Title: Drosophila learn about properties of objects through physical interaction

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Abstract:

Animals interact with unfamiliar objects to learn about their properties and guide future behavior, but the underlying neurobiological mechanism is not well understood. Here, we developed a behavioral paradigm in which freely walking *Drosophila melanogaster* are repeatedly guided to spherical objects using a visual cue. Flies exhibited diverse and structured object interaction motifs, including "ball pulling", and "ball walking", that evolved over time. Notably, flies developed a strong preference for immobile over mobile objects, despite their near identical appearance, suggesting they learn about object stability through physical interaction. This preference was impaired by silencing specific hΔ neurons in the fan-shaped body, a circuit known for spatial navigation through vector computations. hΔ neurons also modulated object interaction motifs and fidelity of following visual guidance cues, pointing to a role in balancing goal-directed and exploratory behaviors. These findings establish *Drosophila* as a model for investigating how internal representations and multimodal feedback contribute to adaptive object interaction.

Introduction:

Across the animal kingdom, organisms must contend with physical challenges unique to their local environments. A key challenge many species face is deciding which objects, among a multitude of animate and inanimate elements in their surroundings, are safe to approach and which should be avoided. The ability to physically engage with unfamiliar objects (hereafter "object interactions") enables organisms to acquire information about an object's properties and is widespread across taxa (Glickman and Sroges 1966; Lobo et al. 2014; Christensen et al. 2021; Galpayage Dona et al. 2022). Many species exhibit an intrinsic bias toward exploring novel objects, a behavioral trait thought to facilitate learning and environmental assessment (Berlyne 1950; Ibañez et al. 2023; Kidd and Hayden 2015; Ogasawara et al. 2022). Through such interactions, animals can gather critical data, such as whether an object poses a threat or offers a benefit, that cannot be inferred from vision or scent alone (Galpayage Dona et al. 2022; Lambert et al. 2017). Once familiar with an object, organisms can then choose among context-appropriate behavioral responses that enhance survival or reproductive success (Lambert et al. 2021; Liu et al. 2009; Schrauf, Huber, and Visalberghi 2008). Indeed, object interactions underpin a wide range of adaptive behaviors, including foraging, courtship display, and nest construction building (Bird and Emery 2009; Fayet, Hansen, and Biro 2020; Kelley and Endler 2012; Laumer et al.

2017; McCoy et al. 2019; Mizuuchi et al. 2018). Despite their broad ecological relevance, however, the neural mechanisms that regulate how animals engage with novel objects remain poorly understood.

The common fruit fly (*Drosophila melanogaster*) is a leading model for investigating the biological basis of complex behaviors, owing to its extensive genetic toolkit and accessibility. Much of what we know about *Drosophila* complex behavior comes from studies of social interactions such as courtship, aggression, and group dynamics (Alekseyenko et al. 2014; Billeter et al. 2009; Coen et al. 2014; Deutsch et al. 2019; Hoopfer et al. 2015; Jiang et al. 2020; Schretter et al. 2020; Hindmarsh Sten et al. 2025; Sun et al. 2020; Wang et al. 2011; Dukas 2020). In contrast, far less is understood about how flies interact with the physical environment beyond the social context. Particularly, how they physically engage with objects, whether they can learn about physical properties of objects, and how such learning may influence future decisions.

Flies are known to form associations between spatial locations and sensory cues, including visual (Ofstad, Zuker, and Reiser 2011) and gustatory stimuli (Corfas, Sharma, and Dickinson 2019; Kim and Dickinson 2017; Behbahani et al. 2021). However, it remains unclear whether similar associations extend to the physical attributes of objects themselves. During flight, flies tend to avoid small objects, presumably to minimize risk of collision or predation (Cheng, Colbath, and Frye 2019; Maimon, Straw, and Dickinson 2008; Mongeau et al. 2019). Interestingly, this aversion can be reversed when small objects are paired with attractive sensory cues (Cheng, Colbath, and Frye 2019). Flies also show a preference for perching on taller structures, possibly to gain visual vantage points for environmental assessment, mirroring behaviors seen in naturalistic settings (Robie, Straw, and Dickinson 2010). Together, these findings suggest that flies can flexibly evaluate and respond to object features in a context-dependent manner. However, whether and how such object-related decision-making occurs during terrestrial exploration remains largely unexplored. This gap is due, in part, to the difficulty of eliciting repeated, sustained interactions between freely walking flies and inanimate objects.

We previously demonstrated that the movement of freely walking flies can be precisely guided toward a spatial target using a dynamic visual cue (Iwasaki et al. 2025). In the present study, we extend this approach to direct flies toward discrete objects, enabling repeated and sustained interactions suitable for detailed behavioral analysis. Using this visual guidance paradigm, we found that flies are capable of complex and structured object interactions with a small spherical target (ball). These interactions consisted of multiple distinct motor motifs, some involving coordinated, multi-step behaviors not previously described in *Drosophila*. Flies flexibly modulated their interactions based on object properties: they consistently preferred immobile over mobile balls and were able to discriminate between the two even when interacting with both repeatedly in the same session. To investigate whether circuits involved in spatial navigation contributes to this preference, we examined the role of hΔ neurons in the central complex – cells known to participate in vector-based navigation (Hulse et al. 2021; Lyu, Abbott, and Maimon 2022). Silencing specific hΔ subtypes disrupted the development of object preference and altered the expression of distinct motor motifs. These findings point to a previously unrecognized role for internal spatial computations, possibly involving vector

arithmetic, in guiding object-directed interactions. Together, our results uncover new layers of physical and cognitive sophistication in *Drosophila* behavior and provide a framework for mechanistic studies of adaptive object engagement.

Results:

Visually guided flies engage in persistent interactions with a spherical object

Previous work has shown that freely walking flies can be visually guided to specific spatial destinations using a rotating "pinwheel" cue, which dynamically adjusts its rotation based on the fly's position and orientation (Iwasaki et al. 2025). To test whether this visual navigation system could promote interactions with discrete objects, we used it to repeatedly direct flies toward a small spherical object (2.38 mm in diameter, 10 mg mass; Figure 1A). The guidance cue was deactivated once the fly approached within a 5-mm radius of the object, allowing the fly to freely decide whether to interact (Figure 1B). Flies were not required to engage with the object and could leave without penalty.

We found that most guided flies initiated active, repeated interactions with the ball, often displacing it across the arena over extended periods (Figures 1C–1D). By contrast, flies that were not visually guided interacted with the ball only rarely. Guided flies moved the ball on approximately 26% of their visits, with some reaching a maximum ball-pushing probability of approximately 50% (Figures 1F–1H).

To test whether the increase in object interaction could be explained by passive exposure to the visual cue or simple proximity to the ball, we conducted two control experiments. One possibility was that the presence of the visual stimulus itself might enhance the salience of the nearby ball, thereby increasing the likelihood of interaction. First, presenting stationary (non-rotating) pinwheels (centered either on the fly or on the ball) did not increase interaction frequency, although it slightly reduced average fly-to-ball distance (Figures S1A, S1B). Second, reducing the arena size to force proximity between flies and balls failed to produce comparable levels of interaction (Figures S2). These results indicate that dynamic, trajectory-coupled visual guidance, not mere pinwheel presence or spatial closeness, is critical for promoting sustained fly-ball interactions.

Guided flies exhibit a repertoire of distinct interaction motifs with a spherical object

During initial experiments, we observed that guided flies interacted with the ball using a range of distinct strategies (Videos 1–4). These included walking across the ball without displacing it, displacing the ball while jumping off it, and pulling or pushing the ball from the side while walking on the ball. These observations suggested that flies engage with the object through a variety of structured behaviors. To quantify this diversity, we measured three kinematic features for each interaction: ball displacement during interaction (before the fly exited the ball), ball displacement after interaction (after exit), and fly movement after interaction (following exit). Fly movement after interaction was analyzed over a 200-millisecond window, based on the finding that most jump events occurred within this period (Figures S4A, S4B).

We applied k-means clustering to 29,982 interaction events collected from 48 male flies, identifying five distinct behavioral motifs (Figure 2A). These included “walk-off” events, in which flies crossed over the ball without substantial movement of either the fly or the ball; “ball pull” events, where the fly displaced the ball while dismounting with minimal post-interaction fly movement; and “ball walk” events, characterized by the fly walking along the side of the ball while rolling it. Two additional clusters captured jump-like behaviors, defined by rapid post-interaction fly movement with varying degrees of ball displacement (Figure 2B). Importantly, these interaction motifs were not restricted to particular individuals: most flies contributed to all five clusters, with no single fly accounting for more than 12% of the events in any one group (Figure S4C).

Interactive motifs follow distinct patterns of temporal development

Having identified multiple fly-ball interaction motifs through clustering (Figure 2), we next asked whether these motifs were used consistently throughout the experiment or evolved differently over time. Divergence in their temporal trajectories would suggest that different interaction strategies may be governed by distinct regulatory processes in the nervous system.

To address this, we focused on two distinct behavioral motifs: “ball pull” and “ball walk.” Ball-pull events were defined by displacement of the ball after the fly departed, whereas ball-walk events involved the fly actively moving the ball while walking along its side (Figure 3Ai–iv). Full definitions and classification criteria for these motifs are detailed in the Methods. These two behaviors exhibited markedly different temporal profiles. Ball-walk frequency gradually increased over the course of the experiment, peaking in the later hours, whereas ball-pull frequency rose initially but plateaued early (Figure 3B). This divergence was confirmed both in raw event counts and in normalized comparisons of early and late time bins: flies performed significantly more ball walks in the second half of the session, while the frequency of ball pulls remained unchanged (Figures 3C, 3D; see also Figures S5A, S5B).

A cluster-based analysis confirmed the same pattern, independently supporting the conclusion that these motifs follow distinct temporal trajectories (Figures S5E, S5F). Notably, the distance the ball was displaced during ball walks increased significantly over time (Figure 3G), indicating that flies engaged in longer ball walks as the experiment progressed. In contrast, the magnitude of displacement during ball pulls remained stable (Figure 3F). These findings suggest that the increase in ball-walk frequency and displacement is not merely due to increased activity.

We also observed that the frequency of ball walks peaked as the frequency of jumps declined (Figures S5B to S5D), suggesting a possible shift in behavioral state. Together, these results indicate that interactive motifs are not only behaviorally distinct but also follow different patterns of temporal development, consistent with the idea that they may be under separate regulatory control.

Flies develop preference for immobile objects

In earlier experiments, the plastic balls used were fully mobile and could be readily displaced by most test flies. This raised a key question: how does object mobility influence fly behavior? Does

the ability of the ball to move enhance interaction by providing sensorimotor feedback, or could it instead discourage engagement by making the object unpredictable or harder to evaluate? To address this, we directly tested how object mobility affects fly-ball interactions by comparing fly behavior toward two visually identical balls – one free to move and the other fixed in place using a small amount of glue. To control for potential olfactory differences arising from the glue or ball itself, we blocked olfactory input by covering both antennae with UV glue (Figure 4A).

Flies responded differently to the two ball types. Interactions with the mobile ball were marked by frequent jumping, whereas flies rarely jumped from the immobile ball (Figure 4B,C). Mounting events were significantly more common for the immobile ball (Figure 4D,E). Notably, the decision to mount the ball was made autonomously by the fly and likely reflects a combination of visual and mechanosensory evaluation during close-range inspection, shaped by prior experience.

Flies displayed a strong and persistent preference for the immobile ball, as reflected in their interaction scores across visits (Figure 4F,G). This preference was also observed in flies with an intact olfactory system (Figure S6) and remained significant when directly compared across conditions (Figure S7). Interestingly, flies with functional olfaction exhibited even higher interaction scores, suggesting that antennal olfactory or mechanosensory cues, or a combination of both, may enhance fly–object interactions.

Finally, flies spent significantly more time freezing beside immobile balls (Figure S8), providing further evidence that they are capable of distinguishing physical object properties through direct interaction.

Flies' preference for immobile objects persists even when they interact with two objects

The experiments described above involved a single object per experiment. To examine whether flies could discriminate between objects presented in parallel, we designed a two-object assay in which one ball was immobile and the other mobile. Flies were guided alternately between the two balls using our visual pinwheel cue, with each target switched only after the fly had completed an interaction with the current ball (Figure 5A). To eliminate the influence of olfactory cues on preference, we UV-glued the antennae in all experiments.

As in the single-ball experiments (Figure 4), flies showed a clear behavioral distinction between the two object types. They frequently jumped off mobile balls but rarely from immobile ones (Figure 5B,D). Mounting occurred more often on immobile balls (Figure 5C), and this difference was consistent across experiments, yielding a persistent preference for immobile objects (Figure 5F). This preference stabilized after approximately 40 visits (Figure 5G), suggesting that repeated exposure supports the formation of a stable preference. Similar trends were observed in flies with intact olfactory systems (Figure S9), indicating that the preference is not dependent on antennal ablation.

To control for the potential confound that the mobile ball could drift into less favorable regions of the arena – such as near walls, where flies might be less inclined to interact – we performed a positional control experiment. We fixed two immobile balls in the arena: one in the standard

location used previously (ball A), and a second positioned at various distances along the axis between ball A and the arena wall (Figure S10A). Interaction scores did not differ significantly across these conditions (Figure S10A), indicating that wall proximity alone does not account for object preference. Additionally, the average distance between the mobile ball and the closest wall was comparable to that of the immobile ball (Figure S10B). Together, these results confirm that the fly's preference for immobile objects cannot be attributed to ball displacement or spatial bias within the arena.

Role of hΔ neurons in the central complex in mediating object preference

Having established that flies interact differently with mobile and immobile balls, we next asked: how do flies distinguish between two objects that are visually and olfactorily identical? One key distinction is spatial position of the objects. Given that *Drosophila* are capable of forming spatial memories (Corfas, Sharma, and Dickinson 2019; Kim and Dickinson 2017; Ofstad, Zuker, and Reiser 2011), we hypothesized that flies may associate spatial locations with object properties, and that these associations could guide future interactions.

To test this idea, we conducted a targeted silencing screen using Kir2.1 to inhibit activity in a group of fan-shaped body neurons known as hΔ neurons, previously implicated in spatial vector computations (Hulse et al. 2021; Lyu, Abbott, and Maimon 2022; Wolff et al. 2025). Among these, hΔB neurons encode the fly's allocentric travel direction, while other hΔ subtypes share similar anatomical positioning and projection patterns, suggesting they may represent or store additional spatial vectors potentially relevant to object interactions. We also silenced EPG neurons, which encode body-centered heading direction (Seelig and Jayaraman 2015) and provide input to spatial computation circuits (Lyu, Abbott, and Maimon 2022), reasoning that their inhibition might broadly disrupt spatial reasoning in *Drosophila*.

We screened 13 hΔ neuron subtypes, using our two-ball pinwheel-guided assay to evaluate whether silencing any subtype impaired the fly's ability to develop a preference for the immobile ball (Figure 6A). Silencing five hΔ lines – hΔA, hΔJ, hΔM, hΔK, and hΔI – significantly reduced preference, as measured by the difference in interaction scores between the immobile (ball A) and mobile (ball B) objects (Figure 6B).

To further dissect this effect, we examined the likelihood of interaction with each ball type. Silencing hΔE, hΔF, hΔG, hΔA, hΔM, hΔB, hΔD, hΔI, or hΔK significantly reduced interactions with the immobile ball (Figure 6C). In contrast, only a subset of these lines – hΔI, hΔD, hΔG, and hΔK – showed significantly reduced interaction with the mobile ball (Figure 6D). These results suggest that a broader network of hΔ neurons supports interaction with immobile objects, and that these neurons may contribute to the spatial representations that underlie object evaluation. Interestingly, silencing EPG neurons had no effect on the flies' ability to develop a preference for immobile objects, suggesting that the egocentric heading direction system is not involved in this behavior.

hΔ neurons influence specific fly-ball interaction motifs and guidance behavior

Beyond their role in object preference, hΔ neurons also contributed to the expression of specific interaction motifs. Silencing hΔM significantly reduced the likelihood that flies would perform ball-pull maneuvers (Figure 7A). In contrast, silencing hΔJ led to an increase in ball-walk behavior, in which flies remained on the ball while walking along its surface (Figure 7B). Silencing hΔC resulted in a higher probability of jumping from mobile balls (Figure 7C), though no hΔ lines significantly altered jump probability from immobile balls (Figure 7D).

Interestingly, we also observed that silencing several hΔ subtypes – hΔK, hΔC, hΔE, hΔL, and hΔJ – led to significantly increased fidelity in following the opto-motor based visual guidance cue (Iwasaki et al. 2025). This suggests that activity in these neurons may normally compete with opto-motor responses, and that their inhibition allows flies to more readily follow externally driven visual guidance.

Discussion:

Our results demonstrate that repeated visual guidance of *Drosophila melanogaster* toward a small object promotes voluntary and sustained interaction. Flies engaged in a range of behavioral motifs – from mounting and dismounting to more complex patterns like "ball walking," where they maintained balance while locomoting atop a moving surface. These interactions were self-initiated, indicating agency rather than artifact, and evolved over time, suggesting that flies progressively refine their actions through sensorimotor integration.

