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# Mushroom Bodies Are Required for Learned Visual Navigation, but Not for Innate Visual Behavior, in Ants

## Highlights

- Ants learn to navigate to a food source relative to a visual cue
- Mushroom bodies are required for visual navigation to a learned location in ants
- Mushroom bodies are not required for innate orientation toward a visual cue

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## In Brief

Buehlmann et al. show that insect mushroom bodies are required for visual navigation to a learned location, but not for innate orientation toward a visual cue, in ants. The dissociation between innate and learned visual responses provides direct evidence for a specific role of the mushroom bodies in navigational memory in insects.

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# Mushroom Bodies Are Required for Learned Visual Navigation, but Not for Innate Visual Behavior, in Ants

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## SUMMARY

Visual navigation in ants has long been a focus of experimental study [1–3], but only recently have explicit hypotheses about the underlying neural circuitry been proposed [4]. Indirect evidence suggests the mushroom bodies (MBs) may be the substrate for visual memory in navigation tasks [5–7], while computational modeling shows that MB neural architecture could support this function [8, 9]. There is, however, no direct evidence that ants require MBs for visual navigation. Here we show that lesions of MB calyces impair ants' visual navigation to a remembered food location yet leave their innate responses to visual cues unaffected. Wood ants are innately attracted to large visual cues, but we trained them to locate a food source at a specific angle away from such a cue. Subsequent lesioning of the MB calyces using procaine hydrochloride injection caused ants to revert toward their innate cue attraction. Handling and saline injection control ants still approached the feeder. Path straightness of lesioned and control ants did not differ from each other but was lower than during training. Reversion toward the cue direction occurred irrespective of whether the visual cue was ipsi- or contralateral to the lesion site, showing this is not due simply to an induced motor bias. Monocular occlusion did not diminish ants' ability to locate the feeder, suggesting that MB lesions are not merely interrupting visual input to the calyx. The demonstrated dissociation between innate and learned visual responses provides direct evidence for a specific role of the MB in navigational memory.

## RESULTS AND DISCUSSION

### Ants Learn to Navigate to a Food Source Relative to a Visual Cue

Wood ant (*Formica rufa*) foragers were placed at the center of a circular platform within a large, circular white arena (Figures 1A and 1B). A large black rectangular cue was mounted on the wall of this arena and naive foragers walked toward it (Figure 1C). We then trained ants to find food located at the edge of the circular platform, placed 30° to the right of the cue. All 23 ants shown in Figure 1D walked to the center of the feeder ± 10° and their paths showed no bias toward the visual cue (chi-square test; $p = 0.532$). Hence, ants learned to override their innate attraction to the visual cue, and after 3.8 ± 1.1 (mean ± SD) days of training, we started to manipulate and test these well-trained ants.

### Mushroom Bodies (MBs) Are Required for Accurate Visual Navigation

We investigated the role of the ants' mushroom bodies during this visual navigation task by making chemical lesions through the injection of procaine hydrochloride, a local anesthetic that silences neural activity by reversibly blocking voltage-gated channels, including voltage-gated $Na^+$ channels [5, 10–12].

We lesioned the calyces of individual trained ants with procaine hydrochloride, testing the ants' ability to navigate to the learned position of the food source 30 min later. Every procaine hydrochloride injection was accompanied by co-injection of a fluorescent dye (rhodamine), allowing us to ensure these lesions were targeted correctly, through dissections and imaging of the brain immediately after behavioral testing (STAR Methods; Figure S1). We compared bilateral and unilateral lesioned ants to handling controls and to controls in which saline was co-injected bilaterally or unilaterally into the MB calyces with rhodamine. Ants in both these control groups received the same training to the lesioned ants and were similarly returned to the arena for behavioral testing 30 min after injection or handling.

When released in the center of the arena, ants under each of the treatments adopted non-uniform heading directions (Rayleigh test; all $p < 0.001$; Figure 2). To determine if the direction was influenced more by the cue position (innate behavior) or the feeder position (learned behavior), we carried out a combined analysis of all control and lesion ants using a generalized linear model (GLM) on

