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RESEARCH ARTICLE

Visual discrimination transfer and modulation by biogenic amines in honeybees

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ABSTRACT

For more than a century, visual learning and memory have been studied in the honeybee *Apis mellifera* using operant appetitive conditioning. Although honeybees show impressive visual learning capacities in this well-established protocol, operant training of free-flying animals cannot be combined with invasive protocols for studying the neurobiological basis of visual learning. In view of this, different attempts have been made to develop new classical conditioning protocols for studying visual learning in harnessed honeybees, though learning performance remains considerably poorer than that for free-flying animals. Here, we investigated the ability of honeybees to use visual information acquired during classical conditioning in a new operant context. We performed differential visual conditioning of the proboscis extension reflex (PER) followed by visual orientation tests in a Y-maze. Classical conditioning and Y-maze retention tests were performed using the same pair of perceptually isoluminant chromatic stimuli, to avoid the influence of phototaxis during free-flying orientation. Visual discrimination transfer was clearly observed, with pre-trained honeybees significantly orienting their flights towards the former positive conditioned stimulus (CS+), thus showing that visual memories acquired by honeybees are resistant to context changes between conditioning and the retention test. We combined this visual discrimination approach with selective pharmacological injections to evaluate the effect of dopamine and octopamine in appetitive visual learning. Both octopaminergic and dopaminergic antagonists impaired visual discrimination performance, suggesting that both these biogenic amines modulate appetitive visual learning in honeybees. Our study brings new insight into cognitive and neurobiological mechanisms underlying visual learning in honeybees.

KEY WORDS: Visual learning, Classical conditioning, Learning transfer, Dopamine, Octopamine, *Apis mellifera*

INTRODUCTION

The forager honeybee *Apis mellifera* is a well-established model for studies of visual learning and memory. At the behavioral level, much knowledge has been acquired about the ability of honeybees to learn colors, shapes, patterns and motion stimuli, among other visual attributes present in their rich visual environment (Giurfa and Menzel, 1997; Srinivasan, 2010; Zhang et al., 2012). Furthermore, bees possess impressive cognitive abilities in solving higher-level visual problems such as conceptual categorization and rule learning (Avarguès-Weber et al., 2012; Avarguès-Weber and Giurfa, 2013). Virtually all these studies about visual learning in honeybees have so far been performed using appetitive operant conditioning of free-flying animals, a very effective protocol that unfortunately cannot be combined with invasive techniques for studying the underlying neurobiological mechanisms (Avarguès-Weber et al., 2012).

An efficient classical conditioning protocol for training harnessed bees with olfactory cues has been combined with diverse pharmacological and physiological invasive methods for studying the neural basis of olfactory learning and memory (Giurfa and Sandoz, 2012; Matsumoto et al., 2012; Menzel, 2012). However, the so-called classical conditioning of the proboscis extension reflex (PER) (Kuwabara, 1957; Takeda, 1961), which proved to be very efficient in training harnessed bees to learn odor–reward associations (Giurfa and Sandoz, 2012), does not present the same robustness when visual cues are used as conditioned stimuli. In recent decades, attempts have been made to develop protocols of classical visual conditioning of the PER in harnessed bees, but learning performance has always remained poorer than that obtained with olfactory PER conditioning (Avarguès-Weber and Mota, 2016). Curiously, some authors reported that antennae amputation was essential for visual stimulus–reward association to occur in harnessed bees, whereas others showed that such a drastic procedure was not always necessary (Avarguès-Weber and Mota, 2016). Independent of being intact or deprived of their antennae, harnessed honeybees conditioned in a classical appetitive context never presented the same high levels of visual learning usually acquired in operant visual conditioning studies (Avarguès-Weber and Mota, 2016). Considering that the use of visual cues by bees predominates when they are foraging or navigating in flight, an operant free-flying context indeed seems much more natural for associative visual learning than the harnessed situation imposed in classical visual conditioning.

Learning and memory performance does not simply depend on the relevant stimuli used for conditioning – it is also strongly influenced by contextual cues that the animal experiences during these processes (Rescorla et al., 1985). In this framework, the capacity to transfer information learnt in one context to another context is part of the adaptive mechanisms that improve the use of established memories by animals (Balsam, 1985). A fascinating example of information transfer in honeybees is their ability to find food resources during foraging using information gained inside the hive from a returning forager (von Frisch, 1967). This and other complex behaviors displayed by honeybees probably involve transfer of information from classical to operant associative contexts, but the neural basis of these processes remains so far unknown. Previous behavioral studies have shown that honeybees can transfer olfactory information between classical and operant contexts (Bakchine-Huber et al., 1992; Gerber et al., 1996; Sandoz et al., 2000; Chaffiol et al., 2005; Carcaud et al., 2009) but no study

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List of abbreviations

CS	conditioned stimulus
CS–	non-reinforced conditioned stimulus
CS+	reinforced conditioned stimulus
DI	discrimination index
GLM	generalized linear model
PER	proboscis extension reflex
US	unconditioned stimulus
VUMmx1	ventral unpaired median cell of maxillary neuromere 1

has so far investigated this type of transfer for visual information. In the present work, we aimed to fill this gap by developing a visual conditioning protocol in which harnessed bees are first trained to discriminate visual stimuli in a classical context and then confronted with a retention test presenting the same stimuli in an operant context. We also took advantage of bees initially being harnessed to combine this conditioning protocol with pharmacological injections for studying the possible modulation of appetitive visual learning by dopamine and octopamine.

Dopamine and octopamine are biogenic amine neurotransmitters that have been largely implicated in the modulation of reinforcement neural pathways (Schultz, 2002; Wise, 2004). The role of these molecules in associative learning, however, does not seem to be totally equivalent in vertebrates and invertebrates (Perry and Barron, 2013). Dopamine, for instance, has been identified as a major modulator of reward pathways in vertebrates (Schultz, 2002; Wise, 2004), whereas in insects this molecule seems to play a major role in modulation of punishment pathways (Tedjakumala and Giurfa, 2013). The main biogenic amine related to reward pathways in insects is octopamine, a neurotransmitter that presents strong homology with adrenergic vertebrate neurotransmitters (Roeder, 2005; Perry and Barron, 2013). Typically, in different insect models, octopamine strongly modulates appetitive olfactory associations, whereas dopamine appears to be mainly involved in aversive olfactory associations (flies: Schwaerzel et al., 2003; crickets: Unoki et al., 2005, 2006; bees: Vergoz et al., 2007).

A single broad-field octopaminergic neuron in the honeybee brain, the ventral unpaired median cell of maxillary neuromere 1 (VUMmx1) (Hammer, 1993), has been identified as the main neural substrate of appetitive olfactory learning during PER conditioning. Intracellular stimulation of VUMmx1, as well as injection of octopamine into brain regions arborized by this neuron, were able to replace the function of sucrose reward when paired with odor presentations, inducing PER conditioned responses (Hammer, 1993; Hammer and Menzel, 1998). In contrast, injection of octopaminergic antagonists (Vergoz et al., 2007) and inhibition of genes encoding octopaminergic receptors in the bee brain (Farooqui et al., 2003) disrupted appetitive olfactory learning by bees. Dopamine has been implicated in the modulation of aversive olfactory learning but not appetitive olfactory learning in bees, as suggested by pharmacological studies showing that injection of dopaminergic antagonists into the bee brain inhibits the formation of aversive but not appetitive olfactory associations (Vergoz et al., 2007; Wright et al., 2010). Curiously, dopamine was recently found to impair long-term appetitive olfactory memory formation in bees, although no effect was observed during acquisition or on short-term memory (Klappenbach et al., 2013).

Compared with the accumulated knowledge about the role of dopamine and octopamine in olfactory learning (Scheiner et al., 2006; Menzel, 2012; Tedjakumala and Giurfa, 2013), almost nothing is known about how these monoamines modulate visual learning in honeybees. In an aversive framework in which walking honeybees were subjected to spatial-avoidance conditioning, the formation of associations between a colored space and an electric shock punishment appeared to be modulated in different ways by dopamine and octopamine (Agarwal et al., 2011). Whereas dopamine enhanced aversive spatial learning, its antagonist inhibited it. Surprisingly, octopamine inhibited aversive learning in this operant task but its antagonist had no effect on learning performance (Agarwal et al., 2011). While this single work analyzed the effect of biogenic amines in an operant aversive visuo-spatial task, no studies have been performed until now to analyze the role of dopamine and octopamine in appetitive visual learning by honeybees. Here, we accomplished this goal by combining microinjections of dopaminergic and octopaminergic antagonists in the bee brain with a new protocol of classical appetitive visual conditioning followed by an operant retention test. Our work provides valuable new information for the understanding of visual discrimination transfer between distinct associative contexts and the role of biogenic amines in appetitive visual learning by honeybees.

MATERIALS AND METHODS

Animals

Honeybee *Apis mellifera* Linnaeus 1758 foragers were collected from a feeder containing 30% sugar solution, 50 m from outdoor hives kept in the Ecological Station of the Federal University of Minas Gerais, Brazil. The feeder was a transparent container with capacity of 300 ml. The bottom of the container was partially closed by a platform of gray plastic, on which bees could land and collect sugar solution. No scent was added to this feeder. Forager workers were placed in small glass vials, cooled on ice until they ceased movement and then harnessed in plastic tubes using thin pieces of soft masking tape. The wings were protected by a piece of filter paper (Fig. 1A,B).