Unguided flies rarely interacted with the ball, consistent with prior studies showing object aversion during flight or ambiguous object engagement during walking (Maimon, Straw, and Dickinson 2008; Wehner 1972; Bülthoff, Götz, and Herre 1982). This indicates that proximity alone is insufficient to drive engagement. Our results suggest that visual guidance may act as a behavioral priming mechanism – either by increasing arousal, redirecting attention, or creating a closed-loop contingency where flies gain feedback from influencing object movement. Control experiments ruled out passive visual stimulation or spatial closeness as sufficient drivers of interaction, highlighting the importance of active engagement. Repeated safe exposure may also reshape object valence, reducing avoidance over time in a process reminiscent of exposure-based learning (Craske et al. 2014; Cheng, Colbath, and Frye 2019).

Among the most striking behaviors was ball walking, which emerged progressively across trials. This behavior requires balancing on a curved, unstable surface while moving – an act that likely demands the integration of mechanosensory and visual input. Its emergence over time is consistent with gradual skill acquisition, a hallmark of learning in other insect systems (Kriete and Hollis 2022; Loukola et al. 2017; Dukas 2008; Behmer 2005). One possibility is that ball walking may lie on a behavioral continuum with ball pulling, in which flies displace the ball while dismounting. Ball pulls may represent an earlier stage of motor coordination, with flies initially shifting the ball upon exit before learning to stabilize and actively locomote across it. This sequence hints at progressive refinement in motor control.

While ball walking may reflect growing motor competence, its spontaneous and persistent nature across trials also invites speculation that it may resemble play. Play-like behaviors, once thought exclusive to mammals and birds (Diamond and Bond 2003; Kaplan 2024), have been proposed

in invertebrates (Burghardt 2005; Triphan, Ferreira, and Huetteroth 2025; Galpayage Dona et al. 2022). Play is generally considered to promote cognitive flexibility, sensorimotor coordination, and exploratory problem-solving. The expression of non-goal-directed, physically demanding behaviors such as ball walking may reflect similar functions in flies, even in the absence of external reinforcement.

Flies may also interpret or respond to object interactions with motivational states shaped by internal drives. Some ball engagement, particularly mounting, pushing, or “wrestling”, could recruit motor programs that overlap with courtship or mating behaviors (Yamamoto and Koganezawa 2013). Interacting with a persistently present object in a confined environment may activate latent affiliative or reproductive motor patterns, especially in socially isolated males.

Interaction patterns were also strongly modulated by object stability. Flies exhibited a robust preference for immobile balls over mobile ones, despite being visually and olfactorily indistinguishable. This suggests that flies can detect and evaluate mechanical properties through direct physical interaction. Mechanosensory input – from structures such as campaniform sensilla or vibration-sensitive neurons (Tuthill and Wilson 2016; Lee et al. 2025), likely plays a central role. Alternatively, flies may infer stability through mismatches between expected and observed visual flow, engaging circuits within the central complex to detect these discrepancies (Turner-Evans et al. 2017; Lyu, Abbott, and Maimon 2022).

One possibility is that object stability facilitates memory formation. Spatial learning in flies is well established (Ofstad, Zuker, and Reiser 2011; Kim and Dickinson 2017; Behbahani et al. 2021), and our results suggest that learning about object features may be embedded within spatial representations. The mushroom body and fan-shaped body, which mediate associative learning and spatial integration respectively (Aso et al. 2014; Mussells Pires et al. 2024), are well positioned to support this function. Recent connectomic evidence linking mushroom body output neurons to central complex circuitry (Li et al. 2020) further supports this possibility.

From an ethological perspective, object exploration may allow flies to evaluate environmental affordances such as shelter, elevation, or navigational cues – a strategy widely seen across animals including seed-caching birds, octopuses, and rodents (Mather and Anderson 1999; Brodin 2005; Diamond et al. 2008). Flies exhibit a strong preference for elevated surfaces (Robie, Straw, and Dickinson 2010) and can orient along gravity gradients (Kamikouchi et al. 2009; Armstrong et al. 2006; Kladt and Reiser 2023), suggesting that elevation-seeking is an adaptive strategy. Interacting with a small, raised object may thus align with conserved drives for environmental monitoring and predator avoidance.

Mechanistically, we found that specific hΔ neurons in the fan-shaped body regulate fly preference for immobile versus mobile balls. Neurons most critical for object preference (hΔJ, hΔI, hΔA, hΔK, hΔM) were more interconnected than others (Figure S11A), suggesting a functionally coherent subnetwork. These neurons also received strong input from hΔB neurons, which encode allocentric travel direction (Lyu, Abbott, and Maimon 2022), implicating vector-based computation in object evaluation. Under this framework, flies may assess object mobility by comparing their own movement vector with that of the object – potentially storing the stable location of the immobile ball across trials. Objects that remain in the same position may thus

afford stronger spatial memory encoding, consistent with an “object permanence” heuristic (Baillargeon 1987; Pepperberg, Willner, and Gravitz 1997).

Beyond preference, hΔ neurons also influenced behavioral motifs. Silencing hΔJ increased ball-walking behavior. Notably, hΔJ provides substantial input to FC2 neurons (Figure S13), which have been implicated in spatial goal representation (Mussells Pires et al. 2024). This connectivity suggests that hΔJ may contribute to internal goal-setting processes, and that its silencing may bias flies toward more exploratory, less goal-directed behaviors. Silencing hΔC increased jumping, which may reflect agitation or escape-like behavior (von Reyn et al. 2014; Dombrovski et al. 2023), especially given flies' reduced willingness to mount mobile balls. Alternatively, jumping could result from low-level aggression toward an obstructive or unpredictable object. hΔC’s input from FB6A neurons, implicated in sleep regulation (Hulse et al. 2021), suggests a role in arousal or state regulation, though no changes in overall locomotion were observed (Figure S14).

Finally, silencing specific hΔ neurons (hΔK, hΔC, hΔE, hΔL, hΔJ) enhanced flies' ability to follow visual guidance cues. This may reflect reduced competition between internally generated goals and externally imposed cues. All but hΔK strongly influence FC2 neurons (Figure S13), which encode goal heading (Mussells Pires et al. 2024). Thus, disrupting internal goal circuits may unmask latent opto-motor reflexes, improving guidance fidelity.

In summary, our findings reveal that flies use physical interaction to extract object properties, integrating visual, mechanosensory, and spatial feedback to guide future decisions. These behaviors, previously attributed largely to vertebrates, demonstrate that even small insect brains are capable of structured learning and adaptive exploration. Our experimental paradigm opens new opportunities to dissect the neuronal algorithms underlying object recognition, learning, and memory in *Drosophila*. Given the genetic tools available for circuit dissection in flies, future studies can illuminate how compact neural architectures achieve complex behavioral outcomes through the integration of sensory feedback, motor practice, and associative learning. More broadly, uncovering the principles by which flies learn about and interact with their environment may provide fundamental insights into the neural bases of exploration, memory formation, and adaptive behavior across species.

Author contributions:

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Funding acquisition: AR

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Competing interests: Authors declare that they have no competing interests.

Acknowledgements:

We thank Rosy Hosking and Andrew Murray for helpful comments on the manuscript. We thank Chenxu Zhang, Chris Stokes, Kevin Gozzi, Buck Trible, Leigh Needleman, Michael Burns and Ben de Bivort for helpful discussions. We thank Rowland staff for their assistance with instrumentation. This work was funded by Rowland Institute at Harvard.

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Figure 1

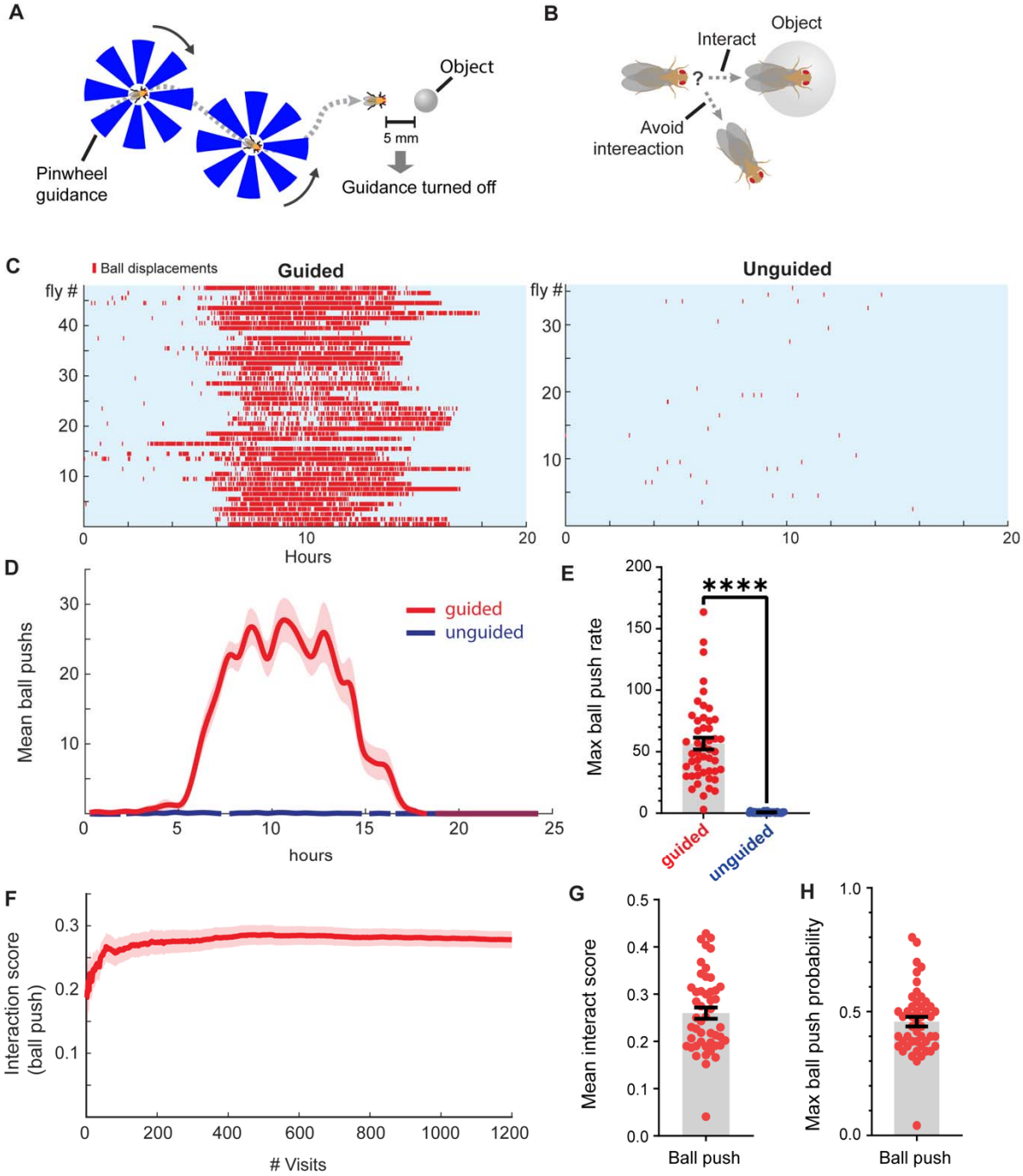
A
Pinwheel guidance
Object
5 mm
Guidance turned off
B
Object
Interact
Avoid intereaction
C
Ball displacements
Guided
Unguided
fly #
Hours
D
Mean ball pushs
guided
unguided
hours
E
Max ball push rate
****
guided
unguided
F
Interaction score (ball push)
Visits
G
Mean interact score
Ball push
H
Max ball push probability
Ball push

Figure 1. Visual guidance enables repeated fly-object interactions with novel spherical targets.

(A) In the "pinwheel-guided" assay, flies are directed toward a spherical object using a visual stimulus (the "pinwheel"). Guidance is turned off when the fly reaches within a 5-mm radius of the object.
(B) The decision to engage with the object is made autonomously by the fly.
(C) Guided flies frequently engage with and move the balls, while unguided flies rarely do so (*n* = 48 guided flies, 36 unguided flies).
(D) Mean frequency of ball-pushing events over time for guided and unguided flies (n = 48 guided, 36 unguided). The delayed onset of interactions (beginning around hour 5) parallels the rise in general locomotor activity observed in Figure S3.
(E) Maximum ball-pushing rate (pushes per 30-minute bin) for each group. *Mann–Whitney test*, two-tailed (*n* = 48 guided flies, 36 unguided flies).
(F) Cumulative interaction scores (pushes) over number of visits. Only flies with >1,200 visits included (*n* = 32 flies).
(G) Mean interaction score (pushes per visit) across flies (*n* = 48 flies).
(H) Maximum ball-pushing probability achieved per fly. Probability was calculated over a sliding window of 50 visits, and the highest value recorded for each fly was used (*n* = 48 flies).

Figure 2

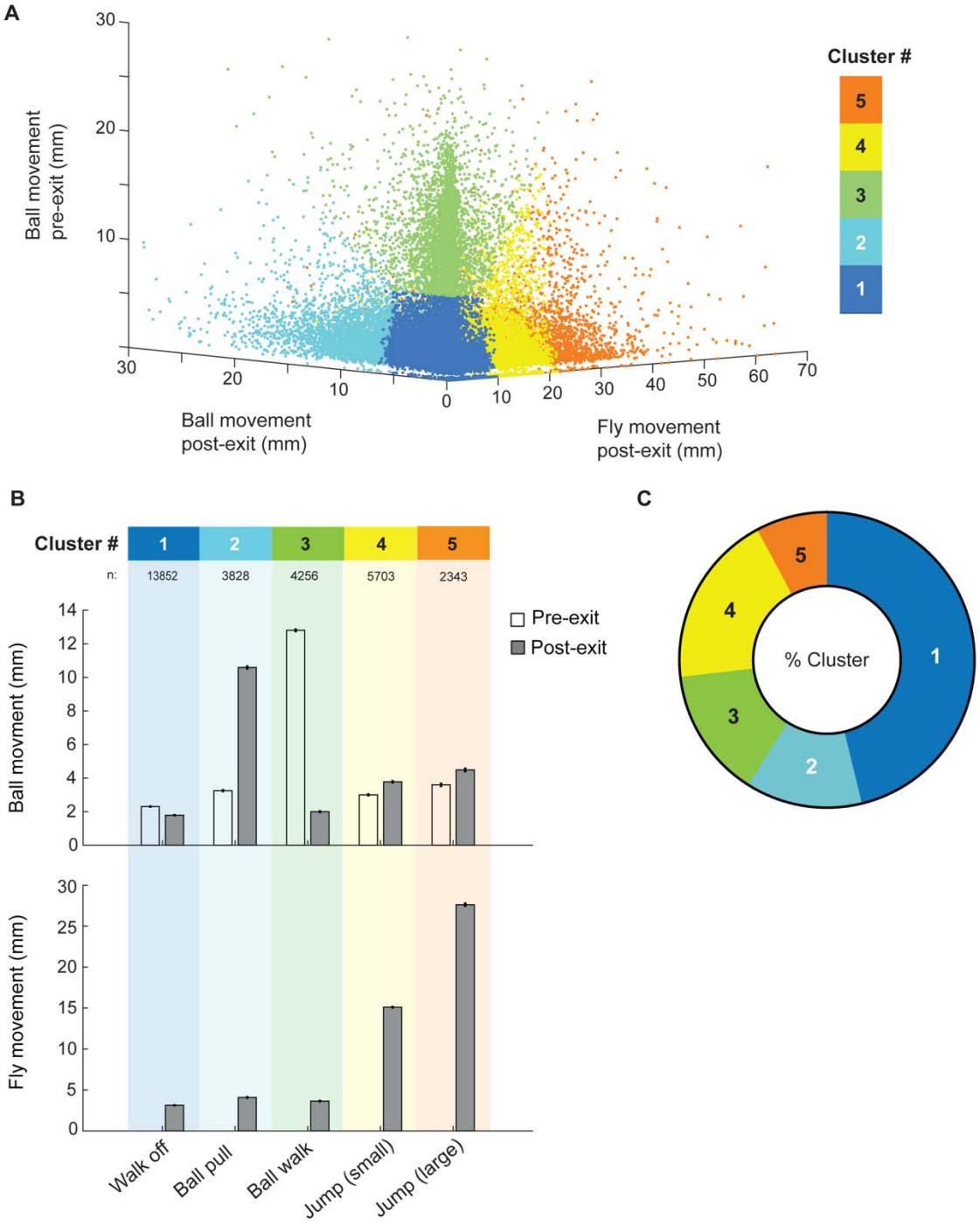
A
Ball movement pre-exit (mm)
Ball movement post-exit (mm)
Fly movement post-exit (mm)
Cluster #
5
4
3
2
1
B
Cluster #
n:
13852
3828
4256
5703
2343
Pre-exit
Post-exit
Ball movment (mm)
Fly movement (mm)
Walk off
Ball pull
Ball walk
Jump (small)
Jump (large)
C
% Cluster

Figure 2. Unsupervised clustering reveals distinct categories of fly-ball interaction motifs.

