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Vertical Lobes of the Mushroom Bodies Are Essential for View-Based Navigation in Australian *Myrmecia* Ants

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SUMMARY

Prior to leaving home, insects acquire visual landmark information through a series of well-choreographed walks or flights of learning [1–4]. This information allows them to pinpoint goals both when in their vicinity [5–7] and from locations they have not previously visited [8–10]. It is presumed that animals returning home match memorized views to their current view for successful view-based navigation [11]. While view-based navigation strategies have been incorporated into several navigation models [8, 12, 13], we still know little about how this behavior is performed by the insect brain. Mushroom bodies are essential for visual learning and memory [14–16], and therefore we investigated their role in view-based navigation in a visually oriented ant, *Myrmecia midas*. We injected the local anesthetic procaine [15, 17, 18] into the mushroom body vertical lobes (VLs) to selectively inhibit neural activity in this region. We compared the behavior of VL-procaine-treated ants with three groups: untreated control, VL-saline, and off-target (antennal lobe) procaine. Experienced foragers were collected, treated, and released in their familiar environment where we documented their behavior. Animals with procaine-inactivated VLs had tortuous paths and were unable to find their nest, whereas ants from the untreated and off-target procaine groups were well directed and were the most successful at returning home. Untreated animals walked faster when their gaze was directed toward home, and this behavior was eliminated by anesthetizing the VL region. Our data provide neurobiological evidence that the mushroom body vertical lobes are necessary for retrieving visual memories for successful view-based navigation.

RESULTS AND DISCUSSION

Australian bull ants, *Myrmecia*, are visually oriented animals that travel individually and establish a narrow foraging corridor to navigate between their nest and a specific tree on which they forage [19]. Although they use the pattern of polarized skylight to obtain compass information [20, 21], they primarily rely on terrestrial visual landmarks for navigation [21–24]. We aimed to identify the brain region involved in view-based navigation. We targeted the mushroom body vertical lobes (VLs) because they are innervated by the mushroom body calyx collar, which receives direct visual input [25]. Additionally, the VLs are a strong candidate for supporting view-based navigation because of their role in visual learning in heat avoidance [14] and aversive conditioning tasks [15].

We studied experienced workers of *Myrmecia midas* (Figure 1A) that had foraged on the same tree for at least 3 days over a 6-week period. We collected ants returning home close to their nest entrance. This ensured that when they were subsequently released at the base of their foraging tree, they could only use familiar terrestrial visual information to navigate [6]. These ants were transferred in the dark to the lab, where they were randomly allocated to one of four treatment groups: untreated controls (untreated), VL-saline injection (VL-saline), antennal lobe (off-target) procaine injection (AL-procaine), and VL-procaine injection (VL-procaine). Procaine transiently blocks K^+ and Na^+ channels, effectively silencing neurons [15, 17, 18]. In the untreated group, ants were anesthetized by placing them on ice only, while the other groups additionally received bilateral injections in either the VLs (VL-procaine and VL-saline) or the antennal lobes (AL-procaine; Figure 1B). A small quantity (1 nL) of physiological saline or procaine dissolved in saline, along with a fluorescent indicator dye, was injected into the specified region, which allowed us to confirm the injection site (STAR Methods). In honeybees, the same quantity of procaine injected in the VLs has an anesthetic effect on this region [18]; however, there is a possibility that procaine may have diffused into adjacent tissues in the brain.

Post-treatment, we provided ants 45 min to regain mobility, then transferred them in the dark to release them individually at the base of their foraging tree. Procaine remains active for at least 90 min in honeybees [17], which have brains comparable in size to *M. midas* brains [26]. Therefore, we carried out our behavioral experiments within this time frame. We filmed the

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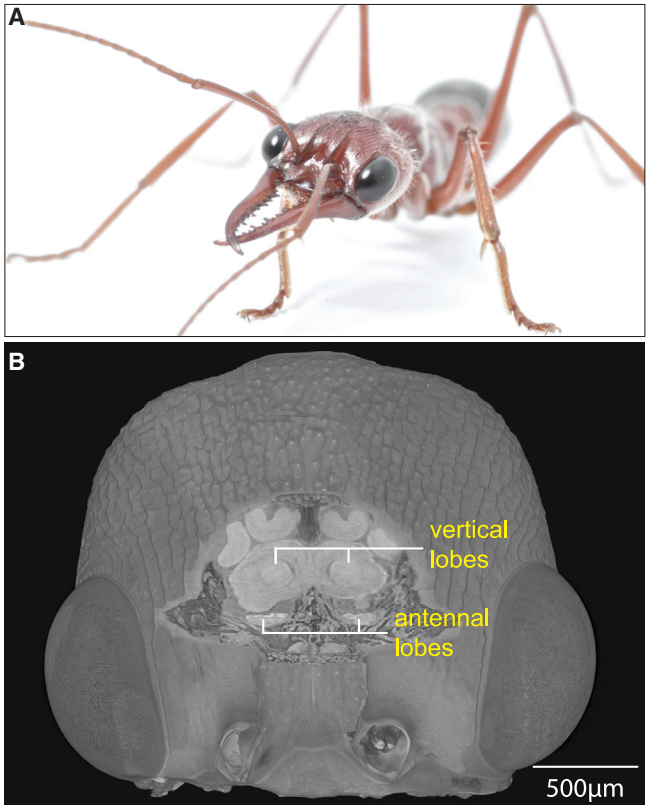