Differential visual conditioning of the PER

To test the effect of the antennae in our classical conditioning paradigm, we cut both antennae of a group of bees with fine scissors at the base of the scapus. Another group was kept with intact antennae. Each bee was fed 1 µl of 30% sugar solution after fixation and then kept for 1 h in a dark chamber with high humidity. Differential visual conditioning of the PER was then performed in a dark room. During conditioning, each harnessed bee was placed in a platform inside one arm of a black Y-maze (Fig. 1C; described in detail in 'Orientation tests in the Y-maze', below). It is important to highlight that inside this arm (Fig. 1A,B), the harnessed bee could not see the overall shape or the other arm of the Y-maze. The plastic tube holding the bee was tilted to 45 deg (Fig. 1A,B) such that the bee's right eye was situated laterally to the visual stimuli presented at a distance of 10 cm. Visual stimuli consisted of an illuminated 20×20 cm screen covered with a chromatic transmission filter (LF124S Dark Green: peak at 535 nm or LF119S Dark Blue: peak at 455 nm; LEE Filters, Panavision, Woodland Hills, CA, USA) and tracing paper for light dispersion. A white-LED light source (E27-5W Cool White; Epistar, Hsinchu, Taiwan) connected to a linear potentiometer provided illumination with controlled intensity behind the colored screen. These large-field chromatic stimuli subtended a visual angle of 90 deg to the right eye of the bee. Taking into account the spectral sensitivities of the honeybee photoreceptors (Peitsch et al., 1992), the green stimulus excited 0%, 15% and 85% of the short- (S), medium- (M) and long-range (L) wavelength photoreceptors, respectively. For the blue stimulus,

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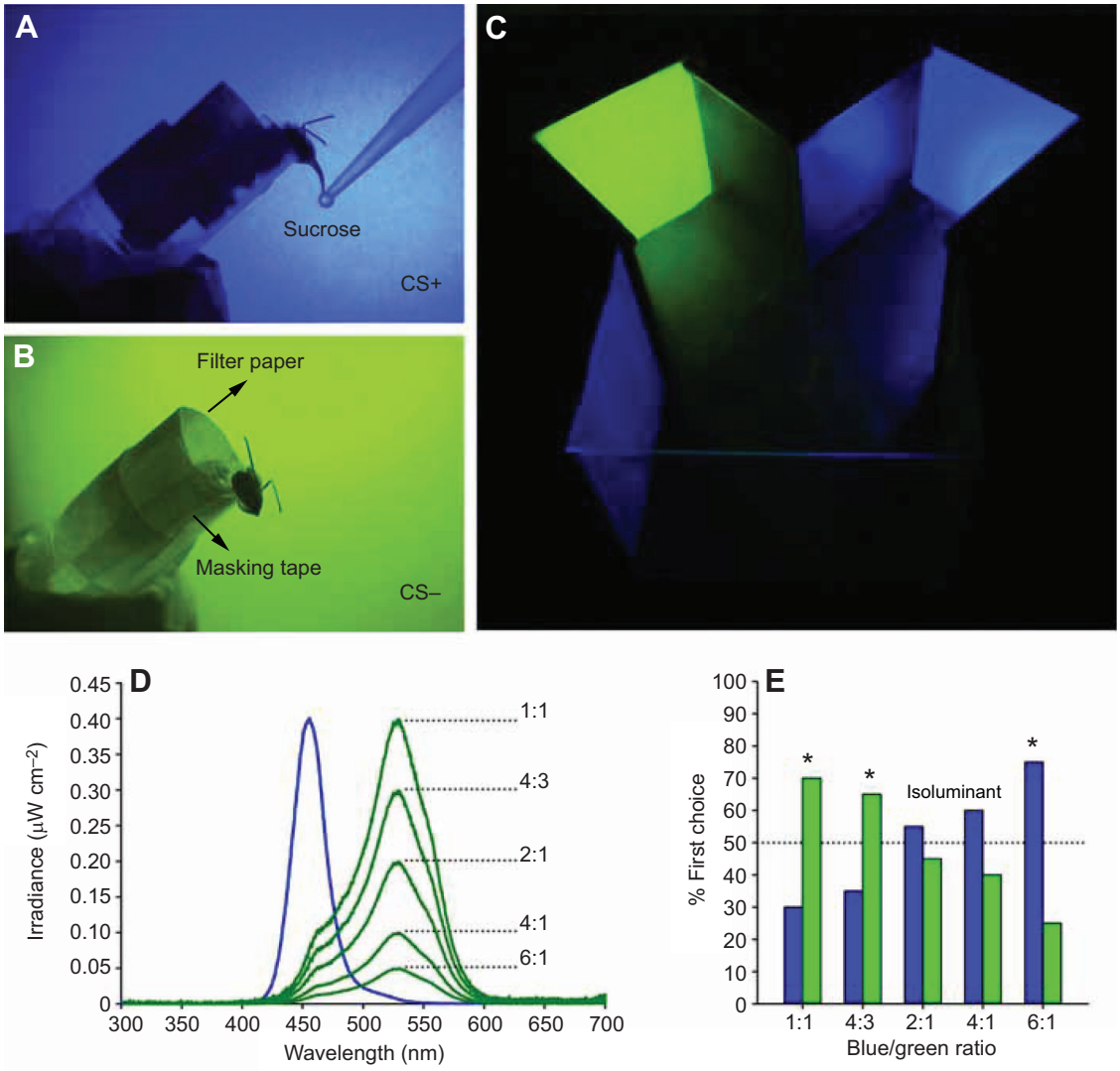

Fig. 1. Conditioning procedures and stimulus calibration for studies on chromatic discrimination by honeybees. (A,B) Differential visual conditioning of the proboscis extension reflex (PER) in harnessed honeybees using a reinforced blue light as the positive conditioned stimulus (CS+; A) and a non-reinforced green light as the non-reinforced conditioned stimulus (CS−; B). During reinforced trials, CS+ presentation was paired with 1 µl of 30% sucrose solution (unconditioned stimulus, US), delivered to the bee by means of a micropipette (A). For these experiments, bees were harnessed in plastic tubes using pieces of soft masking tape. The wings were protected by a piece of filter paper (B). The plastic tube holding the bee was tilted to 45 deg and visual stimulation was presented laterally. (C) Y-maze used for performing orientation tests in free-flying honeybees. The maze was made of opaque black acrylic and its entrance (20×28×20 cm, L×H×W) led to a decision chamber in which the bee could choose between two arms (20×20×20 cm, L×H×W) presenting distinct chromatic stimuli. Classical conditioning of the PER (A,B) prior to the orientation test was also performed inside one of the arms of this Y-maze. (D) Spectral curves of blue and green stimuli presented to naive bees during orientation tests aimed at determining the point of isoluminance. A pair of blue and green stimuli was presented to the bees in the Y-maze at five different proportions of irradiance (1:1, 4:3, 2:1, 4:1, 6:1) from a maximum value of 0.4 µW cm^{-2}. (E) Percentage of first choices for 20 bees independently tested for their orientation in the Y-maze by presenting a blue versus a green light stimulus in different proportions of irradiance. The percentage of bees choosing the blue stimulus was not significantly different from that choosing the green stimulus in experimental groups tested for 2:1 or 4:1 blue–green proportions. Thus, in these groups, blue and green seem to be perceptually isoluminant for the bees in the context of orientation triggered by positive phototaxis. Asterisks indicate distributions that are significantly different from random (binomial exact test).

these values were 2%, 68% and 30%, respectively. The intensity of the blue and the green stimulus was adjusted to a point of isoluminance by using the phototactic response of free-flying bees, as described in 'Visual stimulus calibration and isoluminance', below (Fig. 1D,E). Bees were trained to discriminate between one color (reinforced conditioned stimulus, CS+; Fig. 1A) rewarded with sugar solution (unconditioned stimulus, US) and another unrewarded color (non-reinforced conditioned stimulus, CS−; Fig. 1B). We trained in parallel one subgroup of bees using blue as the CS+ and another subgroup using green as the CS+. The number of bees per subgroup was always equal. Ten trials of CS+ and 10 trials of CS− were presented to each bee in a pseudorandom sequence with an intertrial interval of 10 min. Both visual stimuli (CS+ and CS−) were presented on the same lateral screen, by alternating the chromatic filter covering it during trials. At the beginning of each rewarded trial, the bee was placed in the conditioning setup for 30 s to allow familiarization with the experimental context. Thereafter, the colored screen was illuminated for 7 s. Four seconds after the onset of the CS+, 1 µl of 30% sugar solution was delivered to the bee by means of a micropipette for 3 s (Fig. 1A). Therefore, the interstimulus interval was 4 s and the overlap between the conditioned stimulus (CS) and

US was 3 s. The bee was removed from the setup 23 s after the reward was given, thus completing a total of 60 s per trial. Unrewarded trials followed the same time sequence, but visual stimulation was not paired with a reward (Fig. 1B). The occurrence of proboscis extension was recorded within the first 4 s of CS presentation, as well as during the US presentation. Animals that did not show the PER more than 3 times during the US presentation (<5%) were excluded from our analysis, as they may present impairment of the muscular reflex and/or sucrose responsiveness. At the end of the differential conditioning, each bee with intact antennae was carefully removed from the tube and placed inside a small glass vial. This procedure was performed inside a dark room illuminated by a low-intensity red-light source (peak at 660 nm). Glass tubes with bees were then kept for 1 h in the dark room.

Orientation tests in the Y-maze

One hour after differential visual conditioning of the PER, each bee with intact antennae was individually released in the entrance of a black Y-maze for an operant orientation test towards the same pair of visual stimuli formerly used as the CS+ and CS−. Animals that died during this 1 h period (<10%) were excluded from analysis to allow comparison of performance between classical conditioning and the operant orientation test in equivalent subjects. Bees deprived of their antennae could not be tested in the Y-maze because antenna deprivation impairs flight. The Y-maze (Fig. 1C) was made of opaque black acrylic and its entrance (20×28×20 cm, L×H×W) led to a decision chamber in which the bee could choose between the two arms (20×20×20 cm, L×H×W). Visual stimuli were presented over the entire surface of the back walls of the maze arms (20×20 cm) at a distance of 20 cm from the decision chamber (Fig. 1C). They thus subtended a visual angle of 53 deg to the center of the decision chamber, in both the vertical and the horizontal direction. Therefore, these visual stimuli were large enough to recruit the chromatic pathways of the honeybee visual system (Giurfa et al., 1996). The entire maze was covered by a removable lid of transparent acrylic. Each bee was released just once in the Y-maze and its first choice was then recorded. After making a choice, the free-flying bee was captured at the chosen arm by means of an insect aspirator (pooter). Although each bee was tested only once in the Y-maze, we constantly alternated the position (right or left) of the blue and green filters for different tests with different bees.

Visual stimulus calibration and isoluminance

In order to measure the spectral properties and adjust the irradiance of the monochromatic stimuli, we used a spectrophotometer (USB2000+UV-VIS-ES, Ocean Optics, Dunedin, FL, USA) radiometrically calibrated using a deuterium/tungsten light source (DH-2000-BAL, 220–1050 nm, Ocean Optics). Absolute irradiance was measured using an optical fiber (QP600-2-UV-VIS, Ocean Optics) coupled to a cosine corrector with Spectralon diffusing material (CC-3-UV-S, Ocean Optics) that was fixed 10 cm from the center of the colored visual screen. The software SpectraSuite (Ocean Optics) was used for acquisition and analysis of spectral curves.

