as the socially transmitted suppression of egg laying in the presence of predatory wasps (*10*) or the reduction in erratic turns during evasive flights when in a group, compared to when alone, in the presence of dragonflies (*14*).

To simulate a predator's attack, we used a looming stimulus (Figure 1A), an expanding dark disc, that mimics an object on collision course and elicits defense responses in visual animals, including humans (reviewed in *12*–*14*). Individually tested fruit flies respond to looming stimuli with escapes in the form of jumps (*18*, *19*), in flight evasive maneuvers (*20*) or running as well as with freezing (*21*, *22*) when in an enclosed environment. In our setup,

the presentation of 20 looming stimuli (Figure 1A) elicited reliable freezing responses for flies tested individually and in groups of up to 10 individuals (Figure 1B-E, Figure S1 shows that running and jumps are less prominent in these arenas). The fraction of flies freezing increased as the stimulation period progressed for flies tested individually and in groups of up to 5 flies; in groups of 6 to 10 individuals, the fraction of flies freezing only transiently increased with each looming stimulus (Figure 1B). The fraction of flies freezing was maximal for individuals and minimal for groups of 6 to 10, while groups of 2 to 5 flies showed intermediate responses (Figure 1B). At the level of each individual fly's behavior, flies tested alone spent more time freezing, 76.67%, IQR 39.75-90.42%, during the stimulation period than flies in any of the groups tested (Figure 1C; statistical comparisons in Table S1). Flies in groups of 2 to 5 spent similar amounts of time freezing (for groups of 2: 31.67%, IQR 9.46-64.38% and for groups of 5: 43.08%, IQR 11.79-76.50%), while flies in groups of 6 to 10 displayed the lowest levels of freezing (for groups of 6: 8.08%, IQR 3.04-17.46% and for groups of 10: 3.33%, IQR 2-7.67%) (Figure 1C; statistical comparisons in Table S1). The decrease in time spent freezing for flies tested in groups of 2 to 5, compared to individuals, was not due to a decrease in the probability of entering freezing after a looming stimulus (Figure 1D; statistical comparisons in Table S2), but rather to an increase in the probability of stopping freezing, i.e. resuming movement, before the following stimulus presentation (individually tested flies: $P(F_{exit})$=0.08, IQR 0-0.21, groups of 2: $P(F_{exit})$=0.31 IQR 0.11-0.78, groups of 5: $P(F_{exit})$=0.54 IQR 0.31-0.90) (Figure 1E; statistical comparisons in Table S3). Flies in groups of 6 to 10, were not only more likely to stop freezing (groups of 6: $P(F_{exit})$=0.93, IQR 0.80-1, groups of 10: $P(F_{exit})$= 1, IQR 0.83-1) (Figure 1E; statistical comparisons in Table S3), but also less likely to enter freezing (groups of 6: $P(F_{entry})$=0.35, IQR 0.20-0.46, groups of 10: $P(F_{entry})$= 0.21, IQR 0.10-0.36) (Figure 1C; statistical comparisons in Table S2) compared to the other conditions. The decrease in persistent freezing with the increase in group size suggests that there is a signal conveyed by the other flies that increases in intensity with the increase in the number of flies tested together.

We next examined whether flies respond to each other. We started by exploring the effect on freezing onset, as freezing has been shown to constitute an alarm cue in rodents, such that one rat freezing can lead another to freeze (*4*). We decided to focus on groups of 5 flies, which showed intermediate freezing levels (Figure 1). The onset of freezing both for individually tested flies and in groups of 5 occurred during and shortly after a looming stimulus (Figure 2A). This window, of ~1s, in principle allows for social modulation of freezing onset. Indeed, the probability of freezing onset at time *t* gradually increased with increasing numbers of flies freezing at time *t-1* (see methods), indicating that flies increase their propensity to freeze the more flies around them were freezing. This synchronization in freezing could result from flies being influenced by the other flies or simply time locking of freezing to the looming stimulus. To disambiguate between these possibilities we shuffled flies across groups, such that the virtual groups thus formed were composed of flies that where not together when exposed to looming. If the looming stimulus was the sole source of synchrony for freezing onset, then we should see a similar increase in probability of freezing by the focal fly with increasing number of 'surrounding' flies freezing in the shuffled group. We found a weaker modulation of freezing onset by the number of flies freezing in randomly shuffled groups compared to that of the real groups of 5 flies (Figure 2B; G-test, $p<0.0001$). We corroborated this result by testing single flies surrounded by 4 fly-sized magnets whose speed and direction of circular movements we could control (Figure 2C-F). During baseline, the magnets moved at the average walking speed of flies in our arenas, 12 mm/s, with short pauses as the direction of movement changed. Stopping the magnets upon the first looming stimulus and throughout the entire stimulation period led to increased time freezing (Figure 2D) and increased probability of freezing entry upon looming (Figure 2E), compared to all

controls – individuals alone, magnets not moving throughout the entirety of the experiment and the exact same protocol (magnets moving during baseline then freezing) but in the absence of looming stimuli. The transition from motion to freezing is thus important, but not sufficient to drive freezing, since flies surrounded by magnets that do not move for the entire experiment froze to individually-tested levels, but flies exposed to magnets that move and then freeze in the absence of looming stimuli did not freeze. Together these results suggest that flies use freezing by others as an alarm cue, which increases their propensity to freeze to an external threat, the looming stimulus.