(A) K-means clustering was applied to 29,982 interactive events from 48 male flies, using features related to ball displacement (before fly exit (pre-exit) and after fly exit (post-exit)) and fly movement following interaction termination (post-exit fly displacement). A five-cluster solution was chosen to isolate "walk-off" events – characterized by minimal ball and fly movement – from other motif types while minimizing redundancy.
(B) The clustering analysis revealed several distinct behavioral motifs, including "walk-off," "ball pull," "ball walk," and "jump"-like events.
(C) Among all classified events, "walk-off" interactions were the most frequent (46%), followed by small jumps (19%), ball walks (14%), ball pulls (13%), and large jumps (8%).

Figure 3

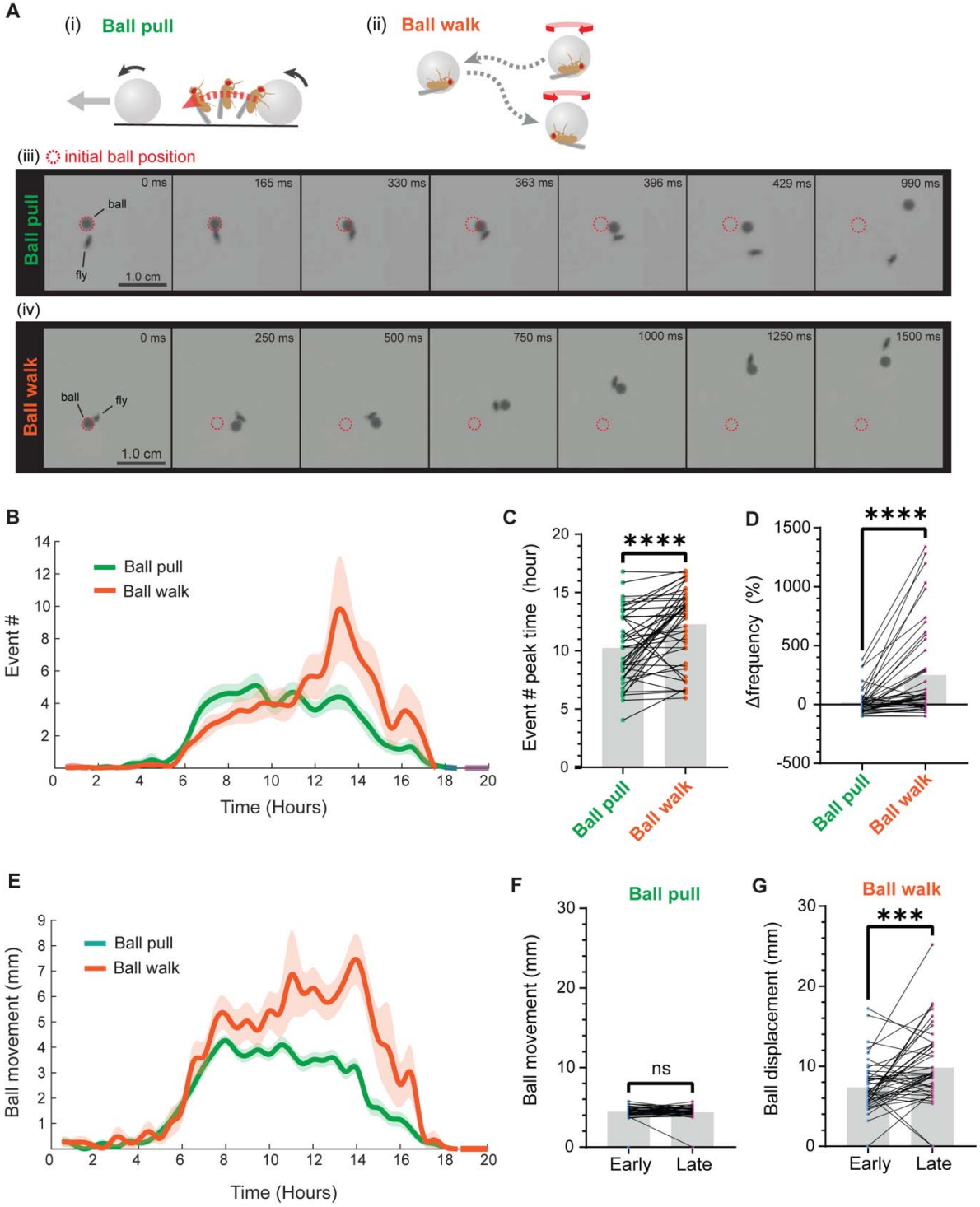
A
(i) Ball pull
(ii) Ball walk
(iii) initial ball position
Ball pull
0 ms
165 ms
330 ms
363 ms
396 ms
429 ms
990 ms
ball
fly
1.0 cm
(iv)
Ball walk
0 ms
250 ms
500 ms
750 ms
1000 ms
1250 ms
1500 ms
ball
fly
1.0 cm
B
Ball pull
Ball walk
Event #
Time (Hours)
C
****
Event # peak time (hour)
Ball pull
Ball walk
D
****
Δfrequency (%)
Ball pull
Ball walk
E
Ball pull
Ball walk
Ball movement (mm)
Time (Hours)
F
Ball pull
ns
Ball movement (mm)
Early
Late
G
Ball walk
***
Ball displacement (mm)
Early
Late

Figure 3: Ball-walk interactions show distinct temporal development from ball-pull events.

(A) Schematic examples of (i, iii) *ball pull* and (ii, iv) *ball walk* events. In ball pull, a fly mounts the ball and pulls it upon dismounting, resulting in a displacement (≥3 mm) without walking on the ball. In ball walk, a fly remains on the side of the ball and displaces it by walking along its surface; these events are defined by coordinated fly-ball movement (>2.5 mm). Definitions are based on movement thresholds exceeding noise levels (<2.5 mm). The dotted circles indicate the ball's original position. In ball walk events, significant displacement occurs while the fly remains on the ball, suggesting active control during locomotion. In contrast, ball pull events are defined by displacement that occurs after the fly dismounts, as the fly appears to "throw" the ball upon exit.
(B) Time-course of motif frequencies across the experimental period (30-minute bins). Ball-walk frequency increases gradually and peaks later, whereas ball-pull frequency plateaus earlier. *Y*-axis: mean number of events; *X*-axis: experimental time in hours. $n = 48$.
(C) Ball pull frequency peaks significantly earlier than ball walk frequency (*Wilcoxon matched-pairs signed-rank test, two-tailed*, $n = 47$ flies; one fly excluded due to no relevant events).
(D) Relative frequency change over time. Ball-walk frequency shows a significantly greater increase from early to late periods than ball-pull frequency. Δ frequency (%) = [(# events $_{\text{late period}}$ - # events $_{\text{early period}}$)/# events $_{\text{early period}}$] x 100. Periods were divided in half per fly. *Wilcoxon matched-pairs signed-rank test, two-tailed*, $n = 48$ flies.
(E) Average displacement distances for ball-pull and ball-walk events. $n = 48$.
(F,G) Event displacement comparisons (*Wilcoxon matched-pairs signed-rank test, two-tailed*) across early vs. late periods for (F) ball pull ($n = 48$) and (G) ball walk ($n = 48$).

Figure 4

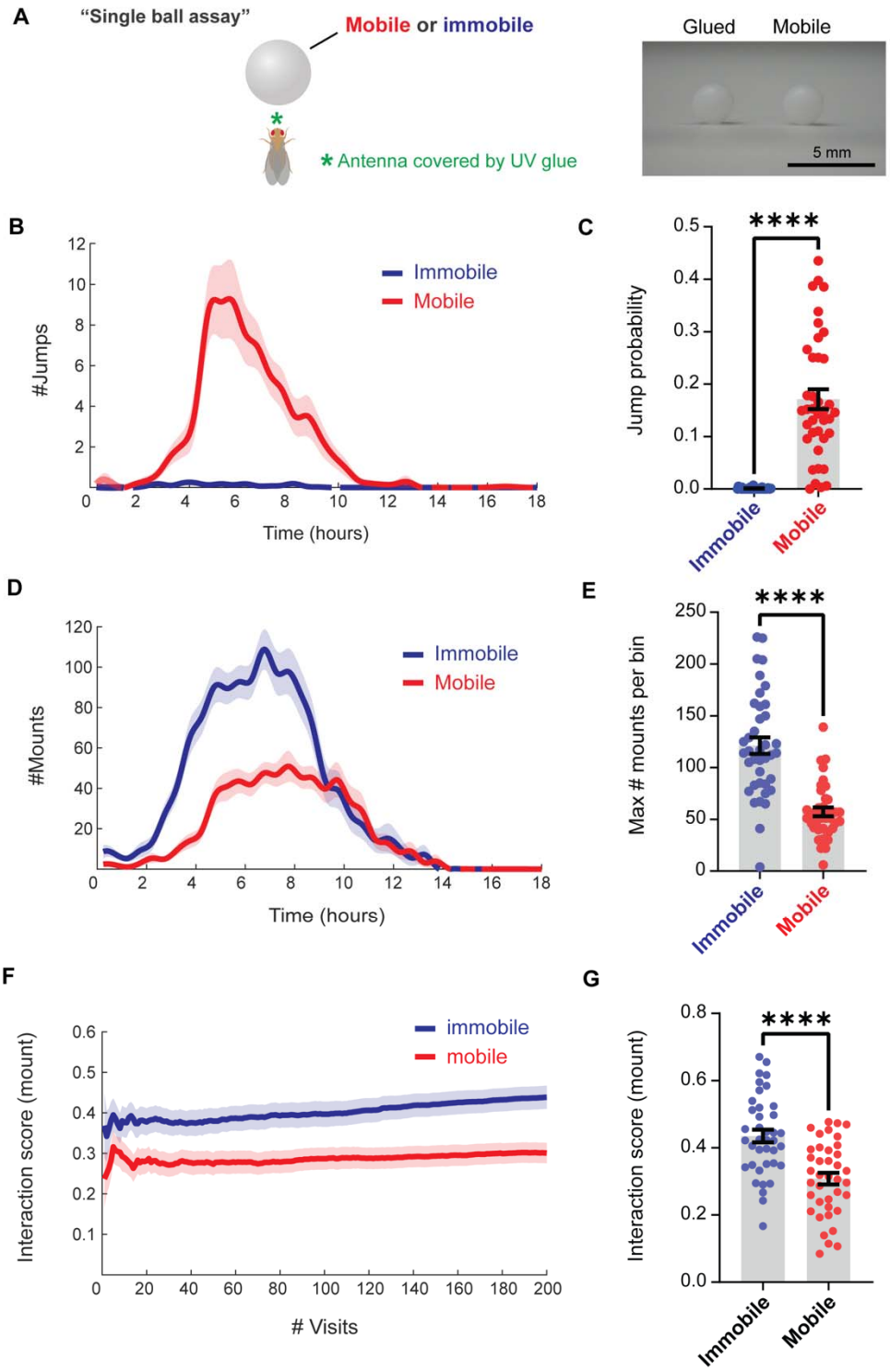
A
"Single ball assay"
Mobile or immobile
* Antenna covered by UV glue
Glued
Mobile
5 mm
B
#Jumps
Time (hours)
Immobile
Mobile
C
Jump probability
****
Immobile
Mobile
D
#Mounts
Time (hours)
Immobile
Mobile
E
Max # mounts per bin
****
Immobile
Mobile
F
Interaction score (mount)
Visits
immobile
mobile
G
Interaction score (mount)
****
Immobile
Mobile

Figure 4. Flies preferentially interact with immobilized balls.

To examine how ball mobility affects interaction behavior, we compared responses to mobile versus immobilized balls in a single-ball assay using flies with UV-glued antennae to block olfactory sensation.
(A) (i) Schematic of the single-ball assay. Flies were guided toward either a mobile or glued (immobile) ball. *(ii)* Photographs of the glued (left) and mobile (right) balls, which are visually indistinguishable.
(B) Flies interacting with mobile balls exhibited a high number of jump events (>5 mm displacement), whereas such jumps were rare when flies interacted with glued balls (n = 39 flies per group; 30-minute bins).
(C) The probability of jumping off a ball was significantly higher when the ball was mobile, indicating that mobility influences behavioral outcomes upon dismount. *Mann-Whitney Test*, two-tailed (n = 39 flies per group).
(D) Mounting frequency over time for mobile versus immobile balls (n = 39 flies per group).
(E) The maximum number of mounts per time bin was significantly higher for immobilized balls (*Mann–Whitney test*, two-tailed; n = 39 flies per group).
(F) Interaction scores across the first 200 visits (only flies with ≥200 visits included; n = 38 flies per group).
(G) Global interaction scores (mounts per visit) were significantly higher for immobile balls. That is, flies were more likely to mount an immobilized ball than a mobile one. Final interaction scores across the entire experiment were used for statistical comparison to avoid bias from arbitrary visit windows (*unpaired t-test*, two-tailed; n = 39 flies per group).

Figure 5

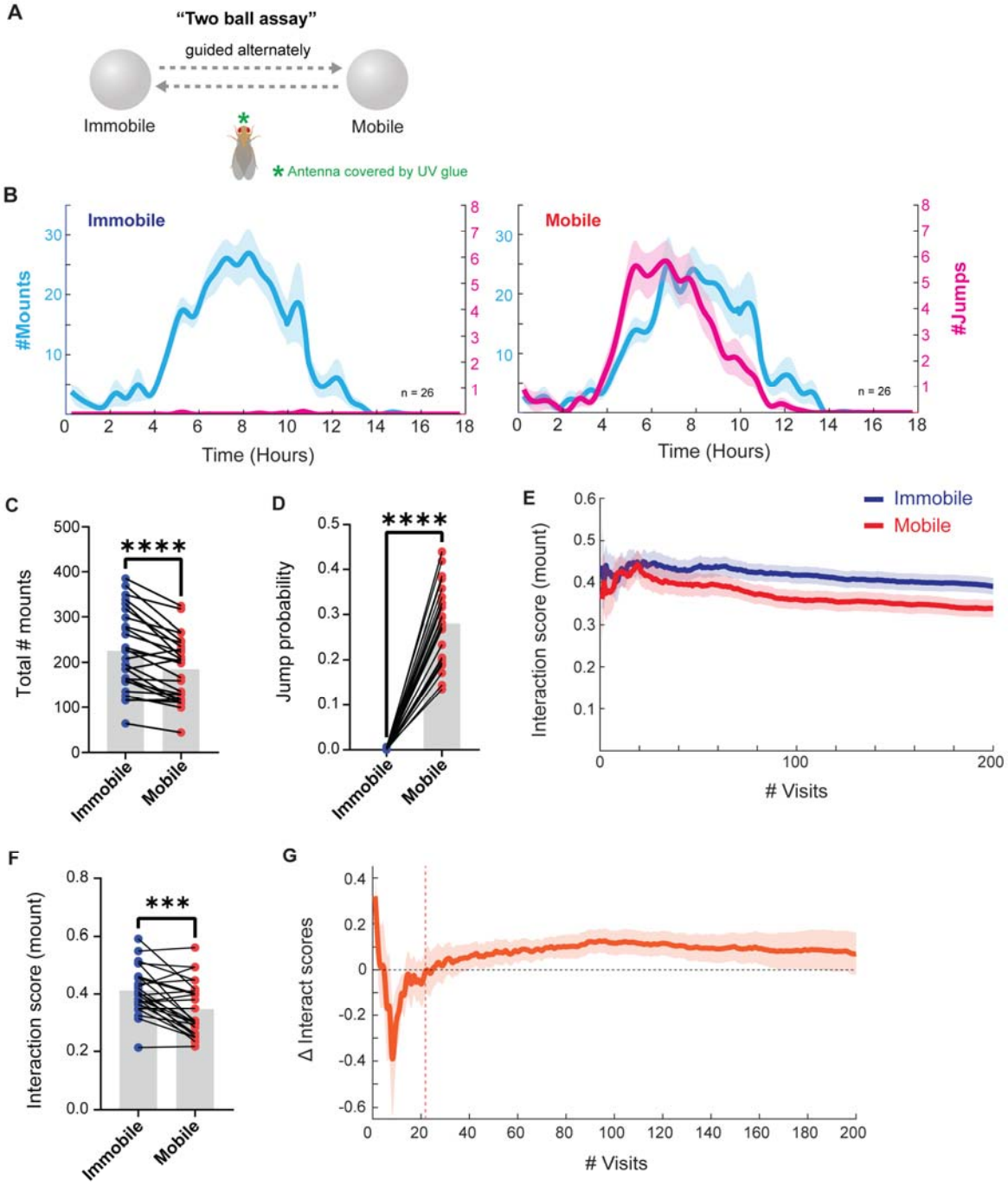
A
"Two ball assay"
guided alternately
Immobile
Mobile
* Antenna covered by UV glue
B
Immobile
Mobile
#Mounts
#Jumps
n = 26
n = 26
Time (Hours)
Time (Hours)
C
Total # mounts
****
Immobile
Mobile
D
Jump probability
****
Immobile
Mobile
E
Immobile
Mobile
Interaction score (mount)
Visits
F
Interaction score (mount)
***
Immobile
Mobile
G
Δ Interact scores
Visits

Figure 5. Flies develop a preference for immobilized balls when alternately interacting with mobile and immobile objects.