The orientation of honeybees in a dark environment in the presence of light stimuli is highly influenced by positive phototaxis, which in turn is strongly triggered by light intensity (Labhart, 1974; Menzel and Greggers, 1985; Erber et al., 2006). As this behavior clearly interferes with the choices of free-flying honeybees in the Y-maze presenting blue versus green light stimuli (Fig. 1C), we performed an experiment to adjust the irradiance of these stimuli to a point of isoluminance using the phototactic responses of naive bees. Bees tend to perceive green light as more intense than blue light, because they have many more photoreceptors for green than blue in their compound eyes (Wakakuwa et al., 2005). Moreover, the overall perceptual intensity (q) of our blue stimulus (q=16), calculated as the sum of all photoreceptor excitations with respect to the background (Peitsch et al., 1992; Giurfa et al., 1996), was 62% of the overall intensity of the green stimulus (q=26). We thus set the intensity of the blue stimulus to an irradiance value of 0.4 µW cm^{-2} and varied the irradiance of the green stimulus from this value to lower ones using a linear potentiometer (Fig. 1D). Five groups of 20 bees were independently tested for their orientation in the Y-maze presenting a blue versus a green light stimulus in different proportions of irradiance (1:1, 4:3, 2:1, 4:1, 6:1). Each bee was kept for 1 h in the dark room inside a small glass vial and then released a single time at the entrance of the Y-maze. The first choice of each individual bee for the blue or the green light was recorded (Fig. 1E). We considered the point of isoluminance had been reached when the proportion of bees orienting towards the blue stimulus was not significantly different from the proportion flying towards the green stimulus (Fig. 1E). After identifying the range of isoluminance, we used for all experiments presented in Figs 2–9 blue and green stimuli in an isoluminant irradiance proportion of 2:1. More specifically, the blue stimulus had an irradiance of 0.4 µW cm^{-2} at 455 nm and the green stimulus had an irradiance of 0.2 µW cm^{-2} at 535 nm (Fig. 1D,E).

Pharmacological injections

For testing the effect of dopaminergic and octopaminergic receptor antagonists in visual learning by bees, harnessed animals received pharmacological injections 30 min prior to classical PER conditioning. Previous experiments in honeybees showed that pharmacological injections of the aminergic antagonists used in our study were effective approximately 30 min after drug application (Vergoz et al., 2007). We used flupentixol as a dopaminergic antagonist and epinastine or mianserine as octopaminergic antagonists (all chemicals from Sigma-Aldrich, São Paulo, Brazil). Flupentixol has been shown to have a high affinity for and significant antagonistic effect on *A. mellifera* dopaminergic receptors 1 and 2 (AmDOP1 and AmDOP2) (Blenau et al., 1998; Mustard et al., 2003). Both mianserine and epinastine proved to be strong antagonists of the *A. mellifera* octopaminergic receptor 1 (AmOA1) (Roeder et al., 1998; Degen et al., 2000) but we decided to test the effect of both these antagonists, because in high doses they may also present some affinity with other aminergic receptors, such as serotoninergic or histaminergic receptors (Roeder et al., 1998; Degen et al., 2000; Beggs et al., 2011).

Chemicals were dissolved in honeybee Ringer (NaCl 130 mmol l^{-1}, KCl 6 mmol l^{-1}, $MgCl_2$ 4 mmol l^{-1}, $CaCl_2$ 5 mmol l^{-1}, Hepes 10 mmol l^{-1}, glucose 25 mmol l^{-1}, sucrose 160 mmol l^{-1}) in the following concentrations: flupentixol 2×10^{-7}/10^{-5}/10^{-3} mol l^{-1}, epinastine 4×10^{-7}/10^{-5}/10^{-3} mol l^{-1}, mianserine 3.3×10^{-7}/10^{-5}/10^{-3} mol l^{-1}. Antagonist solution or Ringer solution alone (control) was injected (200 nl) into the brain through the median ocellar tract using a nanoinjector (Nanoject II, Drummond Scientific, Broomall, PA, USA). During injection, we stabilized the bee's head by fixing the mandibles in the plastic tube using a piece of nylon wire. After injection, bees were placed in the dark room for 30 min and then visual conditioning of the PER followed by an orientation test in the Y-maze was performed exactly as described above (see 'Differential visual conditioning of the PER' and 'Orientation tests in the Y-maze').

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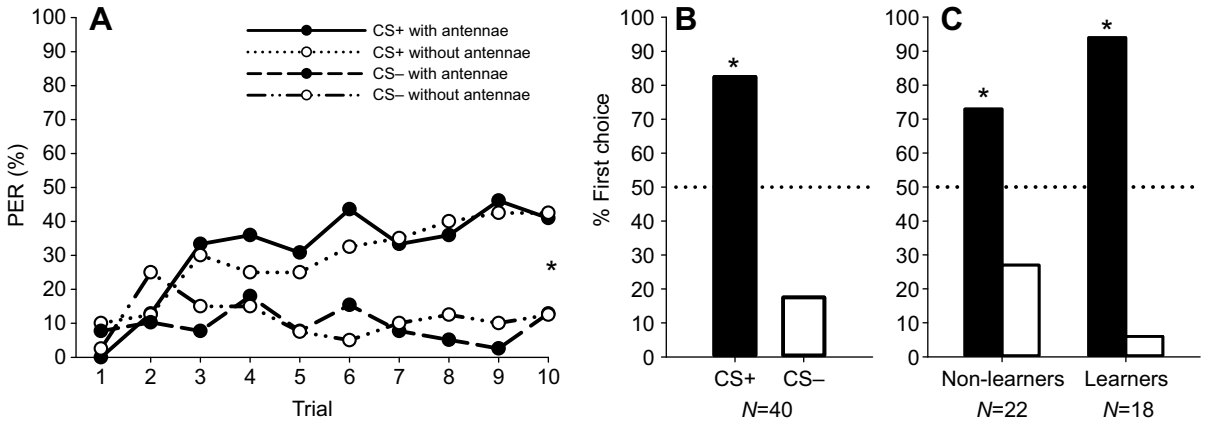

Fig. 2. Classical conditioning of the PER followed by an operant retention test in the Y-maze. (A) Percentage of conditioned PER responses in 10 trials of CS+ and CS− presentation in harnessed bees with or without antennae (*N*=40 bees per group). The asterisk indicates that bees both with and without antennae were able to discriminate between the reinforced (CS+) and the non-reinforced (CS−) chromatic stimulus (GLM ANOVA for repeated measures). *N*=2 bees without antennae and *N*=1 bee with antennae were excluded from analysis because of PER impairment during conditioning. (B) Percentage of bees with intact antennae flying towards the former CS+ or CS− during an orientation test performed 1 h after differential classical conditioning (*N*=40 bees). The percentage of first choices in the Y-maze was significantly higher for the former CS+ than for the former CS−, revealing learning transfer from a classical to a new operant context. (C) Performance in operant retention tests of bees classified as 'learners' or 'non-learners', according to their discrimination indexes (DIs) during classical conditioning. Asterisks in B and C indicate distributions that are significantly different from random (binomial exact test).

As an initial control experiment to test whether injections could affect the ability of bees to fly or their visual orientation towards the pair of light stimuli, we also performed orientation tests in naive bees 30 min after injection for all the pharmacological treatments (Fig. 3A). Orientation tests in control groups of naive bees, as well as in groups of bees previously subjected to visual conditioning of the PER, were performed as described above ('Orientation tests in the Y-maze') using blue and green stimuli in

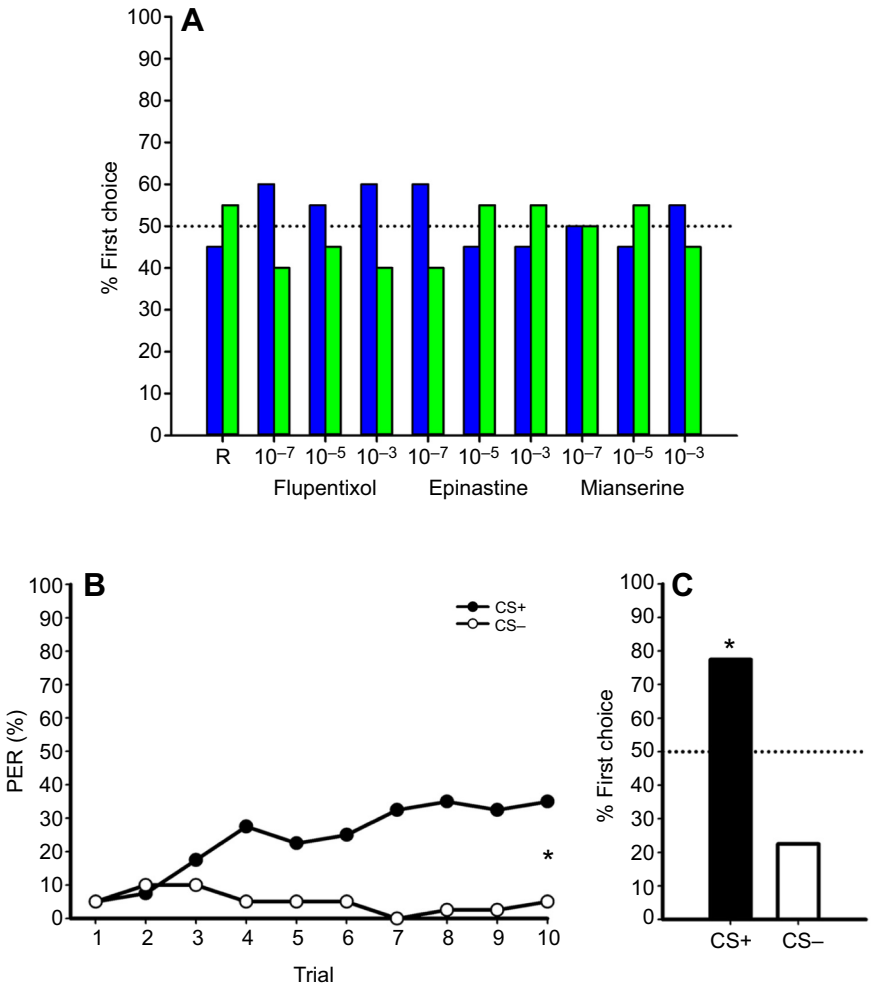

Fig. 3. Control experiments testing the effect of aminergic antagonists on visual discrimination. (A) Orientation tests towards blue and green isoluminant stimuli in groups of 20 naive bees injected with Ringer solution (R) or different concentrations of the aminergic antagonists flupentixol, epinastine and mianserine. This control experiment was performed to evaluate whether the pharmacological injections could interfere with the flight behavior or the choice of chromatic stimuli in the Y-maze. (B) Learning curves of bees injected with Ringer solution during visual differential conditioning of the PER (*N*=40 bees). (C) Percentage of Ringer solution-injected bees choosing the former CS+ or CS− during an orientation test performed 1 h after classical differential conditioning (*N*=40 bees). Asterisks indicate significant discrimination during conditioning (GLM ANOVA for repeated measures) or the orientation test in the Y-maze (binomial exact test). *N*=1 bee was excluded from analysis because of PER impairment during conditioning and *N*=3 bees were excluded because they died between conditioning and the test.

an isoluminant irradiance proportion of 2:1 (for details, see 'Visual stimulus calibration and isoluminance', above). We also conducted additional experiments to evaluate whether the effects of pharmacological treatments in operant orientation tests could be related to interference with memory retention. Groups of harnessed bees injected with Ringer solution, flupentixol or epinastine were subjected to PER retention tests 1 h after visual conditioning of the PER (see 'Differential visual conditioning of the PER', above). Retention tests consisted of an unrewarded presentation of the CS+ and the CS− with an intertrial interval of 10 min. The order of presentation (first or second) of the CS+ and the CS− was randomized between subjects in all experimental groups.