As the strongest effect observed across all group sizes was on freezing exit, i.e. the resumption of movement, we asked whether the propensity to exit freezing was also dependent on the number of surrounding flies that were freezing. To this end, we performed a similar analysis as for freezing onset and found that the higher the number of flies freezing the lower the probability of the focal fly to exit from freezing. This effect was also decreased in shuffled groups (Figure 3A; G-test, $p<0.0001$). We then examined the contribution of mechanosensory signals in the decrease in freezing and found that collisions between flies play a minor role in the observed effect (Figure S2; statistical comparisons in Tables S4-S6), contrary to what happens with socially-mediated odor avoidance (*9*). Next, we explored our intuition that motion cues from the other flies were the main players affecting exit from looming-triggered freezing. We formalized the motion signal (Figure 3B), perceived by a focal fly, as the summed motion cues produced by the other four surrounding flies (we multiplied the speed of each fly by the angle on the retina, a function of the size of the fly and its distance, to the focal fly, Figure 3B). We then analyzed separately the summed motion cue perceived by focal flies during freezing bouts that terminated before the following looming stimulus (freezing with exit) and continuous freezing bouts (with no breaks in between looming stimuli) (representative examples in Figure 3B). Freezing bouts with exit had higher motion cue values (Figure 3C) compared to continuous bouts ($p<0.0001$, Freezing without exit=0.64 IQR: 0.00-2.11, Freezing with exit=2.79 IQR: 1.28-5.08). This difference raised the possibility that motion cues produced by others could constitute a safety signal leading flies to resume activity.

To test whether motion cues from others constitute a safety signal, we manipulated the motion cues perceived by the focal fly, while maintaining the number of flies in the group constant. An increase in the social motion cues, should enhance the group effect, and hence decrease the freezing responses of a focal fly. We compared groups of 5 wild-type flies with groups of 1 wild-type and 4 blind flies (Figure 3D). Blind flies don't perceive the looming stimulus and walk for the duration of the experiment; when a focal fly freezes surrounded by 4 blind flies it is thus exposed to a higher motion signal during the stimulation period than a focal fly in a group of 5 wild-type flies (Figure 3D). When surrounded by blind flies, the fraction of focal flies freezing throughout the stimulation period was lower than the fraction of flies freezing in a group of wild-type flies (Figure 3E). Further, the increase in motion cues in groups with blind flies decreased the amount of time a fly froze compared to that of groups of wild-type flies (6.17% IQR 2.17-15.25% versus 19.58% IQR 8.20-57.12; $p<0.0001$) (Figure 3F). This reduction in freezing resulted mostly from a decreased probability of freezing entry (wild-type groups: $P(F_{entry})=2.57$ IQR 0.15-0.39, groups with blind flies: $P(F_{entry})=0.49$ IQR 0.25-0.61, $p<0.0001$) (Figure 3G) and slightly increased probability of exiting freezing (wild-type groups: $P(F_{exit})= 0.83$ IQR 0.39-1, groups with blind flies: $P(F_{exit})= 0.89$ IQR 0.71-1) (Figure 3H). Hence, a focal fly surrounded by 4 blind flies behaves similarly to flies in groups of more than 6 individuals. Importantly, the decrease in persistent freezing was not due to an increased role of collisions on freezing breaks (Figure S3). We further tested whether any type of visual signal could alter individual freezing in the same manner as the motion cues generated by flies in the group, by presenting a visual stimulus with randomly appearing

black dots with the same change of luminance as the looming stimulus but without motion. (as in *19*) 4.5 seconds after each looming presentation. This stimulus, which could work as a distractor, did not alter the proportion of time freezing nor the probability of freezing entry or exit (Figure S4).