To test whether flies distinguish between objects based on mobility alone, we designed a two-ball assay in which one ball was mobile and the other was immobile. Flies were guided to alternate between the two. Antennae were UV-glued to block olfactory input.

(A) Schematic of the two-ball assay: flies alternately approached a glued (immobile) and a free (mobile) ball within the same experimental session.
(B) Time course of mounting and jumping events for immobile (left) and mobile (right) balls (n = 26 flies per condition; 30-minute bins).
(C) Total number of mounts was significantly higher for immobile balls (*paired t-test*, two-tailed; n = 26 flies).
(D) The probability of jumping after ball interaction was significantly higher for mobile balls (*Wilcoxon matched-pairs signed-rank test*, two-tailed; n = 26 flies).
(E) Final global interaction scores (mounts per visit) for mobile and immobile balls (n = 25 flies).
(F) Interaction scores were significantly higher for immobilized balls (*paired t-test*, two-tailed; n = 26 flies).
(G) Difference in interaction scores (Δ interaction score = score $_{\text{immobile}}$ – score $_{\text{mobile}}$) plotted as a function of visit number. A stable preference for immobile balls emerged after approximately 20 visits on average, following an initial dip (n = 25 flies).

Figure 6

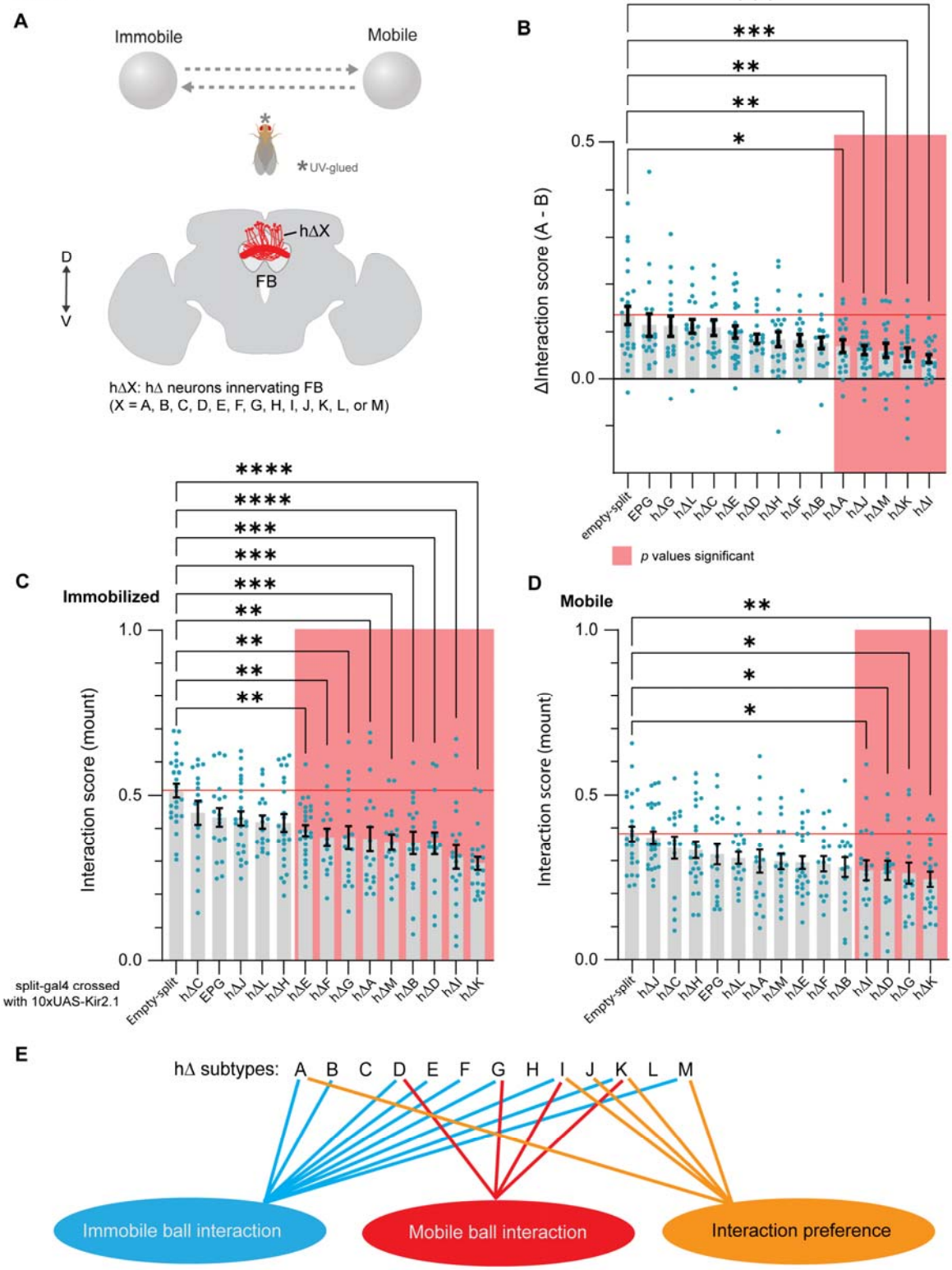
A
Immobile
Mobile
*UV-glued
hΔX
FB
D
V
hΔX: hΔ neurons innervating FB
(X = A, B, C, D, E, F, G, H, I, J, K, L, or M)
B
ΔInteraction score (A - B)
0.5
0.0
empty-split
EPG
hΔG
hΔL
hΔC
hΔE
hΔD
hΔH
hΔF
hΔB
hΔA
hΔJ
hΔM
hΔK
hΔI
p values significant
C
Immobilized
Interaction score (mount)
1.0
0.5
0.0
split-gal4 crossed
with 10xUAS-Kir2.1
Empty-split
hΔC
EPG
hΔJ
hΔL
hΔH
hΔE
hΔF
hΔG
hΔA
hΔM
hΔB
hΔD
hΔI
hΔK
D
Mobile
Interaction score (mount)
1.0
0.5
0.0
Empty-split
hΔJ
hΔC
hΔH
EPG
hΔL
hΔA
hΔM
hΔE
hΔF
hΔB
hΔI
hΔD
hΔG
hΔK
E
hΔ subtypes: A B C D E F G H I J K L M
Immobile ball interaction
Mobile ball interaction
Interaction preference

Figure 6. A subset of hΔ neuron subtypes influence fly-ball interaction probabilities and preference.

(A) To identify neural substrates of object interaction behavior, we silenced 13 distinct hΔ neuron types and EPG neurons using 10xUAS-Kir2.1, and tested the resulting flies in a two-ball interaction assay. In this assay, flies were alternately guided to interact with an immobile and a mobile ball during the same session. Antennae were UV-glued to eliminate olfactory input. Each hΔ split-GAL4 line (hΔA–hΔM), as well as the EPG line (SS00090), were crossed to 10xUAS-Kir2.1. *n* values for each genotype were as follows: hΔA (18), hΔB (17), hΔC (16), hΔD (17), hΔE (26), hΔF (16), hΔG (17), hΔH (24), hΔI (20), hΔJ (25), hΔK (22), hΔL (18), hΔM (19), EPG (18). An empty-split-GAL4 × UAS-Kir2.1 line was used as the control group ($n = 25$).
(B) Silencing of five hΔ subtypes (hΔA, hΔJ, hΔM, hΔK, hΔI) significantly reduced the preference for immobile over mobile balls, as measured by the Δ interaction score (immobile – mobile. *One-way ANOVA, Bonferroni's multiple comparisons test* was used. Significant groups are shaded in light red ($p < 0.05$).
(C) Interaction scores for the immobile ball condition. Nine hΔ lines (hΔE, hΔF, hΔG, hΔA, hΔM, hΔB, hΔD, hΔI, hΔK) showed significantly reduced scores compared to controls (*One-way ANOVA, Bonferroni's multiple comparisons test*).
(D) Interaction scores for the mobile ball condition. Four groups (hΔI, hΔD, hΔG, hΔK) showed significantly reduced scores compared to controls (*One-way ANOVA, Bonferroni's multiple comparisons test*).
(E) Summary diagram highlighting the hΔ subtypes implicated in immobile ball interactions, mobile ball interactions, and interaction preference.

Figure 7

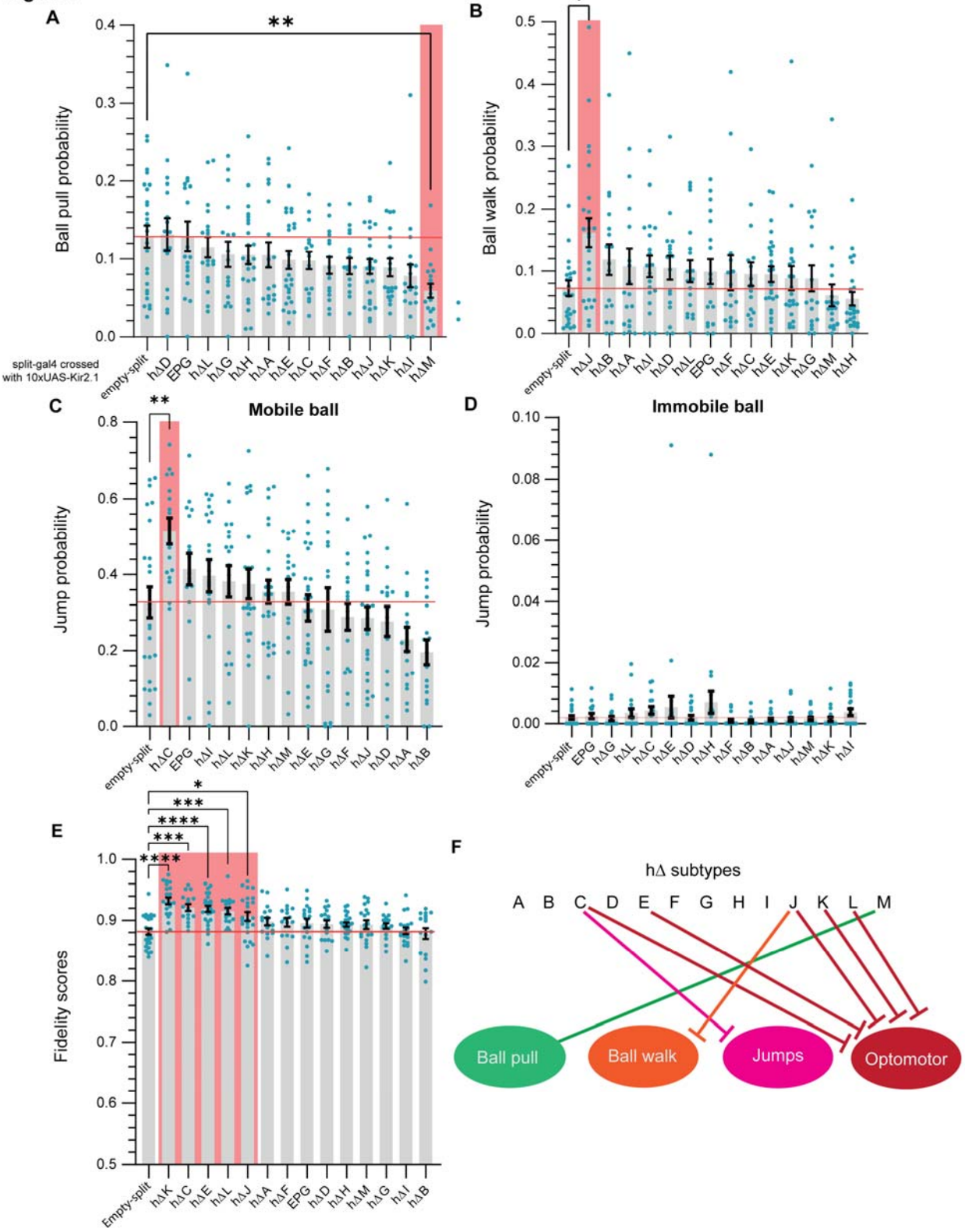
A
Ball pull probability
split-gal4 crossed with 10xUAS-Kir2.1
empty-split
hΔD
EPG
hΔL
hΔG
hΔH
hΔA
hΔE
hΔC
hΔF
hΔB
hΔJ
hΔK
hΔI
hΔM
B
Ball walk probability
empty-split
hΔJ
hΔB
hΔA
hΔI
hΔD
hΔL
EPG
hΔF
hΔC
hΔE
hΔK
hΔG
hΔM
hΔH
C
Mobile ball
Jump probability
empty-split
hΔC
EPG
hΔI
hΔL
hΔK
hΔH
hΔM
hΔE
hΔG
hΔF
hΔJ
hΔD
hΔA
hΔB
D
Immobile ball
Jump probability
empty-split
EPG
hΔG
hΔL
hΔC
hΔE
hΔD
hΔH
hΔF
hΔB
hΔA
hΔJ
hΔM
hΔK
hΔI
E
Fidelity scores
Empty-split
hΔK
hΔC
hΔE
hΔL
hΔJ
hΔA
hΔF
EPG
hΔD
hΔH
hΔM
hΔG
hΔI
hΔB
F
hΔ subtypes
A B C D E F G H I J K L M
Ball pull
Ball walk
Jumps
Optomotor

Figure 7. Distinct hΔ neuron subtypes modulate specific behaviors expressed during fly-ball interactions.

(A) Ball-pull probability, defined as the proportion of mount events followed by a ball pull, across the 15 genetic conditions tested in the two-ball assay (13 hΔ subtypes, EPG neurons, and control). Silencing hΔM resulted in a significantly lower pull probability than control. *One-way ANOVA with Bonferroni's multiple comparisons test.* Sample sizes (*n*) for each condition match those listed in Figure 6A and are identical across all panels in this figure.
(B) Ball-walk probability, defined as the proportion of mounts resulting in a ball walk. Silencing hΔJ significantly increased ball-walk probability compared to control. *Kruskal–Wallis test with Dunn's multiple comparisons* (control distribution was non-normal).
(C) Jump probability for immobile balls, calculated as the number of jumps divided by the number of mounts. Silencing hΔC significantly increased jump probability. *One-way ANOVA with Bonferroni's multiple comparisons test.*
(D) Jump probability for mobile balls. No genotype showed a statistically significant difference from control. *Kruskal–Wallis test with Dunn's multiple comparisons test.*
(E) Fidelity scores, calculated as the proportion of successful visual guidance turns. Silencing hΔK, hΔC, hΔE, hΔL, hΔJ significantly increased fidelity relative to control. *One-way ANOVA with Bonferroni's multiple comparisons test.* One outstanding outlier was removed from hΔE by ROUT method (Q = 0.1%) by GraphPad Prism.
(F) Summary diagram indicating which hΔ neuron subtypes are required for specific motor outcomes. T-bar lines indicate inferred inhibition of behavior.