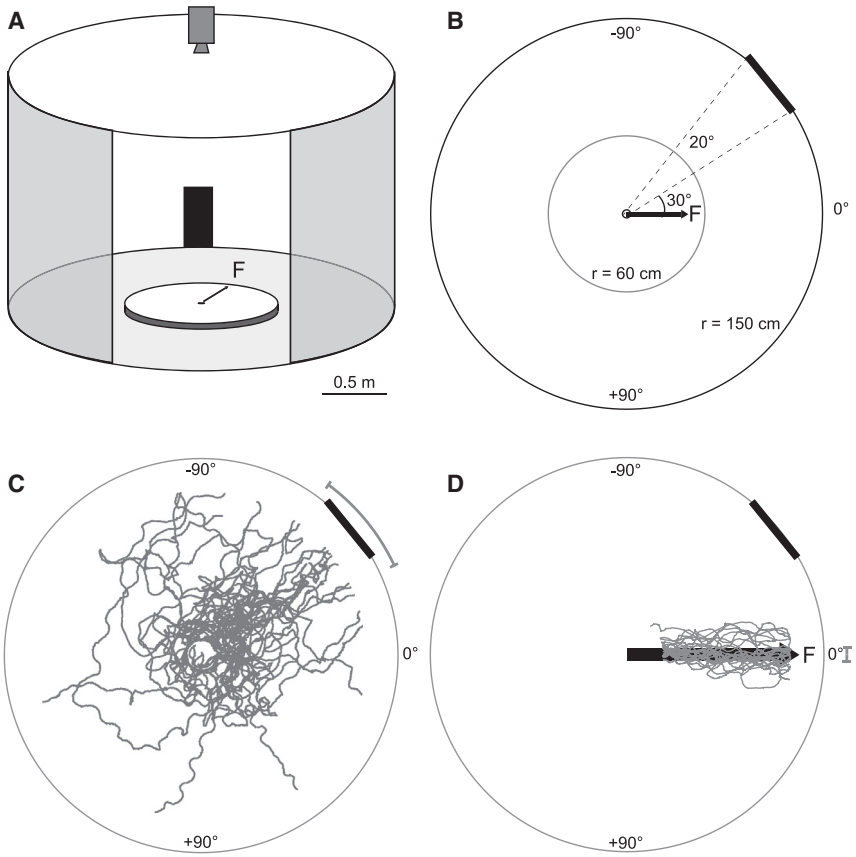


**Figure 1. Ants Learn the Position of Food Relative to a Visual Cue**

(A) The experimental arena in which ants were trained. A feeder (F) was placed at the edge of a circular white platform (radius, 60 cm) at 30° to the right edge of a 20° wide black rectangle (height, 90 cm; width, 52 cm) mounted at the inner wall of the surrounding cylinder (radius, 1.5 m; height, 1.8 m). A camera recorded the ants' paths from above. A small door permitted access to the arena, shown here open and larger for clarity.

(B) A top-down view of the arena shown in (A).

(C) Innate paths of 23 naive ants released from the center of the arena (gray). In this and subsequent figures external arcs show bootstrapped 95% confidence intervals (CIs) of the medians for the heading directions, and the visual cue is shown at the platform edge instead of on the cylinder wall.

(D) Paths of 23 individual ants after training to the feeder (gray). F was placed 30° to the right edge of the visual cue. In this and subsequent figures the black arrow shows the direct path to F. Paths of innate and trained are directed (Rayleigh test; $p < 0.001$).

their heading index (HI; STAR Methods), which reflects whether headings after 50 cm are closer to the feeder position or the visual cue. Comparison of the HIs showed that the lesioned ants differed significantly from saline-injected and handling controls (GLM on HI; $p = 0.011$), whereas the two control groups did not differ from each other (GLM on HI; $p = 0.069$).

Moreover, path straightness of handling controls, saline-injection controls, and lesioned ants did not differ from each other (Figure S2A; Kruskal Wallis with Dunn's post hoc tests; handling control versus saline injection, $p = 0.141$; handling control versus lesion, $p = 0.092$; saline injection versus lesion, $p = 1$). However, paths from all three groups were significantly less straight than the paths of ants during training (Kruskal Wallis with Dunn's post hoc tests; handling control versus training, $p = 0.030$; saline injection versus training, $p < 0.001$; lesion versus training, $p < 0.001$). The ants' walking speed was not affected by any of the treatments (Figure S2B; $p = 0.068$).

## Bilateral Injections Severely Affect the Ants' Navigation Ability

We made both bilateral and unilateral procaine hydrochloride lesions of the MB calyces. In an early study, Vowles [6] reported that bilateral mechanical lesions of the MB calyces severely impaired the ant's navigational ability. Likewise, our bilateral injections (Figure S1A) severely impaired the ants' ability to navigate to the learned feeder position (Figure 2), though the paths of both lesioned and saline-injected control ants were still directed (Rayleigh test; both $p < 0.001$). Although a significant difference in HI shown above demonstrates that lesioned ants differ in their behavior from both control groups, it does not indicate how headings have changed in relation to the learned position of the food source. To explicitly test this, ants were considered to be accurate if they approached the center of the feeder ± 10° (STAR Methods). Few bilaterally lesioned ants still navigated to the learned feeder position (3 of 20); however, saline-injected control ants were similarly unable to navigate to the feeder location (1 of 12). Indeed, there was no significant difference between lesion and control ants in their ability to approach the feeder accurately (chi-square test; $p = 0.581$), indicating that bilateral injections had disrupted the ants' ability to visually navigate to the feeder position. However, in saline-injected control ants, the 95% confidence interval (bootstrap distribution of the median; CI [2.5 percentile values, 97.5 percentile value]; STAR Methods) of the ants' heading directions ([−34.4°, 22.9°]; Figure 2B) encompassed the feeder, whereas the 95% CI of lesioned ants' heading directions ([−45.9°, −14.0°]; Figure 2C) did not encompass the feeder position and was shifted toward the visual cue. The paths of ants subjected to bilateral lesions were significantly biased toward the visual cue (chi-square test; $p = 0.007$). No significant bias was present in the paths of the control ants (chi-square test; $p = 0.564$). Thus, bilateral lesions of the MB calyces prevent ants from accurately approaching the learned feeder position, but the impaired accuracy of control ants suggests that the bilateral injection procedure itself has a substantial effect.