Having identified motion cues of others as the leading source of the group effect on freezing, we decided to test the role of visual projection neurons responsive to the movement of small objects, lobula columnar 11 (LC11) (*23*, *24*) in our paradigm. The behavioral relevance of these neurons was as yet unidentified. We used two fly lines, an *LC11-GAL4* (*24*) and an *LC11-splitGAL4* (*23*), to drive the expression of Kir 2.1 (*25*), a potassium channel that hyperpolarizes neurons. Anatomically, both fly lines encompass LC11 neurons (Figure 4A and B) but *LC11-splitGAL4* is more specific as it does not contain the neurons in the subesophageal zone that descend to the thoracico-abdominal ganglion present in the *LC11-GAL4*. Constitutively expressing Kir 2.1 in LC11 neurons did not alter looming-triggered freezing of flies tested individually, when compared to parental controls (Figure S5). Conversely, for LC11-silenced flies tested in groups of five, the fraction of flies freezing increased throughout the experiment (Figure 4A and B). Moreover, experimental flies in groups of 5 froze longer (~6 fold increase for *LC11-GAL4 >Kir2.1*, and ~2 and 7 fold increase for *LC11-splitGAL4 >Kir2.1* compared to parental controls) (Figure 4C), which was not due to an increase in the probability of freezing entry (Figure 4D), but rather to a decrease in the probability of freezing exit (Figure 4E) (*LC11-GAL4 >Kir2.1* 0.12 IQR 0.05-0.50, *LC11-GAL4/+* 0.786 IQR 0.44-1 and *Kir2.1/+* 0.83 IQR 0.5-1, p<0.; *LC11-splitGAL4 >Kir2.1* 0.24 IQR 0.06-0.67, *LC11-splitGAL4/+* 1 IQR 0.67-1 and *Kir2.1/+* 0.50 IQR 0.26-0.86, p=0.0001). LC11 neurons have been shown to be maximally responsive to moving objects with an angular size of 2.2º and display half amplitude responses to a 4.4º object (*24*) corresponding, in our arenas, to stronger LC11 responses to moving flies further away (a fly at the maximal possible distance, 6.5 cm has an angular size of 2.6º while flies with a 4.4º angular size would be at a distance of ~3.4 cm from a focal fly). Interestingly, silencing these neurons did not affect the use of freezing as an alarm cue, since these flies showed an increased probability of freezing the more surrounding flies were freezing (Figure S6). Finally, expressing Kir2.1 in another LC neuron class, LC20, (*23*) which are not know to respond to small moving objects, does not alter group behavior (Figure S7). In summary, silencing LC11 neurons renders flies less sensitive to the motion of others, specifically decreasing its use as a safety cue that down regulates freezing.

In summary, flies in groups show a reduction in freezing responses that scales with group size. Detailed behavioral analysis together with behavioral and genetic manipulations, allowed us to identify freezing as a sign of danger and activity as a safety cue. These findings are consistent with the hypothesis that safety in numbers may partially be explained by the use of information provided by the behavior of others. Moreover, we show that visual projection LC11 neurons are involved in processing motion cues of others to down regulate freezing. Other LC neurons have been implicated in processing visual stimuli in social contexts, namely *fru+* LC10a while the male follows the female during courtship (*26*). It will be interesting to study to what extent there is specificity or overlap in projection neurons for behaviors triggered by the motion of others.

Motion plays a crucial role in predator-prey interactions. Predator and prey both use motion cues to detect each other using these to make decisions about when and how to strike or whether and how to escape. Furthermore, prey animals also use motion cues from other prey as an indirect cue of a predator's presence (*4*, *27*, *28*). We believe that the current study opens a new path to study how animals in groups integrate motion cues generated by

predators, by their own movement, and that of others to select the appropriate defensive responses.

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Material and methods

Fly lines and husbandry

Flies were kept at 25 °C and 70% humidity in a 12 h:12 h dark:light cycle. Experimental animals were mated females, tested when 4–6 days old.

Wild-type flies used were Canton-S. LC11-splitGAL4 line *w[1118]; P{y[+t7.7] w[+mC]=R22H02-p65.AD}attP40; P{y[+t7.7] w[+mC]=R20G06-GAL4.DBD}attP2*, LC11-GAL4 w[1118]; P{y[+t7.7] w[+mC]=GMR22H02-GAL4}attP2 and LC20-splitGAL4 *w[1118]; P{y[+t7.7] w[+mC]=R35B06-GAL4.DBD}attP2 PBac{y[+mDint2] w[+mC]=R17A04-p65.AD}VK00027* and w[*] norpA[36] were obtained from the Bloomington stock center. *10XUAS-IVS-eGFPKir2.1 (attP2)* flies were obtained from the Card laboratory at Janelia farm. UAS-CD8::GFP; lexAop-rCD2::RFP (*1*) recombined with nSyb-lexA.DBD::QF.AD (obtained from the Bloomington stock center) were obtained from Wolf Huetteroth (University Leipzig).

Behavioral apparatus and visual stimulation

We imaged unrestrained flies in 5 mm thick, 11º slanted polyacetal arenas with 68 mm diameter (central flat portion diameter 32 mm). Visual stimulation (twenty 500 ms looming stimuli, a black circle in a white background, with a virtual object length of 10 mm and speed 25 cm/s (l / v value of 40 ms) as in (*2*)) was presented on an Asus monitor running at 144 Hz, tilted 45º over the stage (Figure 1A). For the experiments with random dots, 4.5 s after the looming presentation we presented a visual stimulus consisting of appearing black dots at random locations on the screen to reach the same change in luminance as the looming stimulus (*2*).

