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Aminergic neuromodulation of associative visual learning in harnessed honey bees

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ABSTRACT

The honey bee *Apis mellifera* is a major insect model for studying visual cognition. Free-flying honey bees learn to associate different visual cues with a sucrose reward and may deploy sophisticated cognitive strategies to this end. Yet, the neural bases of these capacities cannot be studied in flying insects. Conversely, immobilized bees are accessible to neurobiological investigation but training them to respond appetitively to visual stimuli paired with sucrose reward is difficult. Here we succeeded in coupling visual conditioning in harnessed bees with pharmacological analyses on the role of octopamine (OA), dopamine (DA) and serotonin (5-HT) in visual learning. We also studied if and how these biogenic amines modulate sucrose responsiveness and phototaxis behaviour as intact reward and visual perception are essential prerequisites for appetitive visual learning. Our results suggest that both octopaminergic and dopaminergic signaling mediate either the appetitive sucrose signaling or the association between color and sucrose reward in the bee brain. Enhancing and inhibiting serotonergic signaling both compromised learning performances, probably via an impairment of visual perception. We thus provide a first analysis of the role of aminergic signaling in visual learning and retention in the honey bee and discuss further research trends necessary to understand the neural bases of visual cognition in this insect.

1. Introduction

In both vertebrates and invertebrates, biogenic amines act as important regulators of cell functions and as neurotransmitters, neuromodulators, and neurohormones (Libersat & Pflueger, 2004; Huber, 2005; Scheiner, Baumann, & Blenau, 2006). In insects, besides modulating stereotyped behaviours, biogenic amines are also key players in associative learning and memory formation as they may mediate the reinforcing properties of unconditioned stimuli (reward or punishment) (Giurfa, 2006; Scheiner et al., 2006; Perry & Barron, 2013).

A well-established insect model for the study of the role of biogenic amines in learning and memory is the domestic honey bee *Apis mellifera* (Giurfa, 2007; Srinivasan, 2010; Avarguès-Weber, Deisig, & Giurfa, 2011; Menzel, 2012; Avarguès-Weber & Giurfa, 2013). The success of the bee for studies on learning and memory is based on its capacity to learn and memorize multiple sensory cues in standardized conditioning protocols in the laboratory (Giurfa, 2007). In some of these protocols, bees are immobilized, thus enabling efficient stimulus control and the coupling with invasive methods for studying neural and molecular underpinnings of learning and memory (Giurfa & Sandoz, 2012). One of such protocols is the olfactory conditioning of the Proboscis Extension Response (PER) in which a harnessed bee learns to associate a neutral odorant (the conditioned stimulus, CS) with an appetitive reward of sucrose solution (the unconditioned stimulus or US). Sucrose stimulation of the antennae of a hungry bee induces a reflexive extension of the proboscis. After pairing the CS with the US, the bee learns the association between odorant and food, and responds with PER to the odorant itself (Takeda, 1961; Bitterman, Menzel, Fietz, & Schäfer, 1983; Giurfa & Sandoz, 2012; Matsumoto, Menzel, Sandoz, & Giurfa, 2012).

While this protocol has greatly contributed to our current knowledge of the neurobiological mechanisms of honey bee learning and memory (Giurfa & Sandoz, 2012), less is known about the neural underpinnings of visual learning and memory. Studies in this domain have been mostly restricted to the use of free-flying bees, which can be easily trained to choose visual targets paired with sucrose reward (Giurfa, 2007; Srinivasan, 2010; Avarguès-Weber, Deisig, & Giurfa, 2011, Avarguès-Weber, Dyer, & Giurfa, 2011; Avarguès-Weber, Dyer, Combe, & Giurfa, 2012). However, laboratory protocols allowing the study of visual learning in harnessed bees have been difficult to develop. Since the first report of visual conditioning of PER (Kuwabara, 1957), several attempts have revealed the difficulty of achieving fast and robust acquisition performances as commonly observed in olfactory PER

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conditioning. In fact, the resulting learning and retention performances are usually poor, even in simple color-learning tasks (see review in Avarguès-Weber & Mota, 2016). Yet, in some cases (Dobrin & Fahrbach, 2012; Riveros & Gronenberg, 2012; Jernigan, Roubik, Wcislo, & Riveros, 2014; Lichtenstein, Sommerlandt, & Spaethe, 2015), better performances were observed, which inspired the goal of establishing a differential visual conditioning protocol and unravelling the role of biogenic amines in this learning form.

In our protocol, harnessed bees should learn to discriminate a colored stimulus paired with a sucrose reward from a different, non-rewarded colored stimulus. To dissect the contribution of aminergic circuits, we focused on (OA), dopamine (DA) and serotonin (5HT), which are main neurotransmitters and neuromodulators in the insect brain (Libersat & Pflueger, 2004; Huber, 2005; Scheiner et al., 2006).

In honey bees, OA is the neurotransmitter that conveys reinforcement signaling in the brain for various forms of appetitive but not aversive learning (Hammer, 1993; Hammer & Menzel, 1998; Farooqui, Robinson, Vaessin, & Smith, 2003). In this context, OA signaling substitutes directly for sucrose, the appetitive US, in the bee brain (Hammer, 1993). Conversely, DA mediates reinforcement signaling in aversive learning in bees (Vergoz, Roussel, Sandoz, & Giurfa, 2007) but its role in appetitive learning is less clear (Klappenbach, Kaczer, & Locatelli, 2013). In this framework, DA signaling would directly substitute for aversive US, although it may also mediate forms of attention towards this kind of stimulation (Tedjakumala, Aimable, & Giurfa, 2014). The picture is different for the fruit fly *Drosophila melanogaster*, where different subsets of dopaminergic neurons convey both appetitive and aversive reinforcement signaling in olfactory and visual learning (Kim, Lee, & Han, 2007; Selcho, Pauls, Han, Stocker, & Thum, 2009; Burke, Huetteroth, Owald, Perisse, & Krashes, 2012; Liu, Plaçais, Yamagata, Pfeiffer, & Aso, 2012; Vogt et al., 2014; Rohwedder, Wenz, Stehle, Huser, & Yamagata, 2016; Yamagata, Hiroi, Kondo, Abe, & Tanimoto, 2016). In the fly, OA signaling participates in appetitive reinforcement signaling but as a first relay towards dopaminergic neurons, which are crucial to convey this information to higher-order brain centers (Burke et al., 2012; Liu et al., 2012). Finally, the role of 5-HT is less clear in honey bee associative learning as it does not seem to signal the presence of particular forms of reinforcement. It affects, nevertheless, olfactory PER conditioning and retention (Mercer & Menzel, 1982; Erber, Kloppenburg, & Scheidler, 1993; Erber & Kloppenburg, 1995) and aversive shock responsiveness (Tedjakumala et al., 2014). 5-HT is also a major neurotransmitter of the bee's visual system and participates in different forms of visual processing (Schürmann & Klemm, 1984; Brüning, Kaulen, Scheidler, & Erber, 1987; Erber, Kloppenburg, & Scheidler, 1991; Kloppenburg & Erber, 1995).

Here we studied the involvement of OA, DA and 5-HT in visual PER conditioning using a combination of behavioural and pharmacological analyses. We studied the effect of specific agonists and antagonists of these biogenic amines on visual discrimination learning and mid-term retention. We thus provide a first analysis of the role of these amines in an appetitive learning task in the visual domain of bees.

2. Material and methods

Experiments were conducted at the Research Center of Animal Cognition (CRCA), Toulouse, France, from January to July 2016. Winter bees were caught from hives kept in a heated building in order to maintain them active. Honey bee workers (*Apis mellifera*) from the CRCA apiary were collected in the morning at the hive entrance and placed on ice to immobilize them. Bees were then individually harnessed in plastic tubes by means of two pins placed around their neck (Dobrin & Fahrbach, 2012). Fifty min after fixation, they were fed with 30 μL of sucrose solution (30% weight/weight) and kept in a dark and humid chamber at 25 °C for 24 h. Conditioning was thus performed the day after the bees were captured.

2.1. Experimental setup and visual stimulation

The conditioning setup was composed of a row of five individual cubic chambers (10 × 10 × 10 cm) that were covered by a removable red Plexiglas roof, which isolated the bees from light stimulation. The row of chambers was mounted on wheels in order to position each bee in front of a 10 × 10 cm screen made of tracing paper and distant of 4 cm. A monochromator (Polychrome V®, Till Photonics, Germany) projected via an optic fiber a colored light ambiance and a colored disk 2 cm in diameter on the screen. The disk subtended a visual angle of 28° to the bee eye, thus ensuring that it was perceived in terms of its chromatic contents (Giurfa, Vorobyev, Kevan, & Menzel, 1996; Giurfa, Vorobyev, Brandt, Posner, & Menzel, 1997; Hempel de Ibarra, Giurfa, & Vorobyev, 2002). Custom software allowed controlling the stimulus wavelength, its duration and the intertrial- and interstimulus intervals. Two different monochromatic lights peaking respectively at 465 nm (Blue, B) and 525 nm (Green, G) were used as conditioned stimuli. They were perfectly discriminable for the bee's visual system (Menzel & Backhaus, 1991).

2.2. Visual PER conditioning procedure

Conditioning was performed in a dark room, under a weak red-light illumination, which was invisible for the bees. Before training, PER integrity was checked by stimulating both antennae with a toothpick soaked in sucrose solution (50% w/w). Only the bees that extended their proboscis upon this stimulation were kept for the experiment (only 5% of the bees were excluded following this check).

Five bees were conditioned in parallel. Each bee was placed in an individual chamber of the experimental device for a 30-min habituation phase. The bees had to learn the association between one monochromatic light (conditioned stimulus, CS+) and the sucrose reward (unconditioned stimulus, US) and between the other monochromatic light (CS−) and the absence of reward. To balance the experiment, a group of bees was trained with a B+/G− contingency (blue rewarded/green unrewarded) while a different group was trained in parallel with a B−/G+ contingency (blue unrewarded/ green rewarded).

The differential conditioning phase consisted of 10 trials (5 CS+ and 5 CS− presentations in a pseudo-random sequence) separated by an inter-trial interval of 5 min. The CS+ was paired with a sucrose reward (40% w/w) delivered on the proboscis after antennal stimulation by a toothpick while the CS− was never rewarded. Each CS presentation lasted 16 s. On CS+ trials, the sucrose reward was delivered during 4 s, 14 s after the beginning of the visual stimulus. The bees thus experienced 14 s of CS presentation alone, followed by 2 s of overlap between CS and US and by 2 s of US alone. In CS+ trials, the soaked toothpick was kept outside of the conditioning chamber during the 14-s period to avoid undesirable responses to water vapor (Kuwabara, 1957). Conditioned responses were recorded during the 14 s preceding US delivery in the case of the CS+, and during the same corresponding period in the case of the CS−.

After the conditioning phase, the bees were left within the individual chambers for 1 h. Thereafter, a retention test was performed in which the CS+ and the CS− were presented for 16 s each, in the absence of reinforcement. The sequence of CS presentation varied randomly from bee to bee. After the end of the retention test, PER integrity was checked for each bee by touching its antennae with sucrose solution. Animals that did not respond to this stimulation were excluded from the analysis (< 5%).

2.3. Pharmacological treatments

Learning and retention performances were compared between bees treated either with the biogenic amines of interest (OA, DA, 5-HT) or with antagonists of the receptors of these neurotransmitters.

Octopamine hydrochloride (OA), dopamine hydrochloride (DA) and

serotonin hydrochloride (5-HT) were used as agonist molecules. The respective receptor antagonists chosen were epinastine hydrochloride (Roeder, Degen, & Gewecke, 1998), flupentixol dihydrochloride (Blenau, Erber, & Baumann, 1998) and methiothepin mesylate (Blenau & Thamm, 2011). All substances were obtained from Sigma-Aldrich (France). Epinastine was selected for its high affinity for OA receptors (Roeder et al., 1998), in particular for the AmOA1 receptor of honey bees (Farooqui et al., 2003). Flupentixol was chosen as antagonist of AmDOP1 and AmDOP3 dopaminergic receptors, due to its high affinity for D1 and D2 dopaminergic receptors, the respective vertebrate homologous of AmDOP1 et AmDOP3 (Kokay & Mercer, 1996; Blenau et al., 1998; Hearn et al., 2002; Mustard et al., 2003; Vergoz et al., 2007; Tedjakumala et al., 2014). Methiothepin was chosen as serotonergic antagonist because it binds to Am5-HT1A, Am5-HT2α et Am5-HT7 serotonergic receptors of the honey bee (Thamm, Rolke, Jordan, Balfanz, & Schiffer, 2013). The three receptor antagonists have been successfully used in experiments on appetitive and aversive responsiveness as well as on olfactory learning and memory in bees (Vergoz et al., 2007; Tedjakumala et al., 2014). Drugs were topically applied on the thorax, a method that has proved to be effective for short-term, systemic pharmacological treatments in the honey bee (Barron, Maleszka, Vander Meer, Robinson, & Maleszka, 2007; Søvik et al., 2016).

Substances were dissolved in dMF (N, N-dimethylformamide), a solvent allowing them to penetrate the cuticle, then to pass into the hemolymph and finally to reach the brain (~1% of the total concentration) (Barron et al., 2007; Søvik et al., 2016). For each molecule, 1 μL of the final solution was applied locally on the center of the thorax with a micropipette. For OA, DA and 5-HT, 2 mg of these molecules were dissolved in 100 μL dMF. In the case of epinastine, flupentixol and methiothepin, 1 mg of these substances was dissolved in 100 μL dMF. Final concentrations were 0.105 $mol{\cdot}L^{-1}$ OA and DA, 0.094 $mol{\cdot}L^{-1}$ 5-HT, 0.035 $mol{\cdot}L^{-1}$ epinastine, 0.020 $mol{\cdot}L^{-1}$ flupentixol and 0.022 $mol{\cdot}L^{-1}$ methiothepin. Each molecule was tested a second time (on different groups of bees) with a concentration increased by a factor of 10. Each biogenic amine was tested in parallel to its respective receptor antagonist and to a control group in which bees were treated with dMF only (dMF-control group). Treatments were performed 30 min before conditioning, which corresponds to the time required for the substances to be biologically effective (Blenau et al., 1998; Barron et al., 2007; Vergoz et al., 2007; Tedjakumala et al., 2014; Nouvian, Mandal, Jamme, Claudianos, & d'Ettorre, 2018).

2.4. Phototaxis test

To evaluate whether the drugs used may affect visual processing, we measured their impact on bees' sensitivity to light in a standard phototaxis assay. Novel groups of bees were used to this end. They were tethered, fed and kept in dark conditions for 24 h as explained above. Topical applications were performed as in the previous experiments, after which bees were released individually in Eppendorf tubes (volume 1.5 mL) 30 min before the phototaxis test. During this period, drugs exerted their action and bees could recover their mobility (Barron et al., 2007).

The phototactic response of bees was measured within an arena (30 × 30 × 4.5 cm) conceived to this end. Each bee was released individually in one corner of the arena opposite to the corner in which a light source (200 lx) produced by a cold-light generator (Cold Light L-150) was visible (Bergougnoux, Treilhou, & Armengaud, 2013). A camera placed above the arena recorded the displacement of the bee during 2 min. Video recordings (15 pictures/sec) were analyzed using custom tracking software (Tosia, M. Combe, CRCA, Toulouse, France) measuring the latency before the bee reached the light source and the total distance travelled. Potential effects of the three neurotransmitters (OA, DA, 5-HT) and of their respective receptor antagonists, epinastine, flupentixol and methiothepin, at the highest doses used in the visual conditioning experiments were determined. These doses were chosen, based on their effective modulation of visual learning performances. A dMF-treated group and an untreated group were run in parallel as controls to check for potential effects of the solvent alone (no dMF) on light responsiveness. The arena was cleaned with 90% ethanol solution between tests.

2.5. Sucrose sensitivity

To evaluate whether the drugs used affect sucrose processing, we analyzed their impact on the bees' sensitivity to sucrose solution. A standard test for measuring sucrose responsiveness was used to this end (Pankiw & Page, 1999; Scheiner, Page, & Erber, 2004). A novel group of bees was used for each drug treatment. The highest dose of agonists and antagonists was used in these experiments. A dMF-treated group and an untreated group were run in parallel as controls to check for potential effects of the solvent alone (no dMF) on sucrose sensitivity.

After being collected, the bees were harnessed, fed and kept in the dark for 24 h and the pharmacological treatment was applied 30 min before the start of the sucrose responsiveness test. The test consisted in touching the bees' antennae with increasing sucrose concentrations (0.1%, 0.3%, 1%, 3%, 10% and 30%, w/w) every 4 min and measuring the presence (1) or absence (0) of a proboscis extension response within the first 5 s of each stimulation (Scheiner et al., 2004). Multiple responses during a sucrose stimulation were considered as a single response. To avoid potential sensitization due to the repeated stimulations with increasing sucrose concentrations, water was delivered on the antennae 2 min after each sucrose presentation (control stimulation). The presence or absence of PER to water was then recorded. At the end of the experiment, PER integrity was checked by stimulating the bees' antennae with a 50% sucrose solution. Non-responsive bees and inconsistent bees (responding only to low sucrose concentrations) were discarded from the analysis (< 5%).

2.6. Statistical analysis

During conditioning, a conditioned response (PER) was recorded if it occurred after the onset of the visual CS+ and before the onset of sucrose stimulation. For the visual CS−, a response was recorded if it occurred during the same corresponding period of visual stimulation. Responses were scored as 0 (no response) or 1 (PER response). Multiple responses during a visual stimulus presentation were considered as a single response. Acquisition performances were represented in terms of the percentage of bees responding to the CS+ and the CS−. They were analyzed using generalized linear mixed models (GLMM, R 3.1.2 (R Core team), lme4 package) with a repeated-measure design in a binomial family in which *pharmacological treatment*, *rewarded color* (Blue+ or Green+), *trial category* (CS+ or CS−) and *trial number* were treated as fixed effects; individuals were considered as a random factor. An interaction term between *trial category* (CS+ or CS−) and *trial number* was included. Non-significant terms were dropped sequentially and the significance of each factor was assessed with Likelihood Ratio tests (see Suppl. File S1).

In the retention tests, responses to the CS+ and to the CS− were compared within a group using a McNemar test. Bees were categorized as non-responsive (no response to either CS), learners (response to the CS+ and not to the CS−), non-learners (response to the CS− and not to the CS+) and generalists (responses to both CS). Between-groups comparisons were performed using a Fisher's exact test.

In the phototaxis tests, the number of bees that reached the light source within 2 min was compared between a given treatment and the control conditions using a Fisher exact Test. The locomotion speed was compared between treatment groups using a Kruskal-Wallis test.

Data from the sucrose-responsiveness tests were analyzed using generalized linear mixed models with a repeated-measure design in a binomial family. *Pharmacological treatment* and *sucrose concentrations*

were included as fixed effects while individuals were considered as a random factor (Suppl. File S1). Besides quantifying population responsiveness to increasing sucrose concentrations, we evaluated individual responsiveness via a gustatory sucrose score, which corresponds to the number of sucrose concentrations to which a bee responded (Scheiner et al., 2004). Thus, a non-selective bee responding to all six sucrose concentrations received a score of 6, while a selective bee responding only to the highest sucrose concentration received a score of 1. The number of responses of each bee to the water presentations was also quantified by means of a score varying between 0 and 6. Scores were compared between treatment groups using a Kruskal-Wallis test. A significance threshold of $p < 0.05$ was adopted for all analyses.

3. Results

3.1. Visual associative learning and retention in harnessed bees

Harnessed honey bees (n = 37) were conditioned to differentiate a rewarded color (CS+: Blue [B] or Green [G] in a group-balanced design) from a non-rewarded color (CS−: G or B, respectively). No significant difference was observed between the performance of bees trained with the B+/G− contingency (blue rewarded/green unrewarded) and that of bees trained with the G+/B- contingency (green rewarded/blue unrewarded). Neither during the acquisition (GLMM: *color* effect: df = 12, $\chi^2 = 0.23$, p = 0.63) nor during the retention test (Fisher's exact test: p = 0.50) did these groups differ. Results were, therefore, pooled and presented as a CS+/CS− discrimination (Fig. 1).

A significant proportion of bees learned to respond preferentially to the CS+ during the conditioning phase (GLMM: *CS+/CS−*trials*: df = 11, $\chi^2 = 14.61$, $p < 0.01$; Fig. 1A). This successful discrimination was confirmed by the retention test performed 1 h after conditioning (Fig. 1B) where bees responded significantly more to the CS+ than to the CS− (McNemar test; $\chi^2 = 5.14$; p = 0.02), with 18.9% of bees responding to the CS+ and not to the CS− (learners).

We conclude that our conditioning procedure results in appetitive visual-discrimination learning and retention. This protocol was then used to study the influence of different biogenic amines on the visual associative abilities of bees.

3.2. Octopaminergic modulation of visual associative learning and retention

The potential influence of octopaminergic signaling on visual-discrimination learning and memory was investigated by comparing the performance of control bees treated with a topical application of the solvent dMF with that of bees treated with a topical application of OA (0.105 mol·L^{-1} or 1.05 mol·L^{-1}; Fig. 2AB) or epinastine, an octopaminergic antagonist (0.035 mol·L^{-1} or 0.35 mol·L^{-1}; Fig. 2CD). All groups were run in parallel to allow direct comparison.

In all groups and treatments, the bees' responses did not vary significantly according to which color was rewarded or non-rewarded (n = 201; Acquisition: GLMM, *color* effect: df = 16, $\chi^2 = 0$, p = 1; Test: Fisher's exact test: p = 0.59). Performance was therefore analyzed as a CS+ vs. CS− discrimination. We found a significant influence of treatment on performances (GLMM, *treatment* effect: df = 15, $\chi^2 = 25.69$, $p < 0.001$). Bees from the control group (dMF treatment) specifically increased their responses to the CS+ across trials (n = 40; GLMM, *CS+/CS−*trials* effect: df = 11, $\chi^2 = 10.37$, p = 0.03; Fig. 2AC), thus showing significant visual learning performances. In the retention test, we observed significantly more learners (15%) than generalists (2.5%) and non-learners (0.5%) (n = 40; McNemar test, $\chi^2 = 4.17$; p = 0.04; Fig. 2B).

Honey bees treated with OA prior to conditioning also learned the visual discrimination, regardless of the concentration used (Acquisition: OA 0.105 mol·L^{-1}, n = 46; *CS+/CS−*trials* effect: df = 11, $\chi^2 = 30.44$, $p < 0.001$; OA 1.05 mol·L^{-1}, n = 36: df = 11, $\chi^2 = 25.90$, $p < 0.001$; Fig. 2A). In the retention test, a higher proportion of learners was observed after both OA treatments (OA 0.105 mol·L^{-1}, n = 46, 30.4% of learners, 6.5% of generalists and 2.17% of non-learners; $\chi^2 = 9.60$; $p < 0.01$; OA 1.05 mol·L^{-1}: n = 36, 38.8% of learners, 5.5% of generalists and 0.5% of non-learners; $\chi^2 = 12.07$; $p < 0.001$; Fig. 2B). Comparison with the control dMF

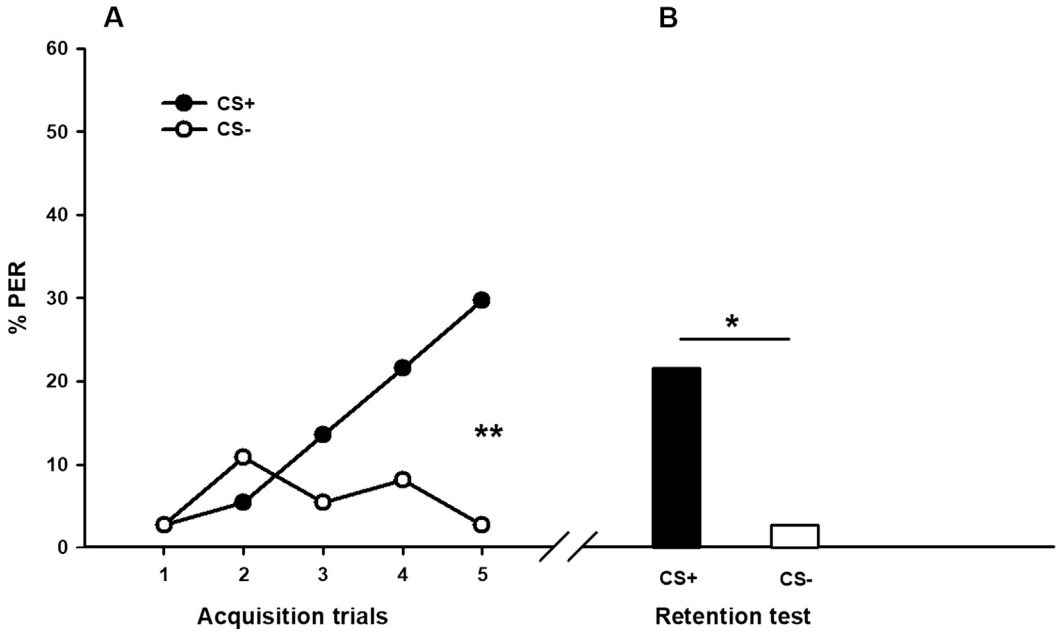

Fig. 1. Visual conditioning of the proboscis extension response (PER) in restrained honey bees. (A) Percentage of bees (n = 37) extending their proboscis (% PER) to a monochromatic light paired with sucrose (rewarded conditioned stimulus CS+; black circles) and to a different, non-rewarded monochromatic light (unrewarded conditioned stimulus CS−; white circles) during 10 conditioning trials (5 CS+ and 5 CS− trials). (B) Percentage of bees (n = 37) responding with PER to the conditioned stimuli during a non-rewarded retention test performed 1 h after the last conditioning trial (black bar: responses to the CS+; white bar: responses to the CS−). A significant proportion of bees learned to solve the discrimination task during the acquisition phase (GLMM: CS+/CS−*Trial effect: **: $p < 0.01$) and showed successful differentiation in the subsequent retention test (McNemar test: *: $p < 0.05$).

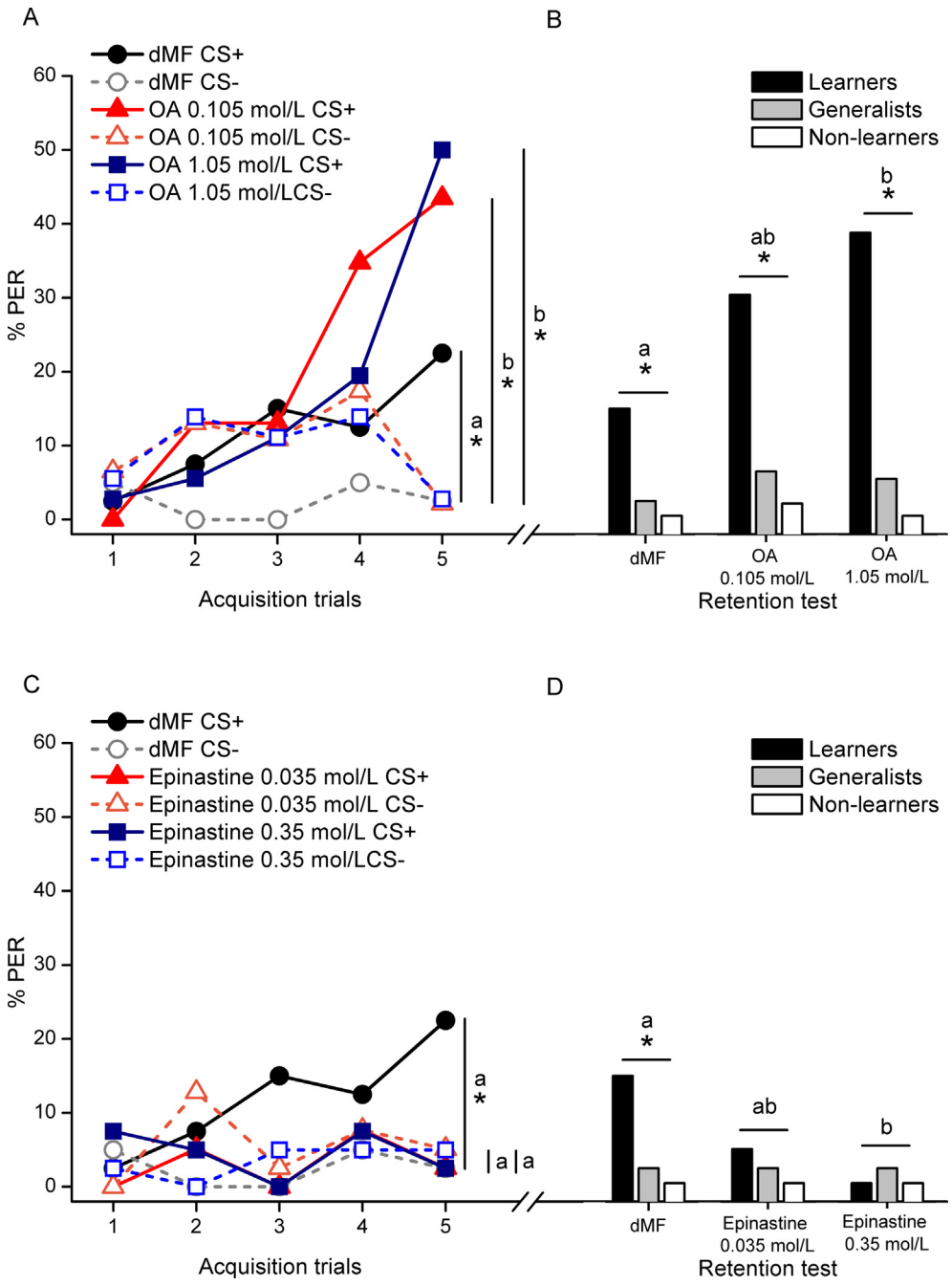

Fig. 2. Octopaminergic modulation of visual associative learning and retention in honey bees. (A) Visual learning performances (% PER to the CS+ and the CS−) during acquisition of the control group treated with dMF (n = 40) and of the two groups treated with two different doses of OA (0.105 mol·L^{-1}: n = 46; 1.05 mol·L^{-1}: n = 36). Dashed vertical lines at the end of acquisition indicate the discrimination level reached by each group. (B) Retention performances of the dMF group and of the two OA groups (0.105 mol·L^{-1} and 1.05 mol·L^{-1}) during a test presenting both the CS+ and the CS− without reward 1 h after the last conditioning trial. Bars represent the proportion of bees within three categories: learners (bees responding to the CS+ and not to the CS−); generalists (bees responding to both the CS+ and the CS−) and non-learners (bees responding to the CS− and not to the CS+). (C) Same as in (A) but for bees treated with dMF or with the two doses of the OA-receptor antagonist epinastine (0.035 mol·L^{-1}: n = 39; 0.35 mol·L^{-1}: n = 40). (D) Retention tests performed 1 h after conditioning for bees treated with dMF or with the two doses of the OA-receptor antagonist epinastine (0.035 mol·L^{-1}: n = 39; 0.35 mol·L^{-1}: n = 40). Bars represent the proportion of bees within three categories: learners, generalists and non-learners. These results suggest that OA treatment improved visual learning and retention while epinastine impaired them. Significant learning performance (Acquisition: GLMM: *CS+/CS−*Trial* effect; Test: McNemar test; $p < 0.05$) are indicated by an asterisk. Significant performance level differences between groups (Acquisition: GLMM: *Treatment* effect; Test: Fisher's exact test; $p < 0.05$) are indicated by different letters.

group showed that both OA concentrations improved the acquisition of the visual discrimination (GLMM, *treatment* effect: dMF vs. OA 0.105 mol·L^{-1}: df = 12, $\chi^2 = 6.30$, p = 0.01; dMF vs. OA 1.05 mol·L^{-1}: df = 12, $\chi^2 = 4.69$, p = 0.03; Fig. 2A). In the test, only the treatment with the highest OA dose increased significantly the proportion of learners compared to the dMF control group (OA 0.105 mol·L^{-1} vs. dMF: p = 0.13; OA 1.05 mol·L^{-1} vs. dMF: p = 0.02; Fig. 2B). Thus, enhancing OA signaling improves acquisition and retention performances in visual-discrimination learning.

By contrast, blocking OA signaling with epinastine impaired learning and retention performances (Fig. 2CD). Independent of the dose applied, epinastine-treated bees were not able to learn the discrimination between the CS+ and the CS− during the acquisition phase (*CS+/CS−*trials* effect: epinastine 0.035 mol·L^{-1}: n = 39, df = 11, $\chi^2 = 1.62$, p = 0.80; epinastine 0.35 mol·L^{-1}: n = 40, df = 11, $\chi^2 = 7.69$, p = 0.10; Fig. 2C). In the retention tests, the proportion of learners did not differ from that of non-learners and generalists (epinastine 0.035 mol·L^{-1}: n = 39, 5.1% of learners, 2.5% of generalists and 0.5% of non-learners; $\chi^2 = 0$; p = 1; epinastine 0.35 mol·L^{-1}: n = 40, 0.5% of learners, 2.5% of generalists and 0.5% of non-learners; $\chi^2 = 0$; p = 1; Fig. 2D). No difference between the epinastine groups and the control group was observed during acquisition (epinastine 0.035 mol·L^{-1} vs. dMF: df = 12, $\chi^2 = 0.98$, p = 0.32; epinastine 0.35 mol·L^{-1} vs. dMF: df = 12, $\chi^2 = 1.54$, p = 0.21; Fig. 2C) but a significant difference was observed in the retention test for the highest dose of epinastine (epinastine 0.035 mol·L^{-1} vs. dMF: p = 0.37; epinastine 0.35 mol·L^{-1} vs. dMF: p = 0.02; Fig. 2D).

Taken together these results show that treatments with OA and with an OA receptor antagonist induce an enhancement and an impairment of visual learning and retention, respectively. The octopaminergic pathway is thus involved in visual associative learning in honey bees.

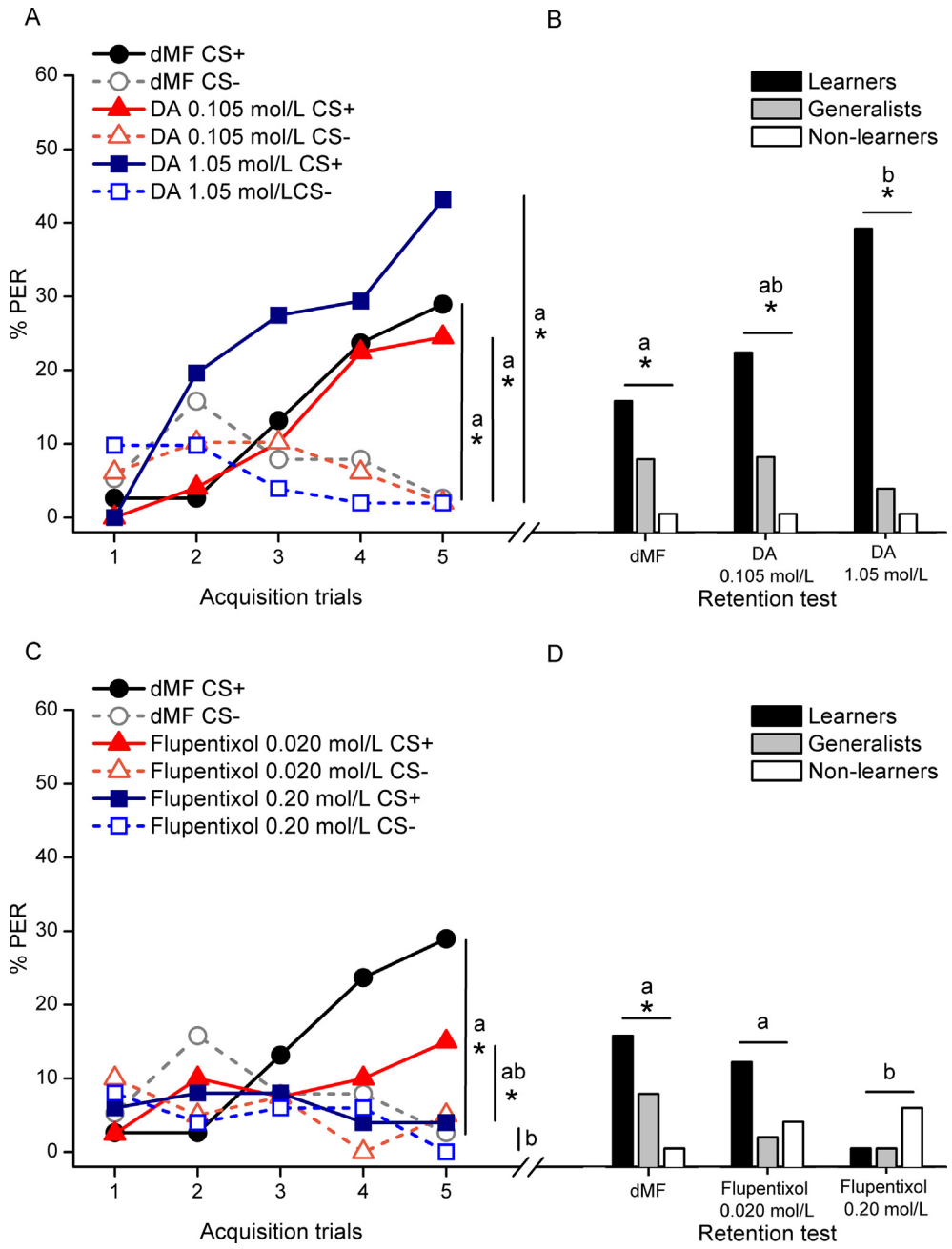

Fig. 3. Dopaminergic modulation of visual associative learning and retention in honey bees. (A) Visual learning performances (% PER to the CS+ and the CS−) during acquisition of the control group treated with dMF (n = 38) and of the two groups treated with two different doses of DA ($0.105\,mol{\cdot}L^{-1}$: n = 49; $1.05\,mol{\cdot}L^{-1}$: n = 51). Dashed vertical lines at the end of acquisition indicate the discrimination level reached by each group. (B) Retention performances of the dMF group and of the two DA groups ($0.105\,mol{\cdot}L^{-1}$ and $1.05\,mol{\cdot}L^{-1}$) during a test presenting both the CS+ and the CS− without reward 1 h after the last conditioning trial. Bars represent the proportion of bees within three categories: learners; generalists and non-learners. (C) Same as in (A) but for bees treated with dMF or with the two doses of the DA-receptor antagonist flupentixol ($0.020\,mol{\cdot}L^{-1}$: n = 49; $0.20\,mol{\cdot}L^{-1}$: n = 50). (D) Retention tests performed 1 h after conditioning for bees treated with dMF or with the two doses of the DA-receptor antagonist flupentixol ($0.020\,mol{\cdot}L^{-1}$: n = 49; $0.20\,mol{\cdot}L^{-1}$: n = 50). Bars represent the proportion of bees within three categories: learners, generalists and non-learners. These results suggest that DA treatment improved visual learning and retention while flupentixol impaired them. Significant learning performance (Acquisition: GLMM: *CS+/CS−*Trial* effect; Test: McNemar test; $p < 0.05$) are indicated by an asterisk. Significant performance level differences between groups (Acquisition: GLMM: *Treatment* effect; Test: Fisher's exact test; $p < 0.05$) are indicated by different letters.

3.3. Dopaminergic modulation of visual associative learning and retention

The role of dopaminergic signaling on visual-discrimination learning and retention was studied by comparing the performance of control bees treated with solvent with that of bees treated with topical application of DA ($0.105\,mol{\cdot}L^{-1}$ and $1.05\,mol{\cdot}L^{-1}$; Fig. 3AB) or with flupentixol, a dopamine receptor antagonist ($0.020\,mol{\cdot}L^{-1}$ and $0.20\,mol{\cdot}L^{-1}$; Fig. 3CD).

In all groups and treatments, the bees' responses did not vary significantly according to which color was rewarded or non-rewarded (n = 237; Acquisition: *color* effect: df = 16, $\chi^2 = 1.27$, p = 0.26; Test: p = 0.30). Performances were therefore pooled and treated as a CS+ vs. CS− discrimination. The different pharmacological treatments had an influence on learning and retention abilities (*treatment* effect: df = 15, $\chi^2 = 13.83$, $p < 0.01$). Control bees (dMF) learned to respond more to the CS+ than to the CS− during the acquisition phase (n = 38; *CS+/CS−*trials* effect: df:11, $\chi^2 = 16.64$, $p < 0.01$; Fig. 3AC). They also showed successful differentiation in the retention test where the proportion of learners was higher than that of generalists and non-learners (n = 38, 15.8% of learners, 7.9% of generalists and 0.5% of non-learners; $\chi^2 = 5.14$; p = 0.02; Fig. 3BD). They thus learned and memorized the association between the CS+ and the sucrose reward and between the CS− and the absence of reward.

Bees treated with both concentrations of DA (Fig. 3AB) were also able to learn the discrimination as shown by the significant increase of selective responses to the CS+ during the acquisition phase (DA $0.105\,mol{\cdot}L^{-1}$, n = 49; *CS+/CS−*trials* effect: df = 11, $\chi^2 = 21.55$, $p < 0.001$; DA $1.05\,mol{\cdot}L^{-1}$, n = 51: df = 11, $\chi^2 = 37.09$, $p < 0.001$; Fig. 3A). In both cases, performance was not significantly different from that of the control group (DA $0.105\,mol{\cdot}L^{-1}$ vs. dMF: *treatment* effect: df = 12, $\chi^2 = 0.14$, p = 0.70; DA $1.05\,mol{\cdot}L^{-1}$vs. dMF: *treatment* effect: df = 12, $\chi^2 = 1.70$, p = 0.19) even if responses to the CS+ were always higher in the DA $1.05\,mol{\cdot}L^{-1}$ group than in the control group (Fig. 3A). One hour later, DA-treated bees remembered better the

discrimination as the proportion of learners increased with the dose of DA (DA 0.105 mol·L^{-1}: n = 49, 22.4% of learners; $\chi^2 = 9.09$; $p < 0.01$; DA 1.05 mol·L^{-1}: n = 51, 39.2% of learners; $\chi^2 = 18.05$; $p < 0.001$; Fig. 3B). Retention of the DA 1.05 mol·L^{-1}group was significantly higher than that of the control group (DA 0.105 mol·L^{-1} vs. dMF: p = 0.67; DA 1.05 mol·L^{-1} vs. dMF: p = 0.01; Fig. 3B). Thus, DA had a significant positive impact on the retention of a visual discrimination.

Blocking dopaminergic signaling with the highest dose of flupentixol impaired visual learning as in this case bees could not learn the CS+ vs. CS− discrimination (flupentixol 0.20 mol·L^{-1}, acquisition, *CS+/CS−*trials* effect: n = 50: df = 11, $\chi^2 = 3.82$, p = 0.43; Fig. 3C). Therefore, in the retention test, only 0.5% of learners, 0.5% of generalists and 6% of non-learners were found (n = 50, $\chi^2 = 1.33$, p = 0.25; Fig. 3D). On the contrary, bees treated with the lower dose of flupentixol learned the visual discrimination (flupentixol 0.020 mol·L^{-1}, n = 49; *CS+/CS−*trials* effect: df = 11, $\chi^2 = 10.47$, p = 0.03; Fig. 3C). The retention test showed a higher proportion of learners (12.2%) but it was not sufficient to observe significant differences with generalists (2%) and non-learners (4.1%) (n = 49; $\chi^2 = 1.13$, p = 0.29; Fig. 3D). Only the highest dose of flupentixol induced a significant decrease in performance compared to the control group, both for the acquisition (flupentixol 0.020 mol·L^{-1} vs. dMF: df = 12, $\chi^2 = 1.43$, p = 0.23; flupentixol 0.20 mol·L^{-1} vs. dMF: df = 12, $\chi^2 = 4.45$, p = 0.03; Fig. 3C) and the retention (flupentixol 0.020 mol·L^{-1} vs. dMF: p = 0.19; flupentixol 0.20 mol·L^{-1} vs. dMF: $p < 0.001$; Fig. 3D).

Thus, while enhancing DA signaling improved retention in the case of the highest DA dose, blocking it with the highest dose of flupentixol impaired both acquisition and retention. This suggests that dopaminergic signaling participates in visual associative learning and retention in honey bees.

3.4. Serotonergic modulation of visual associative learning and retention

We finally studied the role of 5-HT in visual-discrimination learning and retention. To this end, we treated bees with either 5-HT (Fig. 4AB) or with methiothepin (Fig. 4CD), a 5HT antagonist. Performance of these groups was compared to that of a control group treated with dMF solvent. As in previous experiments, the color contingency (B+/G− or G+/B−) did not have an impact on the bees' performance (n = 199; Acquisition: *color* effect: df = 16, $\chi^2 = 1.49$, p = 0.22; Retention Test: p = 0.70) thus allowing the pooling of each group's performance in terms of a CS+ vs. CS− discrimination.

Treating the bees with either 5-HT or methiothepin resulted in different levels of visual associative learning performances compared to control bees (*treatment* effect: df = 15, $\chi^2 = 13.05$, p = 0.01). Control bees treated with dMF learned the visual discrimination during the acquisition phase (n = 43; *CS+/CS−*trials* effect: df = 11, $\chi^2 = 17.87$, $p < 0.01$; Fig. 4AC) but, contrarily to the other control groups tested in parallel to OA/epinastine- and DA/flupentixol-treated bees, they did not respond more to the CS+ than to the CS− in the 1 h retention test. In fact, the proportion of learners in the test was lower than what was expected given the level of discrimination reached in the last conditioning trial (n = 43, 9.3% of learners responding selectively to the CS+; $\chi^2 = 0$, p = 1; Fig. 4BD).

Treating bees with the higher 5-HT dose impaired acquisition as these bees did not differentiate the CS+ from the CS− contrary to control bees (5-HT 0.94 mol·L^{-1}, n = 39; *CS+/CS−*trials effect*: df = 11, $\chi^2 = 6.66$, p = 0.15; 5-HT 0.94 mol·L^{-1} vs. dMF: *treatment effect*: df = 12, $\chi^2 = 4.95$, p = 0.03; Fig. 4A). By contrast, bees treated with the lower dose of 5HT learned to discriminate the CS+ from the CS− (5-HT 0.094 mol·L^{-1}, n = 42; CS+/CS−*trials effect: df = 11, $\chi^2 = 9.63$, p = 0.04; 5-HT 0.094 mol·L^{-1} vs. dMF: *treatment* effect: df = 12, $\chi^2 = 3.13$, p = 0.08; Fig. 4A). Similar results were observed in the retention test (Fig. 4B), as a higher proportion of learners was only found for the lowest dose of 5-HT (5-HT 0.094 mol·L^{-1}: n = 42, 19% of learners, 0.5% of generalists and 0.5% of non-learners; $\chi^2 = 6.13$, p = 0.01; 5-HT 0.94 mol·L^{-1}: n = 39, 17.9% of learners, 0.5% of generalists and 7.7% of non-learners; $\chi^2 = 0.90$, p = 0.34; Fig. 4B).

Interestingly, blockade of serotonergic signaling via methiothepin also impaired visual associative learning and retention (Fig. 4CD). Bees treated with both methiothepin doses did not learn the visual discrimination (methiothepin 0.022 mol·L^{-1}: Acquisition: n = 38, df = 11, $\chi^2 = 4.41$, p = 0.35; methiothepin 0.22 mol·L^{-1}: Acquisition: n = 37, df = 11, $\chi^2 = 6.45$, p = 0.17; Fig. 4C). Accordingly, for both doses, the proportion of selective responses to the CS+ during acquisition was significantly lower than in the control group (methiothepin 0.022 mol·L^{-1} vs. dMF: df = 12, $\chi^2 = 11.10$, $p < 0.001$; methiothepin 0.22 mol·L^{-1} vs. dMF: df = 12, $\chi^2 = 6.92$, $p < 0.01$; Fig. 4C). During the retention tests (Fig. 4D), no significant enhancement of learners could be observed for both methiothepin doses (methiothepin 0.022 mol·L^{-1}: n = 36, 11.1% of learners, 5.5% of generalists and 2.8% of non-learners; $\chi^2 = 0.25$, p = 0.62; methiothepin 0.22 mol·L^{-1}: n = 37, 10.8% of learners, 10.8% of generalists and 0.5% of non-learners; $\chi^2 = 2.15$, p = 0.13; Fig. 4D). Test performances did not differ from that of the control group, which as mentioned above, was deficient in terms of discrimination (methiothepin 0.022 mol·L^{-1} vs. dMF: p = 0.57; methiothepin 0.22 mol·L^{-1} vs. dMF: p = 0.50; Fig. 4D). We conclude that both enhancing and inhibiting serotonergic neurotransmission impaired the learning and retention of visual associations.

3.5. Aminergic modulation of phototaxis

In order to evaluate whether the observed effects of the aminergic treatments on visual learning performance could be due to enhanced or depressed visual sensitivity, we measured bees' responsiveness to light in a standard phototaxis test after treating them with the biogenic amines of interest or with the respective receptor antagonists. For these tests, bees were placed within an arena where attraction towards the corner displaying a light source was measured. Only the higher dose of each compound was used in these experiments as significant effects on associative learning and retention were only observed for such doses. A group treated with the solvent alone (n = 10) and an untreated group (no dMF; n = 10) were used as controls to check for potential effects of the solvent alone on light responsiveness.

Most control bees (100% of the untreated bees and 90% of the dMF group) were able to reach the light source within the two minutes of the test (Fig. 5A). Treatment with the dMF solvent, OA, epinastine, DA and flupentixol did not affect phototactic behaviour (Fisher's exact test: no dMF vs. dMF: p = 1; dMF vs. OA: p = 1; dMF vs. epinastine: p = 1; dMF vs. DA: p = 0.27; dMF vs. flupentixol: p = 1; Fig. 5A). Yet, 5-HT and methiothepin diminished significantly the percentage of bees reaching the light source within the two minutes of the test (Fisher's exact test: dMF vs. 5-HT: p = 0.02; dMF vs. methiothepin: p = 0.02; Fig. 5A). This deficient phototaxis was not the consequence of an impaired locomotion, since the mean walking speed did not differ between treatment groups (Kruskal-Wallis tests; no dMF/dMF/OA/epinastine: K = 5.35, df:3, p = 0.15; no dMF/dMF/DA/flupentixol: K = 5.88, df:3, p = 0.12; no dMF/dMF/5-HT/methiothepin: K = 7.94, df:3, p = 0.05; Fig. 5B). Thus, enhancing and inhibiting serotonergic signaling had a detrimental impact on the bees' responsiveness to the light source. This impairment may be the result of a defective visual perception, which could explain the decay of visual learning and retention found upon interference with 5-HT signaling.

3.6. Aminergic modulation of sucrose responsiveness

We finally determined if our pharmacological treatments have a direct influence on the bees' sensitivity to sucrose by comparing sucrose responsiveness to increasing sucrose concentrations between groups treated with OA, DA, 5-HT and with the respective receptor antagonists.

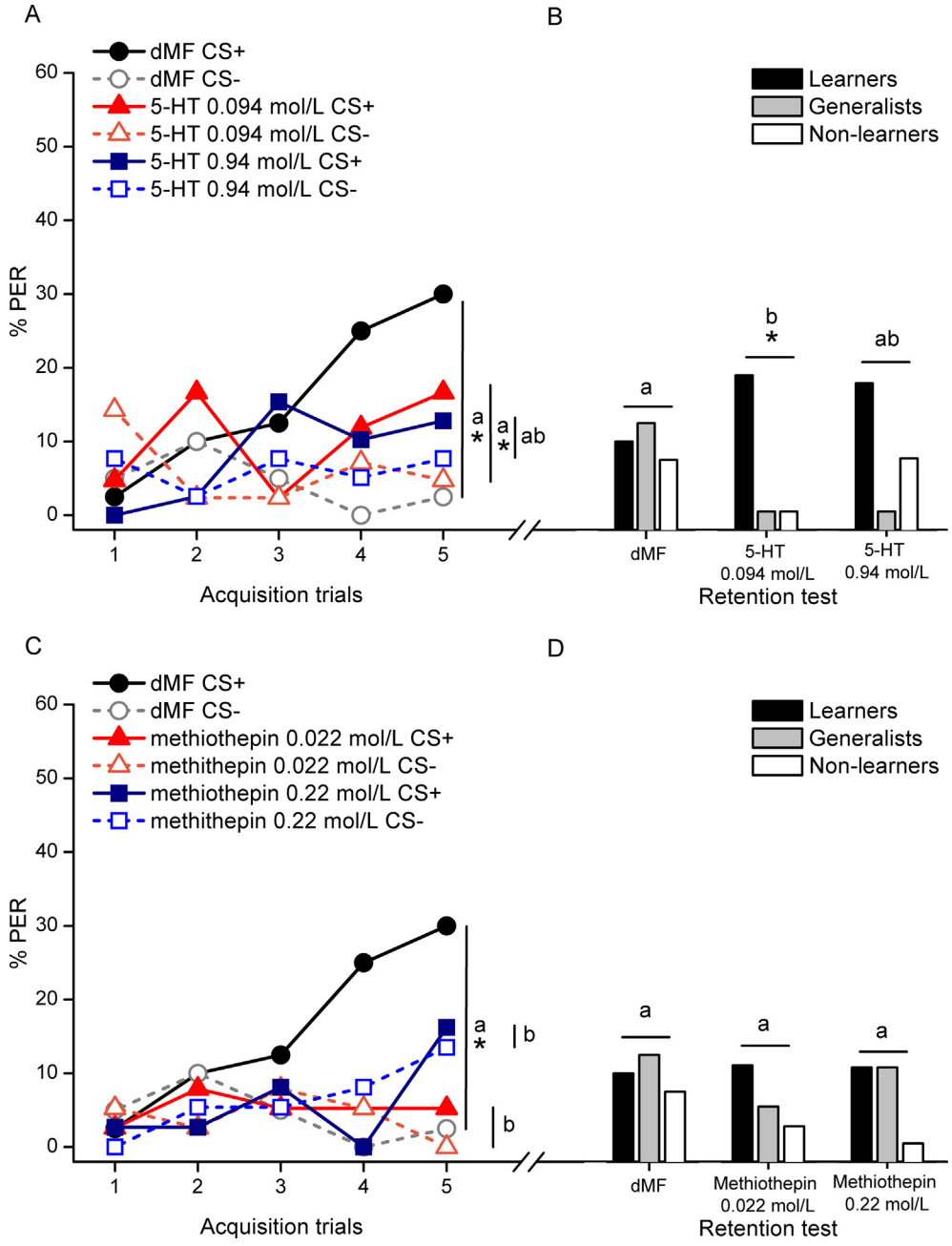

Fig. 4. Serotonergic modulation of visual associative learning and retention in honey bees. (A) Visual learning performances (% PER to the CS+ and the CS−) during acquisition of the control group treated with dMF (n = 43) and of the two groups treated with two different doses of 5-HT ($0.094\ mol{\cdot}L^{-1}$: n = 42; $0.94\ mol{\cdot}L^{-1}$: n = 39). Dashed vertical lines at the end of acquisition indicate the discrimination level reached by each group. (B) Retention performances of the dMF group and of the two 5-HT groups ($0.094\ mol{\cdot}L^{-1}$ and $0.94\ mol{\cdot}L^{-1}$) during a test presenting both the CS+ and the CS− without reward 1 h after the last conditioning trial. Bars represent the proportion of bees within three categories: learners; generalists and non-learners. (C) Same as in (A) but for bees treated with dMF or with the two doses of the 5-HT-receptor antagonist methiothepin ($0.022\ mol{\cdot}L^{-1}$: n = 38; $0.22\ mol{\cdot}L^{-1}$: n = 37). D) Retention tests performed 1 h after conditioning for bees treated with dMF or with the two doses of the 5-HT-receptor antagonist methiothepin ($0.022\ mol{\cdot}L^{-1}$: n = 38; $0.22\ mol{\cdot}L^{-1}$: n = 37). Bars represent the proportion of bees within three categories: learners, generalists and non-learners. These results suggest that both 5-HT and methiothepin treatments impaired visual learning and retention. Significant learning performance (Acquisition: GLMM: *CS+/CS− *Trial* effect; Test: McNemar test; $p < 0.05$) are indicated by an asterisk. Significant performance level differences between groups (Acquisition: GLMM: *Treatment* effect; Test: Fisher's exact test; $p < 0.05$) are indicated by different letters.

Sucrose responsiveness increased with sucrose concentration in all groups (Fig. 6A). No significant effect of OA/epinastine or 5-HT/methiothepin treatments was found (GLMM: *treatment*sucrose concentration* effect: no dMF/dMF/OA/epinastine: n = 123, df:25, $\chi^2 = 18.67$, p = 0.23; no dMF/dMF/5-HT/methiothepin: n = 129, df:20, $\chi^2 = 18.93$, p = 0.22; Fig. 6A), although there was a tendency for the OA-treated group to show a higher level of responses for intermediate sucrose concentrations. A significant variation of sucrose responsiveness was observed for the DA/flupentixol treatment (no dMF/dMF/DA/flupentixol: n = 128, df:10, $\chi^2 = 26.30$, p = 0.03; Fig. 6A). This effect was due to flupentixol (flupentixol vs. dMF: n = 86, df:13, $\chi^2 = 18.64$, $p < 0.01$; DA vs. dMF: n = 85, df:13, $\chi^2 = 3.90$, p = 0.56; no dMF vs. dMF: n = 73, df:7, $\chi^2 = 0.004$, p = 0.94; Fig. 6A).

The analysis of individual responsiveness scores confirmed the absence of effects in the OA/epinastine, the DA/flupentixol groups and the 5-HT/methiothepin groups (Kruskal-Wallis tests; no dMF/dMF/OA/epinastine: K = 1.62, df:3, p = 0.65; no dMF/dMF/DA/flupentixol: K = 1.50, df:3, p = 0.68; no dMF/dMF/5-HT/methiothepin: K = 0.97, df:3, p = 0.81;Fig. 6B). Thus, the differences found in the DA/flupentixol groups at the population level were not maintained in this individual analysis.

Finally, aminergic treatments did not affect water responsiveness (not shown), which was low in all groups (average water response scores of 1; no dMF/dMF/OA/epinastine: K = 7.18, df:3, p = 0.07; no dMF/dMF/DA/flupentixol: K = 1.50, df:3, p = 0.68; no dMF/dMF/5-HT/methiothepin: K = 0.05, df:3, p = 0.99),). Taken together, our findings revealed that our drug treatments had no or marginal influence on sucrose and water responsiveness.

4. Discussion

We combined visual conditioning of harnessed bees with pharmacological analyses in order to study the role of biogenic amines as neuromodulators of appetitive visual learning and retention. We first

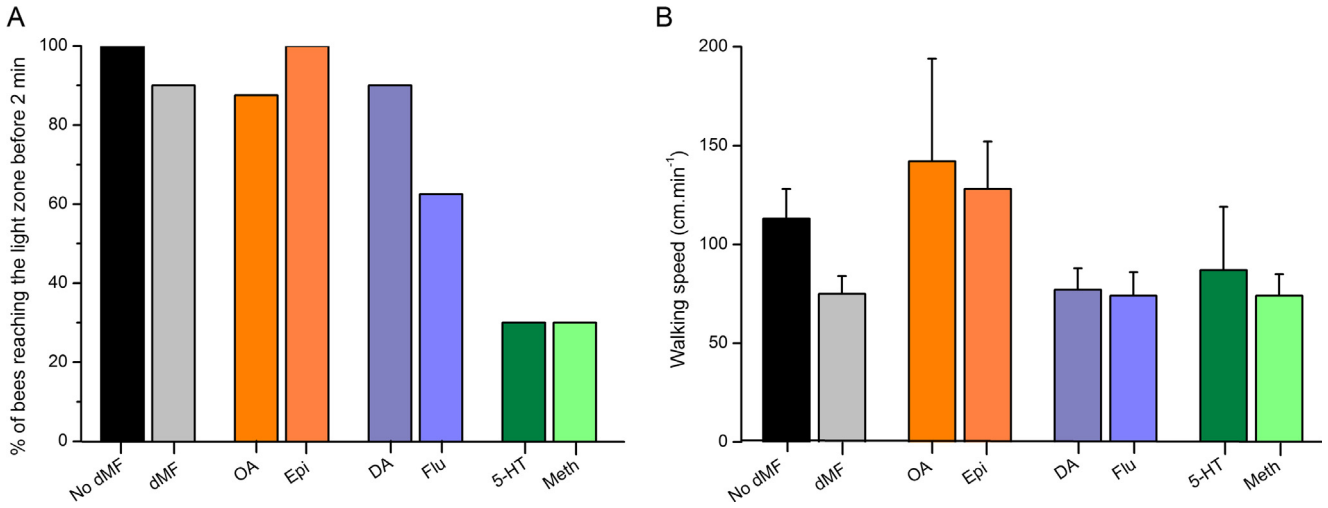

Fig. 5. Aminergic modulation of phototaxis in honey bees. (A) Percentage of bees reaching the light zone of an experimental arena within the 2 min of a phototaxis assay. Bees were untreated (no dMF, n = 10) or treated with either dMF (n = 10) or with the highest dose of OA, DA, 5-HT or their respective receptor antagonists, epinastine (Epi), flupentixol (Flup) and methiothepin (Meth) used in visual conditioning experiments (OA: n = 10; epinastine: n = 8; DA: n = 8; flupentixol: n = 10; 5-HT: n = 10; methiothepin: n = 10). Only bees treated with 5-HT or methiothepin showed phototaxis deficits compared to the control dMF group, as a significantly lower percentage of these bees oriented towards the light. Different letters indicate significant differences between groups (Fisher's exact test; $p < 0.05$). (B) Walking speed ($cm{\cdot}min^{-1}$) of bees in the arena under the different pharmacological treatments (sample sizes and drug concentrations as above). No difference in walking speed was observed between groups, thus showing that the drugs had no effect on locomotion but affected instead visual processing. Different letters indicate significant differences between groups (Kruskal-Wallis tests; $p < 0.05$).

showed that control bees learn to discriminate a rewarded color from a non-rewarded color and retrieve the discrimination memory one hour after acquisition. Our pharmacological analyses showed that enhancing octopaminergic signaling improved visual learning and retention, while inhibiting it impaired these capacities (Fig. 2). Similarly, enhancing dopaminergic signaling improved visual retention, while blocking it impaired both acquisition and retention (Fig. 3). Importantly, these treatments did neither affect visual responsiveness in a phototaxis assay (Fig. 5) nor sucrose responsiveness (Fig. 6), thus suggesting that OA and DA, and their respective antagonists, acted on the association between colors used as CS and sucrose used as US, rather than on their separate processing. A different conclusion can be reached for 5-HT as in this case both enhancing and inhibiting serotonergic signaling induced a comparable impairment of visual learning (Fig. 4). The fact that a similar result was obtained in a phototaxis assay (Fig. 5) indicates that in this case the pharmacological treatments may have interfered with visual processing rather than with the CS−US association.

4.1. The role of OA in visual associative learning and retention

Our results showed that application of an OA agonist or antagonist induced, respectively, an enhancement or an impairment of visual learning and retention (Fig. 2), which is consistent with the role of OA as a neurotransmitter mediating the reinforcement properties of sucrose in honey bee appetitive learning (Hammer, 1997). Interfering with US signaling is expected to affect learning about that US (Scheiner et al., 2006), and this is what we found in our experiments. Yet, we did not find that OA or epinastine affected sucrose responsiveness (Fig. 6), contrary to previous findings (Scheiner, Plückhahn, Öney, Blenau, & Erber, 2002; Scheiner et al., 2006). Nevertheless, a tendency to increase sucrose responsiveness upon OA treatment was observed (Fig. 6A).

A number of studies support the notion that OA mediates sucrose reinforcing properties in the honey bee brain. For instance, a single identified octopaminergic neuron, VUMmx1 (for "ventral unpaired median neuron of maxillary neuromere 1"), conveys the reward signal to olfactory regions in the bee brain (Hammer, 1993). The impact of OA and epinastine on visual learning and retention suggests that one or more octopaminergic neurons may convey information about the sucrose reward to visual areas of the bee brain. However, none of the ten VUM neurons described anatomically in the bee brain project to the optic lobes nor the collar (receiving visual input) of the mushroom bodies (Schröter et al., 2007). It is nevertheless possible that other non-identified octopaminergic neurons convey the reward signal to the visual neuropiles of the bee brain. OA receptors have been indeed identified in these neuropils although they may be expressed in other types of neurons, in particular in GABAergic, inhibitory neurons (Sinakevitch et al., 2011).

OA has been repeatedly associated with appetitive US representation in visual and olfactory learning of bees and crickets (Hammer, 1993; Hammer & Menzel, 1998; Farooqui et al., 2003; Unoki, Matsumoto, & Mizunami, 2005). Experiments on innate responsiveness (PER) to sucrose solution in bees have also shown that sucrose sensitivity depends on OA signaling (Scheiner et al., 2002; Pankiw & Page, 2003; Scheiner et al., 2006). Why then did we not observe any clear effect of OA or epinastine on this same responsiveness? A main difference between these works and our experiments lies in the method of drug delivery. While experiments showing an enhancement of sucrose responsiveness following OA delivery injected this amine in the thorax (Scheiner et al., 2002) or added it in the food (Pankiw & Page, 2003), we applied it topically onto the thorax to avoid damaging the bees with an injection. Although this method has proved to be an effective non-invasive method for short-term, systemic treatment with OA (Barron et al., 2007), it may result in less drug passing through the cuticula into the nervous system. This could explain why we would not observe significant effects but only a tendency towards enhanced sucrose responsiveness upon OA treatment (Fig. 6A). The same rational could apply for our results on the absence of a significant effect of phototaxis following OA treatment in contradiction with previous findings (Scheiner et al., 2014).

4.2. The role of DA in visual associative learning and retention

In the honey bee, DA is considered as the neurotransmitter mediating aversive reinforcement signaling in the brain (Vergoz et al., 2007; Tedjakumala & Giurfa, 2013). Yet, its role in appetitive learning is less clear (Klappenbach et al., 2013). A similar picture emerges from research on cricket associative learning (Unoki et al., 2005; 2006; Mizunami et al., 2009; Awata et al., 2015; Awata et al., 2016).

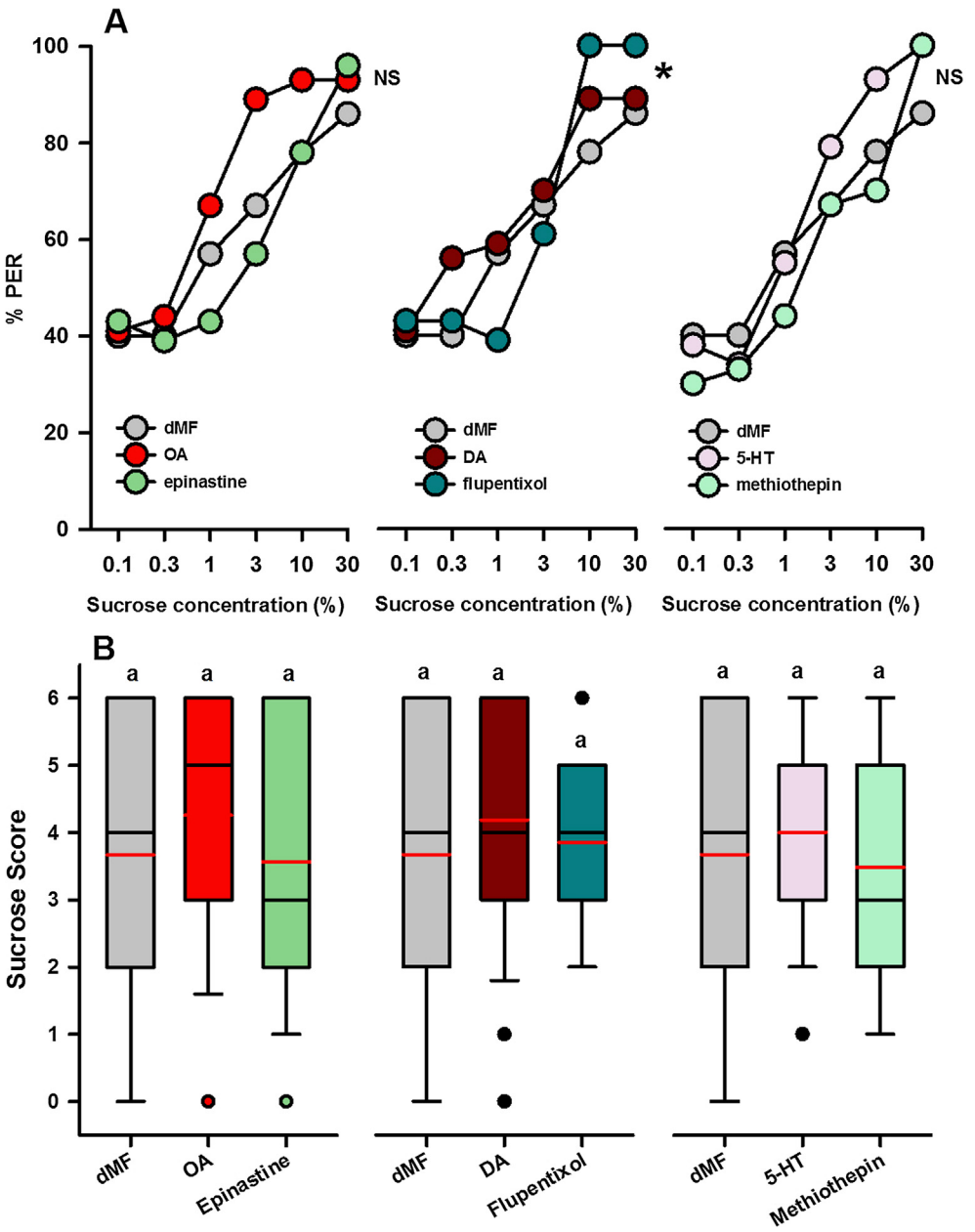

Fig. 6. Aminergic modulation of sucrose responsiveness in honey bees. (A) Cumulative proportions of bees showing PER when presented with the six sucrose solutions of increasing concentration (0.1%, 0.3%, 1%, 3%, 10% and 30% w/w). Control bees were untreated (no dMF, n = 15) or treated with dMF (n = 58). The other groups were treated with the highest dose of OA, DA, 5-HT or their respective receptor antagonists, epinastine (Epi), flupentixol (Flup) and methiothepin (Meth) used in visual conditioning experiments (OA: n = 27; epinastine: n = 23; DA: n = 27; flupentixol: n = 28; 5-HT: n = 29; methiothepin: n = 27). In all cases, responsiveness increased with sucrose concentration. Significant differences between drug groups and the control groups (GLMM: *treatment***sucrose concentration* effect: $p < 0.05$) are indicated by an asterisk. B) Individual sucrose scores. Median, quartiles and max and min (upper and lower whiskers) sucrose score values of bees subjected to the different drug treatments. The red line in each box represents the mean sucrose score value. For each bee, the sucrose score was established by measuring PER to a series of six sucrose solutions of increasing concentration (0.1%, 0.3%, 1%, 3%, 10%, and 30% w/w). Values ranged between six (bees responding to all six concentrations) and 0 (bees not responding to any concentration). Different letters indicate significant differences in scores between groups (Kruskal-Wallis tests; $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

However, in the fruit fly, DA is acknowledged as the main neurotransmitter signalling the appetitive US. In this insect, a subset of dopaminergic neurons (neurons of the PAM cluster, for 'Paired Anterior Medial'), which project to the mushroom bodies, were identified as conveying the sucrose reward signal, thus allowing an association between an olfactory or a visual stimulus with a sucrose reward (Burke et al., 2012; Liu et al., 2012; Vogt et al., 2014).

Our results showed that enhancing DA signaling improved visual retention (in the case of the highest DA dose) while blocking it (with the highest dose of flupentixol) impaired both visual learning and retention (Fig. 3). This suggests that dopaminergic signaling participates in visual associative learning and retention in honey bees. These results were unexpected according to prevailing scenario for US signaling in the bee brain (Vergoz et al., 2007; Tedjakumala & Giurfa, 2013; Tedjakumala et al., 2014). Yet, they could be understood if we assume that dopaminergic signaling either contributes to US representation, as in the case of the fruit fly, or mediates vision-related processing. As no effect of DA/flupentixol treatments was found on bees' responsiveness to light (Fig. 5), while a weak effect was observed for sucrose responsiveness (Fig. 6A), appetitive US signals conveyed to visual brain regions of the bee could be mediated by dopaminergic neurons, as demonstrated for olfactory learning in the fruit fly.

In the honey bee brain, several clusters of dopaminergic neurons have been described (Tedjakumala et al., 2017), some of which innervate key visual regions such as the central complex and the mushroom bodies (Tedjakumala et al., 2014; Tedjakumala et al., 2017), and even the lobula. Although this innervation pattern does not constitute a proof for a role in appetitive-reinforcement signaling, it shows the tight connectivity between visual and dopaminergic circuits, which could provide a value labeling for visual signals. If some of these dopaminergic neurons provided an appetitive signal, the absence of 'appetitive' OA neurons projecting directly to the visual areas of the bee brain (see above) could be understood.

Alternatively, our results may also reflect the role of dopaminergic neurons in attentional visual processes as demonstrated in *Drosophila* (van Swinderen & Andretic, 2011; Aptekar, