Numerical discrimination in Drosophila melanogaster

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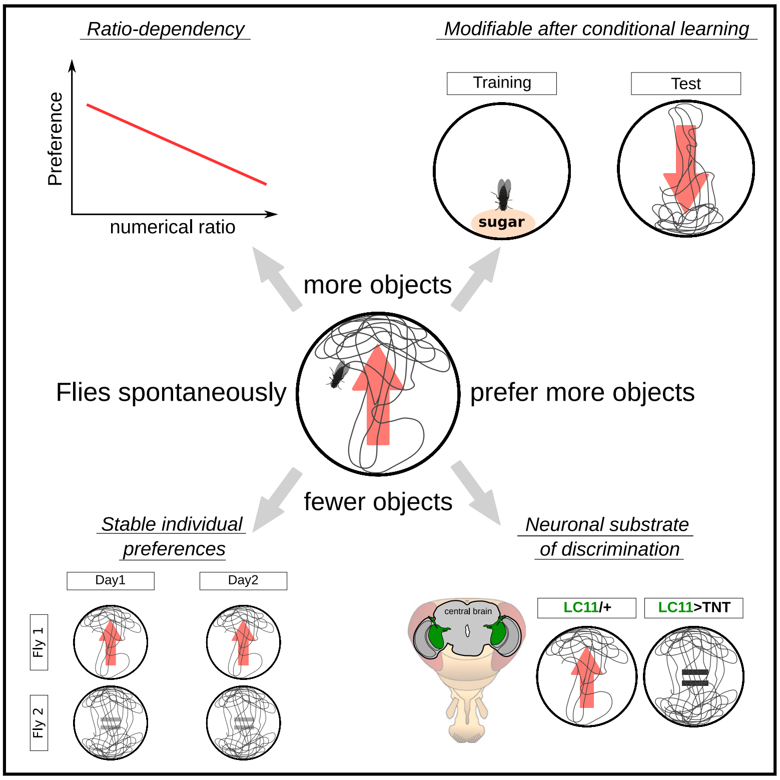
Graphical abstract



Highlights

- Flies discriminate quantity depending mainly on numerical ratio
- Flies display stable individual preferences between numerical quantities
- Flies can learn to associate a numerical quantity with a reward
- Spontaneous and learned numerical discrimination require visual LC11 neurons

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In brief

Bengochea et al. present *Drosophila* as a model system for the behavioral and neurogenetic analysis of numerical discrimination. Flies spontaneously prefer more objects but can reverse their preference upon reward conditioning. Silencing a group of visual neurons involved in small-object detection (LC11) diminishes the spontaneous and learned numerical discrimination.

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Numerical discrimination in *Drosophila melanogaster*

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SUMMARY

Sensitivity to numbers is a crucial cognitive ability. The lack of experimental models amenable to systematic genetic and neural manipulation has precluded discovering neural circuits required for numerical cognition. Here, we demonstrate that *Drosophila* flies spontaneously prefer sets containing larger numbers of objects. This preference is determined by the ratio between the two numerical quantities tested, a characteristic signature of numerical cognition across species. Individual flies maintained their numerical choice over consecutive days. Using a numerical visual conditioning paradigm, we found that flies are capable of associating sucrose with numerical quantities and can be trained to reverse their spontaneous preference for large quantities. Finally, we show that silencing lobula columnar neurons (LC11) reduces the preference for more objects, thus identifying a neuronal substrate for numerical cognition in invertebrates. This discovery paves the way for the systematic analysis of the behavioral and neural mechanisms underlying the evolutionary conserved sensitivity to numerosity.

INTRODUCTION

The cognitive capacity to make decisions based on numerical information is not restricted to humans. By estimating numbers, animals can dilute predation risks, increase predation efficiency,[1] and maximize food intake.[2] They can also perceive the number of social companions[3] and better communicate with conspecifics.[4] Accordingly, sensitivity to numbers has been documented in a wide variety of vertebrate species from primates and other mammals[5,6] to birds,[7,8] amphibians,[9,10] and fish[11,12] (reviews are available elsewhere[13–15]).

The combination of numerical tasks with simultaneous neural activity recordings allowed the exploration of the neural basis of brain functions for numbers (reviewed in Lorenzi et al.[16]). It was shown that single neurons in the prefrontal and posterior parietal neocortices for humans[17,18] and primates[19] spontaneously respond to specific numerosity. Similarly, "number neurons" have been reported in the telencephalic nidopallium caudolaterale of corvids.[20] In fish, there is evidence for broad activation of the caudal telencephalon during numerosity changes.[21,22] Understanding how the brain computes numerical information will require not only the recording of brain areas but also the manipulation of specific brain regions during the execution of the numerical task. Although a lesion study in primates[23] and several electrical stimulation studies in humans[24–26] have been performed, the study of the neurophysiological basis of numerical cognition in vertebrates has thus far proved to be experimentally difficult due to a combination of challenges of accessibility, complexity, and lack of tools.

Insects have long been used to explore the neuronal computations of complex behaviors (reviewed in Kinoshita and Homberg[27]). Recent evidence shows that numerical skills also provide fitness advantages to invertebrates,[28–30] suggesting that numerical ability may have evolved in common ancestors of insects and mammals. Number judgments allow increased reproductive opportunities in beetles,[31,32] improved predation strategies in spiders[33,34] and ants,[35] estimation of the distance traveled through step counting in desert ants,[36,37] and enhanced foraging strategies in bees.[38] Several tasks have been developed to document the numerical abilities of insects. The most common approach consists in simultaneously presenting two stimuli that differ in numerosity. Observations suggest that animals often prefer larger sets of items. For example, individual carpenter ants spontaneously discriminate between two piles of dummy cocoons,[39] while crickets spontaneously choose the set with the larger number of shelters.[40] Furthermore, it has been shown that honeybees have a spontaneous preference for multiple flowers only in comparisons where the number 1 was the lower quantity and where the ratio between the lower and higher quantity was at least 1:3.[41] The numerical abilities of bees have also been demonstrated using associative learning paradigms upon extensive training (30–100 trials[42–47]).

Despite significant behavioral evidence of numerical tasks in invertebrates, the neuronal basis remains unknown. To our knowledge, numerosity perception has not been studied in *Drosophila*. The fruit fly would offer an excellent experimental platform to uncover the genetic and neurobiological processes for numerical cognition mainly thanks to the availability of tools to label, manipulate, and record activity of neurons and decipher their connectome. Recent studies suggest the existence of magnitude judgment in *Drosophila*. Flies adjust their defensive behavior depending on the number of conspecifics in the group. In response to inescapable threats, flies' freezing behavior decreases with increasing group size.[48] Another study showed that fruit flies also tune their social interactions to group size and density.[49] Whether such changes in behavioral strategies actually reflect a response to number or other continuous dimensions of the stimuli is unknown.

Here, we report evidence of numerical discrimination in *Drosophila*. We show that flies display robust preference for more numerous sets of visual objects, and this discrimination depends mainly on their ratio. Each fly deploys one of three dynamical behavioral patterns during numerical decisions and sustains this behavioral strategy over time. Furthermore, spontaneous preference can be modified by a single training trial of classical Pavlovian conditioning. Finally, we report that silencing lobula columnar neurons 11 (LC11) causes a decrease in spontaneous and learned numerical preference. These findings thus identify a neuronal component of the circuitry required for robust numerical discrimination in *Drosophila*.

RESULTS

Drosophila show a spontaneous preference for larger numbers of items

To evaluate whether flies show spontaneous preference between sets of objects that differ in numerical size, we modified the classical Buridan paradigm[50] (Figure 1A). We measured the amount of time each fly spent near a "preference zone" (i.e., occupancy) in the vicinity of each stimulus (Figure 1B) and used the difference of durations divided by their sum as a preference index (PI).

We first evaluated the spontaneous PI for a set of three stripes versus a single stripe. Flies stayed significantly longer in the preference zone of the arena corresponding to the set of three stripes (Figure 1C). The same preference prevailed when the position of the stimuli was rotated 180° relative to the external environment (Figure S1 A1 and A2) and no differences were found between sexes (Figure S1 B1–B3). Interestingly, when examining the individual trajectories of single flies, we observed "loop paths" around the edges of stripes next to the higher numerosity, which may indicate that at least some flies track the edges of each stripe separately (Figure S1C). Still, this preference among sets differing in numerosity may result from a variety of potential confounds, including density, overall area of the display, dark area, and size of the individual shapes. When flies were given a choice between three stripes and a single wide stripe occupying the same horizontal extension, they still preferred the three stripes (Figure 1D) suggesting that the preference for the larger set was not due to the horizontal extent of the stimuli or to the total dark area, as the wide stripe had a larger dark area than the set of three stripes.

To test these findings with different displays, we replaced stripes with squares. Flies confronted with two single squares (1vs1) showed equal preference (Figure S1D). However, similarly to our previous observations with stripes, flies confronted with 1vs3 squares preferred the more numerous set (Figure 1E). Again, control experiments demonstrated that the preference was not due to the horizontal extent of the set (Figure 1F), nor to the total dark area (Figure 1G). Moreover, the pattern of preference remained the same when using discs (Figure S1E). Flies also showed a significant preference for the more numerous set in 1vs4 (Figure S1F) and 1vs2 (Figure S1G) squares tests.