Figure 1. Injection Sites in the Australian Bull Ant *Myrmecia midas*
(A) Image of an *M. midas* worker (photo credit: Ajay Narendra).
(B) A µCT scan of the dorsal view of the head of a *Myrmecia* sp. ant segmented to reveal the location of the injection sites, mushroom body vertical lobes, and antennal lobes (visualization credit: Zachary Sheehan).

initial trajectories of ants in a 92 × 103 cm area (Figure 2A) and carried out a frame-by-frame analysis at 40 ms inter-frame interval to track the head and pronotum positions. We used this to determine differences in orientation, path straightness (sinuosity), and walking speed between different treatments. Once ants departed from the filming area, we followed them until they reached about 10 cm from their nest, or for 15 min from the time of release (STAR Methods).

Following the behavioral experiments, each ant was captured. Injection sites were verified using the fluorescent indicator dye either at the time of injection or following behavioral experiments with histological processing. Only ants that received successful bilateral injections and maintained natural walking posture during testing were included in the analyses.

Success of Returning Home Diminishes in Ants with Lesioned Vertical Lobes

All of the ants from the untreated group returned home successfully (n = 32). The proportion of ants that returned to the nest decreased slightly in the AL-procaine group (80%, n = 10) and more substantially in the VL-saline group (23.07%, n = 13). No ants from the VL-procaine group returned home (n = 16). This decrease in success rate in the VL-procaine group was also evident from the final heading direction of individual ants at the end of each trial (Figure 2B).

Anesthesia of the Vertical Lobe Region Weakens the Ability of Ants to Determine Nest Direction

Ants collected at the nest and released at their familiar foraging location head toward home using visual landmarks. We hence compared the heading direction of ants from the different groups. Initial heading directions of ants at 30 cm did not align well with their true heading direction (Figures 2A and S1) because at the first instance they move and look around extensively to identify the nest direction. Therefore, we used the bearing of ants from the release point to the location at the end of the recording period as an accurate estimate of their orientation.

The bearings of the ants from the VL-procaine group were uniformly distributed ($Z = 2.58$, $p < 0.07$, Rayleigh test; Figure 2B) and these ants were not oriented toward the nest ($V = 0.164$, $p = 0.18$, V test). The bearings of the ants from the other three groups had non-uniform distributions and were oriented toward the nest (untreated, $Z = 31.07$, $p < 0.0001$; $V = 0.97$, $p < 0.0001$; VL-saline, $Z = 5.14$, $p < 0.01$; $V = 0.56$, $p < 0.01$; AL-procaine, $Z = 6.17$, $p < 0.0001$; $V = 0.78$, $p < 0.0001$; Figure 2B). Since the bearings of the VL-procaine ants were uniformly distributed, their mean direction was not biologically meaningful and this group was excluded from further analyses. We determined whether mean heading directions differed between the remaining three groups. The untreated group differed from the VL-saline group ($W = 13.26$, $p < 0.001$, Mardia Watson-Wheeler test). We found no significant difference in the mean direction between the untreated and AL-procaine groups ($W = 0.014$, $p = 0.99$) or between the AL-procaine and VL-saline groups ($W = 5.86$, $p = 0.053$).

Our results show that anesthesia of the VL region profoundly weakens the ability of ants to use visual landmark information to determine their heading direction toward the nest. The off-target injection of procaine into a non-visual region did not affect their final heading direction, and the effect was similar to injecting saline into the VLs. The VL-saline group of ants was well oriented toward the nest (Figure 2B) yet had a reduced success rate of returning home. Within the recording duration, the VL-saline ants that did not reach the nest traveled an absolute distance of 1.6 ± 0.29 (mean ± SEM) times the tree-nest distance, which is significantly farther than the direct path from the release point to the nest. Thus, saline injection in the VLs did not affect the ability of ants to walk long distances. The reduced homing of the VL-saline group may have been caused by physical or electrochemical disturbance from the injection into the VLs. Effects of similar vehicle and injection controls on visual learning have been reported in other insect studies [15, 27].

