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RESEARCH ARTICLE

Appetitive olfactory learning suffers in ants when octopamine or dopamine receptors are blocked

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ABSTRACT

Associative learning relies on the detection of coincidence between a stimulus and a reward or punishment. In the insect brain, this process is carried out in the mushroom bodies under the control of octopaminergic and dopaminergic neurons. It was assumed that appetitive learning is governed by octopaminergic neurons, while dopamine is required for aversive learning. This view has recently been challenged: both neurotransmitters are involved in both types of learning in bees and flies. Here, we tested which neurotransmitters are required for appetitive learning in ants. We trained *Lasius niger* workers to discriminate two mixtures of linear hydrocarbons and to associate one of them with a sucrose reward. We analysed the walking paths of the ants using machine learning and found that the ants spent more time near the rewarded odour than near the other, a preference that was stable for at least 24 h. We then treated the ants before learning with either epinastine, an octopamine receptor blocker, or flupentixol, a dopamine receptor blocker. Ants with blocked octopamine receptors did not prefer the rewarded odour. Octopamine signalling is thus necessary for appetitive learning of olfactory cues, probably because it signals information about odours or reward to the mushroom body. In contrast, ants with blocked dopamine receptors initially learned the rewarded odour but failed to retrieve this memory 24 h later. Dopamine is thus probably required for long-term memory consolidation, independent of short-term memory formation. Our results show that appetitive olfactory learning depends on both octopamine and dopamine signalling in ants.

KEY WORDS: Associative learning, *Lasius niger*, Long-term memory, Neurotransmitters, Short-term memory

INTRODUCTION

There is nothing stable in the world; those that can adapt to changes will prevail. When the profitability of food sources changes over time, it would be advantageous to quickly learn which ones are currently most rewarding. Individuals can do so by associating the food with other stimuli such as odours or colours, i.e. they establish a predictive relationship between two independent cues from the environment (Giurfa, 2007). When a neutral stimulus, such as an odour, is paired with a biologically relevant stimulus (food – the unconditioned stimulus, US), the animal may in the future react to the previously neutral odour (the now conditioned stimulus, CS) as if it were the US. In the case of a bee searching for food, the CS and US are processed by two different pathways. The CS pathway starts at the odorant receptors (ORs) in the antenna. Output from the OR neurons is first processed in the antennal lobe (Giurfa, 2007). From there, projection neurons carry the information to the mushroom bodies, where learning occurs. The US pathway begins at gustatory receptors on the mouth parts. Neurons then project to the suboesophageal ganglion, from where the octopaminergic VUMx1 neuron projects to the antennal lobe and the mushroom bodies (Giurfa, 2007; Galizia and Sachse, 2010; Perry and Barron, 2013). Information about the CS and the US converges in the Kenyon cells of the mushroom body, which act as coincidence detectors and signal to higher brain centres through mushroom body output neurons to eventually elicit behaviours. Coincidence of an odour and an US can lead to changes in the Kenyon cells that will later cause them to signal the detection of the CS to the higher brain centres as if it were the US, even if no US is perceived. The reliance of the US signalling on the octopaminergic VUMx neuron suggests that without octopamine (OA) signalling, the US cannot reach the mushroom body and thus insects cannot learn. Indeed, blocking OA receptors in crickets, flies and honeybees prevented appetitive memory formation and memory retrieval (Schwaerzel et al., 2003; Galizia and Sachse, 2010), while injection of OA could replace the US (Hammer and Menzel, 1998). Similar experiments blocking dopamine (DA) receptors had no effect on appetitive learning but prevented individuals from associating a punishment, such as an electric shock, with odours or colours (aversive conditioning; Schwaerzel et al., 2003; Vergoz et al., 2007; Mizunami and Matsumoto, 2017). It was thus assumed that aversive and appetitive memory are formed in different modules of the mushroom bodies that use different neurotransmitters (Hige et al., 2015). To this day, this seems to hold true for crickets (Mizunami and Matsumoto, 2017), but newer data suggest that aversive conditioning relies on both OA and DA signalling in honeybees and fruit flies (Agarwal et al., 2011; Claßen and Scholz, 2018; Mancini et al., 2018). *Drosophila* GAL4 lines that can be used to knock out specific neurons in the mushroom body have allowed a much more detailed picture of learning and memory retrieval to be painted: in flies, learning in the mushroom body appears to be organized in modules that each consist of Kenyon cells, mushroom body output neurons, and dopaminergic neurons that modulate the valence of the modules (Berry et al., 2012; Hige et al., 2015.) Some of the modules organize attraction to odours, and others repulsion (Rohwedder et al., 2016). The modules further differ in how long the memory lasts – from a few seconds to several hours (also in honeybees: Menzel, 2014). The control of both appetitive and aversive learning by dopaminergic neurons also means that without DA signalling, both types of learning should be hampered, which matches the behavioural evidence from pharmacological receptor blocking and knockouts in *Drosophila* and honeybees (Berry et al., 2012; Perisse et al., 2013; Perry and Barron, 2013; Mancini et al., 2018; Sabandal et al., 2020).

One way to hinder DA- and OA-related signalling is to block their respective receptors pharmacologically. In insects, epinastine is an OA receptor blocker that abolishes the OA-related cAMP

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production in the mushroom bodies (Roeder et al., 1998). Epinastine seems to specifically target the AmOA1 receptor in honey bees (Beggs et al., 2011), the OA1 receptor in crickets (Awata et al., 2015), the DmOA3 receptor in *Drosophila* (Qi et al., 2017) and the NcOA2B2 receptor in the green rice leafhopper *Nephotettix cincticeps* (Xu et al., 2020). In contrast, flupentixol largely targets DA receptors, such as the AmDOP2 receptor in the mushroom bodies of honey bees (Mustard et al., 2003, 2010; Beggs et al., 2011).

Just like honey bees, ants are social insects for whom learning is important in many contexts (Bos et al., 2010). Ants can learn odours of rewarding food sources and also have to learn the specific odour of their own colony (Neupert et al., 2018). Indeed, ants can perform similar learning tasks to bees and other insects (Bos et al., 2010; Guerrieri and d'Ettorre, 2010; Fernandes et al., 2018; Piqueret et al., 2019). In addition to floral odours that might signal food sources, ants were successfully conditioned to respond to hydrocarbons that play an important role in social interactions (Bos et al., 2012; Sharma et al., 2015). For example, hydrocarbons can serve as alarm and queen pheromones, and are used to discriminate nestmates from non-nestmates (Leonhardt et al., 2016). While it has been found that protein synthesis is important for the formation of long-term memory in *Camponotus* and *Formica* ants (Guerrieri et al., 2011; Piqueret et al., 2019), we know nothing about the role different neurotransmitters play in ant learning.

Here, we studied reward learning in the black garden ant *Lasius niger*. This species has previously been shown to rapidly associate floral odours with sucrose rewards (Czaczkes and Kumar, 2020) and to acquire a preference for food of certain odours (Beckers et al., 1994). It can even be taught to ignore its own trail pheromones through differential conditioning (Wenig et al., 2021). First, we established an assay for training the ants to associate a mixture of linear hydrocarbons with a sugar reward. We tested memory retrieval after 5 min and after 1 day using a deep-learning algorithm on videos of reward-searching ants. In a second step, we administered epinastine or flupentixol via either the food or topical application to block OA and DA receptors. That way we could test whether a breakdown of the respective neural pathways would prevent the ants from forming short- and long-term memories of the odour–reward association.