Statistical analysis

Two-way analysis of variance in a generalized linear model (GLM ANOVA) for repeated measures was used to analyze discrimination performance in visual conditioning of the PER. A binomial exact test was used to compare the distribution of animals in each arm of the Y-maze during orientation tests. To compare discrimination success among different experimental groups, we used a discrimination index (DI). For each bee, we computed the difference between its responses to the last five trials of CS+ presentations and the last five trials of CS− presentations [DI=(∑CS+)−(∑CS−)]. GLM ANOVA followed by Tukey's multiple comparisons test was used to compare DI values between experimental groups subjected to different treatments. The McNemar test was used to compare responses to CS+ and CS− in PER retention tests. The alpha level was set to 0.05 (two tailed) for all analyses.

RESULTS

Phototactic orientation in the Y-maze and isoluminance of chromatic stimuli

Fig. 1E shows the percentage of first choices of free-flying naive bees in a Y-maze for blue and green light in different irradiance proportions (Fig. 1D). Blue–green irradiance proportions of 1:1 or 4:3 led to a significantly higher percentage of bees choosing the green stimulus (Fig. 1E; binomial exact test; P=0.03 and P=0.04, respectively). Conversely, a significantly higher percentage of bees choose the blue stimulus when the blue–green irradiance proportion was 6:1 (Fig. 1E; binomial exact test; P=0.01). No significant difference was found between the percentage of bees choosing blue or green when their irradiance proportions were 2:1 and 4:1 (Fig. 1E; binomial exact test; P=0.16 and P=0.12, respectively). In these experimental groups, therefore, blue and green stimuli appear to be perceptually isoluminant for bees. We thus decided to use the blue–green proportion of 2:1 for all the behavioral experiments performed in the present work (Figs 2–9).

Differential visual conditioning of the PER

There were no significant differences between the conditioned PER responses of the group of bees trained to the blue stimulus and the group trained to the green stimulus as CS+, for both intact and antennae-deprived bees [group×stimulus×trial GLM ANOVA for repeated measures; group effect; with antennae: $F_{1,37}$=2.81, not significant (NS); without antennae: $F_{1,38}$=0.43, NS]. Therefore, data from these subgroups were pooled in Fig. 2A. Bees both with and without antennae were able to discriminate between the reinforced (CS+) and the non-reinforced (CS−) chromatic stimulus in our experimental approach (Fig. 2A; stimulus×trial GLM ANOVA for repeated measures; interaction effect; with antennae: $F_{9,333}$=3.43, P<0.001; without antennae: $F_{9,342}$=3.28, P<0.001).

Learning transfer from a classical to an operant context

The percentage of first choices of intact bees in the Y-maze was significantly higher for the former CS+ than for the former CS− (Fig. 2B, binomial exact test; P=0.0002), indicating learning transfer from the classical to the operant context. In order to evaluate in more detail the relationship between individual performance in classical conditioning and in subsequent operant retention tests, we calculated a DI for each bee. This index represented the difference between responses to the last five trials of CS+ and the last five trials of CS− presentations [DI=(∑CS+)−(∑CS−)]. DI values could vary from −5 to 5. Whenever an individual bee responded at least once more to the CS+ than to the CS− in the last five trials (DI≥1), we considered this bee as a 'learner' in differential visual conditioning of the PER. Bees presenting DI values below 1 were considered as 'non-learners'. We then analyzed the proportion of learners and non-learners presenting correct responses in the Y-maze retention tests (Fig. 2C). In the learners group, 94% of the bees chose the correct stimulus in the Y-maze, clearly indicating learning transfer from the classical context (Fig. 2C; binomial exact test; P=0.0006). Furthermore, 73% of the bees from the non-learners group chose the correct stimulus in the Y-maze, meaning that they also presented significantly more choices for the stimulus used previously as the CS+ (Fig. 2C; binomial exact test; P=0.01). This result indicates that even some of the harnessed bees not showing learning during visual classical conditioning were actually able to learn color–reward associations and transfer them to a new operant context.

Effect of dopamine and octopamine on associative visual learning

Control experiments

For each concentration of each pharmacological drug used in this study, as well as for Ringer solution-injected bees, we first performed a control experiment to evaluate whether the injection could interfere with the flight behavior and/or the choice of visual stimuli in the Y-maze (Fig. 3A). None of the treatments changed the behavior of injected naive bees, which were able to fly towards one of the Y-maze arms and presented random choices for the isoluminant stimuli used in our study (Fig. 3A; binomial exact test; Ringer solution: P=0.16; flupentixol 10^{-7} mol l^{-1}: P=0.18, 10^{-5} mol l^{-1}: P=0.16, 10^{-3} mol l^{-1}: P=0.16; epinastine 10^{-7} mol l^{-1}: P=0.12, 10^{-5} mol l^{-1}: P=0.16, 10^{-3} mol l^{-1}: P=0.16; mianserine 10^{-7} mol l^{-1}: P=0.12, 10^{-5} mol l^{-1}: P=0.16, 10^{-3} mol l^{-1}: P=0.12).

Control bees injected with Ringer solution were able to discriminate between the CS+ and CS−, as shown by the significantly higher percentage of conditioned responses (PER) to the rewarded stimulus during trials (Fig. 3B; stimulus×trial GLM ANOVA for repeated measures; interaction effect; $F_{9,342}$=4.46, P<0.001). The learning performance of Ringer solution-injected bees trained to green light as the CS+ did not differ from that of bees trained to blue light as the CS+ (group×stimulus×trial GLM ANOVA for repeated measures; group effect; $F_{1,38}$=0.23, NS), thus we pooled data from these two groups in Fig. 3B. Furthermore, Ringer solution-injected bees chose the stimulus used as the CS+ significantly more often than that used as the CS− during subsequent orientation tests in the Y-maze (Fig. 3C; bimodal exact test; P=0.0002).

Classical conditioning followed by operant transfer tests

We found no difference between the performance of bees trained to green or blue light as the CS+ for any of the pharmacological

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treatments using aminergic antagonists (group×stimulus×trial GLM ANOVA for repeated measures; group effect; flupentixol 10^{-7} mol l^{-1}: $F_{1,38}$=0.18 NS, 10^{-5} mol l^{-1}: $F_{1,38}$=0.47 NS, 10^{-3} mol l^{-1}: $F_{1,38}$=0.45 NS; epinastine 10^{-7} mol l^{-1}: $F_{1,38}$=0.44 NS; 10^{-5} mol l^{-1}: $F_{1,33}$=0.47 NS; 10^{-3} mol l^{-1}: $F_{1,29}$=1.63 NS; mianserine 10^{-7} mol l^{-1}: $F_{1,38}$=0.18 NS; mianserine 10^{-5} mol l^{-1}: $F_{1,38}$=1.41 NS; mianserine 10^{-3} mol l^{-1}: $F_{1,38}$=0.61 NS). Therefore, we pooled data from these two independent groups for each treatment, as presented in Figs 4–6.

Fig. 4 shows the learning curves of bees during differential visual conditioning of the PER, and their choices in subsequent Y-maze orientation tests, when three different concentrations of flupentixol were applied. Bees injected with the lowest concentration of this dopaminergic antagonist (2×10^{-7} mol l^{-1}) were able to significantly discriminate between CS+ and CS− during visual conditioning of the PER (Fig. 4A; stimulus×trial GLM ANOVA for repeated measures; interaction effect; $F_{9,342}$=3.72, P<0.001), and transfer this discrimination to the context of the Y-maze orientation test (Fig. 4A; binomial exact test; P=0.005). The injection of a medium dose of flupentixol (2×10^{-5} mol l^{-1}) into the bee brain reduced, but did not completely impair, the capacity of bees to discriminate between CS+ and CS− during differential conditioning of the PER (Fig. 4B; stimulus×trial GLM ANOVA for repeated measures; stimulus effect; $F_{1,38}$=6.90, P<0.05; interaction effect; $F_{9,342}$=1.35, NS). However, bees of this experimental group presented random choices for the pair of visual stimuli during the orientation test in the Y-maze (Fig. 4B; binomial test, P=0.06). Finally, the injection of a higher dose of flupentixol (2×10^{-3} mol l^{-1}) into the bee brain impaired visual discrimination in both differential visual conditioning of the PER (Fig. 4C; stimulus×trial GLM ANOVA for repeated measures; stimulus effect; $F_{1,38}$=3.38, NS; interaction effect; $F_{9,342}$=1.44, NS) and the Y-maze orientation test (binomial exact test; P=0.12), suggesting a dose-dependent effect of dopaminergic blockage on visual associative learning by honeybees.