Supplemental Information for:

Title: Drosophila learn about properties of objects through physical interaction

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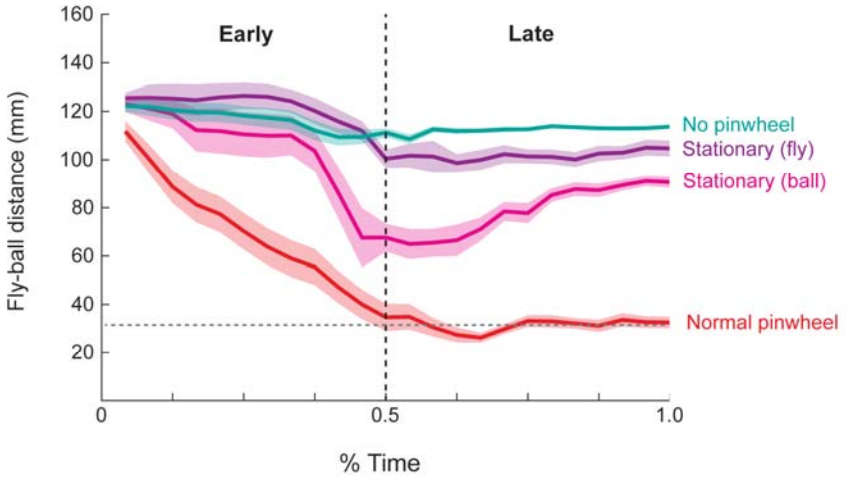
Figure S1

A


Early
Late
Fly-ball distance (mm)
160
140
120
100
80
60
40
20
0
0.5
1.0
% Time
No pinwheel
Stationary (fly)
Stationary (ball)
Normal pinwheel

B

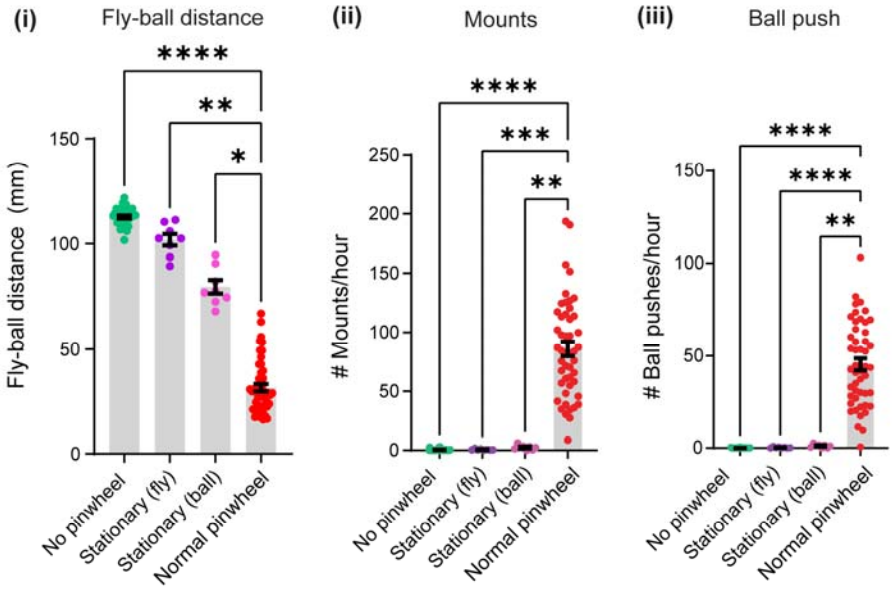

(i)
Fly-ball distance
Fly-ball distance (mm)
****
**
*
150
100
50
0
No pinwheel
Stationary (fly)
Stationary (ball)
Normal pinwheel
(ii)
Mounts
Mounts/hour
****
***
**
250
200
150
100
50
0
No pinwheel
Stationary (fly)
Stationary (ball)
Normal pinwheel
(iii)
Ball push
Ball pushes/hour
****
****
**
150
100
50
0
No pinwheel
Stationary (fly)
Stationary (ball)
Normal pinwheel

Figure S1. Stationary pinwheel cues do not enhance fly-ball interactions

To assess whether visual feedback from the rotating pinwheel influences fly-ball interactions, we compared behavior across conditions using stationary pinwheels affixed either to the fly or the ball.

A) Fly-to-ball distance (mm) across four conditions: no pinwheel, stationary pinwheel fixed to the fly, stationary pinwheel fixed to the ball, and a dynamic (rotating) pinwheel coupled to the guided fly's movement. Flies exposed to stationary pinwheels remained significantly farther from the balls than flies experiencing normal visual feedback (rotating pinwheel). n = 36 (no pinwheel), 8 (stationary-fly), 8 (stationary-ball), 48 (rotating). The final 20% of the session duration was excluded from analysis. Data are binned into 30-minute intervals.

(B) (i) Flies in all stationary or no-pinwheel conditions maintained greater average distances from the ball compared to controls.
(ii) Mounting behavior was significantly reduced in all three experimental conditions relative to the rotating pinwheel control.
(iii) Ball-pushing frequency was likewise reduced in all experimental groups.
Kruskal-Wallis test with Dunn's multiple comparisons test. n = 36 (no pinwheel), 8 (stationary-fly), 8 (stationary-ball), 48 (rotating).

Figure S2

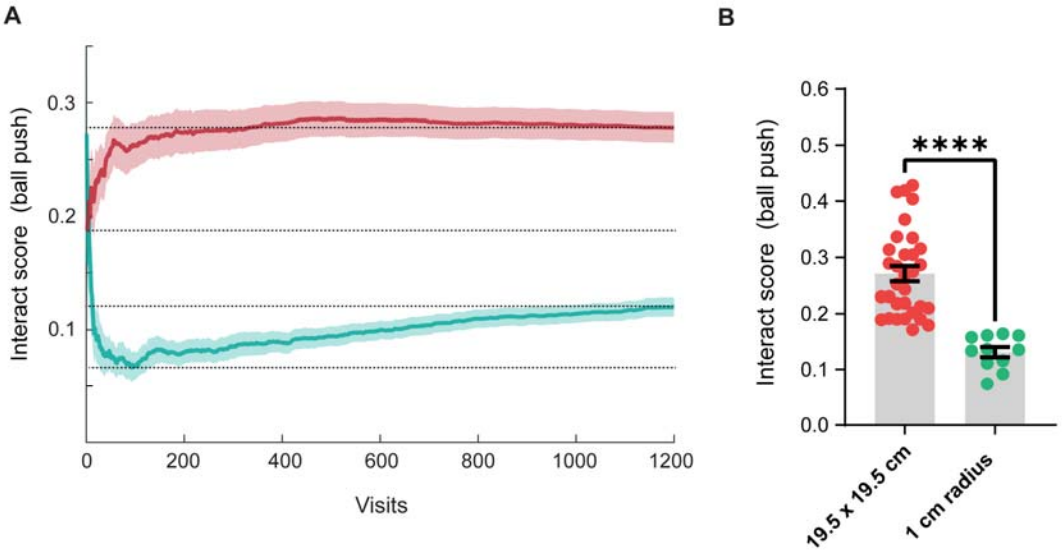

A
Interact score (ball push)
0.3
0.2
0.1
0
200
400
600
800
1000
1200
Visits
B
Interact score (ball push)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
****
19.5 x 19.5 cm
1 cm radius

Figure S2. Reduced arena size does not enhance fly-ball interaction probability.

To determine whether spatial confinement alone increases fly-ball interactions, we compared behavioral metrics across arenas of different sizes.

(A) Ball-pushing scores for the first 1,200 visits in the standard arena versus the 2-cm radius arena (n = 32, 11 flies).
(B) Flies in the standard arena continued to show significantly higher interaction levels on average than those in the smaller arena. *Mann-Whitney test (two-tailed)* (n = 32, 11 flies).

Figure S3

A

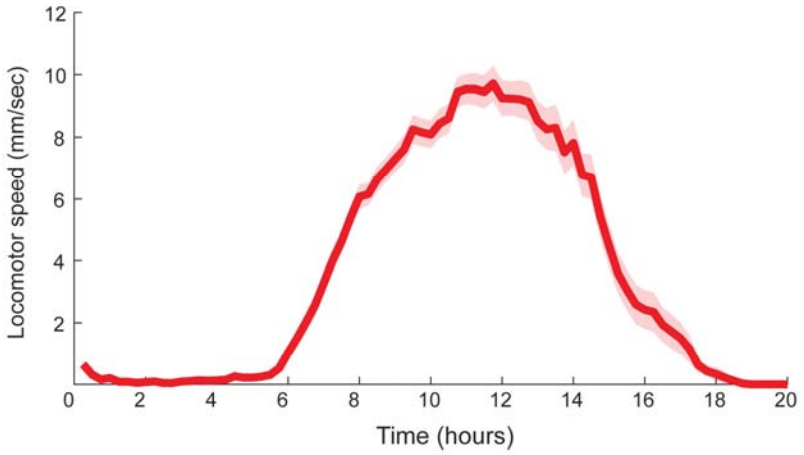

Locomotor speed (mm/sec)
12
10
8
6
4
2
0
2
4
6
8
10
12
14
16
18
20
Time (hours)

Figure S3: The average locomotion speed over time for the flies tested in Fig. 1 (n = 48 flies).

Figure S4

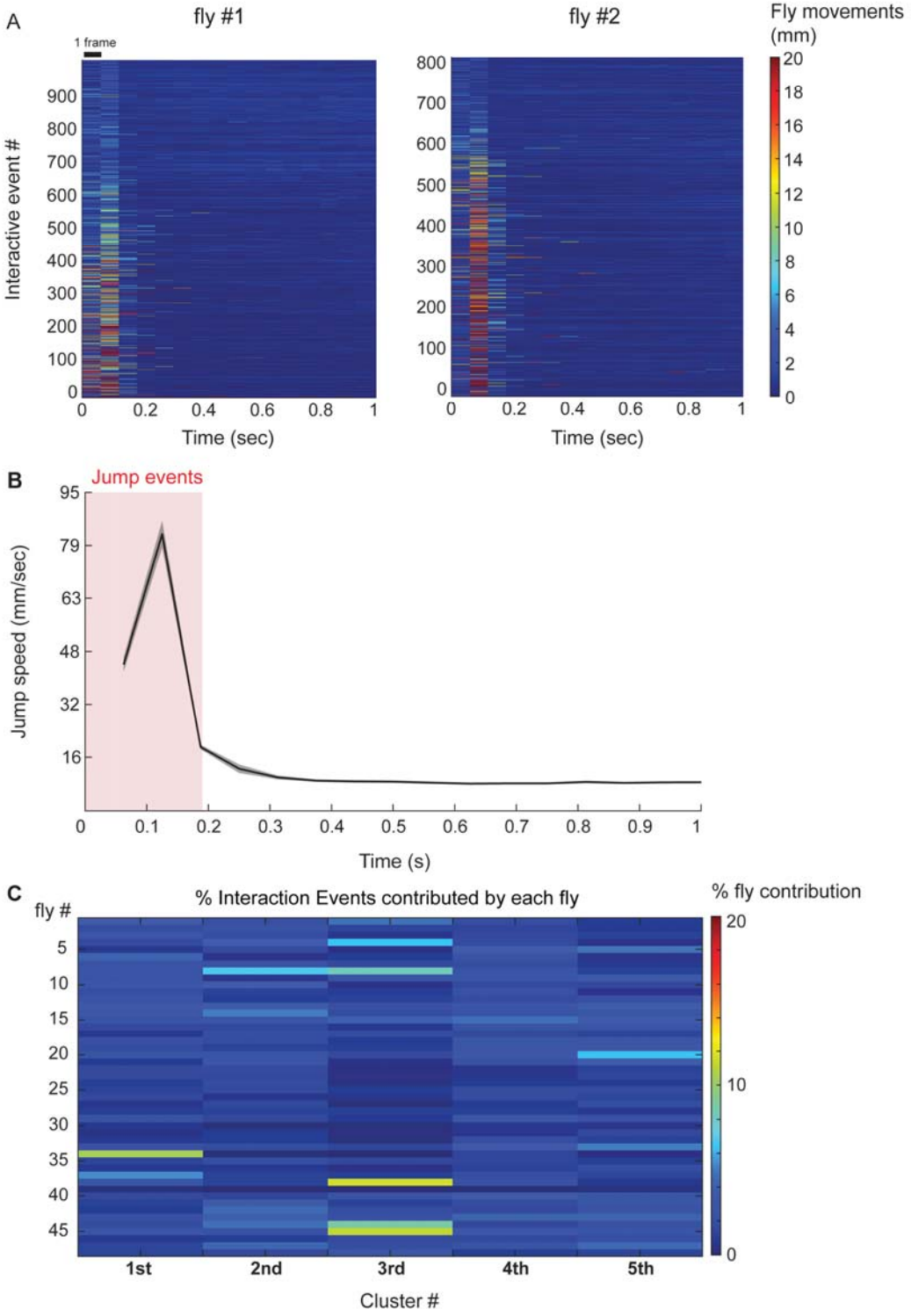

A
fly #1
fly #2
Fly movements (mm)
1 frame
Interactive event #
Time (sec)
B
Jump events
Jump speed (mm/sec)
Time (s)
C
% Interaction Events contributed by each fly
% fly contribution
fly #
Cluster #
1st
2nd
3rd
4th
5th

Figure S4: Jump-like events complete rapidly after fly-ball interactions and are similar across individuals.

(A) Representative examples of "jump" events from two flies (#1 and #2). In both cases, the rapid movement concludes within the first three recorded frames (~188 ms) following the fly's exit from the ball.
(B) Timing of peak jump velocity following interaction termination. Most jump events reach their maximum speed within 200 milliseconds of fly-ball disengagement (n = 48 flies). Number of jump events per fly = 625 ± 310 (standard deviation).
(C) Distribution of event contributions across flies. Individual flies contributed comparably to each of the five behavioral motif clusters described in Figure 2, with no fly accounting for more than 12% of the events in any single cluster.

Figure S5

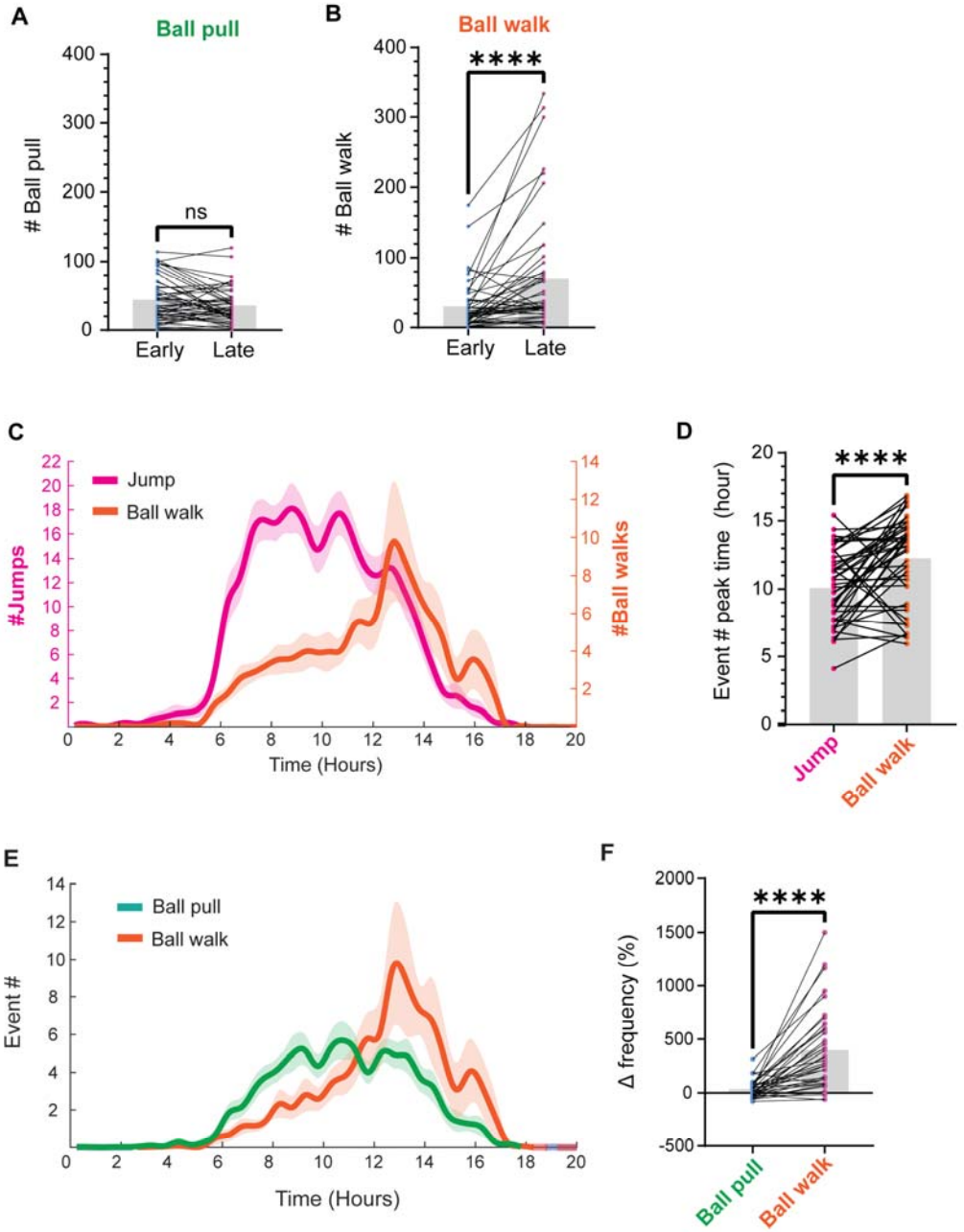

A
Ball pull
Ball pull
ns
Early
Late
B
Ball walk
Ball walk
****
Early
Late
C
Jump
Ball walk
#Jumps
#Ball walks
Time (Hours)
D
Event # peak time (hour)
****
Jump
Ball walk
E
Ball pull
Ball walk
Event #
Time (Hours)
F
Δ frequency (%)
****
Ball pull
Ball walk

Figure S5. Cluster-dependent analysis supports differential temporal dynamics between ball-pull and ball-walk interactions.