The stage contained two arenas, backlit by a custom-built infrared (850 nm) LED array. Videos were obtained using two USB3 cameras (PointGrey Flea3) with an 850 nm long pass filter, one for each arena.

For the experiments with the magnets (Figure 2), we used an electromechanical device developed by the Scientific Hardware Platform at the Champalimaud Centre for the Unknown. It consists of an adapted setup in which a rotating transparent disc with 5 incorporated neodymium magnets moves under the arena. A circular movement is induced by an electric DC gearhead motor transmitted via a belt to the disc. This allows the movement of magnetic material placed on the arena to move around in synchronized motion. The motor is controlled by a custom-made electronic device, connected to the computer and using a dedicated Champalimaud Hardware Platform software. For the experiments of freezing magnets during stimulation, with or without stimulus, the magnets rotated at 12 mm/s with a change in direction every 50 s during the baseline; as soon as the stimulation period started, in synchrony with the first looming stimulus, the magnets ceased movement, until the end of the experiment.

Video acquisition and analysis

Videos were acquired using Bonsai (*3*) at 60 Hz and 1280 width x 960 height resolution. We used IdTracker (*4*) to obtain the position throughout the video of each individual fly. The video and the IdTracker trajectories file were then fed to the 'Fly motion quantifier', developed by the Scientific Software Platform at the Champalimaud Centre for the Unknown (https://bitbucket.org) in order to obtain the final csv file containing not only position and speed for each fly, but also pixel change in a region of interest (ROI) around each fly defined by a circle with a 30 pixel radius around the center of mass of the fly.

321 Data analysis

322 Data were analyzed using custom scripts in spyder (python 3.5). Statistical testing was done
323 in GraphPad Prism 7.03, and non-parametric, Kruskal-Wallis followed by Dunn's multiple
324 comparison test or Mann-Whitney tests were chosen, as data were not normally distributed
325 (Shapiro-Wilk test). Probabilities were compared using the χ2 contingency test in python (G-
326 test).

327 Freezing was classified as 500 ms periods with a median pixel change over that time period
328 < 30 pixels within the ROI. The proportion of time spent freezing was quantified as the
329 proportion of 500 ms bins during which the fly was freezing.

330 We calculated the proportion of freezing entries upon looming and exits between looming
331 stimuli (Figure 1) using the following definitions: 1) freezing entries corresponded to events
332 where the fly was not freezing before the looming stimulus (a 1 s time window was used)
333 and was freezing in the first 500 ms bin after the looming stimulus; 2) freezing exits were
334 only considered if sustained, that is when the fly froze upon looming but exited from freezing
335 and was still moving by the time the next looming occurred, i.e. the first 500 ms bin after
336 looming the fly was freezing and in the last 500 ms bin before the next looming the fly was
337 not freezing.

338 To determine the time of freezing onset or offset (Figure 2A,B and Figure 3A), we used a
339 rolling window of pixel change (500 ms bins sliding frame by frame) and the same criterion
340 for a freezing bin as above). Time stamps of freezing onset and offset were used to calculate
341 the probability of entering and exiting freezing as a function of the number of flies freezing.
342 For freezing entries after looming as well as probabilities of entering and exiting freezing, we
343 considered only instances in which the preceding 500 ms bin was either fully non-freezing or
344 freezing. To determine the numbers of others freezing at freezing entry or exit we used a 10
345 frame bin preceding the freezing onset or offset timestamp.

346 Distances between the center of mass of each fly were calculated using the formula
347 $\sqrt{(x2-x1)^2+(y2-y1)^2}$, and we considered a collision had taken place when the flies
348 reached a distance of 25 pixels. Motion signal was determined as $\sum speed \times$
349 $angle\ on\ the\ retina\ (\theta)$ where $\theta = 2\ arctan\left(\frac{size}{2 \times distance}\right)$.

350 To analyze the motion signal for freezing bouts with and without exit (Figure 3B, C), we
351 defined freezing bouts with exit as bouts where flies were freezing in the 500 ms following
352 the looming stimulus offset and resumed moving before the next looming stimulus (up until
353 the last 500 ms before the looming stimulus onset) and freezing bouts without exit as those
354 where freezing persisted until the next looming.

355

356 Imaging

357 LC11-GAL4>UAS-CD8::GFP; nSyb-lexA>lexAop-rCD2::RFP, LC11-splitGAL4>UAS-
358 CD8::GFP; nSyb-lexA>lexAop-rCD2::RFP and LC20-splitGAL4>UAS-CD8::GFP; nSyb-
359 lexA>lexAop-rCD2::RFP three day-old females were processed for native fluorescence
360 imaging as in (*5*). In brief, brain were dissected in ice-cold 4% PFA and post-fixed in 4% PFA
361 for 40-50 min. After 3x 20 min washes with PBST (0.01 M PBS with 0.5% TritonX) and 2x 20
362 min washes in PBS (0.01 M) brains were embedded in Vectashield and imaged with a 16x
363 oil immersion lens on a Zeiss LSM 800 confocal microscope.