MATERIALS AND METHODS

We trained ants to discriminate two mixtures of n-alkanes, one that was presented with a sugar reward and the other that was unrewarded, during six rounds of training trials. After that, we ran retention tests without the sugar reward to see whether the ants now preferred the previously rewarded odour (conditioned stimulus, CS+) over the unrewarded odour (CS0). A first retention test was conducted 10 min after the final learning trial to test the short-term memory capabilities. The second retention test was performed 1 day later to test the (early) long-term memory (Menzel, 1999).

Before the learning trials, we subjected the ants to treatment with OA receptor blockers, DA receptor blockers or no blockers (controls). The drugs were administered either with food (set 1) or by topical application on the mesothorax (set 2). The two experimental sets also differed in the way we recorded the data (manual versus automated recording, see 'Experimental setup', below).

Experimental organism

Colonies of black garden ants *Lasius niger* (Linnaeus 1758) were collected in the years 2017 and 2019 in Freiburg, Germany. They were transported to the laboratory and kept in plastic boxes at 20°C, under a 12 h:12 h light:dark cycle, and were fed with mealworms (*Tenebrio molitor*) and honey. Test tubes filled with water, cotton wool and an aluminium foil wrapping were placed in the boxes as a refuge and a water source. The walls of the boxes were lined with Fluon® (AGC Chemicals Europe, Ltd). Around 2 weeks before an experiment, the ants were deprived of honey and only fed mealworms, in order to motivate them to forage for carbohydrates. We used four different source colonies for the experiments in set 1, and 11 different source colonies for set 2. All procedures were conducted in concordance with the German Animal Welfare Act.

Stimuli

Two mixtures of three long-chain linear alkanes each were used as the two conditioned stimuli (CS). The first mixture (CS-mixture A) was n-octadecane (n-C18), n-heneicosane (n-C21) and n-heptacosane (n-C27). The second (CS-mixture B) was n-eicosane (n-C20), n-docosane (n-C22) and n-pentacosane (n-C25; all Sigma-Aldrich, Steinheim, Germany). These substances are present on the cuticle of *L. niger* (V.N., unpublished data). We combined them so that the two mixtures had roughly the same average chain length (22 and 22.3, respectively). The hydrocarbons were dissolved in n-pentane (10 µg ml^{-1}) that would evaporate before the experiments. In a preliminary experiment, we used the retention test setup (see below) with untrained ants to see whether they preferred either of the two odour mixtures over the other. The untrained ants spent similar times with the two odour mixtures (n=30, Wilcoxon test V=253, P=0.69; Fig. S1). A 0.5 µl drop of sugar water (50% w/w) or pure water was used as a positive unconditioned stimulus (US+) and neutral unconditioned stimulus, respectively.

Experimental setup

For the first set of experiments (feeding experiment), the test arena was the top of a Petri dish (90 mm in diameter). Another Petri dish lid was used as a roof, and a hole (40 mm in diameter) was cut into the roof to allow the ants to be placed into the arena. The ants were recorded from a 45 deg angle by a Nikon D90 with a 35 mm lens, which was placed in front of the arena on a tripod. For the second set of experiments (topical application), an acrylic glass cylinder (100 mm diameter, 100 mm height) was used as an arena. The arena was covered by a cardboard box lined with aluminium foil to exclude external stimuli and to allow for even illumination by two light sources above the arena. The ants were videotaped with a SM-P600 tablet computer at 1.5× magnification, the lens of which was placed directly above the centre of the arena.

For both sets of experiments, the walls of the arena were coated with Fluon to prevent the ants from climbing. The floor was lined with filter paper that was evenly divided into quarters. A microscope coverslip (24×24 mm) was placed in the centre of each quadrant (Fig. 1A). In each learning and test trial, one of the cover slips was coated with 20 µl of odour A, another with odour B, and the remaining two with pure pentane. The solutions were always applied to the edges of the cover slips at least 2 min before the ants were placed into the arena. After every trial, the filter paper and coverslips were replaced with new ones.

Learning trials

The learning procedure was adapted from Bos and colleagues' (2010, 2012) work on ant learning. From the main colony, around 30 ants that were moving outside of the nest tubes were separated into a box equipped with nest tubes as per the source colonies. Here, ants could perform trophallaxis between the trials, to reduce their sugar storage and increase the motivation to forage again. From each

Journal of Experimental Biology (2021) 224, jeb242732. doi:10.1242/jeb.242732

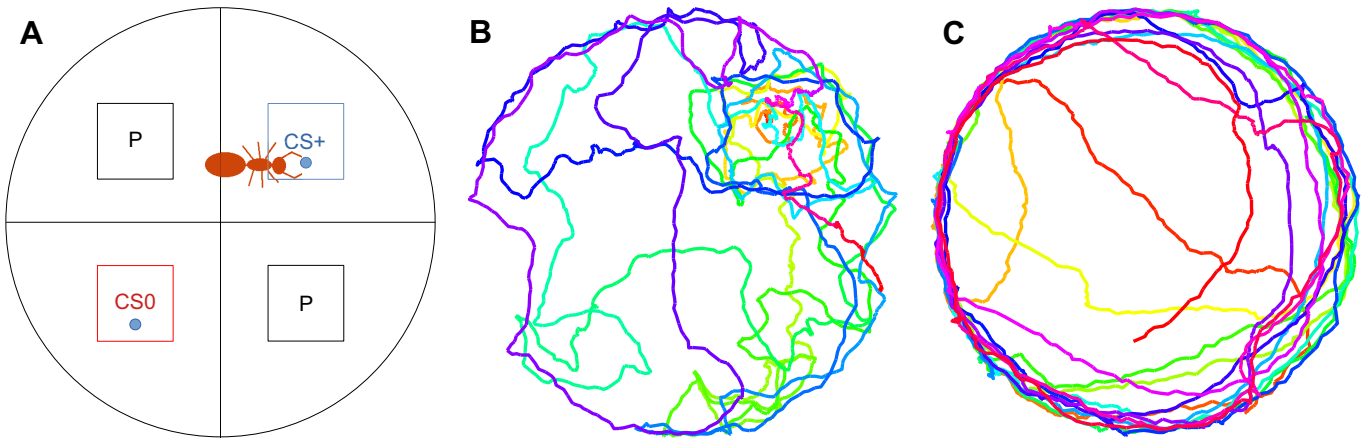

Fig. 1. Experimental setup. (A) Design of the experimental arena used for the learning experiments. The arena was divided into four quadrants, each with a coverslip in it. One of the coverslips was coated with odour A, another one with odour B (conditioned stimulus, CS). The remaining two coverslips were treated with pure solvent (n-pentane, P). During the learning experiments, one of the odours was paired with sucrose solution (CS+) and the other with water (CS0). (B,C) Traces of ants during the retention tests, with the previously rewarded odour in the top right quadrant. The ant in B inspected the CS+; the ant in C did not show any particular interest in the previously rewarded odour. Colours show how the ants moved from the beginning of the test (red) to the end (blue).

experimental subcolony, only one ant entered the experiment. We transferred ants on little pieces of filter paper that they had to climb on actively. The first ant that climbed onto the filter paper was chosen as a test ant. It was marked on the gaster with a small dot of paint (Edding no. 751), so that it was possible to individually track it between learning trials.

For each learning trial, 0.5 µl of sugar solution was added to the coverslips with one of the odours (CS+), while water was added to the coverslip with the CS0. For each ant, the same odour was rewarded in each learning trial, but which odour was rewarded differed randomly between ants. The test ant was transferred into the arena and the time was measured until it began drinking the sugar solution. When the ant stopped drinking, it was removed from the arena, and placed back into the experimental colony where it could perform trophallaxis with the nestmates. After 6 min, when trophallaxis was always complete (i.e. the ant broke contact with its nestmates and was walking around), the ant was brought back into the arena for the next learning trial. In total, there were six learning trials per individual.