(A) Frequency of ball-pull events does not differ significantly between the early and late phases of the active period (*Wilcoxon matched-pairs signed-rank test*, two-tailed; $n = 48$).
(B) In contrast, ball-walk frequency increases significantly in the second half of the experiment (*Wilcoxon matched-pairs signed-rank test*, two-tailed; $n = 48$).
(C) Time-course comparison of jump and ball-walk frequencies. Ball-walk events gradually rise and peak sharply around 12 hours, while jump events peak earlier and decline gradually thereafter. Jumps are defined as fly displacements >5 mm (≥2 body lengths) and ≤75 mm following dismount from a ball. Data are binned in 30-minute intervals ($n = 48$ flies).
(D) On average, ball-walk frequency peaks significantly later than jump frequency (*Wilcoxon matched-pairs signed-rank test*, two-tailed; $n = 47$ flies; one fly excluded due to absence of ball-walk events).
(E) Cluster-dependent analysis confirms the overall pattern observed in Figure 3B: ball-walk frequency increases over time more than ball-pull frequency ($n = 48$; 30-minute bins).
(F) The temporal increase in ball-walk frequency is significantly greater than that of ball-pull frequency. Outlier removal (ROUT method, Q = 0.1%) excluded 12 flies from analysis for clarity ($n = 36$). The result remains significant with or without outliers ($p < 0.0001$, ****; *Wilcoxon matched-pairs signed-rank test*, two-tailed).

Figure S6

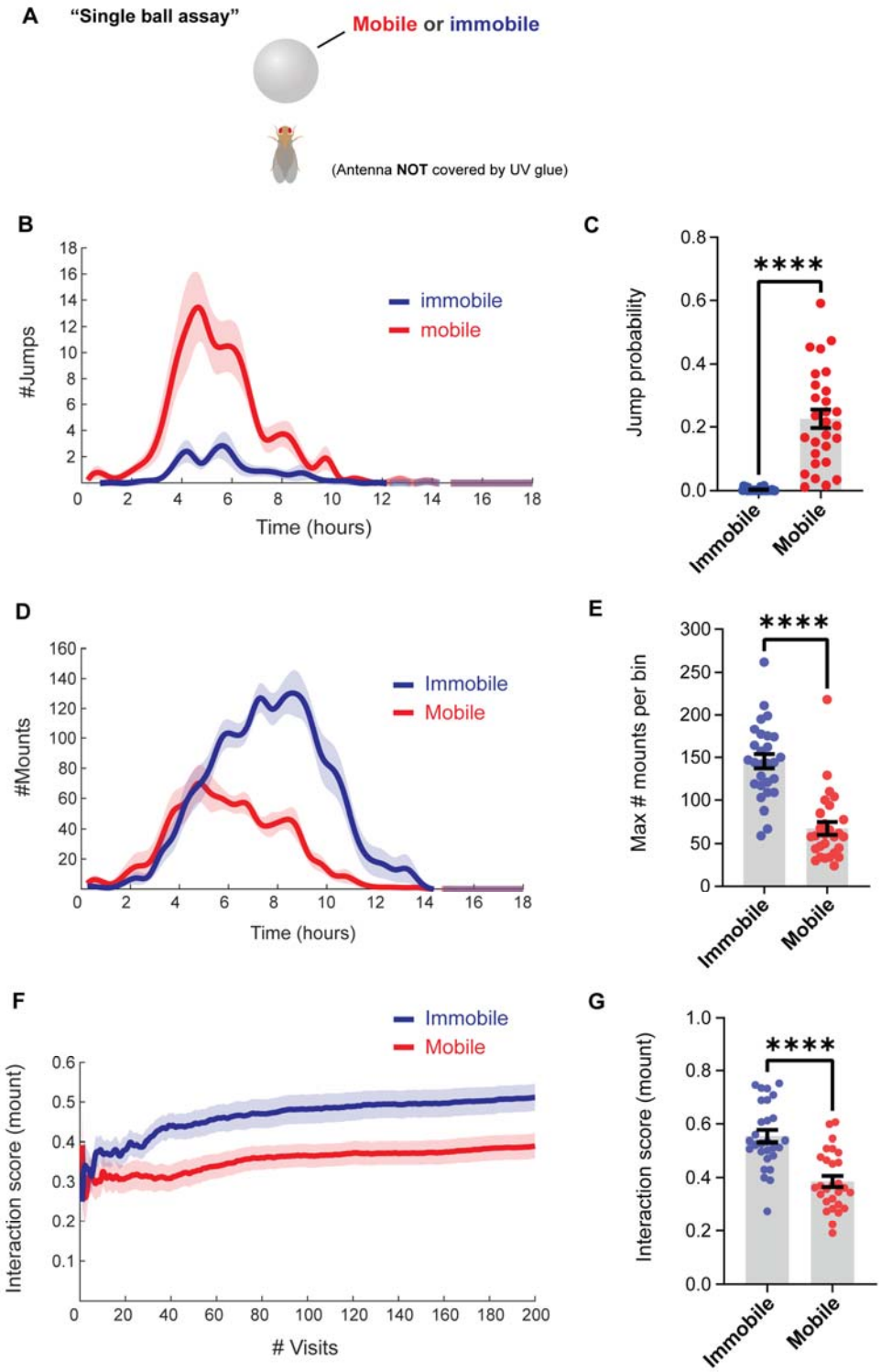

A
"Single ball assay"
Mobile or immobile
(Antenna NOT covered by UV glue)
B
#Jumps
Time (hours)
immobile
mobile
C
Jump probability
****
Immobile
Mobile
D
#Mounts
Time (hours)
Immobile
Mobile
E
Max # mounts per bin
****
Immobile
Mobile
F
Interaction score (mount)
Visits
Immobile
Mobile
G
Interaction score (mount)
****
Immobile
Mobile

Figure S6. Flies exhibit a preference for immobilized balls also in the presence of intact olfactory input.

This control experiment (without antennal UV-gluing) tested whether flies exhibit the same mobility-dependent interaction bias when olfactory input is intact. In contrast, flies in Figure 4 had their antennae glued to eliminate the possibility of distinguishing between the two balls based on olfactory or other antennal cues.

(A) Schematic of the single-ball assay: flies were guided to interact with either a mobile or glued (immobile) ball. Unlike in Figure 4, flies in this experiment retained normal antennal (olfactory) function.
(B) Time course of jump frequency for mobile versus immobile ball conditions (30-minute bins; n = 28 flies per group).
(C) The probability of jumping after interaction is significantly higher for mobile balls (*Mann–Whitney test*, two-tailed; n = 28 flies per group).
(D) Mounting frequency over time for each condition (n = 28 flies per group).
(E) The maximum number of mounts per 30-minute bin is significantly higher for immobile balls (*Mann–Whitney test*, two-tailed, n = 28 flies per group).
(F) Comparison of interaction scores (mounts per visit) for mobile and immobile ball conditions (n = 28 flies per group).
(G) Final interaction scores are significantly higher when flies interacted with immobilized balls, indicating a consistent bias toward mounting immobile objects (*unpaired t-test*, two-tailed; n = 28 per group). Global scores across the entire session were used for analysis to avoid the use of arbitrary visit windows.

Figure S7

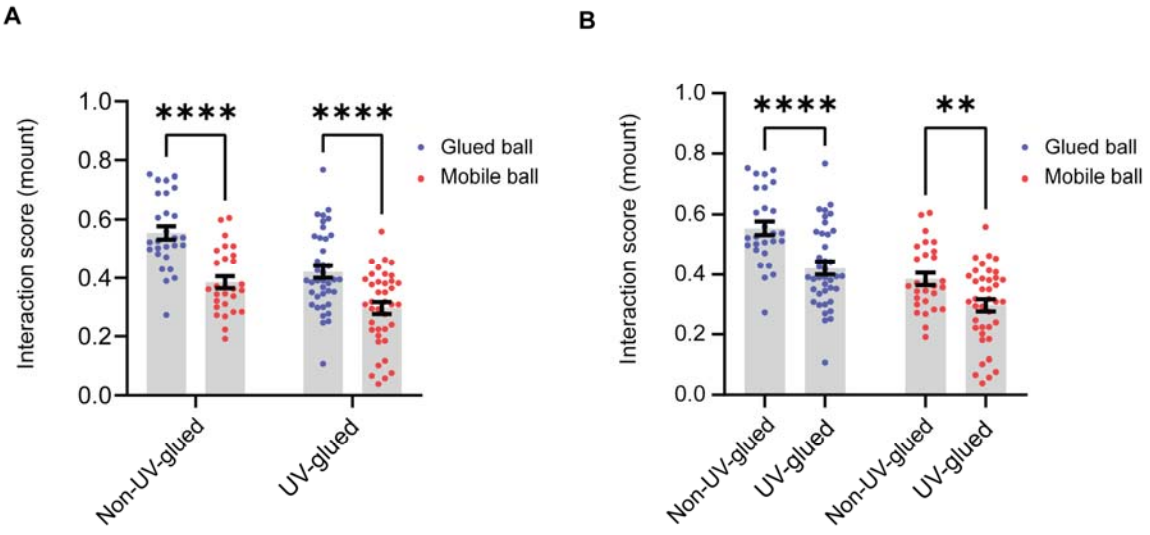

A
B
Interaction score (mount)
1.0
0.8
0.6
0.4
0.2
0.0
****
**
Glued ball
Mobile ball
Non-UV-glued
UV-glued

Figure S7. Flies preferentially interact with immobile balls regardless of antennal UV-gluing.

(A) Flies exhibited significantly higher interaction scores (mounts per visit) for immobilized balls compared to mobile balls, regardless of whether their antennae were UV-glued or left intact. Global interaction scores across the full session were used for analysis. *Two-way ANOVA with Bonferroni's multiple comparisons test*; *n* = 28 flies (non-UV-glued), 39 flies (UV-glued). **(B)** Overall interaction scores for both ball conditions were significantly lower in flies with UV-glued antennae. This reduction may result from loss of olfactory input or from blocking antennal chordotonal neurons. Additional experiments are needed to distinguish between these possibilities. *Two-way ANOVA with Bonferroni's multiple comparisons test*; *n* = 28 flies (non-UV-glued), 39 flies (UV-glued).

Figure S8

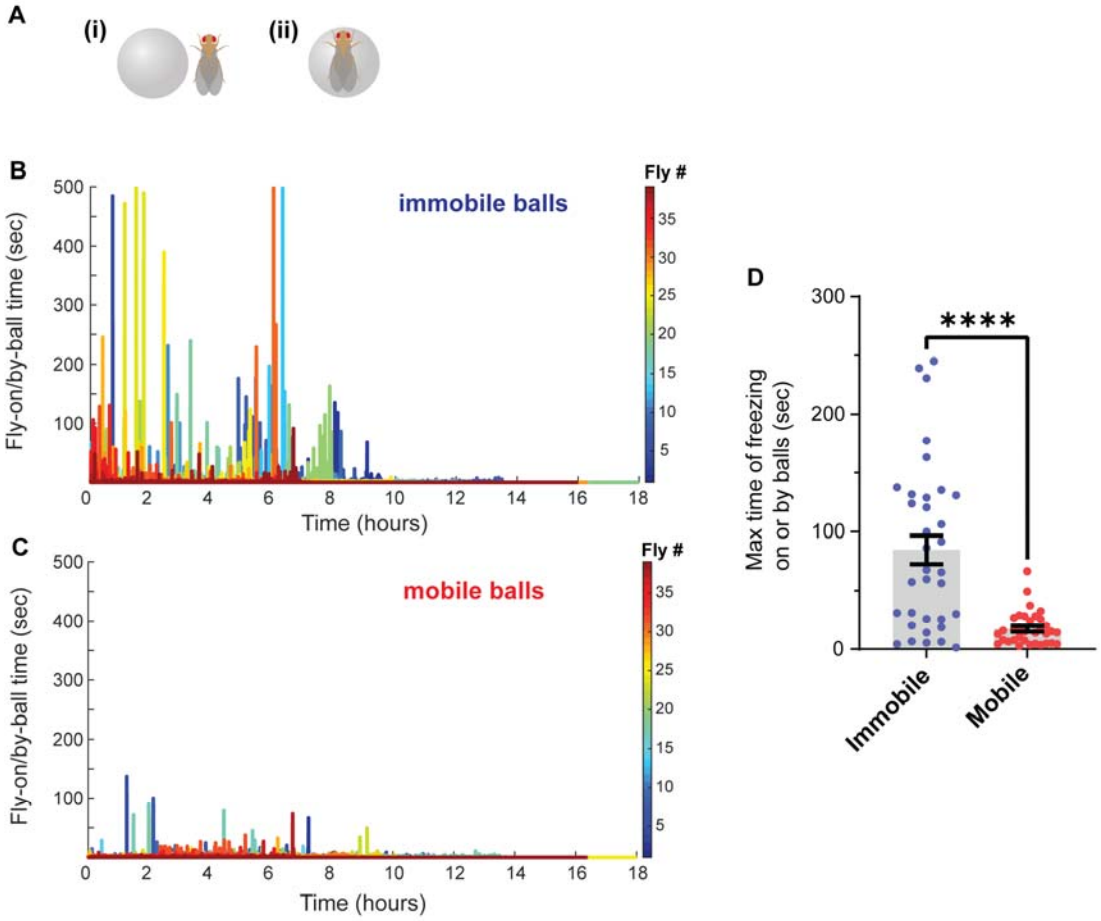

A
(i)
(ii)
B
immobile balls
Fly-on/by-ball time (sec)
Time (hours)
Fly #
C
mobile balls
Fly-on/by-ball time (sec)
Time (hours)
Fly #
D
****
Max time of freezing on or by balls (sec)
Immobile
Mobile

Figure S8. Flies spend significantly more time near immobile balls.

(A) Representative images of flies freezing either on (i) or beside (ii) balls during the experiment.
(B–C) Total time each fly spent by or on the ball across the experiment for (B) immobilized and (C) mobile ball conditions. Each trace represents a different fly (n = 39 flies per group).
(D) Maximum freezing duration (time spent on or near the ball) was significantly greater for immobilized balls. *Mann–Whitney test*, two-tailed (n = 39 flies per group). Five outliers were excluded using the ROUT method (Q = 0.1%, GraphPad Prism v10.0); the p-value remains significant with or without outliers (****, $p < 0.0001$).

Figure S9

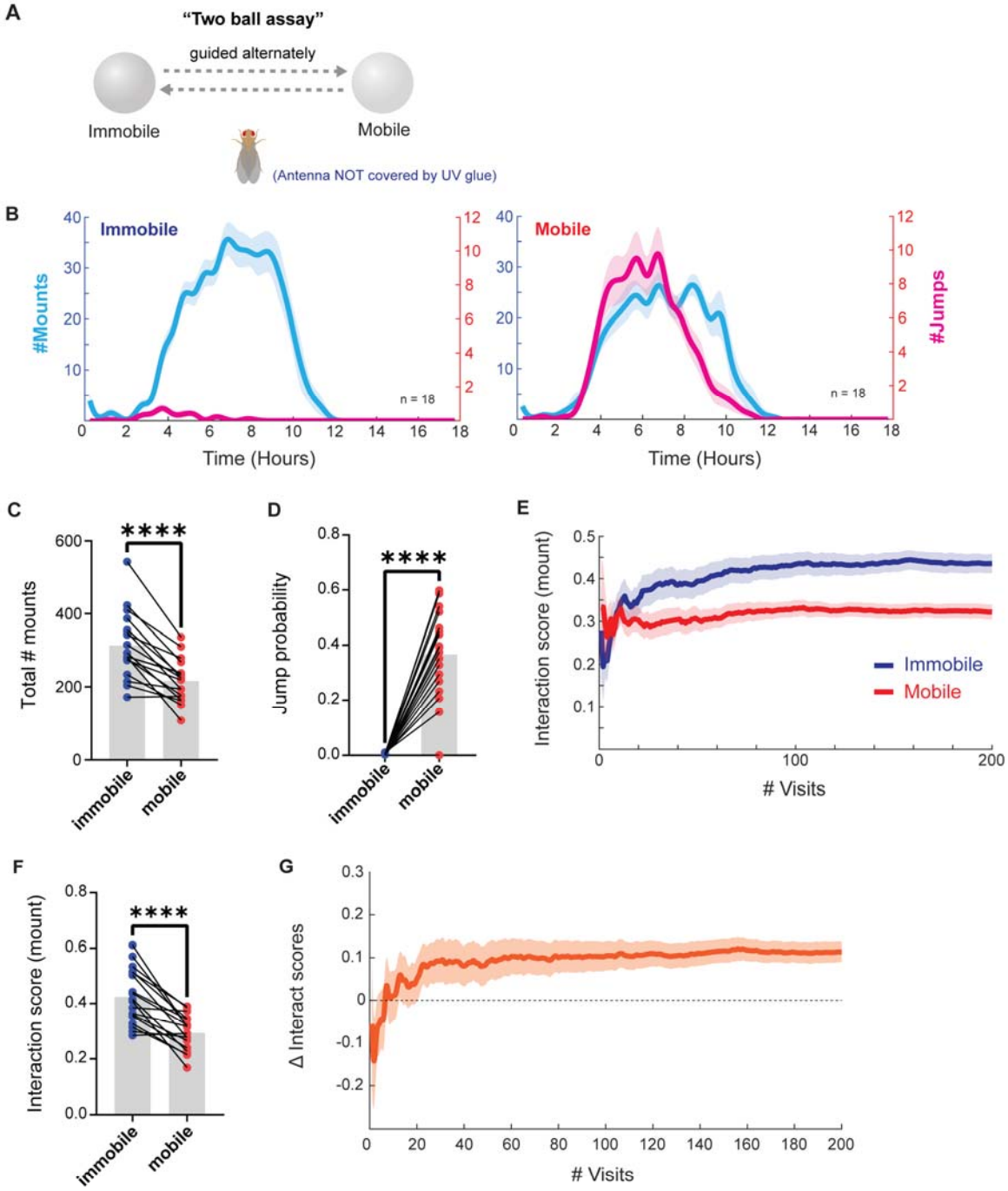

A
"Two ball assay"
guided alternately
Immobile
Mobile
(Antenna NOT covered by UV glue)
B
Immobile
Mobile
#Mounts
#Jumps
n = 18
Time (Hours)
C
Total # mounts
****
immobile
mobile
D
Jump probability
****
immobile
mobile
E
Interaction score (mount)
Immobile
Mobile
Visits
F
Interaction score (mount)
****
immobile
mobile
G
Δ Interact scores
Visits