Ants with Anesthetized Vertical Lobes Do Not Have a Preferred Gaze Direction

For view-based navigation, *Myrmecia* and other Hymenopteran insects compare their current view to views acquired during learning walks and move toward views that provide maximum similarity [1, 11]. When *Myrmecia* are displaced to previously unvisited locations within a 10–15 m radius of the nest, they return home directly with no evidence of search [9]. While their initial bearing from the release point was not always oriented toward

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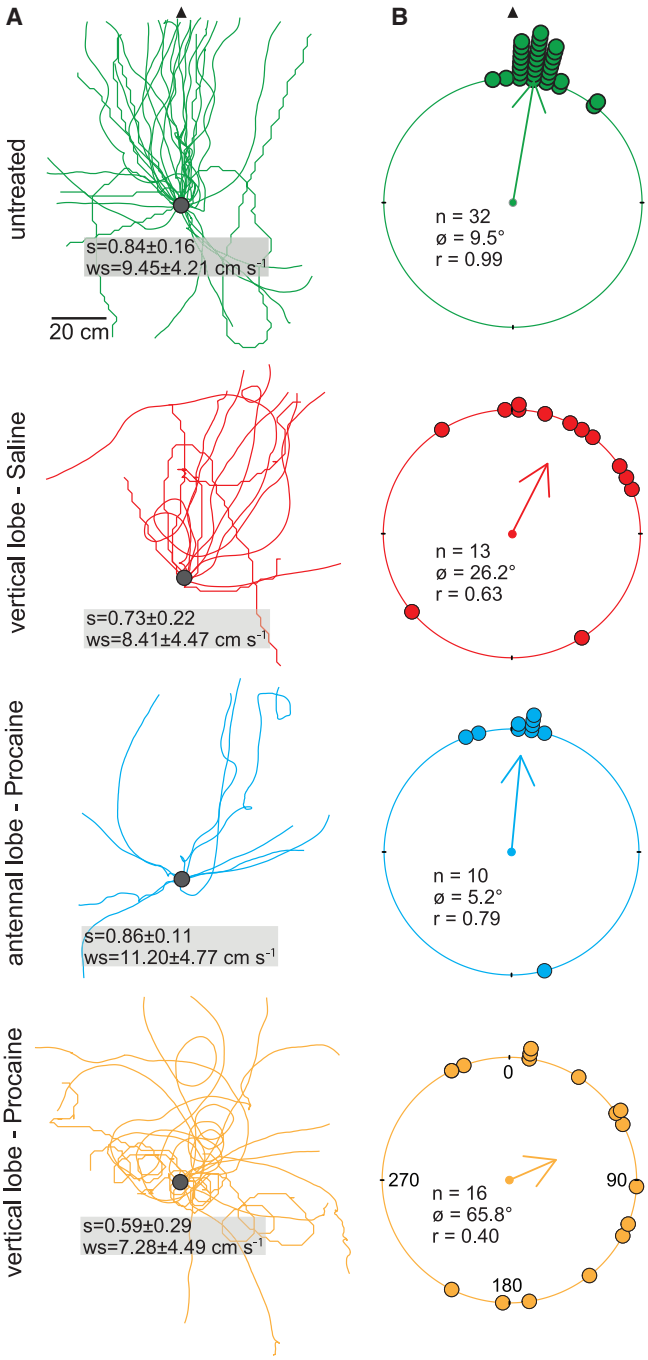

Figure 2. Procaine Injections in Mushroom Body Vertical Lobes of *Myrmecia midas* Affect Visually Guided Navigation

(A) Ant trajectories immediately after release (filled circle) relative to the nest direction (filled arrow) are shown. Sinuosity (s), which ranges from 0 (least straight) to 1 (most straight), and walking speed (ws) are indicated.

(B) Final heading direction of ants (not visible in trajectories) along with sample size (n), mean vector (ø), and length of mean vector (r; arrow) relative to nest direction (0°, filled arrow) are shown.

See also Figure S1.

the nest (Figure S1), their scanning movements served to obtain the best match to their previously learned nest-oriented views [9]. Therefore, we characterized the gaze direction of the animals within the filming area around the release location and the frequency at which they looked in the direction of the nest (Figure 3A). Ants from the untreated and VL-saline groups frequently looked toward the nest (mean + SD, shown in Figure 3A, green and red lines). A similar behavior of predominantly looking in the direction of the nest was also seen in ants from the AL-procaine group (Figure 3A, blue line). Only ants from the VL-procaine group did not exhibit a clear preference in their gaze direction (Figure 3A, yellow line).