## Unilateral Lesions Are Sufficient to Specifically Impair Navigation

To test the impact of unilateral lesions of the MB calyces (Figure S1B) on their visual navigation, we trained new cohorts of ants to the same feeder location. Again, paths from lesioned

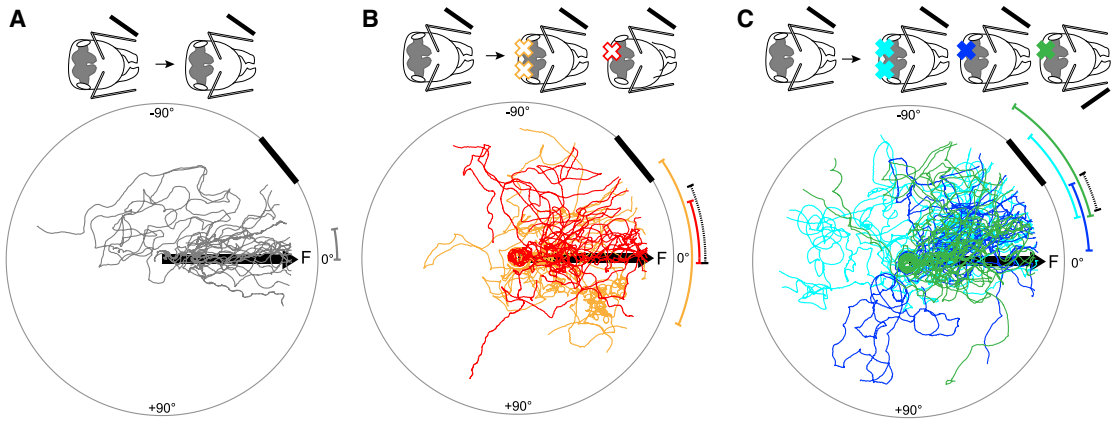


**Figure 2. Mushroom Bodies (MBs) Are Required for Learned Visual Navigation**

(A) The paths (gray) of 19 control ants 30 min after being subjected to handling. In this and subsequent figures the schematic shows an ant head with a black bar to the left representing the position of the visual cue during training and testing.

(B) As in (A) but for saline-injected control ants. Injection sites are marked on the schematic ant heads with an X. Paths of individual saline-injected control ants are shown as orange (bilateral injection; n = 12 ants) and red (unilateral injection; n = 17 ants).

(C) As in (A) but for lesioned ants injected with the local anesthetic. Bright blue, bilateral injections (n = 20 ants); dark blue, unilateral injection ipsilateral to the visual cue (n = 19 ants); green, unilateral injection contralateral to the visual cue (n = 18 ants). Note the injection site was the same, but the cue direction relative to the feeder during training was reversed. The paths of the contralateral injection ants (green) have thus been mirrored about 0° in the plot above to permit comparison. CI for pooled data is shown in black in (B) and (C). See also Figures S1 and S2.

and saline-injected control ants were still directed (Rayleigh test; all $p < 0.001$). The paths of the majority of unilaterally saline-injected control ants (10 of 17) still approached within ± 10° of the feeder, which was not significantly different from the handling control ants (11 of 19; chi-square test, $p = 0.955$). Crucially, both of these control groups showed a significantly higher number of accurate individuals than the bilateral saline-injected control group presented above (1 of 12 ants; chi-square test, both $p = 0.006$). Moreover, in the unilaterally saline-injected control ants and the handling control ants, the 95% CI of the ants' heading directions (saline-injected control ants, CI [−18.4°, 1.5°]; handling control ants, CI [−10.1°, −0.1°]) encompassed the feeder (3.5° either side of 0°). Hence, unilateral saline-injected control ants and handling control ants were still able to accurately navigate to the learned feeder location (Figures 2A and 2B). This shows that handling and the procedures involved in unilateral saline injection, including the removal of the ocelli caused by head opening, did not impair the ants' navigational behavior.

In contrast to the controls, the ants' navigational behavior was impaired when they were subjected to unilateral procaine hydrochloride lesions (Figure 2C). When the MB calyx ipsilateral to the visual cue was lesioned, fewer ants showed paths orientated toward the feeder position. Indeed, just 2 out of 19 ants still approached within ± 10° of the feeder, and the 95% CI no longer encompassed the feeder position ([−25.5°, −3.5°]; Figure 2C) and was shifted toward the visual cue. The paths of ants subjected to unilateral lesions were significantly biased toward the visual cue (chi-square test; $p = 0.039$). No significant bias was present in the paths of the control ants (chi-square test; $p = 0.467$). Hence, lesioned ants have not only lost their ability to navigate to a learned location but also reverted toward their innate attraction to the visual cue.