As the response variable during the learning trials, we recorded the time from the ants entering the arena to them beginning to drink from the sugar solution, when they first touched it with their mouthparts. If an ant had not found the sugar solution within 3 min in set 1, it was removed from the learning arena and returned to the experimental colony until the next learning trial. Ants that could not find the sugar solution in two or more learning trials were excluded from the analysis. The process was similar for set 2, but we gave the ants only 2 min for the second to sixth learning trial, because the results from set 1 showed that periods longer than 2 min are very rare in the later trials. We began set 1 with 169 ants across the six treatments and excluded 11 of those; in set 2, we had to exclude 2 out of 182 ants. The highest proportion of excluded ants came from flupentixol treatments in both sets (Table S1). There was no difference between treatments in the proportion of ants that had to be excluded when we fed the receptor blockers (χ^2_5=8.5, P=0.13, P-value calculated by Monte Carlo simulation). In the topical application experiment, the treatments differed in the proportion of ants that had to be excluded (χ^2_3=10.7, P=0.02), although overall only 2 ants were excluded, both from the flupentixol treatment. In set 1, all ants were trained sequentially, one after the other. In set 2, training and retention tests of two ants were conducted in the same time span: when the first training trial of the first ant was completed and it was back in its experimental colony, we began the first training trial with the second ant, followed by the second trial of the first ant, and so on. In this way we trained and tested 4–8 individuals per day. On each given day, we only administered one of the treatments, but the exact treatments on subsequent days differed. We took care that control treatments were interspersed with blocker treatments and evenly spread throughout the entire experiment, which is why we conducted more replicates for the controls than for any of the blocker treatments. We always conducted experiments with different treatment timings and concentrations of the same receptor blocker sequentially (e.g. the 100 mmol l^{-1} epinastine treatment was only begun after the 25 mmol l^{-1} epinastine treatment had finished).

We tested whether the ants became quicker at finding the sugar solution during the consecutive learning trials with a Poisson MCMC glmm, with time until finding the sugar as the dependent variable and trial number as a continuous predictor, for each treatment separately. We entered the colony ID and individual ant ID as random factors into the model using the R package MCMCglmm with default settings (13,000 MCMC iterations, 3000 burn-in, thinning interval of 10; R version 4.0.3 – https://www.R-project.org/; Hadfield, 2010). Chain convergence was visually inspected by plotting MME solutions. In set 2, we observed the ants for only 2 min in learning trials 2–6, as opposed to 3 min in trial 1. To exclude that the small number of ants taking longer than 2 min in trial 1 caused a downward trend in the learning times across trials, we set the times of all ants that took longer than 2 min to 2 min for the glmm but left all times shorter than 2 min as they were.

Retention tests

When the ants had finished trophallaxis after the sixth learning trial, a first retention test was conducted. Retention tests resembled training trials but no sugar solution or water was added to any of the coverslips. The coverslips with the CS+ and CS0 odours were in opposite quadrants (Fig. 1A). Each ant was recorded for 2 min. Then, a drop of sugar water was placed on the coverslip with the learned odour to prevent extinction before the retention trials on the following day. When the ant had finished drinking the sucrose solution, it was returned to the test colony, where it could perform trophallaxis for 6 min. Then, the position of the coverslips was

Journal of Experimental Biology (2021) 224, jeb242732. doi:10.1242/jeb.242732

changed and the ant was recorded a second time. The time spent in the different quadrants was averaged over the two tests for each ant. All trained ants were kept in an experimental subcolony overnight and tested again the next day to see whether they could still remember the CS+. The interval between the two retention tests was 15–23 h. After the second retention test, the ants were returned into their original colonies, which were not used for further experiments in the same week. Subsequently, we could avoid re-using individuals because they had been colour-marked.

The time the ants spent in the four quadrants was measured from the video recordings. In set 1, times were measured manually using the program etholog (Ottoni, 2000). For set 2, we used DeepLabCut with a ResNet-50-based neural network (Mathis et al., 2018). We manually marked the head in 150 random frames from each of 20 videos (320×240 pixels) of *L. niger* ants. These had been recorded before in the same arenas that we later used for the learning experiments. The network used the pre-labelled frames in 150,000 training iterations to learn recording of the ant position. Then, the algorithm split the pre-labelled frames into a training (95% of the frames) and a test dataset (5% of the frames) and tested its accuracy on the pre-labelled test dataset, reaching an accuracy of 7 pixels (5 pixels in the training dataset), which is within the size of an ant (ca. 11 pixels). After this, we conducted the learning experiments and recorded 756 videos of retention tests, which were processed with the algorithm. We confirmed that there are no obvious errors in the recorded positions by fast forwarding through videos in which the recorded position was highlighted.

From the ant's coordinates, we could calculate in how many frames the ant was identified in each of the four quadrants, and thus how much time it had spent there. We directly compared whether the ants spent more time on one of the two odour mixtures using paired Wilcoxon tests (all statistical tests are two-tailed). In addition, we calculated and visualized (ggplot2; Wickham, 2016) a preference index (PI) as an intuitive measurement of preference and hence learning performance based on the time (t) spent on the different odours (Bos et al., 2010, 2012):

$$\mathrm{PI} = \frac{t_{\mathrm{CS+}} - t_{\mathrm{CS0}}}{t_{\mathrm{CS+}} + t_{\mathrm{CS0}}}. \quad (1)$$

The index ranges from 1 (preference for the CS+) to −1 (preference for the CS0). If the ant spent an equal amount of time on the two odours, the PI would be 0.

Pharmacological manipulations

To test the extent to which neurotransmitters are involved in appetitive learning, the ants were treated with one of two different receptor blockers before the learning trials: epinastine, an OA receptor blocker (Kamhi et al., 2015), or flupentixol, a DA receptor blocker (Agarwal et al., 2011).

For the first set of experiments, ants were fed honey infused with the blocker (50 mmol l^{-1} flupentixol hydrochloride or 20 mmol l^{-1} epinastine hydrochloride; Sigma-Aldrich, Steinheim, Germany), 1–3 h, 5–8 h or 17 h prior to the experiment. The ants were individually fed with a 1 µl drop of this solution, a quantity they can easily take up (Mailleux et al., 2000). The different intervals were chosen because it was not clear how long it would take for the drugs to become effective after feeding. The honey containing the drug was coloured with Neutral Red to control for ingestion of the honey; only ants with a red staining in their gaster were chosen for the experiments. When the ants had finished eating, they were kept in groups with other ants that had received the same drug, until the start of the learning trials. Immediately before the beginning of its learning trial, each ant was marked with the paint marker and moved back to a subcolony of ca. 30 ants set up for this purpose. Control ants were fed pure honey instead of blocker-infused honey.

For the second set of experiments, the drugs were dissolved in dimethylformamide (DMF), and a 0.5 µl droplet was applied to the ant's cuticula on the mesothorax (Barron et al., 2007). The ants were allowed to recover from the treatment for 15 min in isolation, after which they were paint-marked and returned 5 min later to the subcolonies as described above. Another 10 min later, the first learning trial began. For epinastine, the two dosages were 20 and 100 mmol l^{-1} in DMF, corresponding to doses of 1.8 and 9.1 ng μg^{-1} ant body mass, respectively. For flupentixol, the dosage was 250 mmol l^{-1} (39.3 ng μg^{-1}). Control ants received pure DMF.