Since some of the gazes could be sweeping passes, we determined whether the cumulative time spent looking toward the nest (defined as ± 25° from the nest) varied between the four groups. We found a significant difference in the proportion of time ants from each group looked in the nest direction (Kruskal-Wallis test, $\chi^2 = 7.95$, df = 70, $p < 0.05$). The ants that spent the greatest proportion of time looking toward the nest were from the untreated (0.32 ± 0.26, mean ± SD) and VL-saline (0.31 ± 0.23) groups. The ants that spent the least proportion of time looking in the direction of the nest were from the AL-procaine (0.16 ± 0.19) and VL-procaine (0.12 ± 0.15) groups.

Path straightness or sinuosity differed significantly between the four groups (one-way ANOVA, $F_{3,67} = 6.34$, $p < 0.001$; Figure 2A). Ants from the VL-procaine group had the least straight paths: trajectories were often tortuous and consisted of multiple loops. Ants from the untreated, VL-saline, and AL-procaine groups had relatively straight paths. There was no significant difference in the path straightness between the untreated and AL-procaine groups (Tukey-Kramer post hoc test, $p = 0.99$) or between the VL-saline and AL-procaine groups ($p = 0.42$). Ants from both the untreated and AL-procaine groups had significantly straighter paths compared to the VL-procaine group ($p < 0.001$ and $p < 0.01$, respectively). We found that path straightness of the VL-saline ants did not differ from either the untreated ($p = 0.32$) or the VL-procaine groups ($p = 0.28$). Thus saline injections in the VLs were sufficient to slightly decrease path straightness, possibly due to mechanical or electrochemical disruption in this region [15, 27].

Ants with Functional Mushroom Bodies Walk Faster When Facing the Nest

Typically, when ants approach the nest, they reduce their walking speed, which may be a strategy that allows for effective view-based navigation [28]. However, it is unknown whether ants that are farther away from the nest modulate their walking speed when familiar views are detected. We therefore determined the relationship between walking speed and gaze direction of ants between the four groups. We found no significant difference in the average walking speed between groups (one-way ANOVA, $F_{3,67} = 1.84$, $p = 0.15$), suggesting that inactivation of the VLs did not affect general locomotion. We then assessed the relation between walking speed and gaze direction in each group using a generalized linear mixed model. Although walking speed did not significantly differ among groups, we found that variation in walking speed was explained by gaze direction and the interaction between gaze direction and group (Table 1). Ants from the untreated, VL-saline, and AL-procaine groups exhibited a

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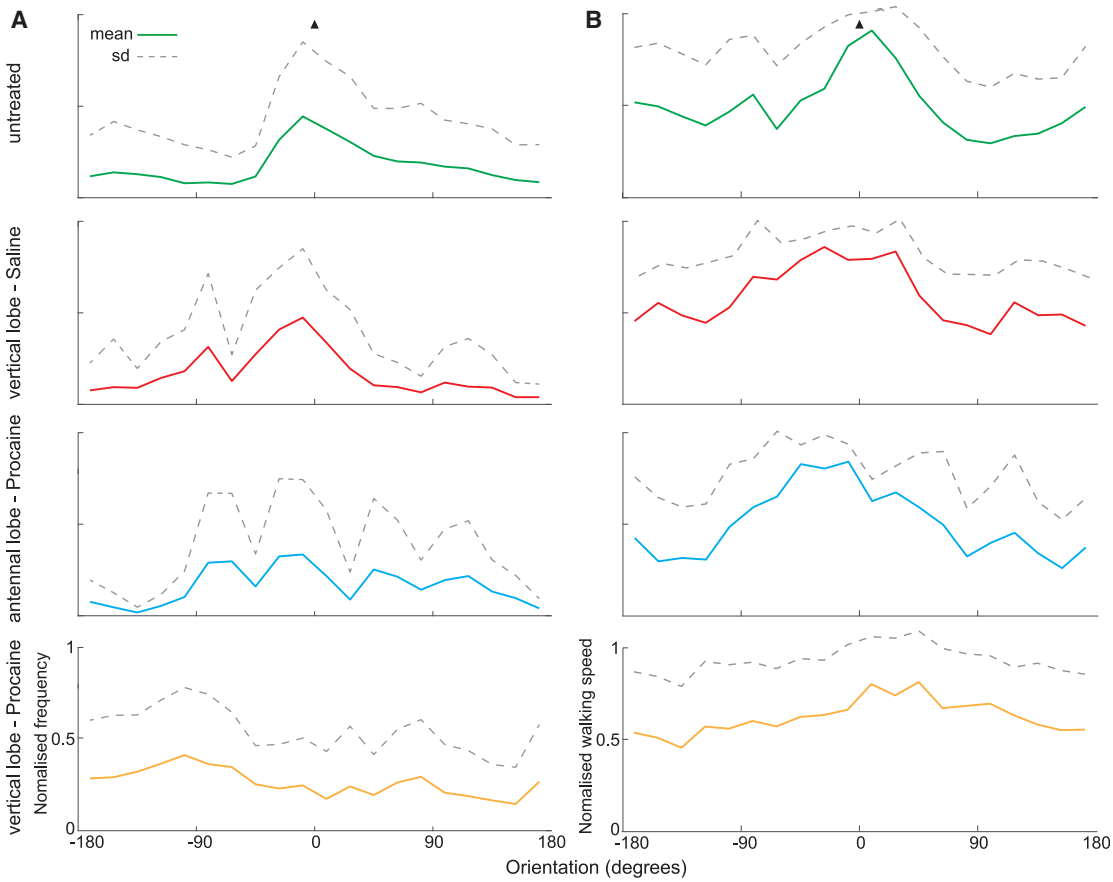