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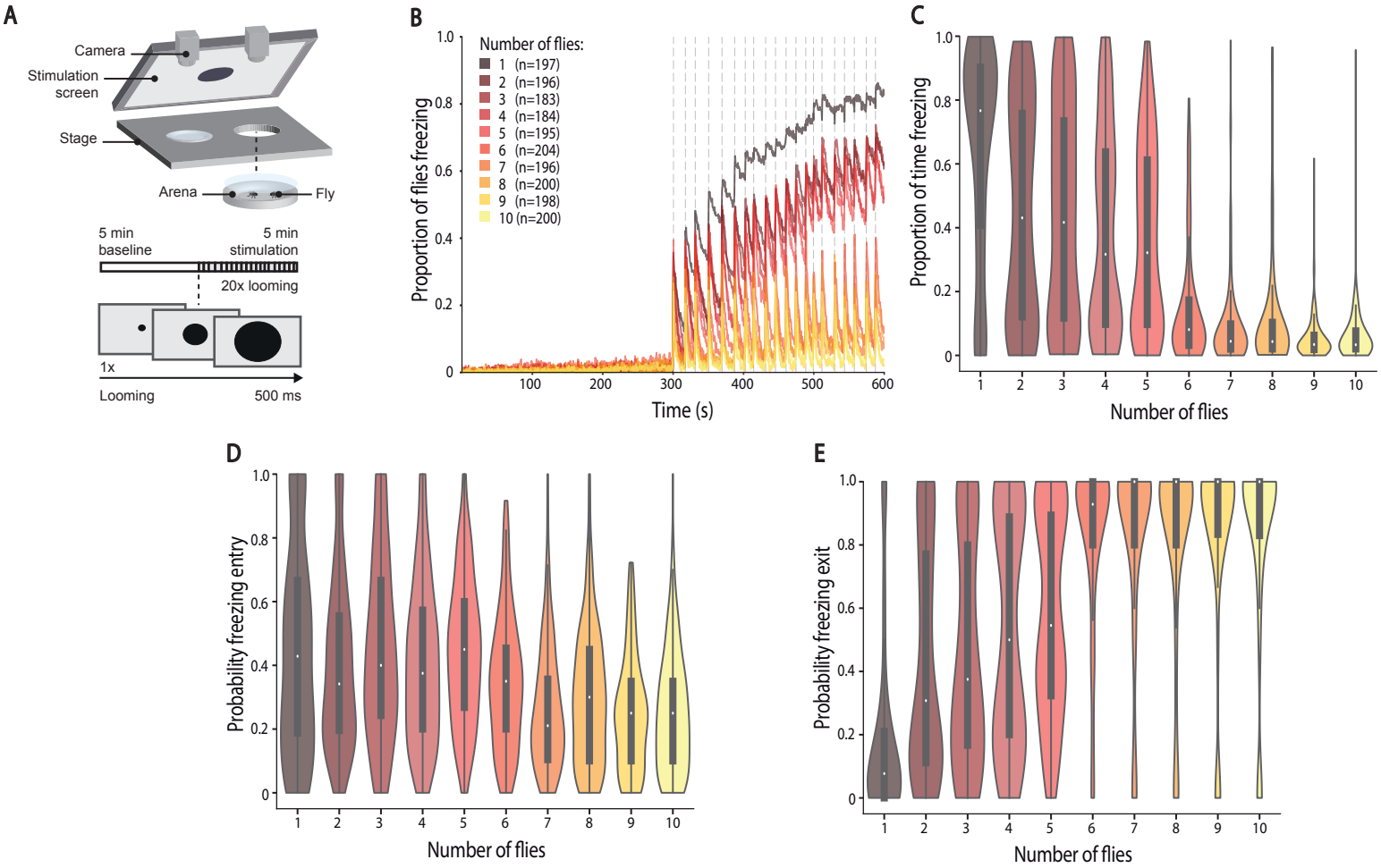

Fig. 1. Analysis of the group effect on freezing responses. A) Experimental setup and protocol. We tested individuals and groups of up to 10 flies in backlit arenas imaged from above. After a 5-minute baseline flies were exposed to twenty 500 ms looming presentations, every 10-20 s, indicated by vertical dashed lines. B) Proportion of flies freezing throughout the experiment. C–E) Violin plots representing the probability density of individual fly data bound to the range of possible values, with boxplots. C) Proportion of time spent freezing throughout the experiment. Statistical comparisons between conditions presented in Table S1. D) Probability of freezing entry in the 500 ms bin following looming presentation. Statistical comparisons between conditions presented in Table S2. E) Probability of freezing exit in the 500 ms bin before the following looming stimulus. Statistical comparisons between conditions presented in Table S3.