We compared the PI among treatments with MCMC glmms, with treatment as the only predictor, so that we could test for each treatment whether it differed from the control. Colony ID was entered into the model as a random factor. In the same manner, we compared the walking speed of ants, calculated from the coordinates generated during set 2, among the different treatments. Code and output of the analyses are given in Supplementary Materials and Methods.

RESULTS

Set 1: administration of receptor blockers via food

Over the successive learning trials, control-treated ants became quicker at finding the sugar reward (Fig. 2, Poisson glmm P=0.034, n=30 ants), indicating that they learned to associate the rewarded odour with the sugar reward. This was also true for all treatments with receptor blockers (epinastine 1–3 h P<0.001, n=27 ants; epinastine 5–8 h P<0.01, n=29 ants; epinastine 17–26 h P=0.034, n=25 ants; flupentixol 1–3 h P=0.034, n=26 ants; Fig. 2), with the exception of the ants that were treated with flupentixol 5–8 h before the first learning trial (P=0.55, n=21 ants).

In the first retention test 10 min after the last learning trial, the ants were able to differentiate between CS+ and CS0, independent of treatment (Fig. 3A; Wilcoxon tests: control P<0.001; epinastine 1–3 h and 5–8 h P<0.01; epinastine 17–26 h and flupentixol 1–3 h P<0.05). Only the ants that were fed with flupentixol 5–8 h before the treatment had difficulty differentiating between CS+ and CS0 (Wilcoxon test P=0.052). In most treatments, the PI was indistinguishable from that of control ants (glmms P>0.15; see Table S2 for details), but there was a trend that the PI of ants treated with epinastine 5–8 h (P=0.058) and flupentixol 1–3 h (P=0.070) before the learning trials might have been lower than those of control ants. In those two cases, the effect sizes indicate that the PI of the control ants (mean posterior estimate PI=0.19) was reduced by roughly half by the epinastine 5–8 h and flupentixol 1–3 h treatment (to PI=0.09; Table S2).

On the second day (Fig. 3B), the control ants (Wilcoxon test P=0.04) and some of the epinastine-treated ants (5–8 h and 17–26 h before the learning trials, both P<0.01) still differentiated between CS+ and CS0. In contrast, ants that were treated with epinastine 1–3 h before the experiment (P=0.9), and the ants from both flupentixol treatments (1–3 h P=0.48; 5–8 h P=0.20), were not able to differentiate between CS+ and CS0. However, there was no difference in the PI between the treatments (glmm all comparisons P>0.1; Table S2). This might have been caused by the relatively small effect of learning visible in the PI of control ants (PI mean estimate=0.08) that made it difficult to see an effect in any reduction of the PI.

Journal of Experimental Biology (2021) 224, jeb242732. doi:10.1242/jeb.242732

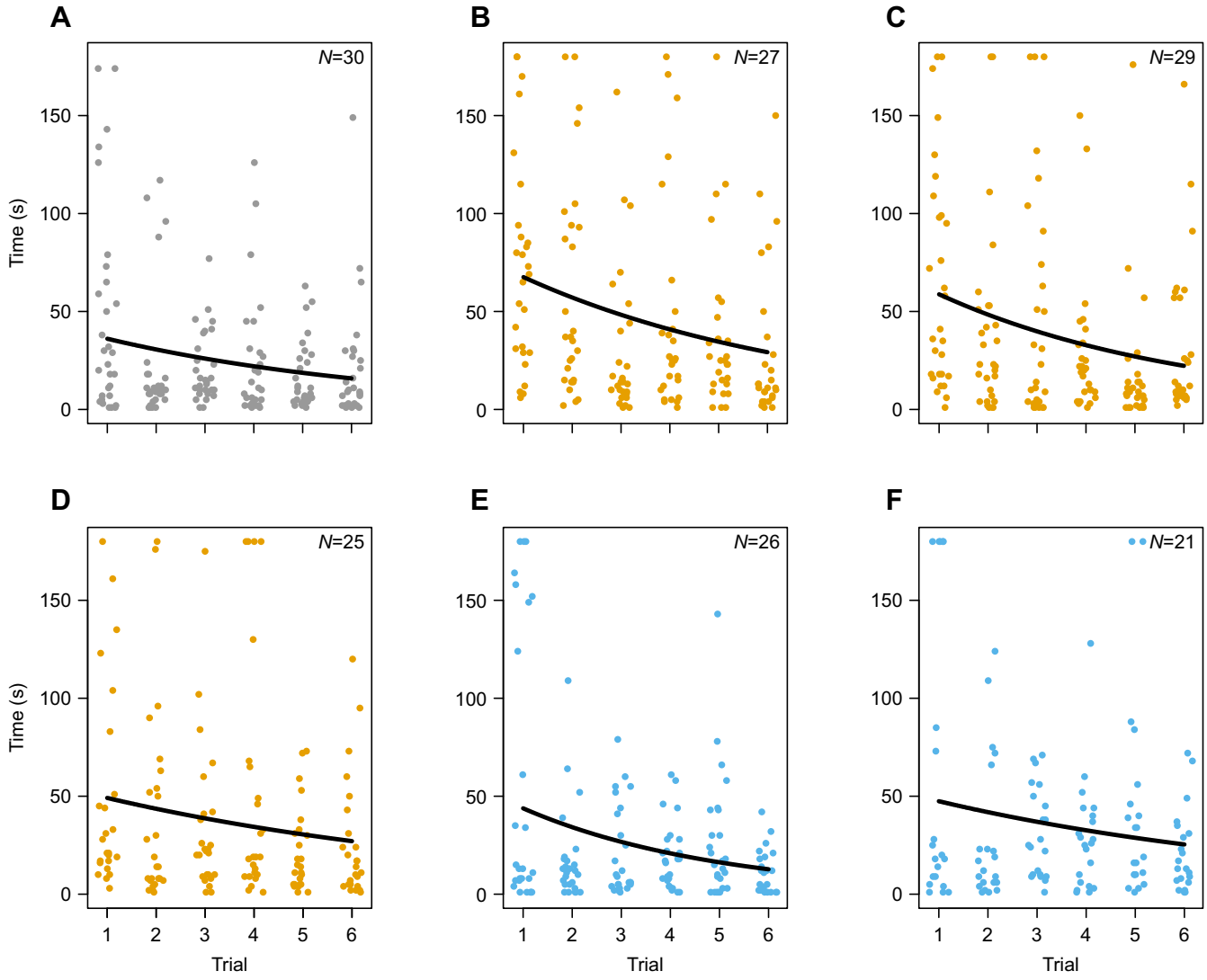

Fig. 2. Time taken for ants in set 1 to find the sugar reward in six consecutive learning trials. Ants were either not treated with receptor blocker (control; A), or received epinastine (B, 1–3 h; C, 5–8 h; D, 17–26 h) or flupentixol (E, 1–3 h; F, 5–8 h) with their food at different intervals before the first learning trials. Individual data points are plotted and slightly displaced along the *x*-axis for visibility; the lines are Poisson regressions.