Figure 3. Procaine Injection in the Mushroom Body Vertical Lobe Affects Nest-Directed Gaze Direction and Walking Speed

(A) Normalized frequency of gaze direction relative to the nest direction at 0°. Gaze direction was determined from the head and pronotum coordinates. Ants from the VL-procaine group did not exhibit a clear preference in their gaze direction.

(B) Relationship between normalized walking speed and gaze direction relative to the nest direction at 0°. Walking speed was determined from the pronotum coordinates. Solid line indicates mean and dashed line denotes standard deviation (SD) in each group. Nest direction is indicated by a filled arrow in the top panels. Each row represents one condition.

distinct increase in walking speed and walked fastest when their gaze was directed toward the nest (Figure 3B). Ants from the VL-procaine group lacked such a distinct increase in walking speed when their views were directed toward the nest. Overall, ants of this group also had the least variation in both gaze direction and walking speed (Figures 3A and 3B). Ants from this group had maximum walking speeds when their views were directed around the nest vicinity; however, this trend was primarily driven by the few ants (3 out of 16) whose views were predominantly directed toward the nest. The remaining ants walked in loops (Figure 2A). Our data therefore suggest that processing involving the vertical lobe region enabled ants to match a learned nest-

Table 1. Results from Generalized Linear Mixed Model of the Interaction of Walking Speed, Group, and Gaze Direction

Group Name	Estimate	SE	T-Statistic	DF	p Value	Lower	Upper
Intercept	9.77	0.76	12.93	26,601	<0.001*	8.29	11.25
VL-saline	−1.09	1.40	−0.78	26,601	0.44	−3.84	1.66
VL-procaine	−1.89	1.31	−1.44	26,601	0.15	−4.45	0.68
AL-procaine	1.69	1.55	1.09	26,601	0.27	−1.34	4.73
Gaze	−0.0029	0.00052	−5.70	26,601	<0001*	−0.0040	−0.0019
VL-saline:gaze	0.0059	0.00095	6.23	26,601	<0001*	0.0041	0.0078
VL-procaine:gaze	0.0075	0.00071	10.52	26,601	<0001*	0.0061	0.0089
AL-procaine:gaze	−0.010	0.0015	−6.96	26,601	<0001*	−0.013	−0.0075

Estimates, standard error (SE), T-statistics, degrees of freedom (DF), p value, and lower and upper confidence intervals of the best fit generalized linear mixed model. Asterisks (*) denote statistical significance.

oriented view with their current view, and when the two views matched, they walked faster.

Vertical Lobes Are Necessary for Retrieving Visual Navigational Memories

Models of insect view-based navigation have presumed an important role for the mushroom bodies in view learning [13, 16, 29]. Here we provide direct neurobiological evidence for the role of the VL region in view-based navigation in an insect's natural foraging environment. Our data show that the VL regions are necessary for retrieving visual memories to compare current views with learned nest-oriented views, and when views match, animals walk faster. The mushroom bodies seem to contribute to the navigational system by associating views with outcomes. Many studies have clearly demonstrated the capacity of the mushroom body to associate visual, olfactory, or multimodal stimuli with reward and punishment [14, 15, 30–34]. In the context of visual navigation, the mushroom bodies have been suggested to support the association of nest-directed views with the rewarding action of walking toward home [16, 35]. Accordingly, the mushroom bodies could support successful navigation in an animal motivated to do so. The central complex, however, likely controls the orientation of the ant relative to the visual panorama [36–38]. There are no direct connections between the central complex and the mushroom bodies, yet in *Drosophila* these regions interact via indirect connections through the superior medial protocerebrum [15, 39, 40]. Future studies of insect navigation should focus on these connections to determine how an animal might affect a turn or an acceleration toward a goal location using directional information supported by the central complex and the recognition of a goal-directed view supported by the mushroom body.