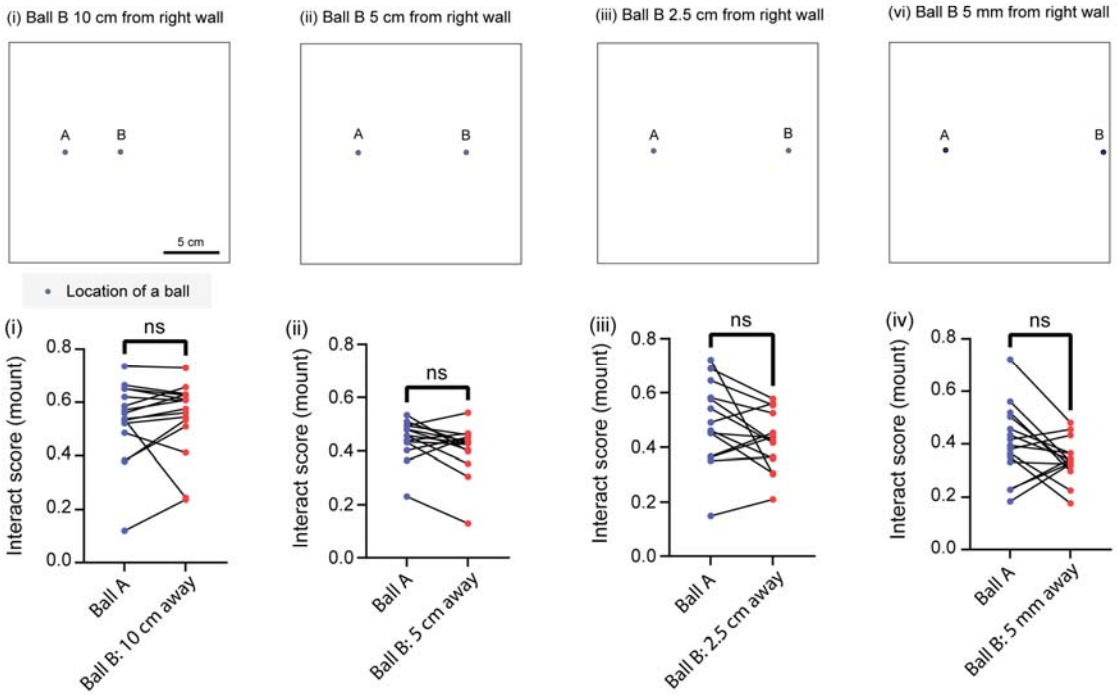
Figure S9: Flies develop a bias toward immobilized balls when alternately interacting with mobile and immobile balls, also with intact olfactory input.

This control experiment mirrors the two-ball assay described in Figure 5, but without antennal UV-gluing.

(A) Schematic of the two-ball assay. Test flies were alternately guided to interact with a mobile and an immobilized (glued) ball. Antennae were left intact (no UV glue).
(B) Time course of mount and jump frequencies for mobile versus immobile balls (n = 18 flies per group; 30-minute bins).
(C) Total number of mounts was significantly higher for immobile balls (*paired t-test*, two-tailed; n = 18 flies).
(D) The probability of jumping after interaction was significantly higher for mobile balls (*Wilcoxon matched-pairs signed-rank test*, two-tailed; n = 18 flies).
(E) Final global interaction scores (mounts per visit) for both conditions (n = 18 flies).
(F) Interaction scores were significantly higher for immobilized balls (*paired t-test*, two-tailed; n = 18 flies).
(G) Δ interaction score (immobile – mobile) plotted as a function of number of visits. A stable preference for immobilized balls emerged after approximately 10 visits on average, following an initial dip (n = 18 flies).

Figure S10

A The effect of ball B's distance from the closest wall



B

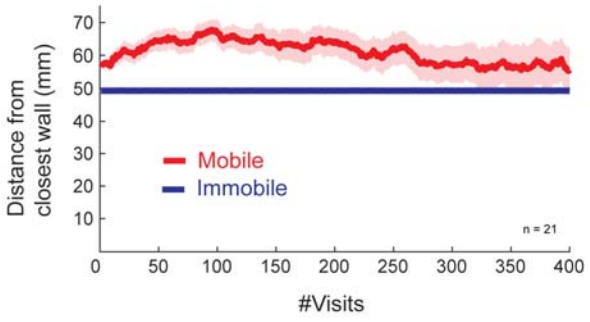


Figure S10. Ball location does not influence interaction scores (mounts) in two-ball experiments.

(A) To determine whether ball position in the arena affects interaction behavior, we conducted four two-ball assays in which the location of Ball B (glued) was varied across the arena (conditions i–vi), while Ball A (also glued) remained fixed. Each experiment was analyzed using a paired comparison of interaction scores (mounts per visit) for Ball A versus Ball B. No significant differences were observed in any condition. *Wilcoxon matched-pairs signed-rank tests* were used for conditions (i) and (ii), and *paired t-tests* for conditions (iii) and (vi) (n = 16, 14, 14, 15 respectively). The two-ball assay enabled controlled within-subject comparisons.
(B) Average distance of each ball from the nearest arena wall. While mobile balls tended to be slightly farther from walls than glued balls, this difference did not correlate with changes in interaction scores, as shown in (A). Data reflect the first 400 visits, a threshold reached by the majority of flies (81%; 21/26); only 42% reached 500 visits.

Figure S11

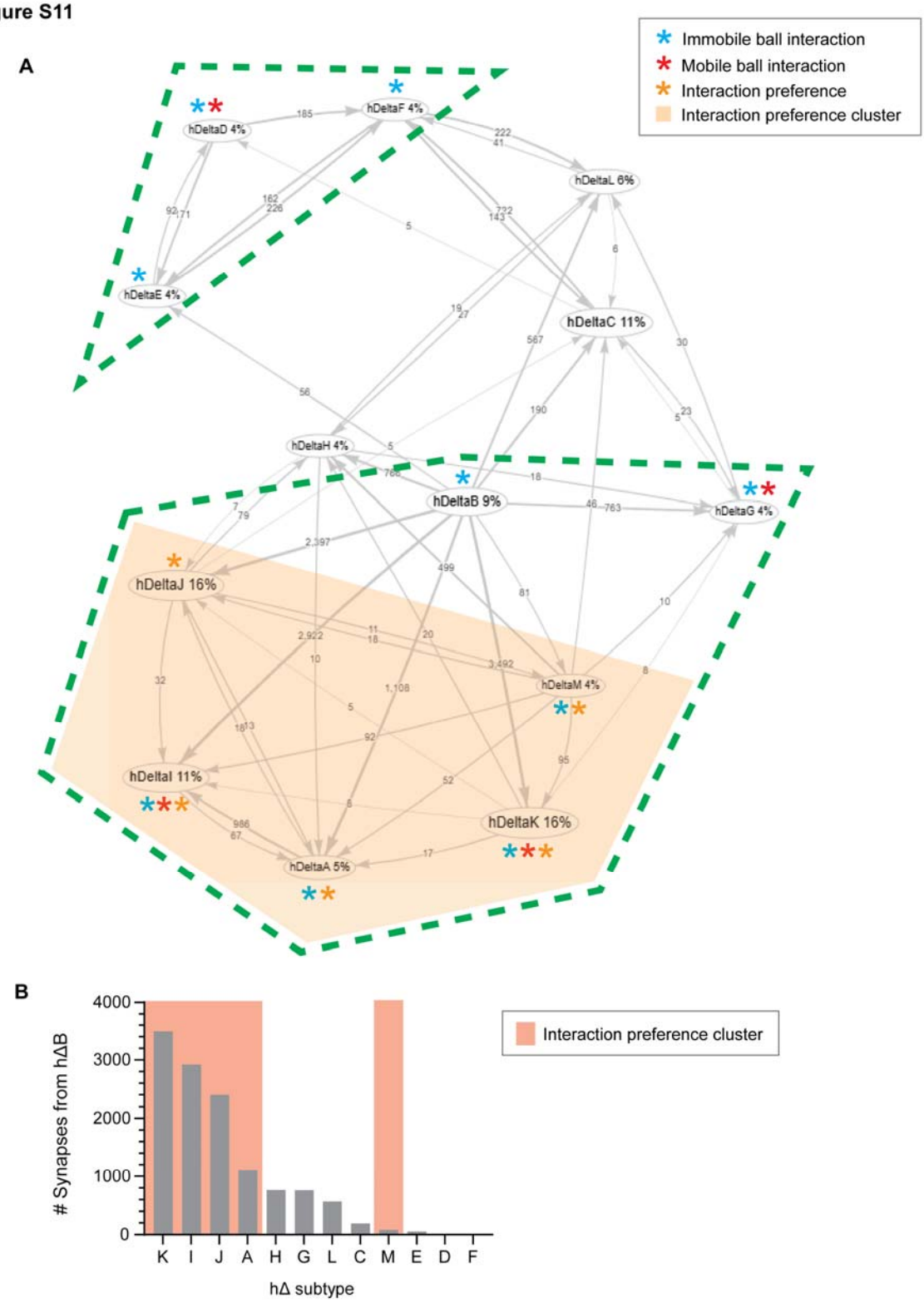

A
Immobile ball interaction
Mobile ball interaction
Interaction preference
Interaction preference cluster
hDeltaD 4%
hDeltaF 4%
hDeltaL 6%
hDeltaE 4%
hDeltaC 11%
hDeltaH 4%
hDeltaB 9%
hDeltaG 4%
hDeltaJ 16%
hDeltaM 4%
hDeltaI 11%
hDeltaK 16%
hDeltaA 5%
B
Synapses from hΔB
hΔ subtype
K I J A H G L C M E D F
Interaction preference cluster

Figure S11. Connectivity among hΔ neuron subtypes implicated in fly-ball interaction behaviors.

(A) A connectivity map was generated using Codex (https://codex.flywire.ai/) (Matsliah et al. 2023; Dorkenwald et al. 2024; Schlegel et al. 2024) for all 13 hΔ neuron subtypes tested in the behavioral screen. Neuron subtypes identified as significant for specific behavioral outcomes are color-coded: blue for immobile ball interaction, red for mobile ball interaction, and orange for interaction preference (Δ interaction score). Force Atlas 2 Dynamic (Codex) was used and top 50 connections are shown with minimum five synaptic connections.
(**B**) Synaptic outputs from hΔB neurons onto downstream partners shown in the network in (A), based on synapse counts.

Figure S12

A

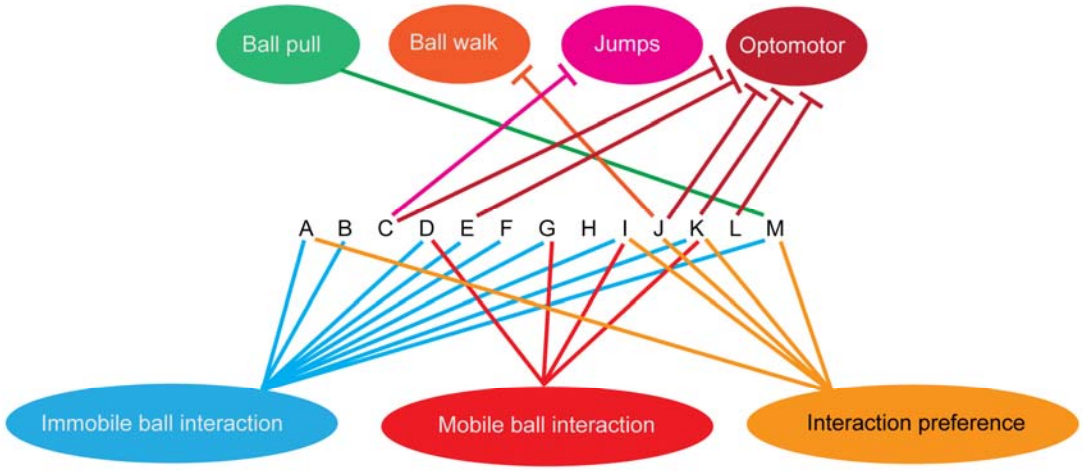
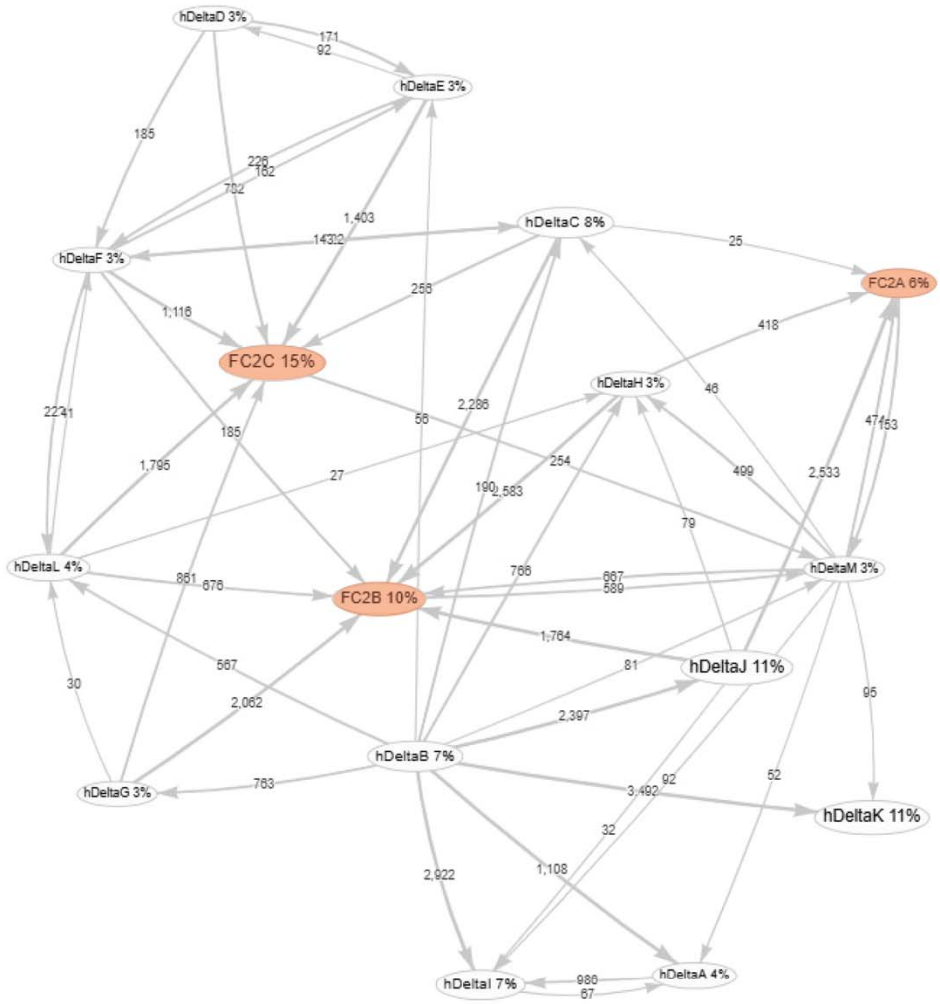

Ball pull
Ball walk
Jumps
Optomotor
A B C D E F G H I J K L M
Immobile ball interaction
Mobile ball interaction
Interaction preference

Figure S12. hΔ neurons collectively influence fly-ball interaction probability and behavior.

(A) Summary diagram indicating which hΔ neuron subtypes are involved in object interaction probability, interaction preference (immobile vs. mobile), and specific behaviors expressed during fly-ball interactions. This summary integrates data from Figures 6E and 7F.