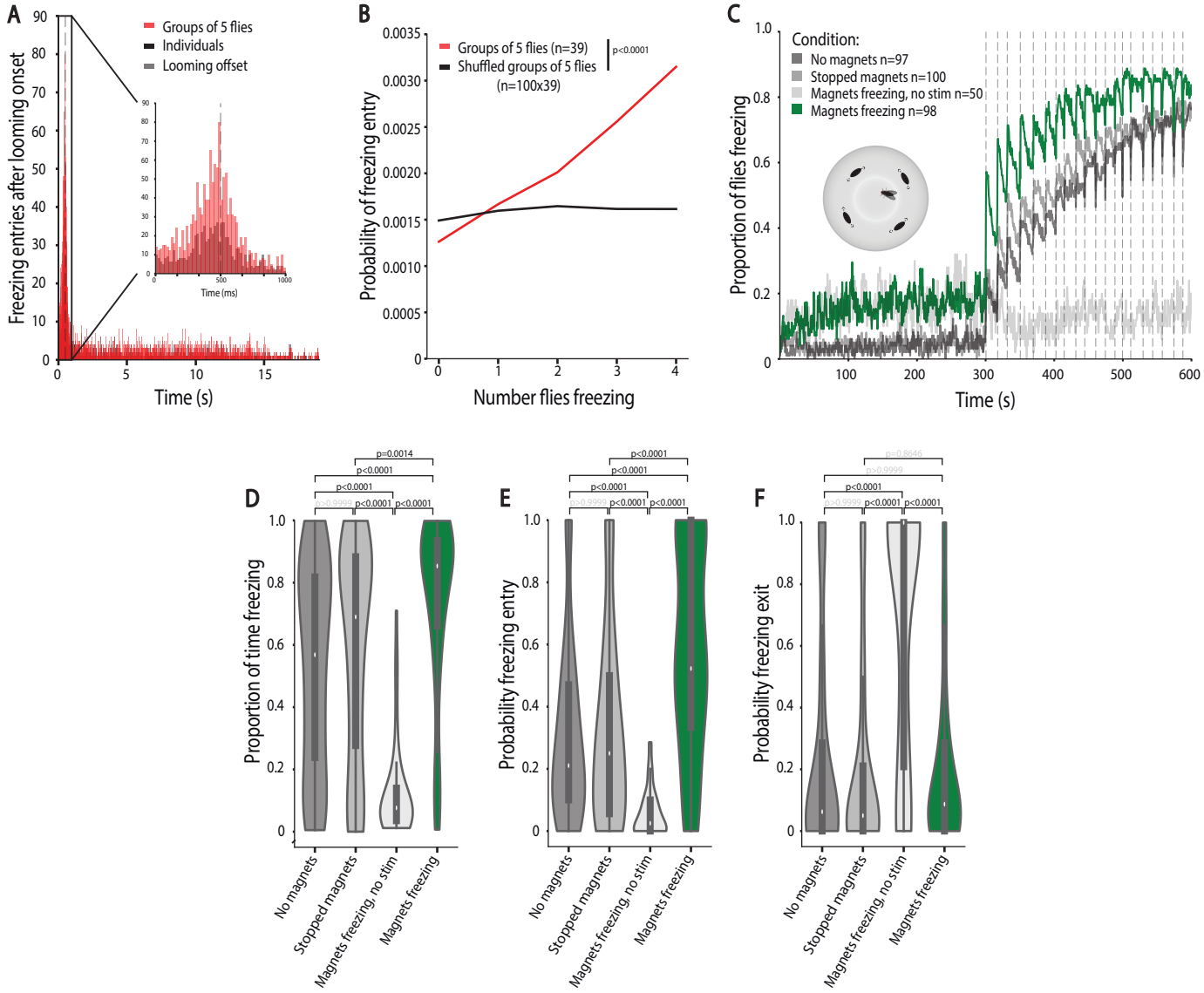

Fig. 2. The group effect on individual freezing entry. A) Distribution of freezing entries after looming onset for flies tested individually and in groups of 5. B) Probability of freezing entry at time t as a function of the number of other flies freezing at t-1 (see methods). C-F) Simulating groups of 5 using movable magnets. C) Proportion of flies freezing throughout the experiment. D–F) Violin plots representing the probability density of individual fly data bound to the range of possible values, with boxplots. D) Proportion of time spent freezing throughout the experiment. E) Probability of freezing entry in the 500 ms bin following looming presentation. F) Probability of freezing exit in the 500 ms bin before the following looming stimulus. P-values result from Kruskal-Wallis statistical analysis followed by Dunn's multiple comparisons test.