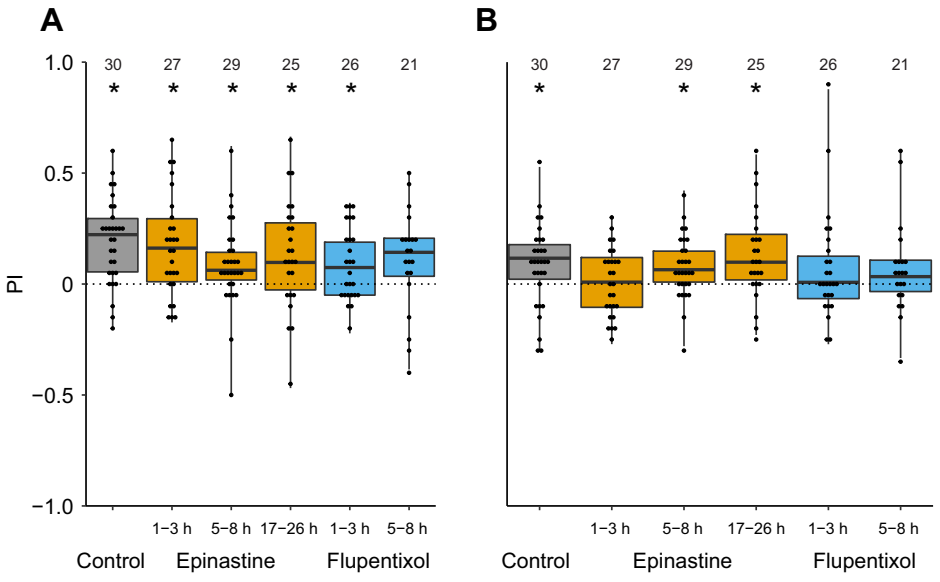

Fig. 3. Preference of ants in set 1 for the previously rewarded odour (CS+) during retention tests. Each ant from the control, epinastine or flupentixol groups (see Fig. 2) completed two retention tests without reward, the first test 5 min after the sixth learning trial (A), and the second test 1 day later (B). The ants could move in an arena in which one quadrant was marked with the CS+ odour and another with CS0. The dotted lines indicate the null hypothesis of ants spending equal time in the CS+ and the CS0 quadrant; positive preference index (PI) values indicate that the ants spent more time in the quadrant marked with the CS+. Numbers above the boxes are sample sizes; asterisks indicate significant differences between the time spent on CS+ and CS0 (Wilcoxon test; **P*<0.05). For each treatment, boxplots (median, interquartile range, total data range) and indices of individual ants (binned to 0.05) are plotted.

Journal of Experimental Biology (2021) 224, jeb242732. doi:10.1242/jeb.242732

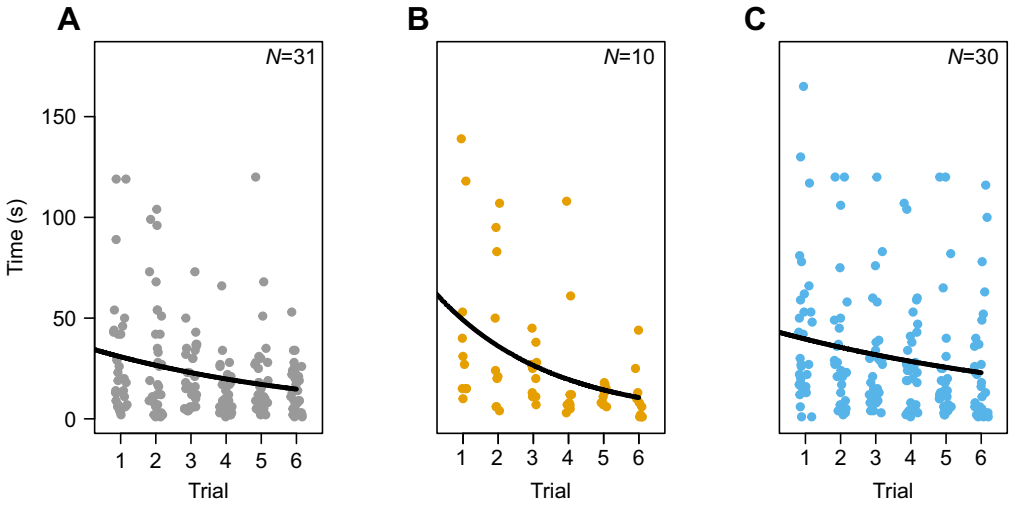

Fig. 4. Time taken for ants in set 2 to find the sugar reward in six consecutive learning trials. Ants were either not treated with receptor blocker (DMF control; A), or topically treated with 100 mmol l^{-1} epinastine (B) or 250 mmol l^{-1} flupentixol (C). Individual data points are plotted and slightly displaced along the *x*-axis for visibility; the lines are Poisson regressions. Note that we could only analyse 10 of the learning trials with 100 mmol l^{-1} epinastine and none of the ones with 20 mmol l^{-1} epinastine.

Set 2: topical application of receptor blockers

The exact dosage of the receptor blockers is difficult to control when feeding the ants the drugs, because the ants may sometimes ingest more and sometimes less of the food. We thus performed a second set of experiments where we topically applied precise doses of the blockers 30 min before the learning trials. During the learning trials, the time it took the ants to find the sugar solution decreased for the control ants (Poisson glmm P=0.006, n=31 ants; Fig. 4A). This effect was also evident for ants that had been treated with 100 mmol l^{-1} epinastine (P<0.001, n=10 ants) or 250 mmol l^{-1} flupentixol (P=0.002, n=30 ants) previous to the learning trials (Fig. 4B,C). Note that we could only analyse 10 of the learning trials with 100 mmol l^{-1} epinastine and none of the ones with 20 mmol l^{-1} epinastine.

In the first retention test, the ants that had been treated with DMF control preferred the CS+ over the CS0 (Fig. 5A; Wilcoxon test P<0.001). The same was true for ants that had been treated with 20 mmol l^{-1} epinastine (Wilcoxon test P<0.001) or 250 mmol l^{-1} flupentixol (Wilcoxon test P<0.001). The ants that had been treated with 100 mmol l^{-1} epinastine did not differentiate between the CS+ and CS0 on the first day (Wilcoxon test P=0.83). The PI of the 100 mmol l^{-1} epinastine treatment was lower than that of the control ants (glmm P<0.001; Table S3) indicating that 100 mmol l^{-1} epinastine had eradicated any effect of learning (mean estimate for the control PI=0.23, for the epinastine treatment PI=0.01; see Table S3). The PIs of the 20 mmol l^{-1} epinastine treatment (P=0.48) and the flupentixol treatment (P=0.16) were not significantly lower than that of the control ants.

One day later, the preference for CS+ was still evident for control ants and ants treated with 20 mmol l^{-1} epinastine (Wilcoxon tests P<0.001), and still absent in ants treated with 100 mmol l^{-1} epinastine (Wilcoxon test P=0.77). There was only a trend for flupentixol-treated ants to discriminate CS+ and CS0 (P=0.09; Fig. 5B). However, all ants appeared to be affected by the receptor blockers, as the PIs of all