Figure S13

A



B

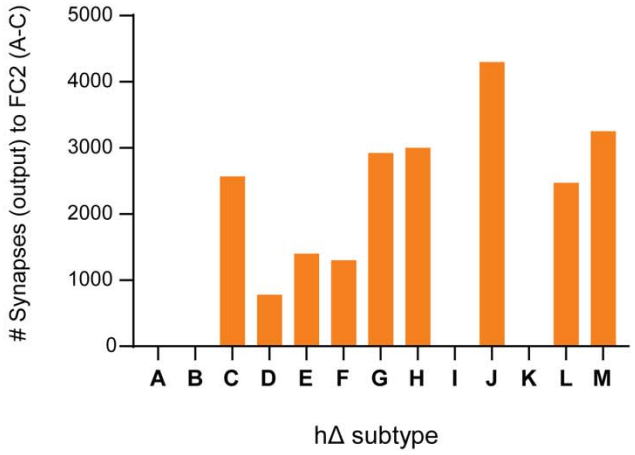
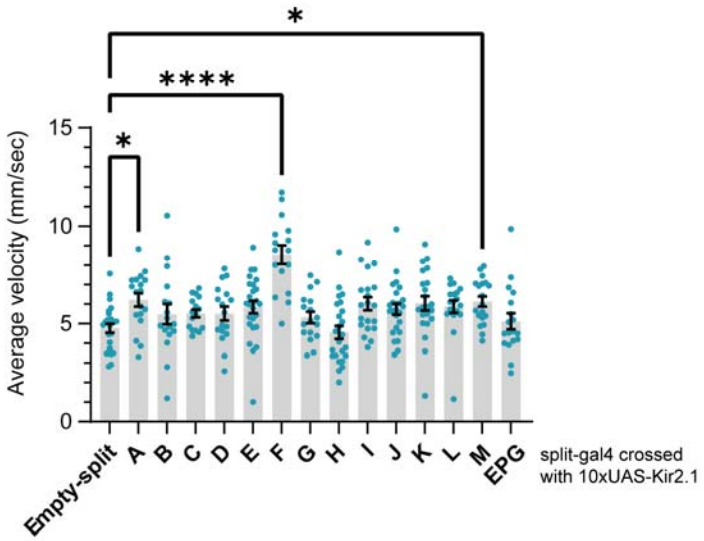


Figure S13. hΔ neuron subtypes project to FC2 neurons.

(A) Network diagram showing synaptic connectivity between hΔ neuron subtypes and FC2 neurons (types A–C). Connections were visualized using Codex (FlyWire) with a ForceAtlas2/Dynamic layout. Only the top 50 connections are shown, with a minimum threshold of 5 synapses per connection.
(B) Quantification of synaptic output from hΔ neuron subtypes to FC2 neurons (types A–C). The y-axis indicates the total number of synapses from each hΔ subtype onto FC2 targets, as represented in (A).

Figure S14

A



B

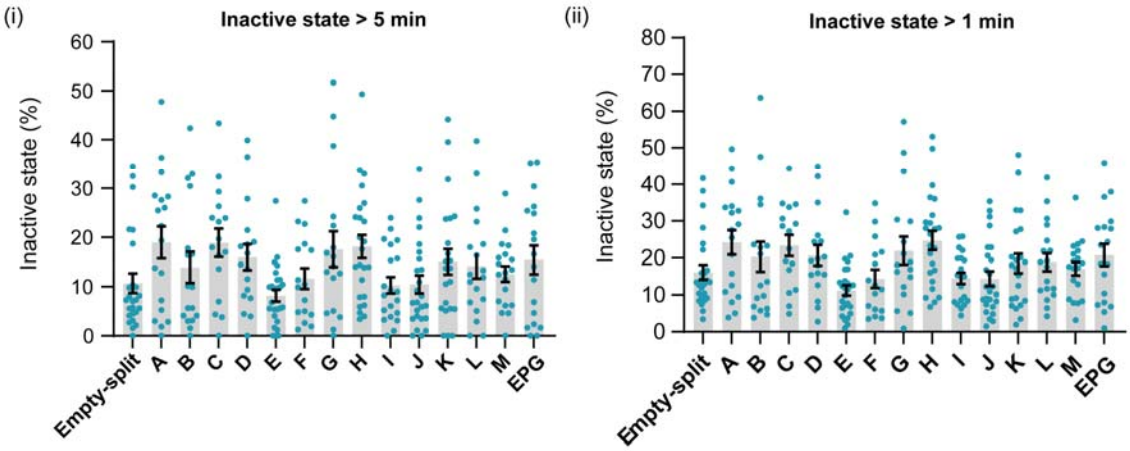


Figure S14. hΔ neuron manipulations do not impair general locomotion or induce prolonged inactivity.

(A) Average locomotor velocity during active periods for each experimental group tested in the hΔ screen. No group showed reduced speed relative to the control. Three groups – hΔA, hΔF, and hΔM – exhibited significantly higher locomotor speeds than control. *One-way ANOVA with Bonferroni's multiple comparisons test.* The sample sizes are as follows: hΔA (18), hΔB (17), hΔC (14), hΔD (19), hΔE (26), hΔF (16), hΔG (17), hΔH (24), hΔI (20), hΔJ (25), hΔK (22), hΔL (18), hΔM (19), EPG (18). An empty-split-GAL4 × UAS-Kir2.1 line was used as the control group (n = 25).

(B) Proportion of time spent in inactive states, calculated using two thresholds: inactivity lasting >5 minutes (i) or >1 minute (ii). No experimental group differed significantly from control under either criterion. Sleep duration was defined as 5 min or more of inactivity (Shaw et al. 2000). *Kruskal–Wallis test with Dunn's multiple comparisons test.* The sample sizes for (i) and (ii) are as follows: hΔA (18), hΔB (17), hΔC (16), hΔD (17), hΔE (26), hΔF (16), hΔG (17), hΔH (24), hΔI (20), hΔJ (25), hΔK (22), hΔL (18), hΔM (19), EPG (18). An empty-split-GAL4 × UAS-Kir2.1 line was used as the control group (n = 25).

Video 1. An example of a ball pulling/pushing behavior

Video 2. Another example of a ball pulling/pushing behavior

Video 3. An example of a ball walk behavior

Video 4. Another example of a ball walk behavior

Methods and Materials:

Fly strains & Maintenance

Flies were reared on standard cornmeal medium (Corn syrup–soy formulation, Archon Scientific) under a 12:12 h light:dark cycle at 25 °C. Stocks were maintained in vials within environmental chambers (Biocold Environmental Inc., Insect Environmental Chamber; Percival Scientific, Model LED-36L2) with relative humidity set to 50%. Genotypes of adult flies used in each experiment are detailed in Tables 1 and 2.

Fly-ball interaction experimental apparatus

Fly–ball interaction experiments were conducted in a custom-built behavioral chamber (43 × 43 cm), subdivided into four individual arenas (19.5 × 19.7 cm each) using 8-mm plastic dividers, as previously described (Iwasaki et al. 2025). Male flies were reared one day post-eclosion from vials containing either Formula 4-24 (Carolina Biological Supply) or Corn Syrup/Soy food (Archon Scientific). All male flies were tested at one day after eclosion. Prior to each experiment, a 2.38-mm diameter, 10-mg white Delrin acetal resin ball (McMaster-Carr #9614K51) was placed at the center of each arena. Flies were briefly anesthetized on ice (20–30 seconds) and positioned in a corner of the arena. An acrylic cover (6 mm thick) was placed atop the chamber to prevent flight, setting the vertical clearance to 5 mm. The arena walls and underside of the cover were treated with SigmaCote (Sigma-Aldrich SL2-25ML) to prevent wall climbing.

Fly behavior was recorded at 16 fps using a FLIR Blackfly S camera (BFS-U3-63S4M). Flies were visually guided toward the ball using a rotating pinwheel cue (480 nm, 2.4–2.6 μW/mm²) projected by an Optoma ZH450. The cue was automatically turned off when the fly entered a 5-mm radius “close-enough zone” around the ball and reactivated upon exit. See our previous study for pinwheel guidance parameters (Iwasaki et al. 2025). Each experiment continued until the fly died. The arena surface was leveled to within 0.05° using a dual-axis digital protractor (TLL-90S, Jingyan Instruments) to prevent unintended ball movement.

Ball immobilization

For the immobilized ball condition, each ball was affixed to the arena floor using a small amount, approximately (0.5 – 1.0 µl) of School Glue (Elmer’s). A thin line of glue was first applied to a sheet of 3M Post-it paper, and each ball was lightly touched to the glue using fine-point tweezers (2A-SA, Romain) before being placed in the arena (Figure 4A). All glued balls were left to dry for at least 30 minutes prior to the start of the experiment. The amount of glue used was sufficient to prevent ball displacement by the fly, while keeping the vertical height nearly identical to that of a free ball (Figure 4A). If a glued ball exhibited any movement during the experiment, suggesting incomplete adhesion, the experiment was excluded from further analysis.

Two-ball assay

In the two-ball assay, a mobile and an immobilized ball were placed 10 cm apart at the center of each test arena. Flies were guided alternately to the two balls using the visual pinwheel navigation cue. Once a fly exited the close-enough zone surrounding one ball, the guidance cue redirected it toward the other.

Control for potential contamination from fly droppings

To assess whether the observed fly-ball interaction motifs, such as ball pushing and ball walking, might be influenced by the accumulation of fly excreta on the ball surface, we conducted a post-experiment contamination control. Twenty 1-day-old male flies were allowed to interact with balls until death. At the conclusion of each experiment, the ball was examined under a stereo zoom microscope (Leica S7 E) for the presence of droppings. Only 1 of the 20 balls showed any sign of contamination: a single fecal spot approximately 0.4 mm in radius. These results indicate that accumulation of fly droppings is rare under our experimental conditions and is unlikely to account for the progressive changes in fly–ball interaction behavior observed over time. All flies displayed ball walking behavior in this experiment.

Fly and ball trajectory analysis

Fly and ball trajectories were extracted from x,y coordinates recorded during the experiment, as described in our previous study (Iwasaki et al. 2025). Behavioral data were recorded at 16 frames per second using a FLIR Blackfly S camera (BFS-U3-63S4M).

Ball Push analysis

Ball push events were identified by first detecting “entry time points” – defined as the time a fly entered the “close-enough zone” (within a 5-mm radius) around a ball. During the experiment, flies were repeatedly guided to this zone to promote fly–ball interactions. To detect push events, ball movement was assessed during the 2.5 seconds following each entry point, based on the observation that most fly-induced displacements occur within this window. A ball push was defined as a displacement of more than 1 mm occurring over at least four consecutive frames (i.e., >250 ms). To minimize false positives due to tracking noise, sequences consisting only of single-pixel displacements (0.25 mm) were excluded. The mean number of ball push events was calculated by averaging event counts within 30-minute time bins across all test flies.

Interaction Score Analysis

Cumulative interaction scores for mounts or ball pushes were calculated by dividing the total number of mount or push events by the number of visits, where a “visit” was defined as an instance in which the fly entered the close-enough zone (within 5 mm of the ball). Maximum interaction scores were determined using a sliding window of 50 visits. For each fly, the highest interaction score observed within any 50-visit window was reported as the maximum.

Cluster Analysis of fly-ball interactive events (Figure 2)

A total of 29,982 fly-ball interaction events were analyzed from 48 *Empty-GAL4* male flies. Events were clustered into five groups using the k-means algorithm (MATLAB), based on three metrics: pre-exit ball movement, post-exit ball movement, and post-exit fly movement (Figure 2). We chose the k-means algorithm for its simple implementation and sufficient capability to capture the diversity of interactive strategies observed in our large dataset. Interactive events were defined as periods during which a fly entered and remained within a 5.0-mm radius "close-enough zone" around a ball. The start of an event was marked by the fly's first contact with the ball; exit was defined as the moment when the fly–ball distance exceeded zero. Post-exit fly movements exceeding velocity – 532 mm/s – were excluded as tracking artifacts. Pre- and post-exit ball movements were measured in the 2 seconds before and after the fly left the ball. Displacements <0.5 mm per 63 ms frame (2 pixels) were filtered out. Post-exit fly movement was calculated over a 188 ms window, based on prior data showing that most exit-related jumps occur within this period (Figures S4A,B).

Five clusters were chosen to isolate "walk-off" events (minimal fly and ball movement) and reduce motif redundancy (Figure 2A). Each cluster was defined by distinct kinematic features and assigned a putative behavioral label (Figure 2B):

- *Walk-offs*: minimal ball and post-exit fly movement
- *Ball pulls*: large post-exit ball movement, low post-exit fly movement
- *Ball walks*: large pre-exit ball movement, low post-exit movement
- *Small jumps*: post-exit fly speed >53 mm/s
- *Large jumps*: post-exit fly speed >133 mm/s

Cluster composition was evenly distributed across flies; no individual contributed more than 12% of events to any single motif (Figure S4C). Overall distribution was as follows: walk-offs (46%), small jumps (19%), ball walks (14%), ball pulls (13%), and large jumps (8%) (Figure 2C).

Analysis of ball walks and ball pulls (Cluster-dependent analysis)

To examine changes in behavior over time, we compared the frequency of cluster-defined ball walk and ball pull events between the first and second halves of each experiment (Figures S5E, S5F). For this analysis, only periods during which flies were actively moving ("active times") were included. Each experiment was divided into 15-minute bins, and total fly movement was calculated per bin. Displacements <0.5 mm per frame (63 ms) were excluded to minimize tracking noise. Bins in which the average fly speed exceeded 2 mm/s were classified as "active." The full active period was defined as the interval between the start of the first active bin and the end of the last active bin. Ball walk and ball pull event counts were then compared between the early and late halves of this active window to assess temporal shifts in behavior.

Analysis of ball walks vs. ball pulls (Cluster-independent analysis)

Ball walks and ball pulls were also analyzed using a cluster-independent approach based on kinematic criteria (Figure 3).

Ball walks were defined by continuous ball displacement while the fly remained on the ball. To qualify, the total displacement had to exceed 2.5 mm, excluding movements likely to result from tracking noise or center-of-mass shifts (which can occur due to the fly's body weight). Additionally, the fly had to remain on the ball for at least one frame (63 ms). Total displacement was calculated by summing inter-frame distances throughout the interaction.

Ball pulls were defined by post-exit displacement of the ball. While the fly remained on the ball, movement had to be <2.5 mm. A ball pull was then detected if displacement after the fly exited the ball exceeded 3 mm. Pull magnitude was calculated as the total distance moved between the moment of fly exit and when the ball came to rest.

To assess behavioral changes over time, active periods were split into early and late halves for comparison.

Interactive jump analysis

Interactive jumps, defined as rapid fly movements immediately following ball interaction, were identified as events in which the fly's speed exceeded two body lengths (5mm or equivalently 27 mm/s during the first three frames – 188 ms) after exiting the ball. To exclude tracking artifacts, fly movements exceeding 532 mm/s were filtered out. To examine how jumping behavior evolved over time, the number of jumps was averaged across all flies within each 30-minute time bin.

Mount analysis

A "mount" interaction was defined as a period during which the fly maintained physical contact with the ball, with a fly–ball distance of zero. The event began when the fly first touched the ball and ended when it exited (i.e., the fly–ball distance became greater than zero). During a mount, the fly could either be standing on top of the ball or beside it while maintaining contact.

Freezing analysis

Freezing was defined as any period during which a fly remained stationary on top of or beside a ball during an interaction. This analysis was based on fly–ball distance measurements to identify when and where a mount occurred. The total duration of each interaction in which the fly remained in contact with or immediately adjacent to the ball was recorded as freezing time.

Tracking artifact filtering

To reduce the impact of tracking errors, data from all analyses were filtered as follows: abrupt drops in fly–ball distance, fly trajectory, or ball trajectory greater than 10 mm within a single frame (63 ms) were removed. Adjacent bins containing similar high-magnitude movements over ≥10 frames (625 ms) were also excluded to account for surrounding tracking instability.

Table 1. The genotypes of flies described in each figure

Figure	Genotypes
1 - 5	*Empty-Gal4 = w[1118]; P(y[+t7.7] w[+mC]=GAL4.1Uw)attP2 (Stock# 68384, BDSC)
6 – 7	Fly strains (Table 2) were crossed with pJFRC49-10XUAS-IVS-eGFPKir2.1 For the control condition, empty stable split* was crossed with 10xUAS-Kir2.1 *empty stable split = w[1118]; P[y[+t7.7] w[+mC]=p65.AD.Uw]attP40; P[y[+t7.7]w[+mC]=GAL4.DBD.Uw]attP2 (Stock# 79603, BDSC)
S1 – S10	Empty-Gal4

Table 2. The genotypes of fly strains used for hΔ neuron & EPG (silencing) screen

Driver	Target cell groups	Source	Stock #
SS59739	hΔA	BDSC	89341
SS64527	hΔB	BDSC	87107
SS02863	hΔC	BDSC	89337
SS64505	hΔD	BDSC	87845
SS90984	hΔE	FlyLight	3044013
SS54903	hΔF	BDSC	87922
SS02857	hΔG	BDSC	87631
SS60474	hΔH	BDSC	88512
SS60919	hΔI	BDSC	87338
SS91768	hΔJ	FlyLight	3045794
SS54676	hΔK	BDSC	89473
SS63922	hΔL	BDSC	87105
SS59766	hΔM	BDSC	87337
SS00090	EPG	BDSC	75849