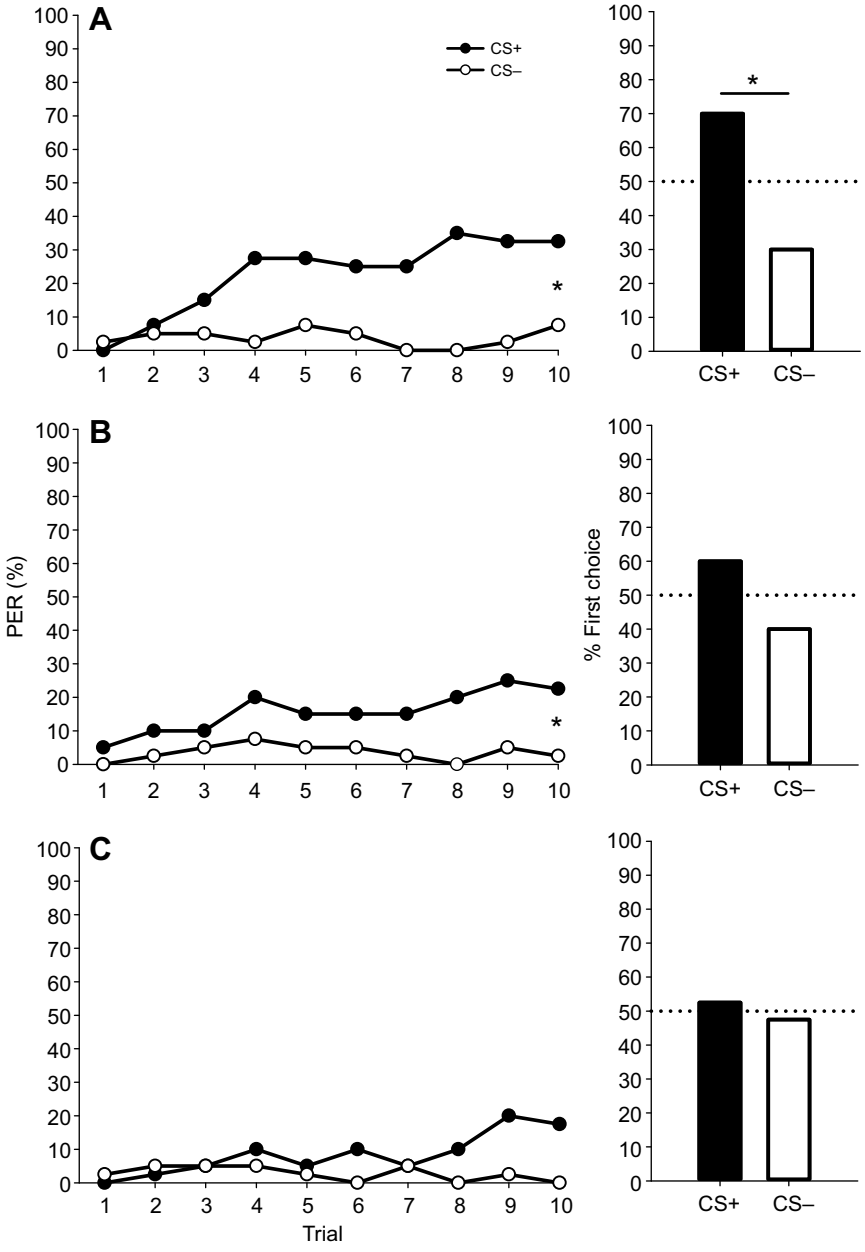

Fig. 4. Learning curves during classical visual conditioning and orientation in an operant test of bees injected with flupentixol solution. The percentage of conditioned PER during conditioning trials (left) and first choices during Y-maze orientation tests (right) is shown for three increasing concentrations of flupentixol: 2×10^{-7} mol l^{-1} (A), 2×10^{-5} mol l^{-1} (B) and 2×10^{-3} mol l^{-1} (C). Asterisks indicate significant discrimination during conditioning (GLM ANOVA for repeated measures) or operant tests (binomial exact test). N=40 bees per concentration. N=2 and 1 bees were excluded from analysis because of PER impairment in B and C, respectively; N=2, 2 and 3 bees were excluded because they died between conditioning and test in A, B and C, respectively.

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Fig. 5 shows the visual learning performance of bees injected with the octopaminergic antagonist epinastine at three different concentrations. Bees injected with the lowest dose of epinastine (4×10^{-7} mol l^{-1}) showed visual discrimination during differential PER conditioning (Fig. 5A; stimulus×trial GLM ANOVA for repeated measures; stimulus effect; $F_{1,38}=0.91$, $P<0.05$; interaction effect; $F_{9,342}=2.05$, $P<0.05$), but presented random choices in the Y-maze test (Fig. 5A; binomial exact test; $P=0.06$). For both the medium and higher epinastine doses (4×10^{-5} mol l^{-1} and 4×10^{-3} mol l^{-1}), injection into the brain completely impaired visual discrimination during PER conditioning (stimulus×trial GLM ANOVA for repeated measures; Fig. 5B: 10^{-5} mol l^{-1}: stimulus effect; $F_{1,33}=1.76$, NS; interaction effect: $F_{9,297}=0.56$, NS; Fig. 5C: 10^{-3} mol l^{-1}: stimulus effect; $F_{1,29}=0.03$, NS; interaction effect; $F_{9,261}=0.51$, NS) and the subsequent orientation test in the Y-maze (binomial exact test; Fig. 5B: 10^{-5} mol l^{-1}: $P=0.09$; Fig. 5C: 10^{-3} mol l^{-1}: $P=0.14$). We also tested the effect of the octopaminergic antagonist mianserine on visual discrimination performance (Fig. 6). In the three different concentrations injected (3.3×10^{-7} mol l^{-1}, 3.3×10^{-5} mol l^{-1} and 3.3×10^{-3} mol l^{-1}), mianserine completely impaired visual discrimination during differential PER conditioning (Fig. 6A–C; stimulus×trial GLM ANOVA for repeated measurements; for all three concentrations: stimulus effect; $F_{1,38}<0.04$, NS; interaction effect; $F_{9,342}<0.51$, NS) and following orientation tests in the Y-maze (Fig. 6A–C; binomial exact test; 10^{-7} mol l^{-1}: $P=0.06$; 10^{-5} mol l^{-1}: $P=0.12$; 10^{-3} mol l^{-1}: $P=0.08$). These results reveal a strong effect of octopaminergic blockage on appetitive visual learning by honeybees.

In order to compare the visual learning performance between bees of all the experimental groups, we calculated their DI (for details, see Materials and methods, 'Statistical Analysis'). Significant differences were found between the DI values obtained in different pharmacological treatments (Fig. 7; treatment×discrimination index GLM ANOVA; $F_{10,415}=5.19$, $P<0.0001$). A clear dose-dependent effect was observed when comparing the DI values obtained for three different concentrations of flupentixol (Tukey test; $P<0.05$ for all

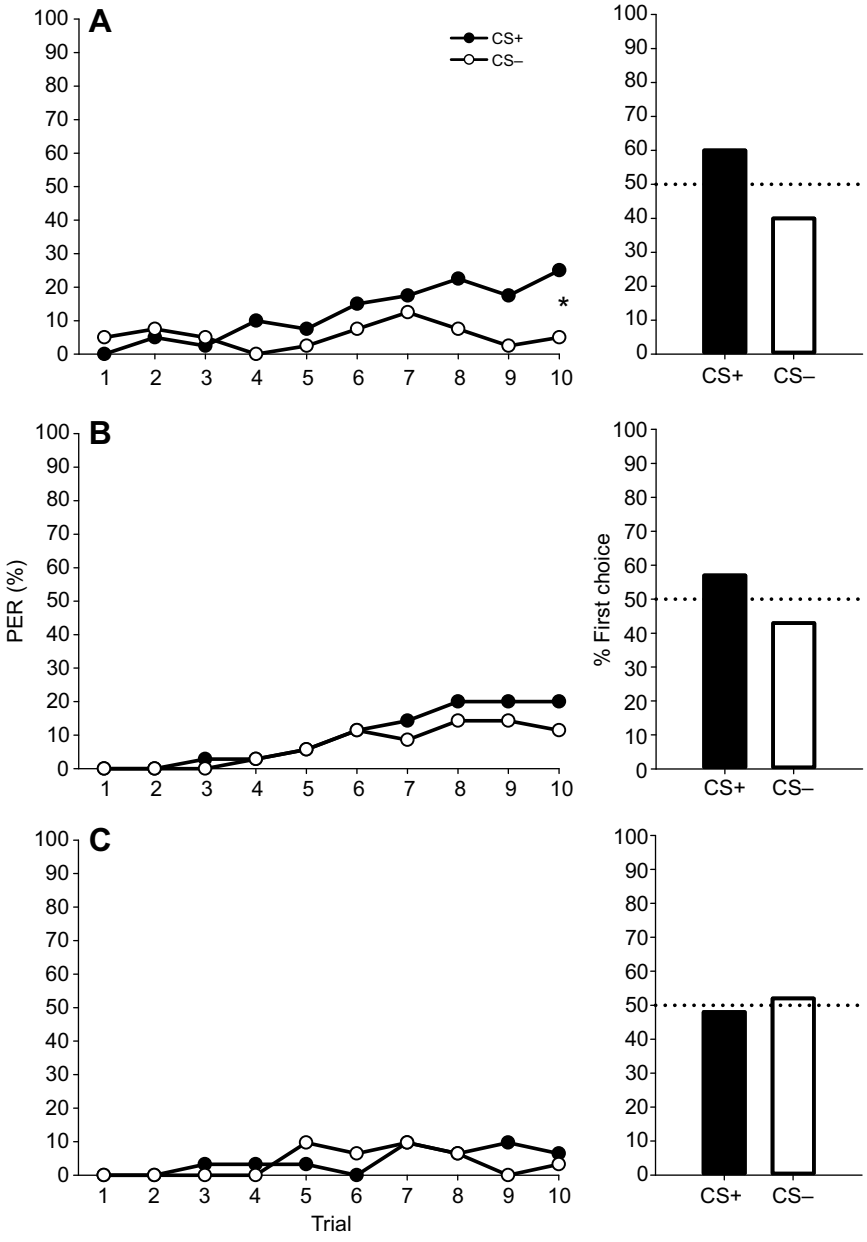

Fig. 5. Performance during visual differential conditioning and further operant test of bees injected with epinastine solution. The percentage of conditioned PER during conditioning trials (left) and first choices during Y-maze orientation tests (right) is shown for three increasing concentrations of epinastine: 4×10^{-7} mol l^{-1} (A; $N=40$ bees), 4×10^{-5} mol l^{-1} (B; $N=34$ bees) and 4×10^{-3} mol l^{-1} (C; $N=36$ bees). The asterisk indicates significant discrimination during conditioning (GLM ANOVA for repeated measures). $N=1$ bee was excluded from analysis because of PER impairment in A and C; $N=2$, 1 and 2 bees were excluded because they died between conditioning and test in A, B and C, respectively.