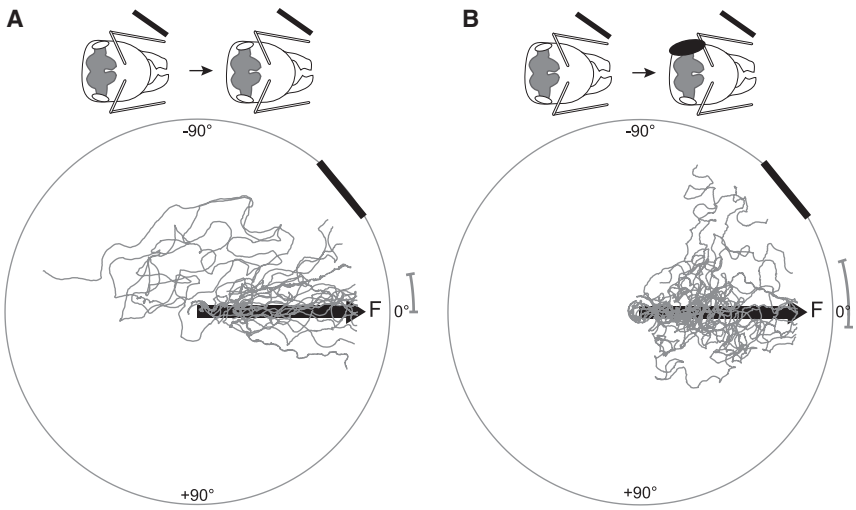


**Figure 3. Monocular Occlusion Does Not Disrupt Visual Navigation**

(A) The paths (gray) of 19 (repeated from Figure 2A) control ants 30 min after being subjected to handling.

(B) As in (A) but for 14 ants that also had the compound eye ipsilateral to the visual cue occluded before the test.

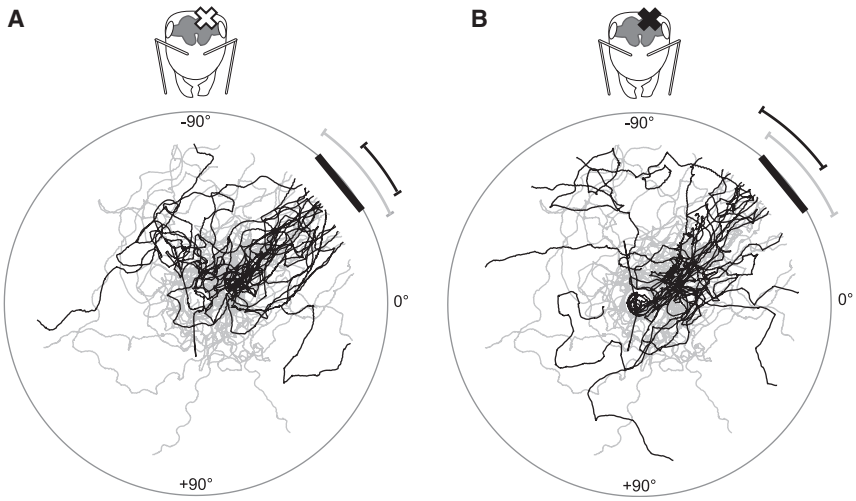


**Figure 4. MB Calyces Are Not Required for Innate Attraction to Visual Cues**

(A) Paths (black) of 18 naive ants tested 30 min after unilateral injection of saline into the MB calyx.

(B) Individual paths (black) of 18 naive ants 30 min after unilateral injection of local anesthetic into the MB calyx. Paths of 23 intact naive ants are shown in gray.

## The Impaired Navigation from Unilateral Lesions Is Not a Motor Bias

We have observed that ants with a unilateral lesion shift their walking direction toward the visual cue rather than approaching the location of the feeder 30° to its right, but this could potentially be due to some leftward bias in motor output, rather than specific disruption of navigational memory. Moreover, many behaviors are lateralized in insects, including aspects of learning and memory (reviewed in [13]), and wood ants are no exception, showing lateralization in behavior [14] and memory formation [15]. Hence, we investigated the effect of lesions contralateral to the visual cue. To this end, we trained a further cohort of ants with the feeder placed 30° to the left of the visual cue. Thus, for these ants the unilateral lesion of the MB calyx was contralateral to the position of the visual cue as the ant approached the feeder. A unilateral lesion contralateral to the visual cue prevented ants from orientating toward the feeder; just 3 out of 18 ants approached the feeder accurately. Additionally, the 95% CI of the ants' heading directions (CI [14.0°, 55.7°]) did not encompass the feeder position (Figure 2C). There was no significant difference in the proportion of accurate ants between ants with lesions contralateral to the visual cue and those with ipsilateral lesions (chi-square test; $p = 0.585$). Moreover, the accuracy of both groups of unilaterally lesioned ants was significantly lower than the saline-injected control ants (chi-square test; ipsilateral procaine injection versus saline-injected controls, $p = 0.002$; contralateral procaine injection versus saline-injected controls, $p = 0.010$). These results suggest that a unilateral MB calyx lesion is sufficient to impair visual navigation and the headings of lesioned ants that had learned the location of a food source shift toward the visual cue, whether this was to the right or left. This is consistent with a reversion toward their innate attraction to conspicuous visual cues and implies that the neural substrate for the learned and innate components of visual navigation is separate.