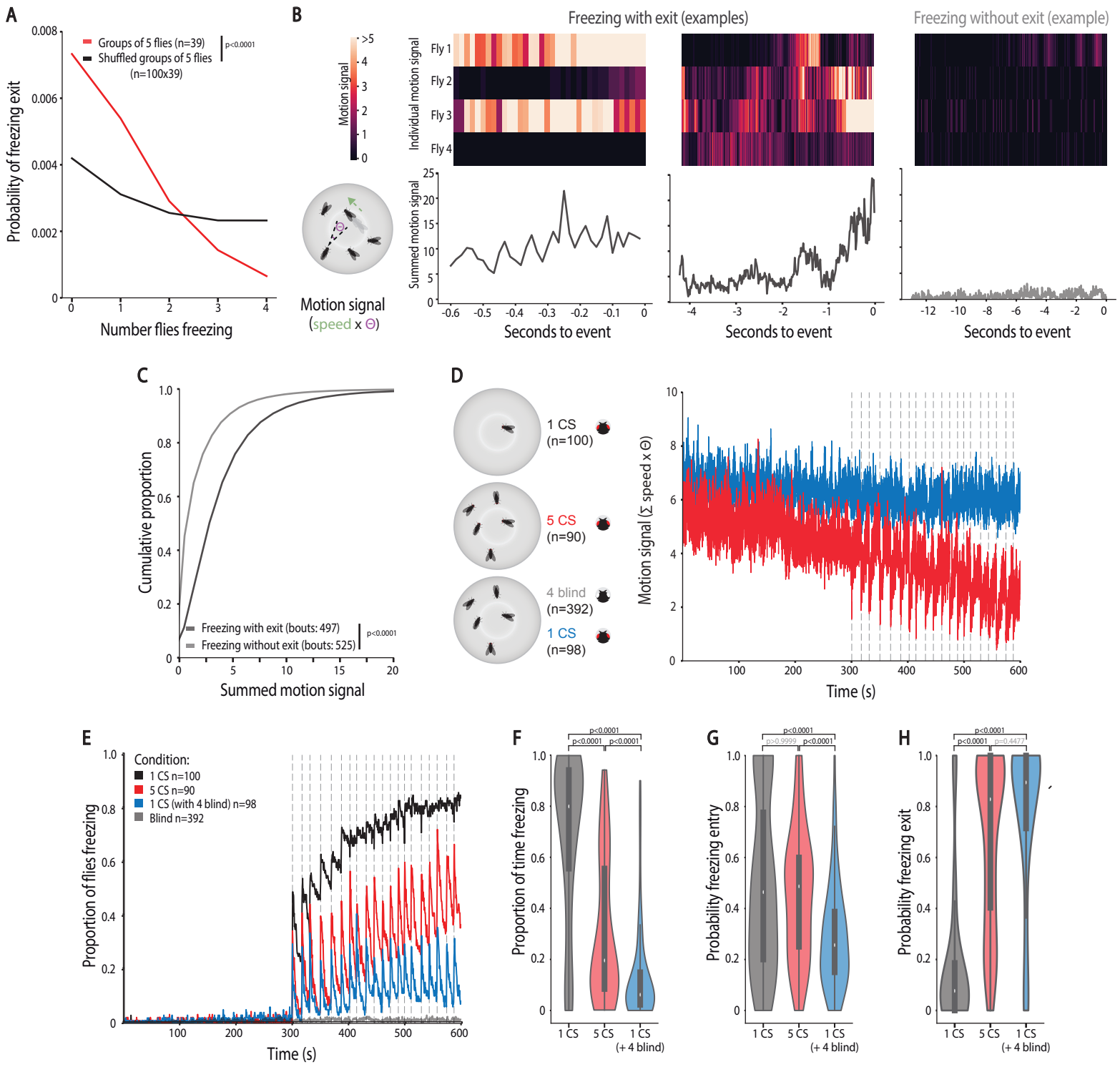

Fig. 3. The group effect on individual freezing exit. A) Probability of freezing exit at time t as a function of the number of other flies freezing at t-1 (see methods). B) The motion signal is formalized as the other fly's speed multiplied by the angle on the retina of in the group (schematic). Representative examples of the motion signal starting in the 500 ms bin after looming offset by a focal fly until freezing exit or the end of the inter-looming period (without freezing exit): heatmaps show the motion signals of the 4 surrounding flies and the line graphs show the summed motion signal. C) Cumulative distributions of the summed motion signals. D-H) Manipulating the motion signal in groups of 5. D) The summed motion signal of surrounding flies for groups of 5 wild-type flies and groups with one wild-type and 4 blind flies. E) Proportion of flies freezing throughout the experiment. F–H) Violin plots representing the probability density of individual fly data bound to the range of possible values, with boxplots. F) Proportion of time spent freezing throughout the experiment. G) Probability of freezing entry in the 500 ms bin following looming presentation. H) Probability of freezing exit in the 500 ms bin before the following looming stimulus. P-values result from Kruskal-Wallis statistical analysis followed by Dunn's multiple comparisons test.