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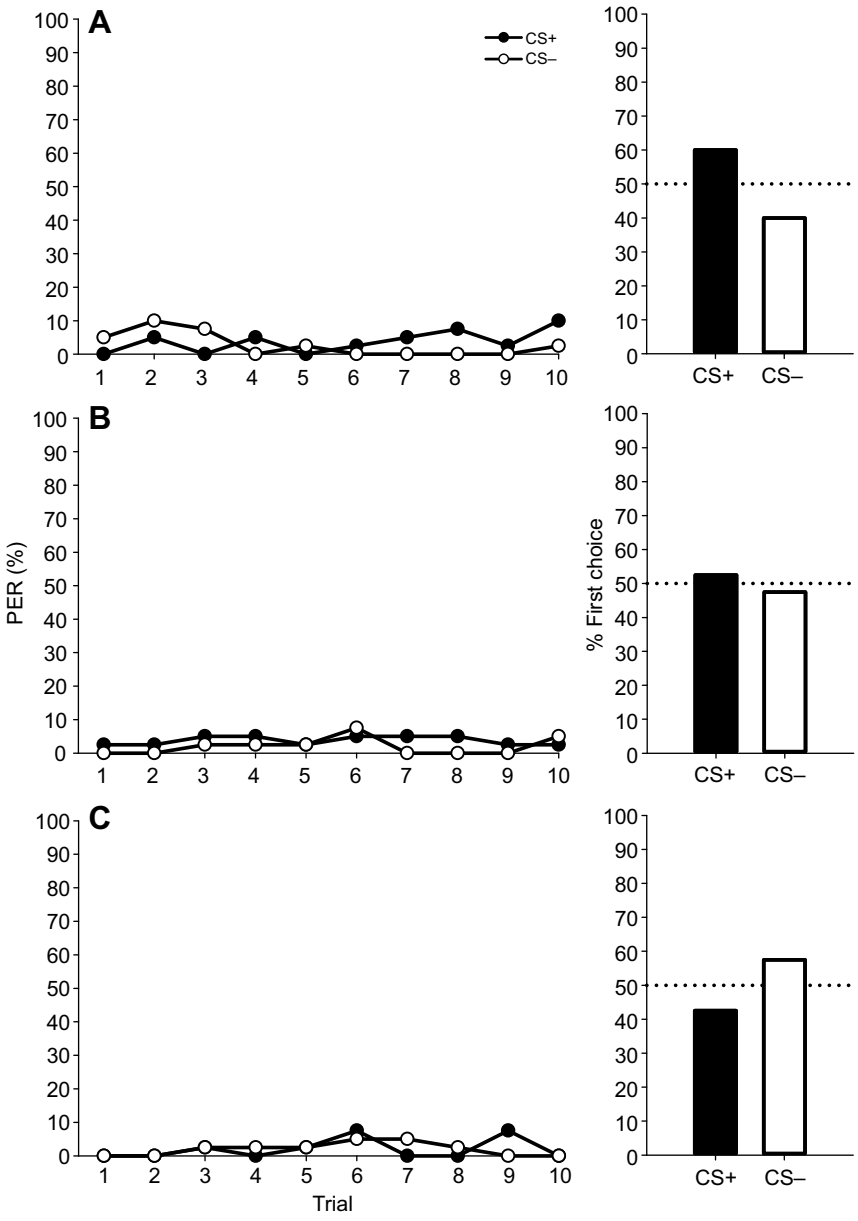

Fig. 6. Performance during visual differential conditioning and further operant test of bees injected with mianserine solution. The percentage of conditioned PER during conditioning trials (left) and first choices during Y-maze orientation tests (left) is shown for three increasing concentrations of mianserine: 3.3×10^{-7} mol l^{-1} (A), 3.3×10^{-5} mol l^{-1} (B) and 3.3×10^{-3} mol l^{-1} (C). *N*=40 bees per concentration. *N*=1, 1 and 2 bees were excluded from analysis because of PER impairment in A, B and C, respectively; *N*=3, 2 and 3 bees were excluded because they died between conditioning and test in A, B and C, respectively.

three comparisons), with a significant decrease in DI following each increase of concentration (Fig. 7). A partial dose-dependent effect was observed for different concentrations of epinastine, with a significantly higher DI for the lowest concentration (Tukey test; 10^{-7} mol l^{-1} versus 10^{-5} mol l^{-1} and 10^{-7} mol l^{-1} versus 10^{-3} mol l^{-1}, $P<0.05$ in both cases) and equivalent DI for the other two concentrations (Tukey test; 10^{-5} mol l^{-1} versus 10^{-3} mol l^{-1}; $P=0.55$, NS). Mianserine injection led to equivalent very low DI values at all three concentrations tested (Tukey test; $P>0.74$, NS; for all three comparisons).

Classical conditioning followed by PER retention tests

These experiments were performed to analyze whether the effects of aminergic antagonists in operant orientation tests were related to interference with memory retention. No differences were found between the performance of bees trained to green or blue light as the CS+ for any of the treatments (group×stimulus×trial GLM ANOVA for repeated measures; group effect; Ringer solution: $F_{1,38}=0.23$, NS; flupentixol 10^{-7} mol l^{-1}: $F_{1,38}=0.16$ NS, 10^{-5} mol l^{-1}: $F_{1,38}=1.25$ NS, 10^{-3} mol l^{-1}: $F_{1,38}=0.12$ NS; epinastine 10^{-7} mol l^{-1}: $F_{1,38}=0.67$ NS, 10^{-5} mol l^{-1}: $F_{1,33}=0.16$ NS, 10^{-3} mol l^{-1}: $F_{1,29}=1.15$ NS). Therefore, we pooled data from these two independent groups for each treatment. As expected, control bees injected with Ringer solution significantly differentiated CS+ and CS– both during conditioning (stimulus×trial GLM ANOVA for repeated measures; interaction effect; $F_{9,342}=4.87$, $P<0.001$) and retention tests (McNemar test; $\chi^2=7.7$, $P<0.01$).

Bees injected with the lowest and the medium dose (2×10^{-7} mol l^{-1} and 2×10^{-5} mol l^{-1}) of the dopaminergic antagonist flupentixol were able to significantly discriminate between CS+ and CS– during visual conditioning of the PER (Fig. 8A,B; stimulus×trial GLM ANOVA for repeated measures; interaction effect; flupentixol 10^{-7} mol l^{-1}: $F_{9,342}=3.24$, $P<0.001$; 10^{-5} mol l^{-1}: $F_{9,342}=3.01$, $P<0.01$), as well as in retention tests (Fig. 8A,B; McNemar test; flupentixol 10^{-7} mol l^{-1}: $\chi^2=5.8$, $P<0.02$; 10^{-5} mol l^{-1}: $\chi^2=4.0$, $P<0.05$). The injection of a higher dose of flupentixol (2×10^{-3} mol l^{-1}) impaired visual discrimination

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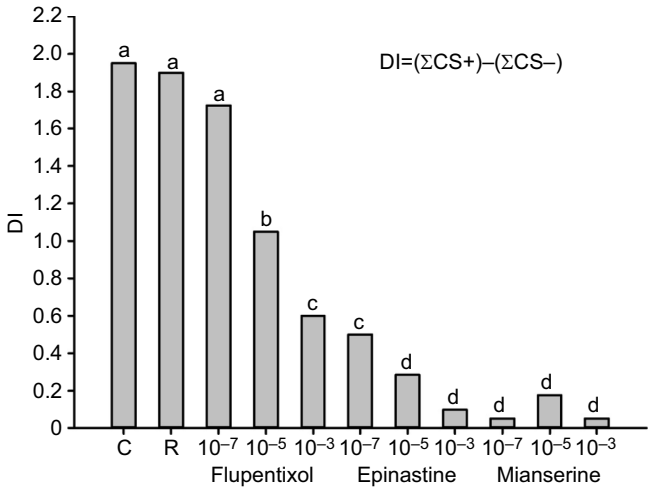

Fig. 7. DI during visual conditioning of bees injected with Ringer solution or different concentrations of the aminergic antagonists flupentixol, epinastine and mianserine. C, control; R, Ringer solution. Letters represent statistical differences between the DI calculated for the different groups (GLM ANOVA).

during both conditioning (Fig. 8C; stimulus×trial GLM ANOVA for repeated measures; interaction effect; $F_{9,342}$=1.23, NS) and retention tests (Fig. 8C; McNemar test; χ^2=0.5, NS).

The group of bees treated with the lowest dose of the octopaminergic antagonist epinastine (4×10^{-7} mol l^{-1}) showed visual discrimination during conditioning (Fig. 9A; stimulus×trial GLM ANOVA for repeated measures; interaction effect; $F_{9,342}$=6.59, P<0.02), as well as in retention tests (Fig. 9A; McNemar test; χ^2=5.2, P<0.05). However, injections of the medium and higher epinastine doses (4×10^{-5} mol l^{-1} and 4×10^{-3} mol l^{-1}) impaired visual discrimination during both PER conditioning (Fig. 9B,C; stimulus×trial GLM ANOVA for repeated measures; 10^{-5} mol l^{-1}: interaction effect: $F_{9,342}$=1.55, NS; Fig. 9C; 10^{-3} mol l^{-1}: interaction effect; $F_{9,342}$=1.19, NS) and retention tests (Fig. 9B,C; McNemar test; epinastine 10^{-5} mol l^{-1}: χ^2=1.4, NS; epinastine 10^{-3} mol l^{-1}: χ^2=0.1, NS).

DISCUSSION

We have shown for the first time that honeybees are able to transfer visual information acquired in classical associative learning to a new operant context during a retention test. The injection of both dopaminergic and octopaminergic antagonists into the bee brain prior to visual conditioning led to impairment of associative learning, revealing that both these monoamines participate in visual stimulus–reward association pathways. Whereas the impairment of visual learning by the dopaminergic antagonist occurred in a clear dose-dependent manner, the injection of the octopaminergic antagonists led to very strong learning inhibition at virtually all doses tested. It seems, therefore, that octopamine is a stronger modulator of appetitive visual learning than dopamine.