STAR★METHODS

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

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.06.030.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.B.B. and A.N.; Methodology, J.F.K., A.B.B., and A.N.; Validation, J.F.K.; Formal Analyses, J.F.K., A.B.B., and A.N.; Investigation, J.F.K. and A.N.; Resources, A.B.B. and A.N.; Writing – Original Draft, J.F.K.; Writing – Review & Editing, J.F.K., A.B.B., and A.N.; Visualization, J.F.K. and A.N.; Supervision, A.B.B. and A.N.; Funding Acquisition, A.B.B. and A.N.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Dextran, Alexa Fluor 568; 10,000 MW, Anionic, Fixable	Life Technologies, Australia	D22912
Chemicals, Peptides, and Recombinant Proteins		
Procaine hydrochloride	Sigma-Aldrich	P9879
DAPI	Sigma-Aldrich	D9542
Experimental Models: Organisms/Strains		
Myrmecia midas	Field site, Macquarie University, Sydney, Australia	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, Ajay Narendra (ajay.narendra@mq.edu.au).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The datasets generated during this study are available at: https://github.com/ajaynarendra/procaine_vertical_lobes_CurrBiol_2020.git

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We studied four colonies of the nocturnal bull ant *Myrmecia midas* that were found on the Macquarie University campus in Sydney, Australia (33°46'09.2'' S, 151°06'39.9 E) between December 2016 and January 2019. Foragers leave their nest about 15 min after sunset, travel to nest-specific trees on which they forage throughout the night, and return to their nest in the morning twilight [19, 24, 41]. The colonies we studied foraged on one or two trees that were 2.4 – 8.62 m from the nest.

METHOD DETAILS

Pharmacological investigation

Identifying experienced foragers

Ants were collected when they arrived at the nest-specific foraging tree. They were marked for identification and released back to their nest. On subsequent nights over a six-week period, returning foragers received an additional colored mark when they revisited the same tree. Ants that visited the same tree for at least three nights were used in our experiments. On their third visit to the same foraging tree, ants were collected at the base of the tree and fed sugar water overnight. Experiments were carried out the following morning 15-30 min before sunrise, which corresponded to the time ants typically return home [19, 24, 41]. These collected animals were transferred in the dark and released at the base of their foraging tree. We followed ants visually as they walked toward their nest and collected them about 10cm away from their nest entrance. This release was carried out to be certain that the ants traveled the distance indicated by their path integrator, thus ensuring that in subsequent releases ants could use only visual landmarks to navigate. By following the ants to the nest, we also confirmed that they belonged to the focal nest.

Preparation

The focal ants collected at the nest were transferred in the dark to the laboratory that was 200 m away from the nests. Here, ants were individually cooled on ice for 15 min and sorted randomly to one of four groups: untreated control (untreated), vertical lobe saline (VL-saline), antennal lobe procaine (AL-procaine), and vertical lobe procaine (VL-procaine). In all groups except the untreated, ants were removed from the ice and their heads were fixed in a custom-made pyrex holder using dental wax. A small ''window'' was cut in the head capsule using four cuts, anterior to the ocelli, posterior to the antennal stems, and between the two compound eyes. To ensure visualization of the vertical lobes, the glands and trachea on the dorsal surface of the brain were carefully pushed aside. The neurolemma directly dorsal to the VL or AL was carefully cut to enable entry of the electrode. After the injection (see *Injection*), the head capsule was carefully replaced and held in place until the endogenous hemolymph sealed it to the surrounding cuticle. Ants from all four groups were allowed 45 min of recovery prior to their release in the field (see Recording Navigational Behavior).