These results may indicate that flies are either sensitive to numerosity or are able to distinguish a single item from sets of several items. To differentiate between these hypotheses, we tested flies in a 2vs4 squares contrast. We found that flies prefer the larger set (Figure 2A). In this experiment, stimuli were equated for density and square size but not for overall area occupied and total dark area. We next used the same numerical contrast to run a set of control experiments manipulating different variables that co-vary with number (Figure 2B). First, to equate overall area and square size, we increased the distance between the two squares such that the outermost edges of the two visual sets matched. Flies still showed a preference for the set of four squares (Figure 2C). Next, we equated the total dark area (while also keeping the horizontal extension equal) by increasing the size of squares in the set of two. Consistent with our previous findings, flies showed a significant preference for the four squares (Figure 2D). Finally, we equated both dark area and overall area (dashed line in Figure 2E) while moving from a linear to a rectangular array of stimuli, providing a significant challenge to the numerical preference. Remarkably, flies still favored the numerically larger set (Figure 2E). In addition, we observed similar responses in settings using 2vs3 squares contrast; flies showed a reproducible preference for the larger set when controlling for density, overall area, dark area, and spatial arrangement of the objects (Figures S2A–S2D). Finally, the preference for three over two objects was maintained when using other shapes, including stripes and discs (Figures S2E and S2F).

Drosophila use the approximate number system to discriminate between numerosities

Animals rely on two different cognitive systems to process numbers in non-symbolic arrays, called the object tracking system (OTS) and the approximate number system (ANS). The OTS reflects the ability to simultaneously represent and track several items (usually up to three or four),[51] thus providing access to exact numerosity for only small arrays. In contrast, the ANS represents the approximate numerosity of sets of objects with no upper bound. Importantly, numerical discrimination by the ANS is governed by Weber's law: the ability to distinguish between two stimulus magnitudes depends on their ratio. Ratio-dependent discrimination has been observed in many species and is considered to be a characteristic signature of ANS.[14,39,52,53]

To test whether flies use the OTS or the ANS, we asked if they have a limit of discrimination at four items, as observed in other insects.[32,40,43,46,54] We tested flies in a 3vs4 squares contrast, this time finding no preference (Figure 3A). We observed the

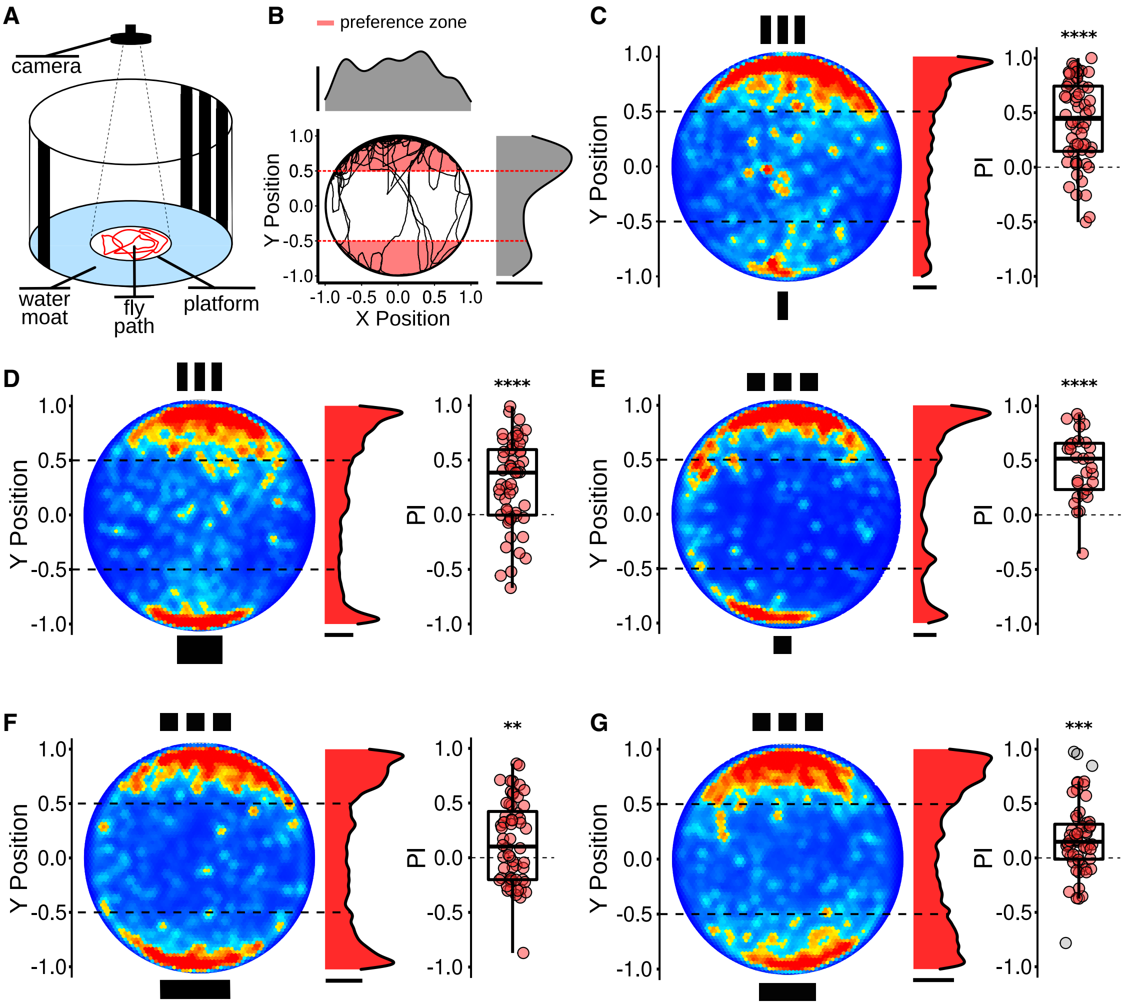


Figure 1. *Drosophila* spontaneously prefers larger numbers of items in a 1vs3 choice test

(A) Schematic of the experimental setup.

(B) Representative example. Red dashed lines illustrate y-limits of the preference zone (red area) for each stimulus. Kernel density plots on the top and right of the platform denote the position permanence of the fly along x and y axes respectively.

(C and D) At population level, flies show a significant spontaneous preference for three stripes over one stripe. (C) Left: heatmap illustrates the relative frequency of the fly location at each position of the platform (red denotes high-frequency permanence, while blue denotes low frequency). Middle: kernel population y-density plot. Right: mean population PI is significantly different from chance preference (n = 60, PI = 0.42 ± 0.38, p = 5.9e−09, Wilcoxon signed rank test). D. Control for overall area occupied (n = 56, PI = 0.29 ± 0.40, $t_{(55)} = 5.41$, p = 1.4e−06, one-sample t test).

(E–G) Flies kept their preference for more objects when tested with arrays of squares. (E) Flies' performance in a 1vs3 squares contrast (n = 29, PI = 0.45 ± 0.31, $t_{(28)} = 7.92$, p = 1.27e−08, one-sample t test). (F) Control for overall area occupied (n = 60, PI = 0.14 ± 0.38, p = 8.7e−03, Wilcoxon signed rank test). (G) Control for total dark area (n = 59, PI = 0.18 ± 0.34, $t_{(58)} = 4.00$, p = 1.8e−04, one-sample t test).

same lack of preference when equating for overall area (Figure 3B) and total dark area (Figure 3C). Importantly, this failure to discriminate three from four was not due to the similarity in total dark area of the two numerical sets: when the equivalent difference in total dark area was presented in a 2vs3 squares contrast, flies showed a significant preference for three squares (Figure 3D), and this preference significantly differed from the response in the 3vs4 experiment ($t_{(108)} = -3.23$, p = 0.0016, Welch two-sample t test).

The failure to discriminate between three and four is compatible with both systems. On one hand, flies may fail because numbers three and four are very close in terms of ratio (0.75), and cannot be distinguished by their ANS. On the other hand, flies may fail because the number four exceeds the capacity of their OTS. To distinguish between these two hypotheses, we investigated whether flies can discriminate numbers larger than four while equating for total dark area and overall area occupied. Flies consistently preferred larger numerosities in contrasts of 2vs6 (Figure 3E), 4vs8 (Figure 3F), 6vs8 tested in two different configurations (Figures 3G and 3H), and 9vs12 (Figure 3I). These data suggest that flies may be using the ANS to perform numerical discrimination.