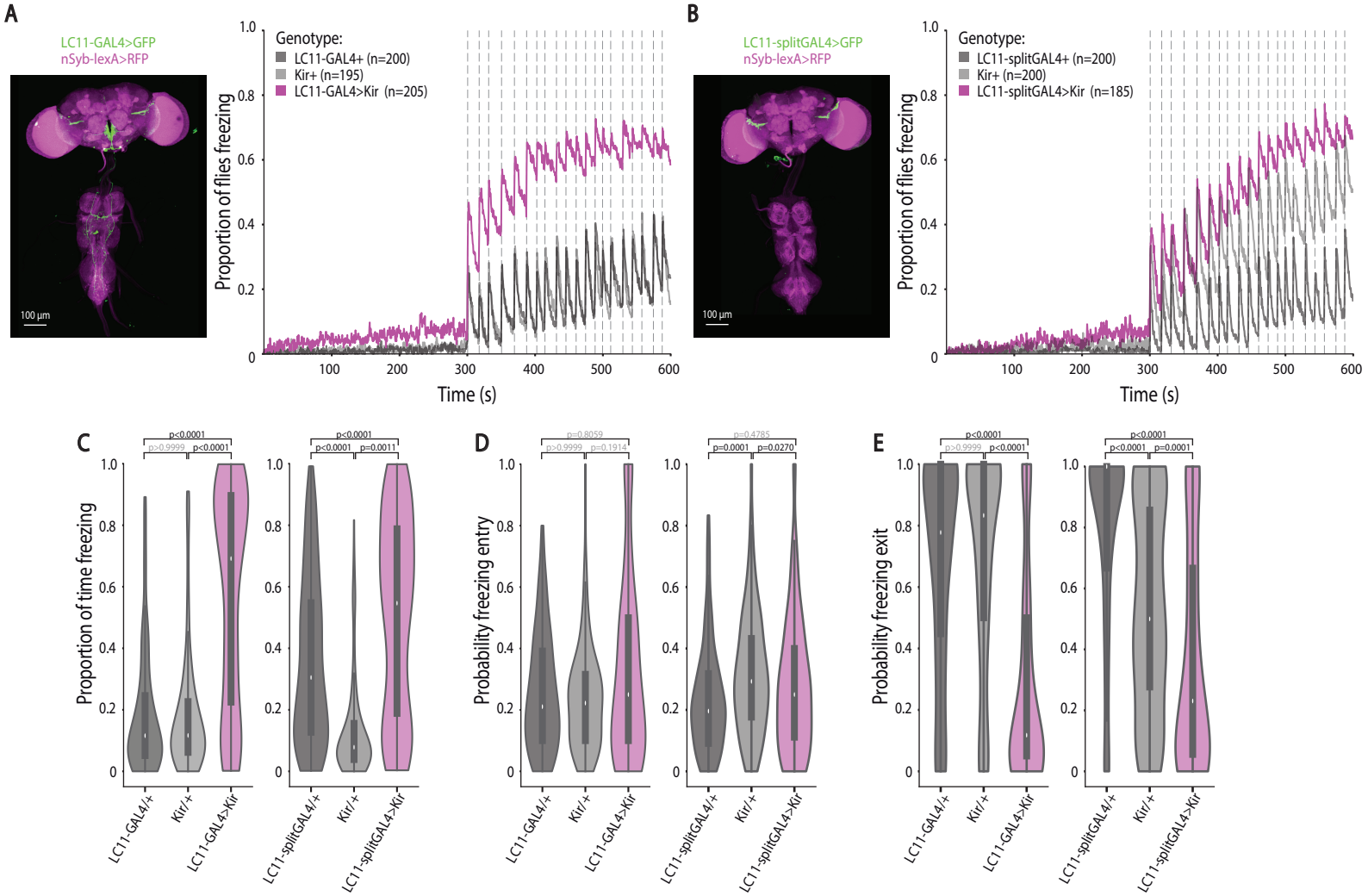

Fig. 4. Manipulating lobula columnar neurons 11 (LC11). A–B) Anatomy (scale bars, 100 µm) and proportion of flies freezing throughout the experiment in groups of 5, for A) *LC11-GAL4>Kir2.1* and B) *LC11-splitGAL4>Kir2.1* depicted in purple and parental controls (grey). C–E) Violin plots representing the probability density distribution of individual fly data bound to the range of possible values, with boxplots. C) Proportion of time spent freezing throughout the experiment. D) Probability of freezing entry in the 500 ms bin following looming presentation. E) Probability of freezing exit in the 500 ms bin before the following looming stimulus. P-values result from Kruskal-Wallis statistical analysis followed by Dunn's multiple comparisons test.