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Injection

We used a well-established technique of inhibiting neural activity in the vertical lobe of the mushroom body using procaine [15, 17, 18]. This method was developed for honeybee brains. The brains of *M. midas* are unusually large among ants and are comparable to the honeybee [26]. The protocol we followed is modified from Plath et al. [15] to cater to the ant brain. A procaine stock solution was prepared by diluting 40% (w/v) procaine hydrochloride (Sigma-Aldrich Australia) solution in physiological saline (7.54 g/L NaCl, 0.448 g/L KCl, 0.872 g/L $MgCl_2 \times 6\ H_20$, 0.735 g/L $CaCl_2 \times 2H_20$, 54.72 g/L Sucrose, 4.95 g/L D-glucose, and 2.38 g/L HEPES, pH = 6.7, 500 mOsm, Sigma-Aldrich Australia). A fresh 20% (w/v) working solution for injections was prepared daily by diluting the stock with saline . We injected procaine into either the vertical lobes or the antennal lobes in both hemispheres. Injection in the antennal lobe was carried out to inhibit neural activity in a non-visual brain region. To test the effect of a non-anesthetic injection in the vertical lobes, we injected physiological saline in the vertical lobes in both hemispheres (VL-saline). Both procaine and saline injection solutions contained 0.5 mg/mL dextran Alexa fluor 568 (10,000 MW, Molecular probes, Life technologies, Carlsbad, CA, USA) for visualization of the injection site. We used glass capillaries (World Precisions Instruments, Sarasota, FL, USA) pulled from an electrode puller (Scientific & Research Instruments, Karnataka, India) with an outer diameter of 10-15μm to inject 1 nL of procaine or saline solution into the VL or AL in each hemisphere at a depth of about 60 μm. We used a pressure microinjecter (Eppendorf, Sydney, Australia) to perform the injections. Injection volume was measured by injecting the solution into mineral oil both before and after injections to calibrate using a graticule. This technique produces a fine bubble of the injector solution with high surface tension. The diameter of the spherical bubble produced in the oil was measured to determine the volume of the injection. We used oil for calibration because both the procaine and tracer solution are not soluble in oil. Additionally, the large difference in refractive index between the solution and the oil provides better optical contrast [42]. To guide the electrode during injections, we used a micromanipulator (Eppendorf, Sydney, Australia or Luigs & Neumann Feinmechanik und Elektrotechnik, Ratingen, Germany or Sensapex). We carried out all injections at a high magnification under a fluorescent stereomicroscope (Leica M205FA), which allowed us to visualize our injection sites.

Injection site confirmation

Injection sites were confirmed either during the injection process under a fluorescent stereomicroscope or after the completion of behavioral experiments under a confocal microscope. For the latter, we followed standard procedure [15] where animals were anesthetized on ice and brains were dissected in physiological saline and immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffered saline (Sigma Aldrich) for 1-2 days. Brains were then permeabilized in 0.2% Triton-X (Sigma Aldrich) in phosphate buffered saline and then rinsed in phosphate buffered saline. The tissue was then incubated in DAPI (0.2mg/mL; Sigma Aldrich) for 2-3 h to label the cell bodies as a background staining. The brains were then dehydrated using an increasing ethanol series (50%, 75%, 90%, 98%, 100% x 2) and cleared first in a 50% methyl salicylate: ethanol solution for 15 min and then in 100% methyl salicylate. Brains were then placed in custom-made 1mm-thick metal slides and allowed to dry overnight. Brains were visualized using an Olympus Fluoview 1000 IX81 inverted confocal microscope to confirm injection sites (10x magnification, 4.51μm step size).

Recording navigational behavior

After treatment and following 45 min of recovery period, ants were transferred in the dark to the field where they were individually released at the base of the foraging tree. Ants were released on a wooden platform (92 × 123cm) that was raised 5mm from the ground. The board was levelled using a spirit level. The platform releases were necessary since distinguishing the ants against the natural leaflitter ground proved difficult. We filmed the entire platform with a high-resolution camera (Sony 4K FDR-AX100) at 25 frames s^{-1} along with a compass to determine nest direction. Once ants left the platform, we followed them by eye for 15 min (or earlier if they returned to the nest) and marked their final position. On each day the behavioral recordings were carried out by different researchers who were blind to the experimental group.

QUANTIFICATION AND STATISTICAL ANALYSIS

We determined the bearing of individual ants from the release location to the last coordinate after each behavioral test. We generated circular plots using the ''circular'' package in RStudio (Version 0.99.892) and carried out circular analyses using Oriana (Version 4.0; Kovach Computing Services, UK). We used the Rayleigh test to assess the uniformity of final bearings, the *V* test to compare heading direction to expected home direction in individual groups, and the Mardia-Watson Wheeler test to compare uniformity between the untreated, VL-saline, and AL-procaine groups.