Next, we wondered which parameter of the visual numerical stimuli best explains the numerical discrimination performance

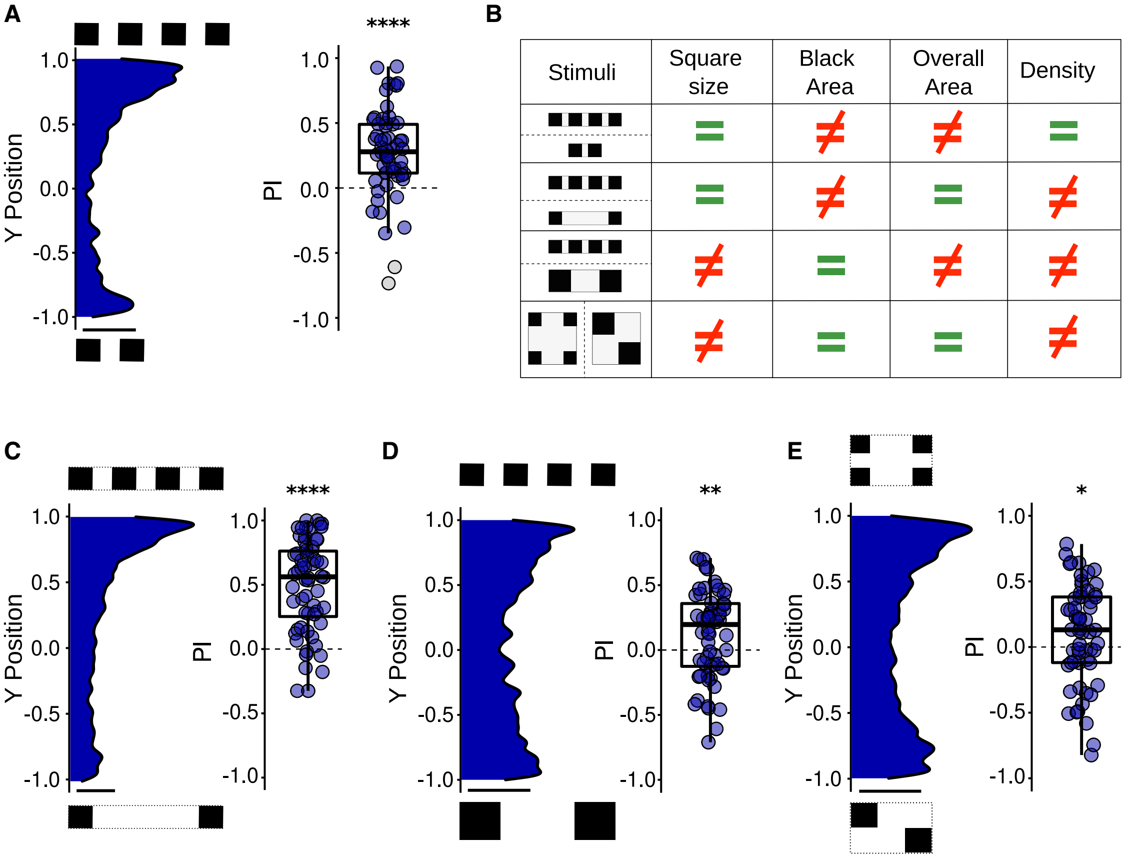


Figure 2. Flies spontaneously present a preference for more units in a 2vs4 numerical discrimination test, irrespective of non-numerical visual cues

(A) Flies preferred the four-squares stimulus (n = 60, PI = 0.27 ± 0.33, $t_{(59)}$ = 6.30, p = 4.13e−08, one-sample t test).

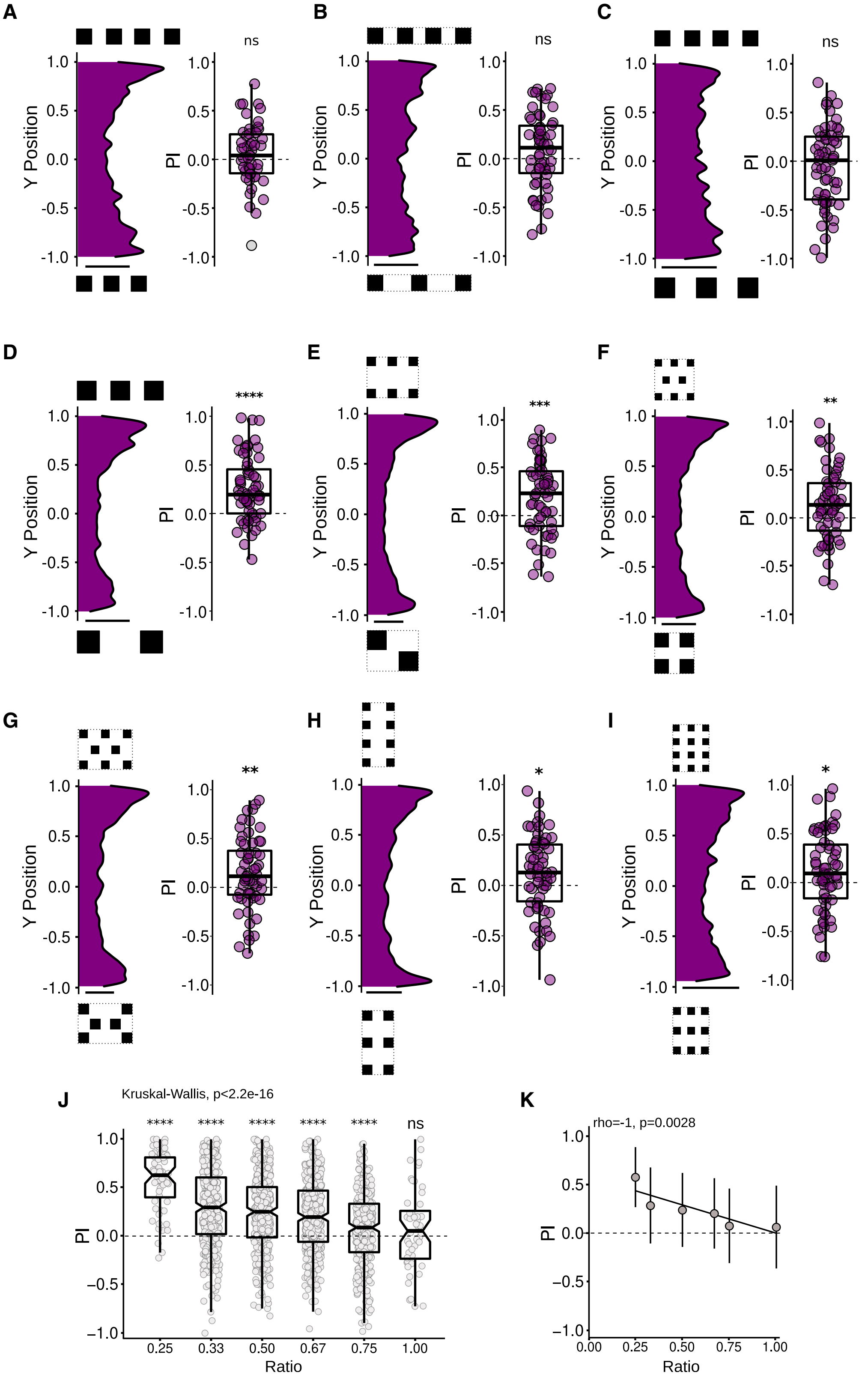
(B) Table illustrating the non-numerical features controlled in successive experiments.

(C–E) Series of experiments showing that the preference for numerically larger arrays is preserved when controlling for non-numerical cues. (C) Control for overall area occupied (n = 60, PI = 0.49 ± 0.36 p = 4.62e−10, Wilcoxon signed rank test). (D) Control for total dark area and horizontal extension of the numerical sets (n = 60, PI = 0.12 ± 0.34, $t_{(59)}$ = 2.74, p = 8.1e−03, one-sample t test). (E) Control for spatial distribution (n = 60, PI = 0.10 ± 0.38, $t_{(59)}$ = 2.09, p = 0.04, one-sample t test).

of the flies. Since it is impossible to control for all confounding variables within a single experiment, we implemented a forward and backward stepwise regression model to examine the statistical relevance of each variable resulting in a selection of variables that fit the numerical performance observed for flies (n = 1,599 flies; see STAR Methods for details). The model predicts that flies primarily rely on the numerical ratio (t = −8.90, p = 1.45e−18), which explains 75.9% of the model's variance and also highlights numerical distance as a variable explaining 5% of the variance (see Table S1 for details). To test the model's prediction, we plotted the PI against the numerical ratio and observed a significant negative correlation (rho = −1, p = 0.0028, Spearman correlation; Figures 3J and 3K). This analysis confirmed that flies principally use numerical parameters to perform the discriminative task. Furthermore, the ratio dependence of their performance strongly suggests that numerosity processing in *Drosophila* is based on the ANS. Interestingly, the model also highlights the total dark area as a continuous variable that can influence preference (t = 4.51, p = 7.07e−06, 19.1% of the variance; Table S1), reflecting the importance of having controlled for it in our experiments (Figures 1G, 2D, 2E, S2C, and S2D).

Flies display three individualized dynamical behavioral patterns for numerical discrimination

To understand the behavioral strategies used by flies to make ratio-based preference decisions, we performed cluster analysis of walking trajectories using all the spontaneous preference data, providing a typology of dynamic behavior during the preference task[55] (Figure 4). We found three main clusters of behavioral patterns: (1) flies that rapidly chose the larger set and remained there over most of the epoch (Figure 4Ai, green line in Figure 4B); (2) flies that first hesitated between the two halves of the arena before eventually choosing the larger set (Figure 4Aii, orange line in Figure 4B); (3) flies with no initial preference but later orienting weakly toward the smaller set (Figure 4Aiii, purple line in Figure 4B). Next, we identified the corresponding cluster for each individual behavior and studied the percentage of animals within each pattern. Animals tested with larger ratio contrasts (close to 0) predominantly display pattern 1, while animals



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tested with ratios closer to 1.0 displayed mainly patterns 2 and 3 (Table S4).