## MB Lesions Are Not Equivalent to an Absence of Peripheral Visual Input

The MB calyces, to which we targeted the chemical lesions, receive inputs from the visual system in ants [16–18]. To ensure that the deficit in visual navigation to the learned feeder location caused by the unilateral lesions could not be explained merely by a deficit or asymmetry in visual inputs, we monocularly occluded trained ants (STAR Methods). The paths of the monocularly occluded ants were directed (Rayleigh test; $p < 0.001$). The majority of the monocularly occluded ants (10 of 14) still approached the position of the feeder, which was not significantly different from the handling controls (11 of 19; chi-square test, $p = 0.424$). Moreover, the 95% CI of their heading directions (CI [−14.4°, 4.1°]) encompassed the feeder's position (Figure 3). Thus, the change in path directions produced by unilateral lesions was not replicated by a large deficit and asymmetry in visual input, suggesting more fundamental deficits to learned visual navigation are caused by the lesion of the MB calyx.

## MB Lesions Do Not Affect Innate Behavior

In contrast to learned navigational behavior, the innate behavior of lesioned naive ants that had not previously experienced the arena or the visual cue was unaffected by unilateral MB calyx lesions (Figure 4; see also STAR Methods). The paths of innate untreated, saline-injected control, and lesioned ants were all directed (Rayleigh test; all $p < 0.001$) and did not differ from each other (GLM on HI; untreated versus saline control, $p = 0.568$; untreated versus lesion, $p = 0.544$; saline control versus lesion, $p = 0.933$). Moreover, the visual cue (cue edges at −30° and −50°) fell within the paths' 95% CI for all three groups (untreated ants, CI [−53.3, −24.9]; saline-injected controls ants, CI [−43.0°, −29.0°]; lesioned ants, CI [−57.7°, −36.6°]). Hence, the innate attraction of wood ants to dark visual cues [19] is unaffected by lesioning an MB calyx, further consistent with the idea that there is a dissociation in brain regions required for innate and learned visual guidance.

## The Role of the MB in Visual Navigation

We have provided direct evidence that the MB calyces are involved in visual navigation to learned locations in ants. This is consistent with previous observations: the MB calyces of ants [16–18] and other Hymenopterans [20, 21] receive direct inputs from the optic lobes; the MB calyces of ants [22, 23], again like those of other Hymenopterans [24, 25], expand at the onset of foraging; and changes in the expression in the MB calyces of a gene associated with learning co-occur with orientation flights in novel environments in honeybees [26]. These findings do not preclude other regions of the MB being involved in visual navigation: the calyces are input regions [16–18, 20, 21], but lesions of

other parts of the circuitry may disrupt MB's associative memory functions or outputs. Indeed, another recent study has shown that the vertical lobes, which are innervated by the calyces, are also required for visual navigation [27]. Computational modeling has shown that the neural circuitry of the MBs is well suited for the storage of navigationally relevant visual information [4, 8, 9], specifically to encode the familiarity of multiple views. A study on cockroaches implicated the MBs in learning a place relative to visual cues, but found MBs were not necessary for directly locating a visual target [7], similar to the dissociation we observe between learned and innate behavior in the current study. However, fruit flies appear to depend upon the central complex to complete a place learning task [28] and some other visual learning paradigms [28, 29, 30], although the MBs have been shown to have a role for some visual associations [31, 32]. This suggests that the locus of visual memories within the insect brain is likely task dependent or may differ among insects from different orders. The innate visual behavior of attraction to a conspicuous cue was not affected by MB lesions in our experiments, providing further evidence that different visual tasks involve different neural pathways.

The results we have presented are consistent with models [4, 8, 9] that suggest visually driven activity across the population of Kenyon cells (KCs) in the MBs can efficiently represent experienced views, learned through dopaminergic reinforcement of the connections between KCs and MB output neurons conveying valence [33, 34] to subsequently guide forward movements or turns. Our results suggest that each MB stores views encompassing the whole visual field, as unilateral lesions affect the behavior in a consistent way (a reversion toward innate attraction to the cue) irrespective of the ipsi- or contralateral location of the cue. Further experiments are needed to explore in fine detail how visual information in different parts of the world is used for navigation, and it remains an open question how MB outputs are translated into steering information for downstream motor control, although several theoretical mechanisms involving output to the central complex have been proposed [35, 36].

## STAR★METHODS

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

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- RESOURCE AVAILABILITY
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  - Materials Availability
  - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Behavioral setup and experimental procedures
  - Chemical brain lesions
- QUANTIFICATION AND STATISTICAL ANALYSIS

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.07.013.

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## AUTHOR CONTRIBUTIONS

Conceptualization, C.B., P.G., J.E.N., and B. Webb; Methodology, C.B., P.G., and J.E.N.; Formal Analysis, C.B. and B. Webb; Investigation, C.B. and B. Wozniak; Writing – Original Draft, C.B.; Writing – Review & Editing, C.B., P.G., J.E.N., B. Webb, R.G., and B. Wozniak; Funding, P.G., J.E.N., and B. Webb.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Deposited Data | | |
| Dataset generated during this study | https://doi.org/10.25377/sussex.12597101 | N/A |
| Experimental Models: Organisms/Strains | | |
| *Formica rufa* L. | Ashdown forest, East Sussex, UK | N/A |

### RESOURCE AVAILABILITY

#### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Cornelia Buehlmann (cornelia.buehlmann@gmail.com).

#### Materials Availability

This study did not generate new unique reagents.

#### Data and Code Availability

The dataset generated during this study will be available from the University of Sussex research repository (hosted by Figshare): https://doi.org/10.25377/sussex.12597101.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

Behavioral experiments were performed with laboratory kept wood ants *Formica rufa* L. collected from Ashdown forest, East Sussex, UK. Ants were kept in the laboratory under a 12 h light: 12 h darkness cycle and constant temperature of 25-27°C. Ants were fed *ad libitum* with sucrose, dead crickets and water. During the experiments, food was limited to a minimum to increase the ants' foraging motivation, although water was permanently available.

### METHOD DETAILS

#### Behavioral setup and experimental procedures

General experimental procedures for behavioral experiments followed those described previously [37]. Wood ant foragers learned to find food at the edge of a circular platform (r = 60 cm) placed either 30° to the right or left edge of a single visual cue (Figures 1A and 1B). After a few days of training, ants had fairly direct paths to the feeder and we started to perform brain lesions to investigate the role of the ants' mushroom bodies during visual navigation. Chemical lesions were performed either bilaterally (Figure S1A) or unilaterally (Figure S1B) in the mushroom body calyces of well-trained individuals by injecting a local anesthetic (procaine hydrochloride) coupled with a fluorescent dye (rhodamine). As control groups, we had saline-injected control ants (injection of rhodamine in saline into the MB calyces) and handling control ants (all the handling steps were undertaken but the head was not opened, and no injection was done). The navigational performance of ants from these three groups was recorded 30 min after the injection or the handling and the duration of the test recording was a couple of minutes only. The location of the lesion was examined directly afterward for each ant.

During training, individually marked ant foragers were taken from the nest and released in the center of a circular platform (120 cm in diameter) that was surrounded by a cylinder (diameter 3 m, height 1.8 m) with white walls. Ants learned to find a drop of sucrose on a 7° wide microscope slide that, from the center of the arena, was located 30° away from the right or left edge of a 20° wide rectangle (height: 90 cm, width: 52 cm) placed on the inner wall of the surrounding cylinder. To remove possible olfactory traces, the surface of the platform was covered with white paper which was rotated after each round of training and was replaced between experiments. Ants performed approximately 10 group training runs before being trained individually. For individual training, ants were put separately into a 6.5 cm diameter, cylindrical holding chamber in the center of the platform. The ant was released from the holding chamber by remotely lowering its wall. Once the ant had reached the sucrose slide and started to feed, the ant was transferred into a feeding box and the next ant was released. Ants were recorded using a tracking video camera (Trackit, SciTrackS GmbH) which provided the ant's position on the platform every 20 ms. All individual training runs were recorded. Ants were considered to be reliable, accurate and ready for testing, if they approached the feeder directly on three consecutive training runs and only such ants were tested. For tests, ants were either chemically lesioned (with procaine hydrochloride), injected with only saline and rhodamine

(saline-injection control), an eye was occluded, or they were considered to be a handling control. In the eye occlusion test, one of the ants' compound eyes was covered with a layer of white enamel paint (Humbrol).

In addition to experiments with experienced ants, we also tested the role of the MBs in naive ants. To do so, we selected active ants from the nest, performed the injection (either procaine lesion or saline-injection control) and recorded the ants' innate response to the rectangular visual cue 30 min after the injection in the same arena as described above.

### Chemical brain lesions

Ants were immobilised on ice for 90 s and harnessed in a custom-made holder keeping their head fixed with plasticine while their body was able to freely move. Antennae were carefully held down with a pin. To access the ants' MB calyces, a small window was cut with a piece of razor blade into the head capsule with four cuts: the lower cut was anterior the medial ocellus, the upper cut was posterior the two lateral ocelli, and the left and right cuts were between the left and right ocelli and the left and right compound eyes, respectively. Hence, the ocelli were removed. Once the MB calyces were exposed, 0.5 nL of solution was injected uni- or bilaterally into the calyces. Injections were performed with a PV820 Pneumatic PicoPump (World Precision Instruments) connected to compressed air. Glass capillaries (Harvard Apparatus; 30-0035; 1.00 mm outer diameter, 0.78 mm inner diameter, 10 mm length) were pulled with a P-97 Micropipette Puller (Sutter Instrument) and then broken manually to a tip size of 10 μm. To get an injection volume of 0.5 nL with each capillary, we injected droplets of the solution into paraffin oil before each lesion to measure the droplet size produced by the capillary and adjusted the settings of the pico pump accordingly. Each capillary was only used once, i.e., two capillaries were prepared and calibrated for bilateral injections. After the solution was injected, the piece of head capsule was put back and fixed with a tiny droplet of super glue (Loctite, Power Flex). After surgery, ants were given 30 min for recovery before their navigational behavior was recorded. It is reported for honeybees that procaine has a functional range of up to 90 min [12], our test recording was well within this time frame.

Test ants were injected with either a control or test solution. For the lesioned ants, we used the transient and local anesthetic procaine that selectively silences the neural activity in the MB calyces by reversibly blocking voltage-gated $Na^+$ and other voltage-gated channels [5, 10–12]. Two stock solutions were prepared in advance and kept in the freezer for up to one month. Solution 1: A 40% procaine solution was prepared by diluting procaine hydrochloride (Sigma Aldrich; CAS 51-05-8) in ant saline (from [23]: 127 mM NaCl (CAS 7647-14-5), 7 mM KCl (CAS 7447-40-7), 1.5 mM $CaCl_2$ (CAS 10043-52-4), 0.8 mM $Na_2HPO_4$ (CAS 7558-79-4), 0.4 mM $KH_2PO_4$ (CAS 7778-77-0), 4.8 mM TES (CAS 7365-44-8), 3.2 mM Trehalose (CAS 6138-23-4), pH adjusted to 7.0; all chemicals from Sigma Aldrich). Solution 2: 6 mM rhodamine B (fluorescent dye; Sigma Aldrich; CAS 81-88-9) was diluted in ant saline. During the experiments, solution 2 was diluted to 3 mM with saline to get the control solution. In order to get the lesion solution, solutions 1 and 2 were mixed in a 1:1 ratio to get a 20% procaine solution in 3 mM rhodamine. Lesion and control solutions were kept in the fridge for up to 3 days.

Directly after the behavioral recordings, the ants' brains were dissected in saline and imaged using a Nikon AZ 100 fluorescent microscope. Only ants with rhodamine-stained calyces were included in the analysis.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Paths were analyzed in MATLAB. Paths were trimmed at r = 50 cm when necessary and the ants' path directions were determined. Directionality of data was tested using the Rayleigh test for circular data [38].

To determine if the direction was influenced more by the cue position (innate behavior) or the feeder position (learned behavior) we carried out a combined analysis of all control and lesion ants using a GLM on their Heading index (HI). Heading index (HI) was the calculated difference between the absolute angle to the center of the visual cue and the absolute angle to the center of the feeder. Scores above 0 mean the path is closer to the feeder and scores below 0 mean the path is closer to the visual cue. Because the data was not normally distributed the values were normalized between 0 and 1 for the beta GLM (betareg package in R).

Although the HI demonstrates how the paths are influenced by the location of the visual cue and feeder, it does not indicate how paths have changed in relation to the learned position of the food source. To explicitly test this, we quantified the accuracy of the ants' approach to the feeder. Ants were considered to accurately approach the feeder if they approached the center of the feeder (0°) +/− 10° [39]. Chi-square tests were used to compare different groups.

Furthermore, to provide a plausible statistic for the heading direction while making minimal assumptions, for each condition we calculated a bootstrap distribution of the median using N = 10,000 samples and report the 95% confidence interval (2.5 and 97.5 percentile values) [40]. We would expect this interval to contain the 7° wide feeder centered at 0° if the ants were directed toward the feeder.

Moreover, chi-square tests were used to test for a symmetrical distribution of the shifts in ants' headings (test heading relative to their training heading).

General walking speed and path straightness (index of straightness = beeline distance / path length) were calculated for paths from r = 11 cm to r = 50 cm (r being distance from the center of the platform). Kruskal Wallis test with Dunn's post hoc and Bonferroni corrections were used to compare ants from different groups.

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## Supplemental Information

# Mushroom Bodies Are Required for Learned Visual Navigation, but Not for Innate Visual Behavior, in Ants

**Cornelia Buehlmann, Beata Wozniak, Roman Goulard, Barbara Webb, Paul Graham, and Jeremy E. Niven**

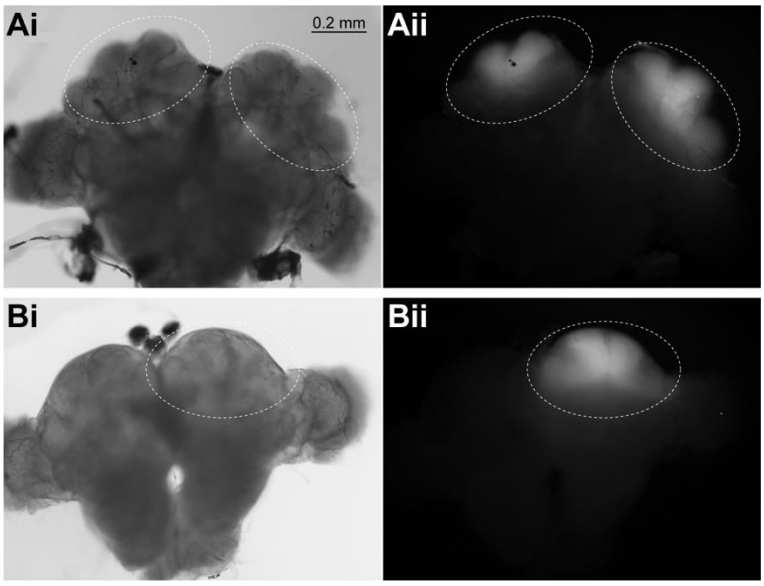


**Figure S1: Microinjection sites in the wood ant brain. Related to Figure 2.** Co-injection of the fluorescent dye rhodamine with saline or anaesthetic (procaine hydrochloride) allowed us to ensure the lesions were targeted correctly. **(A)** Bilateral chemical injection into the MB calyces. **(Ai)** Dissected brain. **(Aii)** Fluorescent dye in calyces of the brain shown in (Ai). Image is shown in black and white with a red filter. White dashed ovals are added to permit comparison of the whole brain in (Ai) with dye injection in (Aii). (**B)** Unilateral chemical injection into the MB calyx. For details see (A).

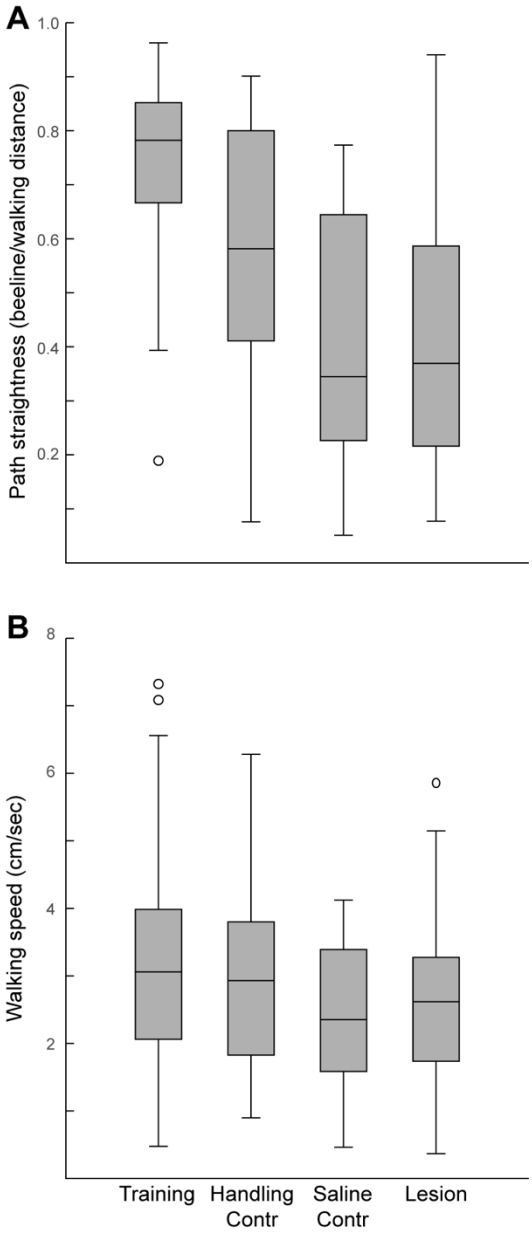


**Figure S2: Path straightness and walking speed of ants with different physiological manipulations. Related to Figure 2. (A)** Handling reduces path straightness in ants. Path straightness of handling controls, saline-injection controls and lesioned ants did not differ from each other (Kruskal Wallis with Dunn's post hoc test and Bonferroni correction; handling control vs saline injection, $p=0.141$; handling control vs lesion, $p=0.092$; saline injection vs lesion, $p=1$). However, paths from all three groups were significantly less straight than the paths of ants during training (Kruskal Wallis with Dunn's post hoc test and Bonferroni correction; handling control vs training, $p=0.030$; saline injection vs training, $p<0.001$; lesion vs training, $p<0.001$). **(B)** Experimental manipulations do not affect walking speed in ants. The ants' walking speed was not

affected by any of the treatments (Kruskal Wallis test; p=0.068). Training, n = 105 ants; Handling control, n = 19 ants; saline-injection control, n = 29 ants; Lesions, n = 57 ants. Boxplots: median, 25th and 75th percentiles (edges of the boxes) and whiskers for extreme values not considered as outliers (circles).