Appetitive visual conditioning in harnessed honeybees

In the present work, we performed an appetitive conditioning protocol that allowed harnessed bees to effectively discriminate between two different isoluminant chromatic stimuli. The growing interest in developing effective visual conditioning protocols in harnessed honeybees has led researchers to adopt diverse harnessing procedures, visual stimulations of different nature and duration, and a varying number of trials and intertrial intervals (Avarguès-Weber and Mota, 2016). In some of these studies, the association between visual stimulus and reward was only possible after amputation of the honeybee antennae, an intriguing fact whose neurobiological basis remains so far unknown (Avarguès-Weber and Mota, 2016). The methodology used here proved to be equally effective in conditioning antennae-deprived and intact harnessed bees to a visual differential task. After adapting (in different ways) previously published protocols for conditioning intact harnessed bees to visual stimuli (Dobrin and Fahrbach, 2012; Jernigan et al., 2014; Balamurali et al., 2015), we believe that some parameters were important to reach this goal in our laboratory: (i) complete darkness of the conditioning environment; (ii) large size and lateral presentation of the visual stimuli; and (iii) inclination of the bee body at 45 deg. Although these seemed to be important parameters for the success of our classical conditioning protocol, further controlled studies are necessary to understand how and to what extent they modulate appetitive visual learning in harnessed honeybees.

Visual information transfer in honeybees

The ability of honeybees to transfer learned information between classical and operant contexts has so far been demonstrated for olfactory cues (Bakchine-Huber et al., 1992; Gerber et al., 1996; Sandoz et al., 2000; Chaffiol et al., 2005; Carcaud et al., 2009), but not yet for visual stimuli. The main reason for this is probably the difficulty of establishing robust and reliable protocols for classical visual conditioning of harnessed honeybees (Avarguès-Weber and Mota, 2016). Although successful visual conditioning of the PER has recently been reported in harnessed bees, the levels of learning reached are typically much lower (30–50%) than those reported in the extensive literature on operant visual conditioning of free-flying bees (80–100%). These data suggest that visual learning by honeybees is more effective in an operant framework than in a classical one. Here, we aimed to test whether harnessed honeybees conditioned in a classical paradigm could transfer visual discrimination to a new operant context when released in a Y-maze during a retention test. We found that virtually all bees (94%) mastering the visual discrimination task in a classical context (learners) chose the correct chromatic stimulus during the orientation tests in the Y-maze, demonstrating that memories acquired by these honeybees in a PER-conditioning paradigm are very resistant to context changes between conditioning and retention tests. Surprisingly, 70% of the bees not achieving the Pavlovian visual discrimination task (non-learners) also made correct choices in the Y-maze during the retention test, indicating that at least some of these bees acquired visual memories during classical conditioning although typically they did not present any conditioned PER responses. These results support the theory that outcomes of associative visual learning in honeybees are much more accessible in an operant context than in a classical one. Therefore, retention tests in a Y-maze after visual PER conditioning appear to be a promising approach for improving the analysis of visual learning and memory performance in honeybees.

It is important to highlight the necessity of performing a precise calibration of visual stimulus irradiance to an isoluminance point for the effectiveness of this new approach. The strong natural phototaxis of honeybees in a dark environment presenting light stimuli tends to guide them to the perceptually brighter side of the Y-maze during orientation tests. This behavior strongly interfered with the response of bees in the Y-maze during the retention test, independent of previous classical visual conditioning. Calibration of stimulus irradiance to an isoluminance point has been effectively used in

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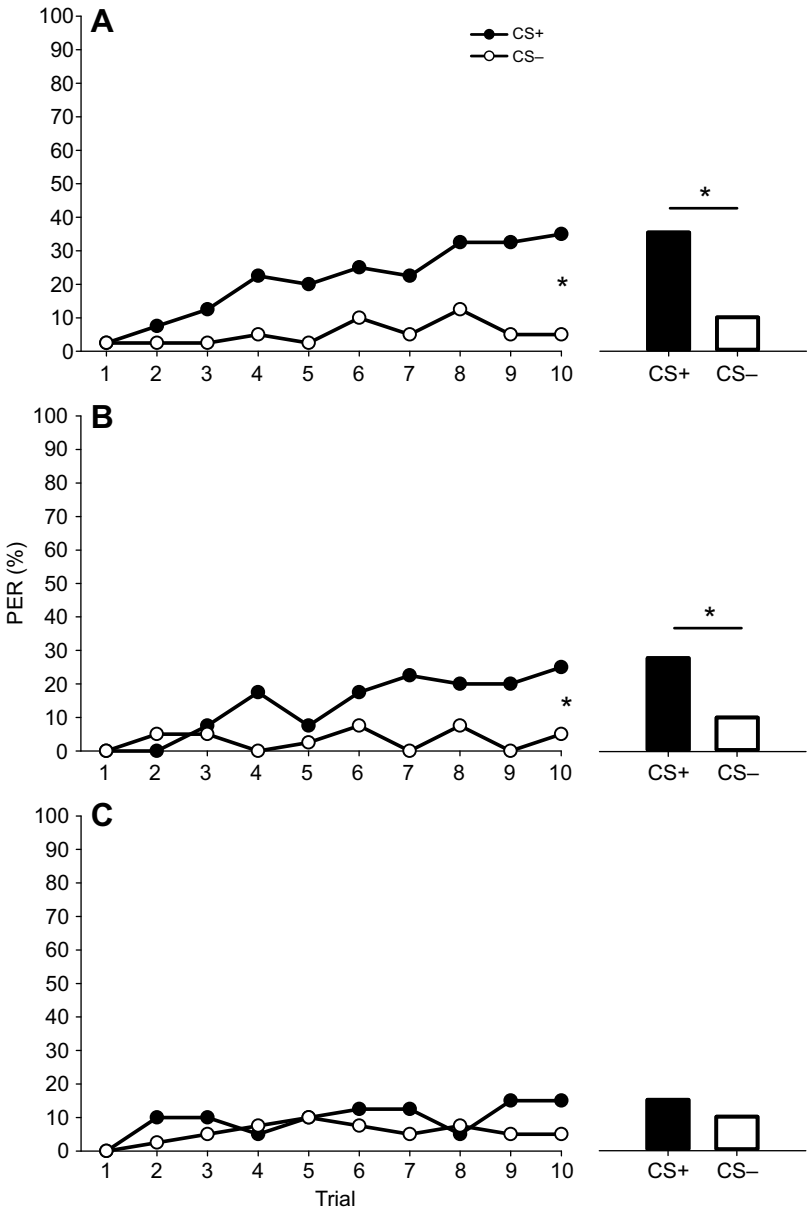

Fig. 8. Classical visual conditioning followed by PER retention tests in bees injected with flupentixol. The percentage of conditioned PER in conditioning trials (left) and retention tests (right) is shown for three increasing concentrations of flupentixol: 2×10^{-7} mol l^{-1} (A), 2×10^{-5} mol l^{-1} (B) and 2×10^{-3} mol l^{-1} (C). Asterisks indicate significant discrimination during conditioning (GLM ANOVA for repeated measures) or retention tests (McNemar test). *N*=40 bees per concentration. *N*=1 and 2 bees were excluded from analysis because of PER impairment in A and C, respectively; *N*=2 bees were excluded because they died between conditioning and test in B and C.

Drosophila for studying chromatic processing without interference from other achromatic visual cues (Yamaguchi et al., 2008; Melnattur et al., 2014). In these studies, the flies' optomotor response in a flight simulator was used to determine a point of isoluminance for blue–green stimuli composed of color-alternating moving bars. As motion processing in flies relies on achromatic contrast and is independent of color vision, the optomotor response is abolished when the blue–green moving bars are set to a point of isoluminance. The positive phototaxis of bees towards a light source is a behavior that also seems to rely exclusively in achromatic cues (Labhart, 1974; Menzel and Greggers, 1985; Erber et al., 2006). Therefore, we used this behavior to set an irradiance ratio for a pair of blue and green lights, in which bees present no preference for one of these two stimuli in a Y-maze. By using this isoluminant pair of stimuli, we were able to not only exclude the influence of positive phototaxis in our visual stimulus–orientation retention tests but also perform differential visual conditioning in harnessed bees that probably relied on 'true' color vision. Thus, using the positive phototaxis to set pairs of spectral stimuli to a point of perceptual isoluminance, although demanding, can be a promising complementary approach for studying chromatic learning and its related neural correlates in honeybees.

The role of dopamine and octopamine in visual learning

Unlike studies on olfactory conditioning of honeybees suggesting that octopamine and dopamine are exclusive modulators of odor–reward and odor–punishment associations, respectively (Vergoz et al., 2007; Wright et al., 2010), our results indicate that both these biogenic amines modulate appetitive visual learning by bees. Significant impairment of appetitive visual discrimination was found when both octopaminergic and dopaminergic antagonists were applied in the honeybee brain. It seems therefore that the octopaminergic role in appetitive reward is conserved across different sensory modalities in honeybees, whereas a dopaminergic role in appetitive reward would mainly exist for the visual modality. Although no effect of dopamine in odor–reward acquisition was observed, this amine appeared to block long-term olfactory appetitive memory formation in bees, while injection of a dopaminergic