To examine whether this reflected stable individualized tendencies, we repeatedly tested individual flies over two consecutive days using a 2vs4 squares contrast. As expected, on average flies chose the larger set on both sessions and the PI did not differ between the 2 days (Figures 4C and 4D). Across flies, there was a strong positive correlation between the PIs in the two sessions (Figure 4E). Finally, we evaluated the stability of the temporal pattern using the clusters defined in the previous analysis. The fact that the clusters are defined on one dataset and tested on an independent dataset is an additional replication of the cluster validity. We assigned the trajectories of each fly on both days independently to the corresponding trajectory cluster. We first validated that cluster 1 was predominant on both days (55.9% of flies on day 1, and 47.5% of flies on day 2). In addition we found that flies were highly consistent in their temporal pattern of preference in between days. In other words, the chosen pattern on day 1 was the most likely pattern on day 2 (Figure 4F, p = 0.0014, Fisher's exact test). These series of analyses suggest that flies have stable individual preferences when making number-based decisions.

Spontaneous numerical preference can be modified by classical conditioning

We further tested the cognitive numerical capacities of flies by asking whether they can associate a learned value with a specific numerosity and change their preferences accordingly. Based on an appetitive classical conditioning paradigm, we endeavored to teach flies to reverse their spontaneous preference for larger numbers by associating sucrose (unconditioned stimulus [US]) with the set containing smaller numbers. Individual wet-starved flies were placed in the arena and were trained in a single trial of 3 min. We paired a sucrose stimulus with the single square (conditioned stimulus [CS+]) in a 1vs3 squares contrast (trained group [TR]). A control group (CT) was run in parallel by pairing the single square with water. For both groups, the set of three squares was water paired (CS−). Two hours after training, flies from both groups were individually tested for 5 min in one non-reinforced test with the same stimuli. In contrast to the control group, flies that were trained to associate sucrose with the single square significantly reduced their initial preference for the set of three squares in the testing session (Figures 5A, S3A, and S3B).

The loss of preference observed after learning could be due to two different causes. First, it may be that the flies simply explored the arena in search for food without learning any association. Second, it could be that flies did learn the association but that there was a competition between the learned response and the strong spontaneous preference for three squares compared to one. To disentangle these possibilities, we trained the flies with a 2vs3 squares contrast that elicits a slightly weaker spontaneous preference, thereby decreasing the competition between the potentially learned and the spontaneous response. In contrast to the control group (Figures 5B and S3D), flies trained to two square-rewarded stimuli showed a switch in their preference during the testing session, preferring the smaller number of items (Figures 5B and S3C). The preferences of the trained and control groups were also significantly different (Figure 5B right panel). Moreover, the conditioned preference for the lower numerosity was significantly different when testing the trained flies in a 2vs3 or a 1vs3 contrast (p = 0.0008, Wilcoxon rank-sum test), suggesting a competition between the natural preference to go for more items and the conditioned response. Importantly, the change in preference toward the smaller numerosity in trained animals was not due to satiation. Trained flies with sugar associated with both numerical sets showed a significant preference for the larger numerosity during the testing session (Figures S3I and S3J).

Did flies learn to respond to numerosity or to other variables of the visual displays? If the reversal of the preference is truly contingent on the numerosity of the trained stimuli, it should not occur in settings where flies are unable to distinguish stimuli based on their numerosity. We thus trained flies using a numerical contrast of 3vs4, which does not elicit a significant spontaneous preference. If flies had learned to respond to confounding variables—such as total dark area—we should find that they again develop a preference for the conditioned stimulus. In contrast, if flies learned to respond to the numerical variable, they should not learn when they cannot discriminate. We found that flies trained to prefer either three or four in a 3vs4 squares contrast show no preference during the testing session after training (Figures 5C, 5D, and S3E–S3H). Finally, conditioned performance between animals trained to prefer two in the 2vs3 squares contrast and animals trained to prefer three or four were significantly different ($2vs3\text{-}3vs4_{(CS+\rightarrow 3)}$, p = 0.013; $2vs3\text{-}3vs4_{(CS+\rightarrow 4)}$, p = 0.003, Wilcoxon rank-sum test). Together, these findings suggest that flies can learn to associate numerical sets with sucrose, and

Figure 3. Flies use ANS to discriminate among numerosities

(A) Flies tested in a 3vs4 squares contrast showed no numerical preference (n = 50, PI = 0.05 ± 0.32, $t_{(49)} = 1.11$, p = 0.27, one-sample t test).

(B) Control for overall area occupied (n = 58, PI = 0.08 ± 0.38, $t_{(57)} = 1.63$, p = 0.11, one-sample t test).

(C) Control for total dark area and horizontal extension of the numerical sets (n = 60, PI = −0.04 ± 0.41, $t_{(59)} = 0.93$, p = 0.36, one-sample t test).

(D) Flies preferred three squares in a 2vs3 contrast with the same dark area as 3vs4 (n = 60, PI = 0.25 ± 0.33, $t_{(59)} = 5.88$, p = 1.96e−07, one-sample t test).

(E) Flies preferred six squares in a 2vs6 contrast (n = 60, PI = 0.16 ± 0.40, $t_{(59)} = 3.15$, p = 0.003, one-sample t test).

(F) Flies preferred eight squares in a 4vs8 contrast (n = 60, PI = 0.13 ± 0.36, $t_{(59)} = 2.82$, p = 0.007, one-sample t test).

(G and H) Flies preferred eight squares in a 6vs8 contrast. (G) n = 60, PI = 0.14 ± 0.37, $t_{(59)} = 3.01$, p = 0.004, one-sample t test; (H) n = 60, PI = 0.12 ± 0.37, $t_{(59)} = 2.49$, p = 0.01, one-sample t test.

(I) Flies preferred 12 squares in a 9vs12 contrast (n = 60, PI = 0.11 ± 0.40, $t_{(59)} = 2.09$, p = 0.04, one-sample t test).

(J) Discriminative performance across numerical ratio (p < 2.2e−16, Kruskal-Wallis rank test. 0.25, n = 60, mean ± SD = 0.58 ± 0.31, p = 4.5e−11; 0.33, n = 384, mean ± SD = 0.29 ± 0.39, p = 1.6e−31; 0.50, n = 360, mean ± SD = 0.24 ± 0.38, p = 8.57e−25; 0.67, n = 399, mean ± SD = 0.20 ± 0.36, p = 5.86e−26; 0.75, n = 348, mean ± SD = 0.08 ± 0.38, p = 1.05e−04; 1.00, n = 48, mean ± SD = 0.06 ± 0.42, p = 0.38, Wilcoxon signed rank test).

(K) Spearman correlation shows that discrimination accuracy decreases as the numerical ratio between quantities becomes closer to 1.0.

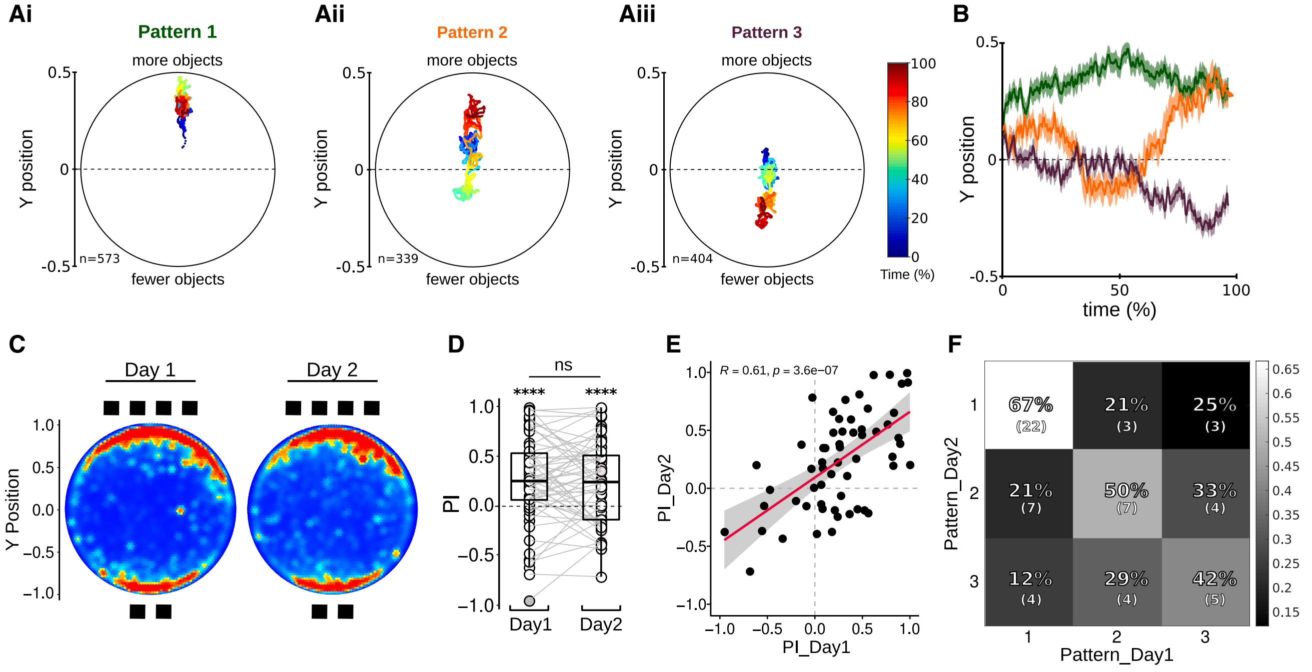

Figure 4. Flies display three main stable behavioral patterns of numerical preference

(A) Mean trajectory for each pattern of behavior. Color code indicates the recording time (%). (i) Flies with stable preference for more items along all recording time; (ii) hesitant flies that finally choose more items; (iii) Flies that show no preference at the beginning but then decide for fewer items.