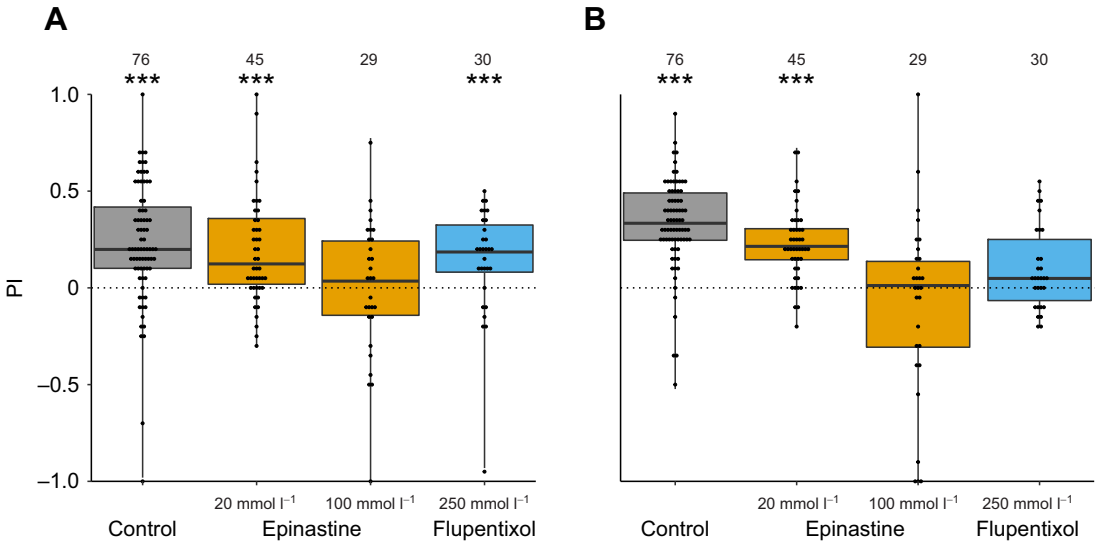

Fig. 5. Preference of ants in set 2 for the CS+ during retention tests. Each ant from the control, epinastine (20 or 100 mmol l^{-1}) or flupentixol (250 mmol l^{-1}) groups (see Fig. 4) completed two retention tests without reward, the first test 5 min after the sixth learning trial (A) and the second test 1 day later (B). The ants could move in an arena in which one quadrant was marked with the CS+ odour and another with CS0. The dotted lines indicate the null hypothesis of ants spending equal time in the CS+ and the CS0 quadrant; positive PI values indicate that the ants spent more time in the quadrant marked with the CS+. Numbers above the boxes are sample sizes; asterisks indicate significant differences between the time spent on CS+ and CS0 (Wilcoxon test; ***P<0.001). For each treatment, boxplots (median, interquartile range, total data range) and indices of individual ants (binned to 0.05) are plotted.

Journal of Experimental Biology (2021) 224, jeb242732. doi:10.1242/jeb.242732

ants treated with receptor blockers were lower than those of the control ants (glmm: 20 mmol l^{-1} epinastine $P<0.03$; 100 mmol l^{-1} epinastine and flupentixol $P<0.001$; see Table S3 for details and effect sizes). This effect was relatively weak in the 20 mmol l^{-1} epinastine treatment group (the PI was reduced by ca. 33%), but stronger in the flupentixol group (72% reduction) and complete in the 100 mmol l^{-1} epinastine treatment group (see Table S3).

To test whether ant learning or memory retrieval may be prevented by a general lethargy caused by the receptor blockers, we tested whether they affected the walking speed of the ants. Indeed, during the first retention test, ants treated with both concentrations of epinastine were slower than control ants (Fig. 6A; glmm $P<0.01$; Table S4), but flupentixol did not affect walking speed ($P=0.25$). However, the effect size of the maximal speed reduction was only in the range of 23% (Table S3) and should not have prevented the ants from encountering the odours. Indeed, only a single ant, treated with 100 mmol l^{-1} epinastine, did not enter the quadrant with the rewarded odour during the retention test. One day later, only the walking speed of 100 mmol l^{-1} epinastine-treated ants was still reduced by ca. 10% (glmm: 100 mmol l^{-1} epinastine $P<0.05$; both other treatments $P>0.6$; Table S4; Fig. 6B).

DISCUSSION

Using a classical conditioning protocol, we were able to show that black garden ant workers can learn to associate blends of linear hydrocarbons with a sucrose reward, and that the memory is stable for at least 1 day. Exposing the ants to the OA receptor blocker epinastine prevented them from learning. In contrast, when we exposed the ants to the DA receptor blocker flupentixol, the short-term memory persisted but the long-term memory was affected. Both OA and DA signalling are thus involved in appetitive learning in ants. Our results further suggest that the active period of the receptor blockers is limited in that learning is possible again a few hours after blocker ingestion.

Associating hydrocarbons with a sucrose reward

During the test trials, the ants spent more time in the quadrant that contained the conditioned stimulus (CS+) than in that containing a neutral stimulus not previously associated with a sucrose reward (CS0). The increase in time spent in the CS+ quadrant is probably a result of the ants searching for the reward. The ants are thus able to learn and discriminate blends of linear hydrocarbons. Most appetitive conditioning assays in hymenoptera rely on volatile odours that are typically emitted from flowers, to closely resemble the natural situation in which learning can lead to flower constancy in bees (Koethe et al., 2020). However, many pheromones of social insects are less volatile hydrocarbons. Our study confirms previous work on carpenter ants and Argentine ants that demonstrates hydrocarbons are also adequate stimuli for appetitive conditioning (Bos et al., 2012; van Wilgenburg et al., 2012; di Mauro et al., 2015; Sharma et al., 2015). It appears that the effects of hydrocarbon learning are less distinct than those of learning volatile odours (cf. Czaczkes and Kumar, 2020), perhaps because the detection of hydrocarbon cues is possible only over very short distances.

Involvement of OA in appetitive learning

Unlike control ants, ants that were treated with 100 mmol l^{-1} epinastine topically to the thorax did not prefer the conditioned hydrocarbon odour over a non-conditioned odour within 5 min of the learning trials, and neither did they prefer the conditioned stimulus after a break of 1 day. As epinastine blocks OA receptors, this suggests that OA signalling is required for olfactory learning of appetitive stimuli in ants, potentially because octopaminergic neurons relay the information about the unconditioned stimulus to the Kenyon cells (e.g. VUMx1 in bees; Rein et al., 2013). Our findings are in line with previous work demonstrating such an effect in fruit flies, honey bees and crickets (Schwaerzel et al., 2003; Giurfa, 2007; Mizunami and Matsumoto, 2017).

Epinastine not only affects memory but potentially also other traits. Under the influence of epinastine, the metabolism of honeybees and their response to sucrose was reduced (Buckemüller et al., 2017), and aggression was modulated in crickets and red wood ants (Rillich and Stevenson, 2011, 2018; Yakovlev, 2018). In our study, the mobility of ants treated with epinastine was reduced during the first retention test. For the high dose of epinastine, this effect was still visible 1 day later, which may indicate that epinastine is only slowly

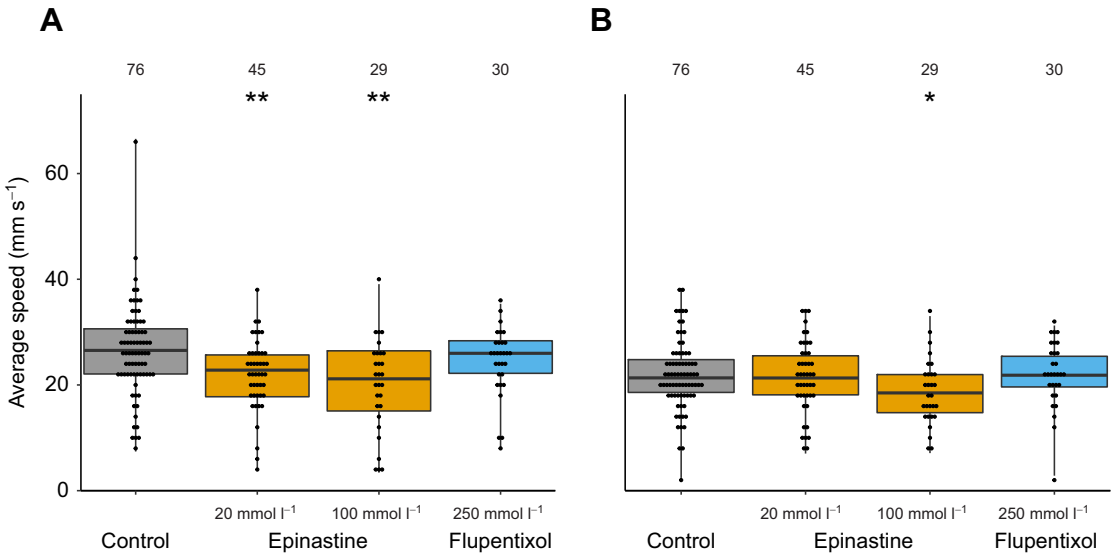

Fig. 6. Walking speed during retention tests of ants from set 2. Ants were from the control, epinastine (20 or 100 mmol l^{-1}) or flupentixol (250 mmol l^{-1}) groups (see Fig. 4). Asterisks indicate treatments that changed the average speed significantly compared with the control treatment (glmm; *$P<0.05$, **$P<0.01$). For each treatment, boxplots (median, interquartile range, total data range) and indices of individual ants (binned to 2 mm s^{-1}) are plotted.