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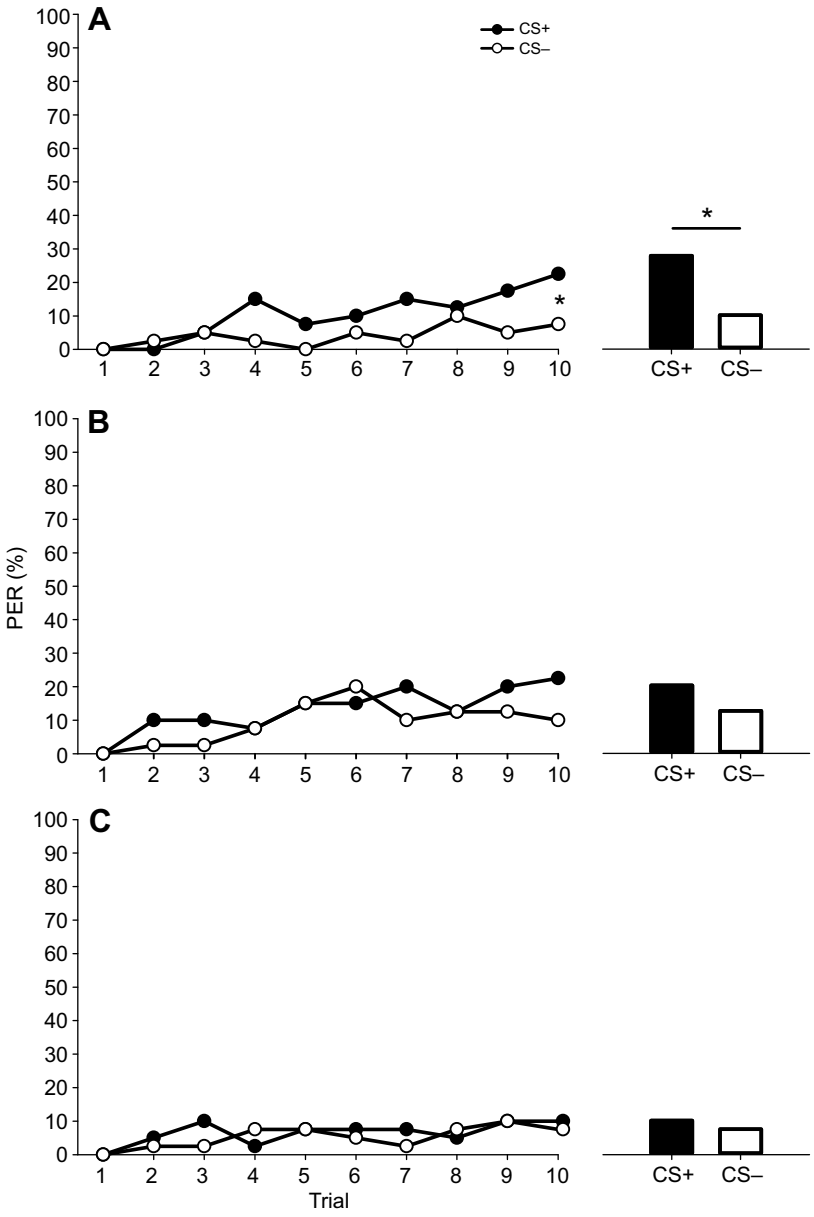

Fig. 9. Classical visual conditioning followed by PER retention tests of bees injected with epinastine. The percentage of conditioned PER in conditioning trials (left) and retention tests (right) is shown for three increasing concentrations of epinastine: 4×10^{-7} mol l^{-1} (A), 4×10^{-5} mol l^{-1} (B) and 4×10^{-3} mol l^{-1} (C). Asterisks indicate significant discrimination during conditioning (GLM ANOVA for repeated measures) or retention tests (McNemar test). *N*=40 bees per concentration. *N*=1, 2 and 2 bees were excluded from analysis because of PER impairment in A, B and C, respectively; *N*=2 and 3 bees were excluded because they died between conditioning and test in B and C, respectively.

antagonist accordingly enhanced it. These results suggest that aversive and appetitive components interact during olfactory memory formation in the bee brain (Klappenbach et al., 2013).

The involvement of both dopamine and octopamine was previously described in an operant visuo-spatial aversive learning paradigm in honeybees (Agarwal et al., 2011). However, contrary to our results in appetitive visual learning, these biogenic amines presented opposite effects in this operant aversive learning approach, with dopamine enhancing and octopamine inhibiting avoidance of punished stimuli (Agarwal et al., 2011). In addition to the effect of the aminergic antagonists on visual differential conditioning, our work suggests that these antagonists can interfere with the capacity of bees to transfer visual information from a classical to an operant context. Bees injected with the medium dose of flupentixol or the lowest dose of epinastine were successful in discriminating CS+ and CS− during classical conditioning, but failed to transfer visual discrimination to an operant context (Figs 4B and 5A). A possible reason for this effect could be that these treatments interfered with memory consolidation or retention, so that 1 h after conditioning, bees would fail to discriminate visual stimuli. However, we found that bees injected with the medium dose of flupentixol or the lowest dose of epinastine did not present impairment of memory retention 1 h after conditioning (Figs 8B and 9A). We thus conclude that these doses of dopaminergic and octopaminergic antagonists impaired the capacity of bees to transfer visual information to a new context.

Studies in crickets have found octopamine and dopamine to be exclusive modulators of appetitive and aversive reinforcement, respectively, both for olfactory and visual stimuli (Unoki et al., 2005, 2006; Mizunami et al., 2009; Nakatani et al., 2009; Matsumoto et al., 2015). Whereas a dopaminergic modulation of appetitive visual learning is apparently absent in crickets, specific dopaminergic neurons involved in this type of modulation have been described in flies (Vogt et al., 2014). Both visual and olfactory appetitive learning in *Drosophila* have been shown to rely on dopaminergic neurons of the mushroom body signaling sugar reinforcement (Kim et al., 2007; Liu et al., 2012; Vogt et al., 2014). Furthermore, other dopaminergic neurons than the ones involved in the formation of appetitive

associations modulate visual and olfactory aversive learning in *Drosophila* (Vogt et al., 2014, 2016). Together with our results, these studies in *Drosophil*a challenge the theory suggested by some authors that dopamine is an exclusive modulator of aversive reinforcement circuits in insects (Schwaerzel et al., 2003; Unoki et al., 2005, 2006; Vergoz et al., 2007).

Whereas dopamine in mammals has for a long time been considered the main neurotransmitter related to reward and motivation (Wise, 2004), it has only recently been associated with rewarding reinforcement in insects (Kim et al., 2007; Krashes et al., 2009; Liu et al., 2012; Burke et al., 2012; Vogt et al., 2014, 2016; present study). In contrast, octopamine has been historically considered the main signal for appetitive reward in different insect models (Hammer, 1993; Hammer and Menzel, 1998; Schwaerzel et al., 2003; Unoki et al., 2005; Vergoz et al., 2007). Interestingly, octopaminergic and dopaminergic neurons interact in the *Drosophila* brain during appetitive olfactory learning to provide reward signaling and memory formation (Liu et al., 2012; Burke et al., 2012). Octopamine was shown to trigger an increase in intracellular calcium in a specific subset of dopaminergic neurons in the mushroom body whose direct activation can substitute for sugar to form appetitive olfactory memory in flies (Burke et al., 2012). Furthermore, octopamine-dependent reinforcement also requires an interaction with other dopaminergic neurons in the fly brain that control appetitive motivation (Burke et al., 2012). Our results in appetitive visual learning coupled to pharmacological injections in the bee brain also suggest a synergic role of octopamine and dopamine in the formation of color–reward associations. Further studies are now necessary to understand which octopaminergic and dopaminergic neurons and receptors in the bee brain are involved in these cognitive processes.

Putative neural correlates of appetitive visual learning in bees

The neural bases of appetitive visual learning remain poorly understood in any insect model. Until recently, virtually all studies aimed at identifying the neural architecture involved in appetitive reinforcement in the insect brain have been focused on olfactory learning substrates. In honeybees, a single neuron called VUMmx1 was found to provide different convergence sites between the olfactory and the sucrose processing pathways in the brain, including the antennal lobes, the lateral horn and the mushroom bodies. Electrophysiological and pharmacological stimulation of this broad-field octopaminergic neuron were able to replace the reward function of sucrose during classical olfactory conditioning (Hammer, 1993; Hammer and Menzel, 1998), proving that this unique neuron is sufficient for the formation of odor–reward associations in the honeybee brain. Behavioral studies on bimodal conditioning of the PER showed that visual stimuli can modulate olfactory learning in bees (Gerber and Smith, 1998; Mota et al., 2011; Hussaini and Menzel, 2013), suggesting that the neural substrates of odor–reward associations can receive direct or indirect input from visual processing regions. Whereas different olfactory neuropils are arborized by VUMmx1, the only brain regions that may provide connection between visual processing substrates and this neuron are the lateral protocerebrum and the basal ring of the mushroom bodies (Hammer, 1993, 1997).

The anatomical study of 11 other ventral unpaired median (VUM) neurons that are possibly octopaminergic (Kreissl et al., 1994) and involved in appetitive learning showed that none of them presents direct connections with the optic lobes or the central complex, but more than one type arborizes lateral and medial protocerebral regions that may provide visual input (Schröter et al., 2007). Interestingly, electrophysiological recordings of the VUMmx1 and the ventral unpaired median cell of the mandibular neuromere 1 (VUMmd1), whose neural architectures are very similar, showed that both these neurons respond to visual as well as olfactory and gustatory stimuli (Schröter et al., 2007). Therefore, these two neurons are strong candidates for participating in octopaminergic modulation of appetitive visual or bimodal learning in the bee brain. Further studies should try to confirm this theory by combining electrophysiological recordings of these neurons with appetitive visual and bimodal conditioning in harnessed bees.

Our pharmacological study shows that not only octopamine but also dopamine participates in the modulation of appetitive visual learning in honeybees. Although dopamine has been found to be an important modulator of aversive olfactory learning in bees, no study has so far described the neurobiological bases of such an associative modulation (Vergoz et al., 2007; Tedjakumala and Giurfa, 2013). Immunohistochemical characterization of dopaminergic neurons in the honeybee brain revealed prominent neural arborizations in the lobula, mushroom bodies, central complex and lateral protocerebrum (Schürmann et al., 1989; Schäfer and Rehder, 1989; Tedjakumala et al., 2017), which may contribute to dopaminergic modulation of visual learning. Considering that neurons involved in learning and memory in the insect brain typically arborize the mushroom bodies, dopaminergic clusters C1, C2 and C3 strongly innervating these higher-order centers in the honeybee brain are good candidates (Tedjakumala et al., 2017). Appetitive visual learning in *Drosophila* has been shown to involve dopaminergic neurons from the paired anterior medial cluster (Vogt et al., 2014), which share anatomical similarities with dopaminergic neurons from clusters C1 and C2 in the honeybee brain (Tedjakumala et al., 2017). Thus, further studies aimed at revealing the neural basis of dopaminergic modulation of appetitive visual learning in bees should be focused on these neural substrates. Our study represents an important step in the comprehension of the neural mechanisms underlying visual learning and memory in honeybees.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.M.; Methodology: A.R.V., N.S., M.B., T.M.; Formal analysis: A.R.V., N.S., M.B., T.M.; Resources: T.M.; Writing - original draft: A.R.V., T.M.; Writing - review & editing: A.R.V., T.M.; Supervision: T.M.; Project administration: T.M.; Funding acquisition: T.M.

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