(B) Y position over time for the three categories of behavioral preference.

(C) Population heatmap corresponding to each day.

(D) For each particular day, flies preferred four squares (day 1, n = 59, PI = 0.26 ± 0.44, $t_{(58)} = 4.48$, p = 3.61e−05, one-sample t test; day 2, n = 59, PI = 0.24 ± 0.42, $t_{(58)} = 4.45$, p = 3.97e−05, one-sample t test). The performance is maintained between consecutive days (p = 0.71; $t_{(58)} = 0.37$, paired t test).

(E) Pearson correlation plot. Gray shadow indicates confidence interval (95%).

(F) Matrix heatmap showing the relationship between patterns of behaviors in day 1 and day 2. The relative and absolute (between parentheses) number of animals with the specific relationship are indicated in each box.

non-numerical aspects of the visual pattern are not the properties of the stimuli that flies associate during the learning process.

LC11 neurons mediate numerical discrimination

To our knowledge, there is no functional evidence linking specific neuronal circuits to numerical abilities in insects. It has been proposed that number-based behavior emerges in higher-order areas of the insect brain such as the mushroom bodies (MBs) or the central complex (CC).[53] Other studies proposed that, in contrast, the number sense is deeply ingrained in the primary sensory system,[29,56] as numerosity is mostly conceived as a primary sensory attribute.[57] We examine these ideas in our model, taking advantage of the neurogenetic toolkit that *Drosophila* offers. We began by silencing several candidate neuronal populations by expressing the tetanus light chain (TNT) in different neuronal subtypes and testing the flies in the 2vs4 squares contrast task. We found that silencing the MBs or the CC respectively did not affect the preference for the larger numerosity (Figures S4A and S4B). Next, we evaluate the requirement of different visual system neurons. We found that silencing object orientation neurons, medulla dorsal cluster neurons (M-DCNs),[58] did not affect the ability of flies to prefer the larger numerosity (Figure S4C). Next, we tested LC11 neurons, which have been implicated in the adjustment of defensive behavior depending on group size, perhaps by making flies less sensitive to the movements of other flies.[48] We wondered whether LC11 is also involved in the detection of group size. We found that flies with silenced LC11 neurons were not able to discriminate between the stimuli (Figure S4D). LC11 neurons have also been shown to be required for small-object response (~10°),[59,60] as were another set of LC neurons called LC10a.[61,62] However, silencing LC10a neurons had no effect on numerical discrimination performance (Figure S4E). Together, these data implicate LC11 neurons in numerical behavior in *Drosophila*.

To further test the requirement of LC11 neurons in numerical discrimination and rule out the potential confounding effect of squares as small objects, we switched to large vertical stripes, as it has been reported that LC11 neurons show weak responses to stripes and blocking them in fact enhances responses to elongated bars.[59,60] We found that silencing LC11 neurons (Figure 6A) reduced numerical discrimination in a 1vs3 stripes contrast (Figure 6B) and abolished the preference in a 2vs3 stripes contrast (Figure 6C). Importantly, LC10a-silenced flies (Figure 6D) tested in 1vs3 stripes (Figure 6E) and 2vs3 stripes contrasts (Figure 6F) were still able to discriminate.

However, in the case of 1vs3 stripes contrast, silencing LC10a showed a decrease in the numerical performance. Unlike for LC11 neurons, silencing LC10a neurons in 2vs3 stripes and

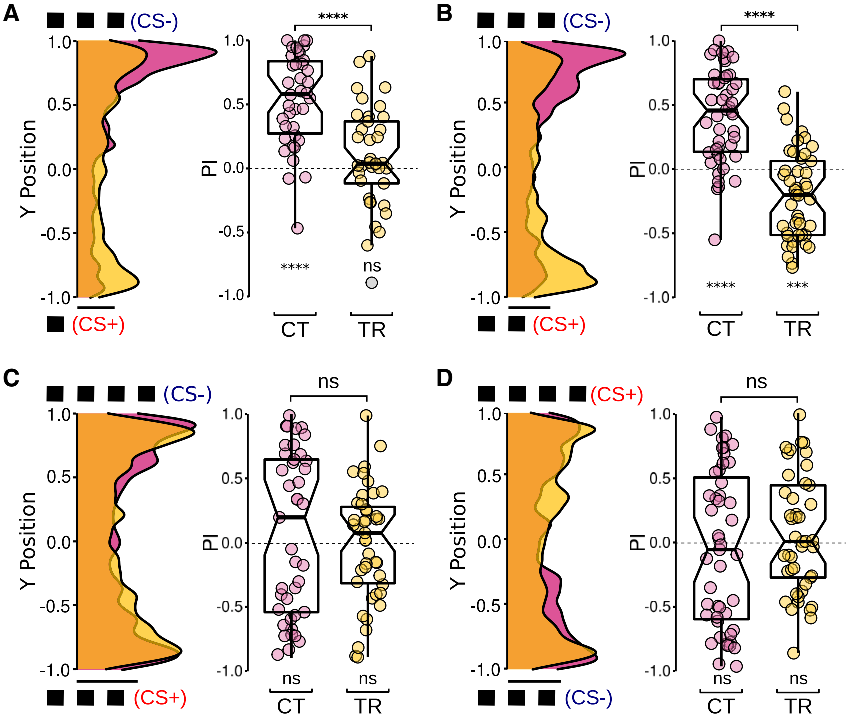

Figure 5. Flies' numerical preference can be modified by associative conditioning

Flies were trained to associate a non-preferred set of squares (CS+) to sugar (US).

(A) Flies trained in a 1vs3 squares contrast showed no preference for three squares in the testing session. Left: testing session kernel density plot for each group (pink, control group; yellow, trained group; orange, overlap of the two curves). Control group (CT) preferred the three squares while the trained group (TR) showed no numerical preference. Right: unlike the control group (n = 38, PI = 0.54 ± 0.36, p = 3.25e−09, Wilcoxon signed rank test), trained flies showed no preference for the set of three squares (n = 37, PI = 0.08 ± 0.36, $t_{(36)}$ = 1.31, p = 0.2, one-sample t test; comparison between groups, p = 1.8e−06, Wilcoxon rank-sum test).

(B) Flies trained in a 2vs3 squares contrast showed inverse numerical preference during the testing session. Trained flies significantly preferred the smaller set of squares (n = 45, PI = −0.20 ± 0.34, $t_{(44)}$ = -3.93, p = 2.9e−04, one-sample t test), opposite to the control group (n = 42, PI = 0.39 ± 0.36, $t_{(41)}$ = 7.19, p = 8.87e−09, one-sample t test; comparison between groups, $t_{(84)}$ = 7.9, p = 7.94e−12, Welch two sample t test).

(C and D) Flies trained with a numerical contrast they could not discriminate (i.e., 3vs4) did not show a change in their preference during the testing session. (C) Flies trained to sugar associate three squares did not show a numerical preference (n = 41, PI = 0.008 ± 0.44, $t_{(40)}$ = 0.11, p = 0.91, one-sample t test), same as the control group (n = 40, PI = 0.08 ± 0.64, p = 0.33, Wilcoxon signed rank test; comparison between groups, p = 0.47, Wilcoxon rank-sum test). (D) Flies trained to associate four squares to sugar did not show a numerical preference in the testing session (n = 45, PI = 0.08 ± 0.47, $t_{(44)}$ = 1.18, p = 0.24, one-sample t test), same as the control group (n = 46, PI = 0.03 ± 0.60, p = 0.71, Wilcoxon signed rank test; comparison between groups, p = 0.25, Wilcoxon rank-sum test).

2vs4 squares conditions did not result in a significant reduction of preference for the larger numerosity. To further examine the effect of the neuronal driver in the performance, a two-way ANOVA was performed. The analysis revealed no statistically significant interaction between the Gal4 driver and the numerical ratio (F(1,225) = 0.11, p = 0.74). Instead, simple main-effects analysis showed that numerical ratio (F(1,225) = 9.36, p = 0.0025) and the silenced neuronal driver (F(1,225) = 4.7, p = 0.03) have a statistically significant effect on the performance (Table S2). This result confirms that the effect of silencing LC11 is stronger than silencing LC10a neurons. Importantly, silencing LC11 neurons did not alter the ability of the flies to see and respond to the stripes per se (Figures S5A and S5B).

Are LC11 neurons required for numerical discrimination or for numerical preference? To answer this question, we trained flies with silenced LC11 neurons. If silencing LC11 neurons reduces preference for larger numerosity, the presence of sugar will create additional motivation and will result in greater preference for the rewarded smaller numerosity after training. If, on the contrary, LC11 neurons are required for numerical discrimination, the presence of reward will have no effect and the flies will not learn what they cannot discriminate, as in the 3vs4 squares learning experiment. We paired a sucrose stimulus with the two-squares set in a 2vs3 configuration. The two-way ANOVA analysis revealed a significant interaction between the silencing and learning effect (F(1,437) = 8.94, p = 0.0029). Importantly, simple main-effects analysis still showed the statistically significant effect of silencing LC11 neurons (F(1,437) = 4.3, p = 0.0387) in this numerical contrast but also the effect of the classical conditioning protocol (F(1,437) = 5.52, p = 0.0192) on the performance of trained flies (Table S3). *Post hoc* comparisons reveal that LC11-silenced flies showed no preference either before or after training, indicating that LC11 are required for numerical discrimination and not for spontaneous preference (Figures 6G and 6H). In summary, these results show that silencing a specific type of visual neurons in the lobula of the fruit flies disrupts numerical discrimination.

DISCUSSION

Drosophila as a model for numerical cognition

Numerical sensitivity is a crucial cognitive ability that is widespread across the animal kingdom. Here, we developed the fruit fly *Drosophila melanogaster* as a model for the neuroscience of numerical cognition. As a result, the powerful and versatile molecular, genetic, and behavioral toolkit of *Drosophila* can now be exploited to unravel the neurobiological underpinnings of this highly conserved adaptive trait while retaining individual-animal resolution. Two main methodologies are described in the literature to study numerical cognition in different animal models: spontaneous discrimination tests and training procedures. In spontaneous choice tests, animals exposed to behaviorally relevant stimuli, such as high-contrast objects in the case of fruit flies, reveal the preferences of animals for numerical sets. On the other

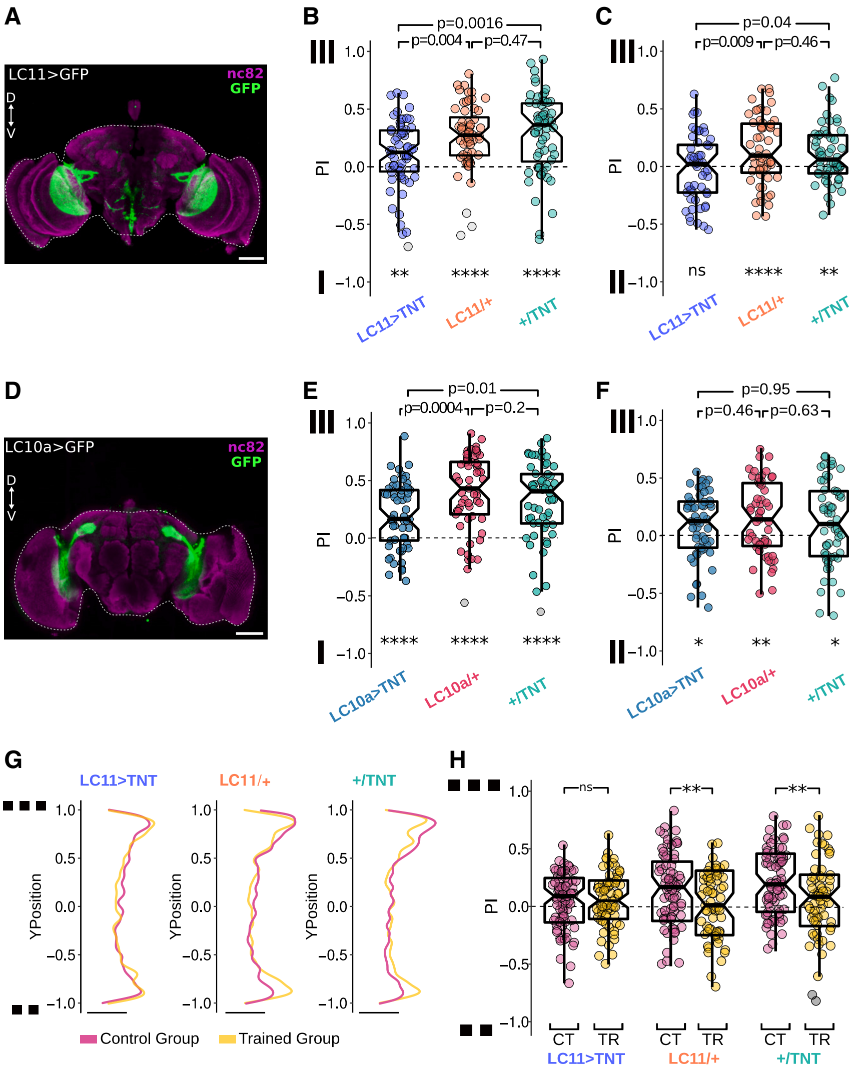

Figure 6. Silencing LC11 neurons diminishes numerical discrimination

(A) Anatomy of LC11 neurons.

(B) Performance of flies with silenced LC11 tested in a 1vs3 stripes contrast. LC11>TNT: n = 60, PI = 0.11 ± 0.30, p = 0.002, Wilcoxon signed rank test. LC11/+: n = 60, PI = 0.27 ± 0.28, p = 3.74e−08, Wilcoxon signed rank test. +/TNT: n = 60, PI = 0.30 ± 0.34, p = 3.43e−07.

(C) LC11-silenced flies tested in a 2vs3 stripes contrast were unable to discriminate in opposition to the control group. LC11>TNT: n = 53, $t_{(52)}$ = -0.47, p = 0.64, PI = −0.02 ± 0.29, one-sample t test. LC11/+: n = 55, $t_{(54)}$ = 3.53, p = 0.0009, PI = 0.14 ± 0.30, one-sample t test. +/UAS: n = 56, $t_{(55)}$ = 3.27, p = 0.002, PI = 0.11 ± 0.25, one-sample t test.

(D) Anatomy of LC10a neurons.

(E) Numerical discrimination performance of flies with silenced LC10a neurons in a 1vs3 stripes contrast. LC10a>TNT, n = 58, $t_{(57)}$ = 5.12, p = 3.93e−06, PI = 0.19 ± 0.28, one-sample t test. LC10a/+: n = 58, p = 6.63e−09, PI = 0.39 ± 0.33, Wilcoxon signed rank test. +/TNT: n = 56, p = 1.67e−07, PI = 0.32 ± 0.33, Wilcoxon signed rank test.

(F) Numerical discrimination performance of LC10a-silenced flies in a 2vs3 stripes contrast. LC10a>TNT: n = 56, p = 0.02, PI = 0.09 ± 0.29, Wilcoxon signed rank test. LC10a/+: n = 57, $t_{(56)}$ = 3.39, p = 0.001, PI = 0.14 ± 0.31, one-sample t test. +/TNT: n = 57, $t_{(56)}$ = 2.10, p = 0.04, PI = 0.10 ± 0.36, one-sample t test.

(G and H) Flies with silenced LC11 neurons trained by pairing two squares with sugar in a 2vs3 contrast. (G) Kernel density plot for each group (pink, control group; yellow: trained group. (H) LC11-silenced flies showed no preference during the testing session (LC11>TNT_CT vs LC11>TNT_TR: p = 0.8, Wilcoxon rank-sum test). In contrast, both control groups significantly diminished the magnitude of their preference after learning (LC11/+_CT vs LC11/+_TR: p = 0.009, Wilcoxon rank-sum test. +/UAS_CT vs +/UAS_TR: p = 0.008, Wilcoxon rank-sum test). Scale in (A) and (B), 50 μm.

hand, training protocols reveal an animal's capacity to extract numerosities by assigning a positive value to a stimulus. We find that flies possess both capacities.

Flies spontaneously discriminate and prefer the more numerous sets with both small and large numerosities. This observation is reminiscent of a study showing that ants can discriminate between larger quantities of cocoons (2vs6, 2vs8).[39] Further, we show that the discrimination is independent of non-numerical continuous variables and that their performance is ratio dependent, a hallmark signature of the ANS, present in beetles[32] and carpenter ants.[39] Interestingly, we also observed a numerical distance effect at 0.75 ratio where accuracy of performance potentially improves as the difference in magnitude between two respective numbers increases. This effect has also been shown in macaque monkeys,[63] crows,[64] and honeybees.[65]

A strict interpretation of Weber's law, which is thought to govern the ANS, might suggest that, when performing numerical discrimination, flies, and likely other animals, predominantly use ratio. Besides, there is evidence that Weber's law approximations may not always apply and that humans and animals use various representations of numerical values that are combinatorially deployed depending on the characteristics of the task.[66] This aligns with our observations that flies also consider continuous properties to discriminate among sets of visual objects. The outcome of the numerical performance observed in flies may be the result of the evaluation of numerical (ratio plus absolute numerical distance) as well as non-numerical (total dark area) properties of the visual set. It has been shown that honeybees[56] and crickets[40] are able to perform relative numerosity judgments without knowing exactly the numerical value, rooting their estimation in non-numerical information. Animals likely use both types of parameters—as shown in fish[11]—depending on ecological needs. Future experiments, such as "equal incongruent" or "incongruent opposite" performed in bees[67] will be needed to deeply explore the importance of non-numerical variables in flies. Importantly, the possibility of manipulating specific neuronal pathways offers a unique opportunity for a mechanistic dissection of how different variables are encoded to produce a coherent behavior.

In addition, we find that flies are able to learn to associate a positive stimulus with numerical sets. Flies can reverse their preference for more items when fewer items are associated with sucrose after a single learning trial in a classical conditioning paradigm. This learning appears to be specifically associated with numerosity. Under conditions where flies cannot discriminate (3vs4), they fail to learn the position of the sucrose. Interestingly, this occurs even though the two sets differ in non-numerical variables, which could potentially be learned. Importantly, a similar discrepancy in dark area and total area occupied did not prevent flies from learning the association in a 2vs3 contrast, where the flies are able to discriminate numerosities. This supports the conclusion that the main criterion for learning under our single-training-session conditions is the numerical ratio.

Visual learning in adult flies has been extensively studied using operant conditioning in the flight simulator assay and in freely walking flies with single-fly and en masse approaches.[68,69] Here, we established a behavioral paradigm for visual classical conditioning in freely walking individual adult *Drosophila*. Our single training trial protocol of 3 min generates a visual memory that lasts at least 2 h, allowing the study of short-term visual memories. It remains to be analyzed whether flies can form long-term numerical memories. In addition, it will be interesting to explore the range of flies' numerical cognitive abilities in flies using more extensive protocols.[42–45]

Stability of numerical preference

One of the great advantages of fruit flies as a cognitive model is the possibility to study behavioral individuality in very large numbers of single flies. By analyzing the temporal dynamics of the numerical preference at individual-fly resolution, we show that flies display three main behavioral patterns of numerical preference. These categories allowed us to study the temporal stability of how flies make their numerical decisions. In a prior study,[58] we established a link between variability in the brain visual system of flies and the emergence of individuality of animal behavior. We find that individual flies show temporal consistency in their numerical choice. Future studies connecting neuronal morphology variation with the different numerical categories of behavior described here will allow us to unravel the neural bases of numerical preference. Moreover, it would be interesting to see whether the progeny of animals with specific numerical traits give rise to a behaviorally homogeneous population or reproduce the population variance, as is the case with object orientation.[58]

Number-processing neural circuitry

A major quest in the field of numerical cognition is to identify neural pathways required for it. Selective manipulation of neuronal populations using available tools enabled testing of a limited number of candidate subtypes involved in visual processing to provide proof-of-concept evidence for numerical processing in the fly brain. We describe the LC11 neuron as a neuronal type required for numerical discrimination, establishing a starting point in the neuronal circuitry of numerical processing in invertebrates. When silencing LC11 neurons, flies showed weaker numerical performance and failed to learn to associate numerosity with reward, resulting in an inability to discriminate rewarded from non-rewarded numerosities. Moreover, silencing a different type of lobula columnar neuron (LC10a)—which, like LC11, also responds to small objects—or the CC involved in visual navigation[70,71] does not abolish spontaneous numerical preference. This suggests not only some level of specificity for the role of LC11 in numerical processing but also the emergence of numerical discrimination relatively early in the visual system. Silencing LC10a neurons resulted in a reduced preference for larger numerosities in one case (1vs3 stripes). This could be due to technical variation (e.g., genetic background) or a ratio-specific effect, with lower ratios computed through different pathways while only the LC11 pathway is used for higher ratios. Future research combining numerical behavior and neuronal recordings will clarify this further.

Recent studies demonstrate that insects achieve cognitive solutions similar to those discovered by artificial neural networks. In accordance with our work, biologically inspired deep neural networks demonstrate that number neurons can spontaneously emerge even without the need for learning.[72] Models as simple as one[73] or four[74] elements (e.g., neurons) have been shown to be capable of solving numerical tasks similar to insects. Vasas and Chitka[74] developed a model that offers a non-countable magnitude estimation in which the success of discrimination is expected to be ratio dependent. This model fits well with our results showing a ratio dependency in spontaneous and learned number discrimination in fruit flies. Based on the model, LC11 would collect input from a neuron that detects brightness changes (e.g., Tm3[75]) and proportionally integrates those changes to accumulate online evidence for the number of objects observed. Testing this model would require the development of novel assays that allow the combination of controlled numerical behavioral tasks while recording neuronal activity, ideally of multiple neurons simultaneously, to investigate how the fly brain computes numerical values. In this regard, *Drosophila* is an ideal model for the future development of such an assay. Eventually, the comparative study of neuronal architectures across animals will unravel the evolutionary origin of number sense.

Limitations of the study

It remains to be tested whether flies are also able to perform more numerical tasks such as the extraction of discrete numerical information. This requires the development of novel protocols for extensive training trials similar to those used for honeybees.[42,44] We showed that a specific type of lobula columnar neuron (LC11) is required for numerical cognition. However, it is highly likely that higher-order brain areas are required for solving more complex numerical tasks, which remains to be explored in future work. Finally, it will be critical to establish the physiological bases of single-cell versus population response to numerosity. This will require the development of novel virtual-reality numerical cognition assays over the next few years that are compatible with physiological recordings, such as calcium imaging, and with optogenetics, as is the case with other visual behaviors. The versatility of *Drosophila* behavioral and genetic tools gives reason to believe that such assays are likely to be developed within a relatively short time to allow experimental testing of the various theoretical models for how the insect brain processes numbers.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - ○ Lead contact
  - ○ Materials availability
  - ○ Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
  - ○ Behavioral arena
  - ○ Visual stimuli
  - ○ Spontaneous numerical preference
  - ○ Trajectory k-means clustering
  - ○ Stability of numerical preference
  - ○ Associative learning experiments
  - ○ Silencing experiments
  - ○ Immunostaining
  - ○ Data analysis & statistics
  - ○ Linear regression model

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2023.112772.

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AUTHOR CONTRIBUTIONS

M.B., L.C., and B.A.H. conceived the study, designed the experiments, and wrote the manuscript. M.B. conducted all behavioral experiments and data analysis. J.D.S. performed cluster and statistical analysis. V.I. and T.P. provided expertise and helped write the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-GFP rabbit polyclonal antibody	Invitrogen	RRID:AB_221569
anti-nc82 mouse monoclonal antibody	Developmental Studies Hybridoma Bank	RRID:AB_2314866
Chemicals, peptides, and recombinant proteins		
Glucose 1.5M	Sigma Aldrich	Cat#S0389
Deposited data		
Raw data and codes	This paper	https://doi.org/10.5281/zenodo.8052276
Experimental models: Organisms/strains		
Drosophila: CantonS	N/A	N/A
Drosophila: w+; P{w[+mC] = UAS-TeTxLC.tnt}E2	Bloomington Drosophila Stock Center	RRID:BDSC_28837
Drosophila: w[1118]; P{y[+t7.7] w[+mC] = GMR22H02-GAL4}attP2	Bloomington Drosophila Stock Center	RRID:BDSC_49304
Drosophila: w[1118]; P{y[+t7.7] w[+mC] = R35D04-p65.AD}attP40; P{y[+t7.7] w [+mC] = VT043656-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	RRID:BDSC_68339
Drosophila: w[1118]; P{y[+t7.7] w[+mC] = GMR20A02-GAL4}attP2	Bloomington Drosophila Stock Center	RRID:BDSC_48870
Drosophila: w[*]; P{w[+m] = Mef2-GAL4.247}3*	Bloomington Drosophila Stock Center	RRID:BDSC_50742
Drosophila: w+; P{y[+t7.7] w[+mC] = VT037804-GAL4.DBD}attP2	Bloomington Drosophila Stock Center	RRID:BDSC_71946
Software and algorithms		
Buritrak	http://buridan.sourceforge.net	N/A
R Core Team (2020)	https://www.R-project.org/	N/A
MATLAB	MathWorks	RRID:SCR_001622
Fiji	http://fiji.sc/	RRID: SCR_002285

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Bassem Hassan (bassem.hassan.icm-institute.org).

Materials availability

Flies used in this study are available upon request to the lead contact.

Data and code availability

- Data reported in this paper is available at https://zenodo.org/record/8052276. The DOI is listed in the key resources table.
- All original code has been deposited at Zenodo and is publicly available as of the date of publication. DOIs are listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Drosophila melanogaster were reared on a standard cornmeal/agar diet (8g Agar, 60g cornmeal, 50g yeast, 20g glucose, 50g molasses, 19mL ethanol, 1.9g Nipagin and 10mL propionic acid in 1L of water). Experimental animals were reared in groups up to 20 until 5 days old at 25°C in a 12/12 h light/dark regime at 60% humidity. On day 5, the wings were cut under CO_2 anesthesia. They were left to recover 48h within individual containers with access to fresh food before being transferred to the experimental set-up.

METHOD DETAILS

Behavioral arena

The behavioral arena used is a modification of the Buridan's Paradigm.[50] The arena consists of a round platform of 119 mm in diameter, surrounded by a water-filled moat (Figure 1A). The arena was placed into a uniformly illuminated white cylinder. The setup was illuminated with four circular fluorescent tubes (Philips, L 40w, 640C circular cool white) powered by an Osram Quicktronic QT-M 1 × 26–42. The four fluorescent tubes were located outside of a cylindrical diffuser (Canson, Translucent paper 180gr/m2) positioned 145 mm from the arena center. The temperature on the platform during the experiment was maintained at 25°C.

Visual stimuli

Accordingly with the particular experiment, different sets of black visual objects varying in width (w), height (h) and distance (d_h and d_v for horizontal and vertical distance respectively) were fixed inside the diffuser drum and opposite presented in the arena. The size of the dark area can be therefore described as: area = w x h. Apart from the stripes -that covered the whole vertical extension of the drum-the lower limit of the different objects were fixed to 30mm subtending an angular high of 11.7° from the middle of the platform. When the objects presented were discs, the diameter was fixed to 35mm. Generally, the distance between objects was 22mm subtending retinal sizes from 6.12° to 16.35° (8.6° in the center of the platform) except for the experiments where we controlled for the horizontal extension and the spatial distribution of the visual sets. Table S5 describes the parameters of the different visual stimuli used in each experiment.

Spontaneous numerical preference

To check for the spontaneous preference of the animals for stimuli of different numerical contrast we placed the flies on the arena for 15 min and tracked the walking path trajectory by using the software BuriTrack (http://buridan.sourceforge.net.).[50] Each fly was tested once on a particular stimulus contrast. For half of the flies tested in each experiment, the drum was rotated 180° to exclude any uncontrolled and systematic influence of other stimuli of the surroundings.

Trajectory k-means clustering

To reveal the animals' prototypical temporal patterns of spatial exploration across experimental conditions, we adapted a clustering approach based on k-means clustering, implemented through MATLAB (MATLAB R2016b and R2020a, The MathWorks, Inc.).[55] Clustering was performed on the temporally resolved Y axis coordinate of each experiment/fly. Spatial trajectories across experiments were fisher transformed and temporally smoothened. All trajectory recordings were temporally aligned from the beginning of the experiment to a maximal length of 26500 samples (14.72 min). In this analysis when a fly's trajectory could not be temporally aligned in more than 90% of the experiment (for example, if the fly made several jumps outside the arena) we discarded that given recording (45 flies rejected, 3.3% of the flies recorded).

Trajectories were iteratively partitioned into 2–16 clusters, in which each fly was assigned to the cluster with the nearest centroid trajectory. In detail, the procedure is as follows: (1) we define N (2–16) initial centroids corresponding to pseudo-randomly chosen individual trajectories (kmeans++ strategy, maximizing distance between initial centroids), (2) we compute the distance of each individual trajectory to the centroids by summing the euclidean distance -time point by time point-between each centroid and the given individual trajectory, (3) the individual trajectory is assigned to the cluster with the minimal distance, (4) the centroids are re-computed as the average -time point by time point-of all the trajectories assigned to that cluster, (5) the process is repeated until an stable assignment is determined for each trajectory, (6) the overall process is repeated 2500 times with different initial centroids and the iteration with minimal intracluster distance is selected.

The optimal number of clusters was determined using the elbow method. The intracluster distance showed a point of inflection for 3 clusters.

Stability of numerical preference

To check whether the natural tendency of the flies was stable over time we tested the spontaneous preference of individual flies over two consecutive days. Flies were tested in a 2vs.4 squares contrast for 15 min. After the first assay, each fly was placed back into their individual vial until the following day. Flies were tested at the same time of the day. Using the pre-defined clusters (learn on independent data, n = 1,599 flies), the behavior of each fly/trajectory was classed to the cluster with the nearest centroid trajectory. We did this analysis for each fly and day separately. Then, we calculated how many flies were classified in each cluster for each day and plotted the percentage of coincidence in the matrix.

Associative learning experiments

For appetitive conditioning, flies were starved by placing them on individual vials with wet filter paper (EVIAN mineral water) 21h before the training trial. The associative training consisted in one single trial of 3 min. Each individual fly was positioned in the arena and was trained once. As with the spontaneous preference test, the arena had two opposite sets of numerical cues. One set was used as sucrose-paired conditioned stimulus (CS+) and the other was used as unpaired conditioned stimulus (CS-). For each training trial, a round filter paper of 100 mm of circumference (Whatman CatNo 1001-110) was placed above the platform to support 2 to 3 drops of high concentrated glucose on the CS + numerical set stimuli (1.5M, Sigma Aldrich G8270) and 2 to 3 drops of EVIAN water on the CS- numerical set condition. Between flies, one new fresh filter paper was used. In parallel, a control group was run in a second behavioral arena. Control flies had the same manipulation as trained ones but during the training trial we presented 2 to 3 drops of EVIAN mineral water on both sets of numerical stimuli. For both groups, flies that visited only one zone of the platform (CS + or CS-) were discarded.

After the training trial, flies were placed back into their individual starved vial. The platform was cleaned with abundant distilled water and ethanol 70%. In order to test the short term conditioning, 2h later each fly was individually positioned into the arena for 5 min. During the testing session, one clean dry filter paper was positioned above the platform. As in the training, between flies the filter paper was renewed. Both groups were run in both set-ups and the position of the visual stimuli were rotated for half of the animals of each group.

Silencing experiments

To drive expression of the silencer (TNT) in targeted tissues the Gal4/UAS system was used.[76] The complete genotypes for each figure are in Table S6.

Immunostaining

Adult brains of the following lines LC11-GAL4>UAS-GFP; LC10a-GAL4>UAS-GFP; MB-GAL4>UAS-GFP; CC-GAL4>UAS-GFP; M-DCN-Gal4>UAS-GFP were processed for GFP expression. Brains were dissected in ice-cold PBS, fixed 20 min at room temperature in 4% PFA. Samples were washed three times with PBST (0.3% PBT) for 20 min. Antibody incubations were performed in PBST, normal anti-GFP rabbit serum 1:1000 (Invitrogen, RRID:AB_221569) and anti-nc82 mouse serum 1:100 (Hybridoma Bank, RRID:AB_2314866), overnight at 4°C for primary antibodies and 2h at room temperature for secondary antibodies. After 3 washes in PBST 0.3%, samples were mounted in Vectashield (Vector Laboratories, CA) and imaged using a 20X objective with a Leica TCS SP8-X white laser confocal microscope. Acquired images are visualized and processed offline using Fiji.

Data analysis & statistics

Statistical data was analyzed using R (https://www.R-project.org/.).[77] Transition plots were done as described before.[50] Briefly, the platform was divided in 60*60 hexagons and fly's position raised the count of each hexagon by one in the arena. The scale starts at 0 (blue) and goes up until a value calculated by the 95%-quantile of the count-distribution (red). The arena was divided into three zones. To calculate the preference index (PI) we sum the density of passage of the hexagons within zones close to the visual stimuli (Red areas in Figure 1B) while the center part of the arena was not analyzed. Values indicate mean ± SD. The preference index was calculated as

$$PI = \frac{totaldensity(largersetarea) - totaldensity(smallersetarea)}{totaldensity(largersetarea)+totaldensity(smallersetarea)}$$

For the statistical analyses we first checked for normal data distribution using the Shapiro-Wilk normality test. Then, we chose the appropriate parametric or non-parametric test. For the spontaneous preference test we compared the PI against 0 (chance preference; 1 indicates preference for the larger set area and -1 indicates preference for the smaller set area) by using one-sample t test or one sample Wilcoxon de of the platform by using paired t test or Wilcoxon Signed Rank Test. For the associative learning experiments, we statistically compared groups with the non-parametric Wilcoxon rank-sum test. PI Boxplot: Each dot indicates the PI for each fly tested. Occupancy boxplots: Each blue dot corresponds to the permanence of a single fly in the area of the platform corresponding to the smaller set and each red dot corresponds to the permanence of the same fly in the larger set area. Boxplot elements: Center line: median; box limits: upper (75) and lower (25) quartiles; and whiskers, 1.5x inter quartile range; gray dots indicate outliers. Scale bar of all the density plots is 0.5 probability density. For all plots, asterisk indicates significance: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, not significant.

Linear regression model

A forward and backward stepwise linear regression was used to identify possible predictors of the outcome PI out of the following candidate variables: total dark area, numerical ratio, total perimeter, larger numerosity, absolute numerical distance and total overall area. At each step, variables were added based on the p value (<0.05) for an F-Test of the change in the sum of squared error that results from adding the term (forward steps), and the p value (>0.1) was used to remove a variable included in the final model (backward steps). With this algorithm the sequence of variables included in the model were: (1) numerical ratio, (2) total dark area, and (3) absolute numerical distance. No variables were removed from the model. At each step of variable inclusion, we computed the R-square of the partial model and compared it to the R-square of the complete model (i.e. R-square of the model with one variable – ratio – versus R-square of the complete three variable model and R-square of the model with two variables – ratio and total dark area - versus R-square of the complete three variable model).