We carried out a frame-by-frame analysis of the video footage. We first converted the videos to image sequences in Final Cut Pro (Version 10.2.3, Apple). In each frame we tracked the head and pronotum position (first segment of the mesosoma) using a custom-written MATLAB based program (courtesy of Jan Hemmi and Robert Parker) and carried out further analyses in MATLAB (2013b, 2019a; Mathworks, Natick, Massachusetts, USA). We smoothed the data using the 'smooth' function over a 5x5 pixel window. Because the head moves independently of the mesosoma in *Myrmecia* [10], we used the *x*, *y* coordinates of the pronotum position to plot trajectories, determine path straightness (sinuosity) and measure walking speed. Path straightness was defined as the absolute distance divided by the total distance traveled. Path straightness between groups was analyzed using a one-way ANOVA and post hoc Tukey-Kramer tests. Differences in walking speed between groups were analyzed using a one-way ANOVA and post hoc Tukey-Kramer tests.

We determined the initial heading direction of each ant at 30 cm from release point, using the *x,y* coordinates of the head position. We determined the gaze direction of each ant from the release point until they left the platform. Gaze direction was calculated using the *x,y* coordinates of the head and pronotum position [9]. Gaze direction was averaged across groups and normalized to the

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maximum in each ant. We calculated the cumulative time spent looking toward the nest (±25°) and expressed it as a proportion for each group. We then used a Kruskal-Wallis test to compare the proportion of time ants looked toward the nest between all four groups.

We analyzed the relationship between walking speed and gaze direction using a generalized linear mixed model (dependent variable: walking speed; independent variables: gaze direction, treatment, and the interaction of gaze direction and treatment; random factor: individual ant). The model including the interaction between gaze direction and treatment was a better fit than excluding this interaction.

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Supplemental Information

Vertical Lobes of the Mushroom Bodies Are Essential for View-Based Navigation in Australian *Myrmecia* Ants

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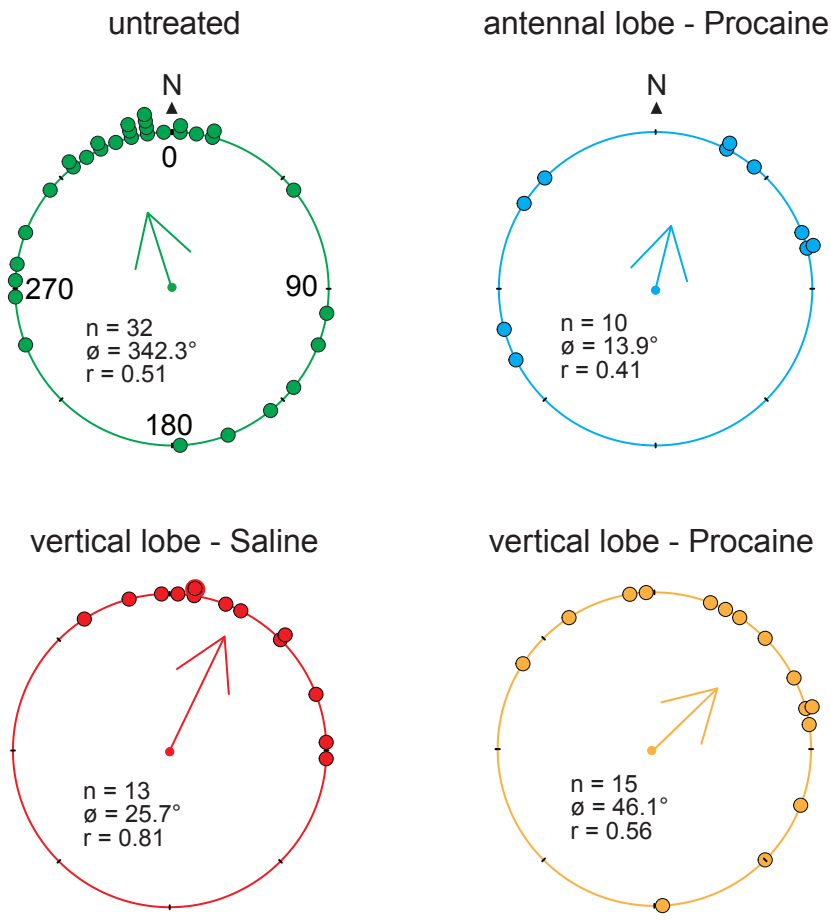

Figure S1. Initial heading direction of ants were not uniform. Related to Figure 2.
Heading directions of ants at 30cm from the release point. Sample size (n), mean vector (ø), and length of mean vector (r; arrow) relative to nest direction (N) are shown. One individual from the vertical lobe - procaine group was excluded because she did not travel 30cm from the release point. Corresponding trajectories and final heading directions are shown in Figure 2.