degraded. The ants still visited all quadrants of the arena during the retention tests, so the failure to identify the CS+ in the retention tests is not simply due to an inability to walk. The ants also found and typically completely ingested the sucrose solution droplet during the learning trials. In fact, in our learning trials with epinastine-treated ants, the time until the ants found the sucrose solution decreased. There is thus no evidence from our results that epinastine decreased the motivation of the ants to collect sucrose, which could then decrease their motivation to learn. However, with our experiments, we cannot fully exclude this possibility, and the issue deserves more attention in the future.

In moths, OA plays a modulatory role in sex pheromone perception, and epinastine has been shown to affect the signalling in odorant receptor neurons (Pophof, 2000; Hillier and Kavanagh, 2015). OA signalling also seems to affect olfactory sensitivity to brood pheromones in bees (Spivak et al., 2003). We do not currently know whether similar effects also occur in neurons for hydrocarbon detection in ants, but it is possible that at least part of the effect of epinastine on learning is caused by a reduced sensitivity to odorants, both in our studies and in others investigating olfactory conditioning. A failure to learn after epinastine treatment may thus be due to information about both the CS and the US not reaching the Kenyon cells.

Ants topically treated with a lower dose of epinastine were not impaired in their learning ability. More interestingly, the effects were still visible when we fed the ants with epinastine, but weaker than when we directly applied the drug to the thorax. It is possible that only part of the dose is entering the haemolymph when the drugs are taken up with the food. Ant workers possess a crop used to store food that is later regurgitated to feed nestmates (Mailleux et al., 2000). Some epinastine entering the crop may thus never reach the OA receptors in the brain, exposing them to lower doses than intended. It may take a while until epinastine is taken up into the haemolymph and reaches the brain, meaning that it will take action only after a certain delay (but see Barron et al., 2007: OA delivered through the food reaches the honey bee brain in less than 1 h). We thus fed the ants epinastine at different intervals before subjecting them to the learning trials. However, longer intervals only reduced the effect of epinastine, and ca. 20 h after being fed the receptor blockers, the PIs of epinastine-fed individuals were no different from those of control ants. Finally, when ants were fed epinastine, we found only weak effects on short-term memory retention. Given that there was a clear effect on the short-term memory when epinastine was topically applied, this might have to do with long- and short-term memory being formed in different parts of the brain (e.g. different populations of Kenyon cells; Sachse and Galizia, 2003) and potentially at different times. If short-term memory formation is quicker than long-term memory formation, epinastine may have reached the brain before long-term memory formation, but after short-term memory formation was complete.

Involvement of DA

In our experiments, flupentixol strongly affected long-term memory as well, but not necessarily short-term memory. After topical application, we did not find any evidence of flupentixol affecting short-term memory. Flupentixol blocks DA receptors, so our observation suggests that dopaminergic neurons are not required in ants to relay the unconditioned stimulus to the Kenyon cells (unlike in fruit flies and bees; Schwaerzel et al., 2003; Giurfa, 2007). However, we found weak effects of flupentixol on short-term memory when we fed it to the ants, which may indicate that a functional dopaminergic system may improve learning performance.

In contrast to short-term memory, long-term memory was always affected when we administered flupentixol via either food or topical application. It is particularly noteworthy that even ants that clearly preferred the rewarded stimulus in the short-term memory retention tests after topical application of flupentixol did not recall this information 1 day later. This indicates a necessary involvement of dopaminergic neurons in the consolidation phase of long-term memory formation. This process would be independent of short-term memory formation and cannot be rescued by octopaminergic neurons, which are still functioning when only flupentixol is administered. The dopaminergic neurons may thus not be relaying the unconditioned stimulus to the learning centres, but be part of feedback loops or modulation pathways (Eschbach et al., 2020). Long-term memory formation is a more complex process than short-term memory formation (e.g. Menzel, 1999; Villar et al., 2020) and memory consolidation might rely on additional neural circuits that could be dopaminergic. For example, some lateral neurons in the antennal lobes are dopaminergic (Galizia and Sachse, 2010) and might be used to improve the perception of relevant odours such as the CS+. In the mushroom body of fruit fly larvae, Kenyon cells receive feedback from the primary protocerebral anterior cluster via dopaminergic neurons (Lyutova et al., 2019).

Blocking DA receptors does not only influence appetitive and aversive learning. Blocking the AmDOP2 receptor leads to reduced motor behaviours and an increase of grooming in honey bees (Mustard et al., 2010). In *Drosophila*, social interactions and temperature preferences seem to be affected (Verlinden, 2018). In our study, there was no evidence of effects of flupentixol on motor behaviour, as walking speed of flupentixol-treated ants did not differ from that of control ants. This is in line with results from weaver ants (Kamhi et al., 2015). Interestingly though, flupentixol fed 5–8 h before learning, the treatment that affected both short- and the long-term memory, also prevented an improvement in the time required for ants to find the reward during the learning trials (Fig. 2). Also, in the flupentixol-treated groups, some individuals did not find the sucrose reward during the 2 min of the learning trial (Table S1). One possible explanation could be that blocking DA signalling interferes with the motivational state of the ants. If lack of DA signalling prevents effective hunger signals, the ants might not be primed to seek the sucrose reward in the learning trials, but may still be drawn to the conditioned stimulus in the retention tests because of its familiarity. Indeed, DA is important for hunger signalling in *Drosophila* (Krashes et al., 2009; Siju et al., 2021). Hunger affects the motivational state, which is important for memory formation and retrieval because satiated flies do not seek food. However, dopaminergic neurons appear to signal satiation rather than hunger in *Drosophila*, so blocking them should improve motivation (Krashes et al., 2009). If the neural circuits were similar in ants, hunger could thus not explain the pattern we found when we blocked DA receptors. However, the motivation of ants as social beings is perhaps regulated less by their personal physiological hunger and more by that of their colony, so that entirely different regulatory circuits might be involved here (e.g. Cholé et al., 2019). In any case, such a lack of hunger or motivation could only explain an effect of flupentixol on short-term memory in the feeding trial: in the topical application experiment, ants did initially learn in learning trials and remembered in the first retention test, but clearly failed to remember on the next day. This observation indicates an involvement of DA in the consolidation of long-term memory, independent of short-term memory formation and motivation.

Conclusions

Lasius niger ants can learn to associate mixtures of linear hydrocarbons with a sugar reward and remember the association for at least 24 h. Both OA and DA appear to be involved in appetitive learning, with strong effects of a pharmacological OA knockout on both short- and the long-term memory. DA signalling is required for the formation of long-term memory but not necessarily for short-term memory.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: V.N.; Methodology: M.W.; Validation: V.N.; Formal analysis: M.W., V.N.; Investigation: M.W.; Resources: V.N.; Data curation: M.W.; Writing - original draft: M.W.; Writing - review & editing: V.N.; Visualization: M.W.; Supervision: V.N.; Project administration: V.N.; Funding acquisition: V.N.

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Data availability

The datasets generated during the study are available from the figshare data repository: https://doi.org/10.6084/m9.figshare.14884860.v1

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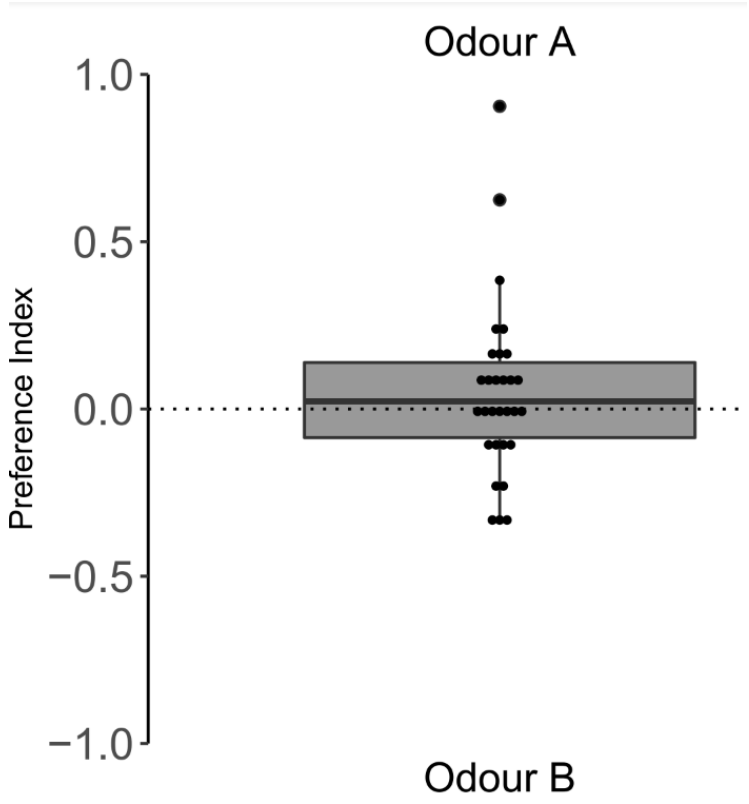

Fig. S1. The preference index of untrained ants for odour mixture A over odour mixture B when presented on glass slides. The odour mixtures were later used as rewarded stimulus (CS+) and unrewarded stimulus (CS0) in the learning experiments. Both odour mixtures contained equal proportions of three n-alkanes (odour A: n-C18, n-C21, and n-C27; odour B: n-C20, n-C22, n-C25), and the ants did not prefer either (n = 30, wilcoxon test V = 253, p = 0.69).

Table S1. The number of ants that entered the learning trials, and the sample size of the following retention tests after removing ants that did not find the sugar solution during at least two learning trials, or died before the second retention test.

Experiment	Treatment	Sample Size	Sample Size – Adjusted
Application	DMF – Control	76	76
	Epinastine 20mM	45	45
	Epinastine 100mM	29	29
	Flupentixol 250mM	32	30
Feeding	Untreated – Control	30	30
	Epinastine 1-3 Hours	30	27
	Epinastine 5-8 Hours	29	29
	Epinastine 17-26 Hours	27	25
	Flupentixol 1-3 Hours	28	26
	Flupentixol 5-8 Hours	25	21

Table S2. MCMCglmm on preference indices during retention tests of ants fed with receptor blockers. The table contnains the results of two independent models for day 1 and day 2. Each model contained colony ID and ant ID as random factors. For each factor level we report the effect (mean of the posterior distribution, i.e. the effect size of treatment compared to the control, measured in PI units), the limits of its 95% confidence interval (CI), the effective sample size as a measure of model convergence, and the p value derived from ther posterior distribution.

Response Variable	Treatment	Effect	Lower 95% CI	Upper 95% CI	Effective Sample	p
Preference Index Day 1	Control PI	0.19	0.11	0.26	1205	< 0.001
	Epinastine 1-3 Hours	-0.02	-0.14	0.08	1000	0.656
	Epinastine 5-8 Hours	-0.10	-0.21	0.01	1000	0.058
	Epinastine 17-26 Hours	-0.06	-0.18	0.05	1000	0.300
	Flupentixol 1-3 Hours	-0.10	-0.22	0.00	1000	0.070
	Flupentixol 5-8 Hours	-0.09	-0.20	0.03	1000	0.158
Preference Index Day 2	Control PI	0.08	0.02	0.15	1128	0.018
	Epinastine 1-3 Hours	-0.08	-0.17	0.02	985	0.102
	Epinastine 5-8 Hours	-0.00	-0.10	0.09	1000	0.962
	Epinastine 17-26 Hours	0.05	-0.05	0.14	1075	0.332
	Flupentixol 1-3 Hours	-0.01	-0.11	0.09	1000	0.852
	Flupentixol 5-8 Hours	-0.02	-0.12	0.08	1000	0.758

Table S3. MCMCglmm on preference indices during retention tests of ants that received a topical application of receptor blockers. The table contnains the results of two independent models for day 1 and day 2. Each model contained colony ID and ant ID as random factors. For each factor level we report the effect size (mean of the posterior distribution, i.e. the effect size of treatment compared to the control, measured in PI units), the limits of its 95% confidence interval (CI), the effective sample size as a measure of model convergence, and the p value derived from ther posterior distribution.

Response Variable	Treatment	Effect	Lower 95% CI	Upper 95% CI	Effective Sample	p
Preference Index Day 1	Control PI	0.23	0.15	0.29	1000	< 0.001
	Epinastine 20mM	-0.04	-0.15	0.07	1000	0.480
	Epinastine 100mM	-0.22	-0.34	-0.08	1000	0.002
	Flupentixol 250mM	-0.09	-0.22	0.04	1000	0.164
Preference Index Day 2	Control PI	0.33	0.26	0.39	1000	< 0.001
	Epinastine 20mM	-0.11	-0.20	-0.00	1562	0.032
	Epinastine 100mM	-0.42	-0.55	-0.31	1071	< 0.001
	Flupentixol 250mM	-0.24	-0.36	-0.13	1000	< 0.001

Table S4. glmm on the walking speed of ants in the retention tests after topical application of receptor blockers. The table contnains the results of two independent models for day 1 and day 2. Each model contained colony ID and ant ID as random factors. For each factor level we report the effect (mean of the posterior distribution, i.e. the effect size of treatment compared to the control, measured in mm/sec), the limits of its 95% confidence interval (CI), the effective sample size as a measure of model convergence, and the p value derived from ther posterior distribution.

Response Variable	Treatment	Effect	Lower 95% CI	Upper 95% CI	Effective Sample	p
Average Speed Day 1	Control PI	26.4	24.6	28.5	1000	< 0.001
	Epinastine 20mM	-4.4	-7.2	-1.3	902	0.002
	Epinastine 100mM	-6.0	-9.6	-2.5	409	0.001
	Flupentixol 250mM	-1.9	-5.3	1.5	784	0.294
Average Speed Day 2	Control PI	22.5	20.1	25.2	1000	< 0.001
	Epinastine 20mM	-0.6	-2.9	1.6	1000	0.624
	Epinastine 100mM	-2.9	-5.6	-0.4	895	0.030
	Flupentixol 250mM	-0.6	-3.4	2.2	1000	0.694

Supplementary Materials and Methods. (pdf) Code and output for the statistical analyses presented in the paper, as conducted in R.

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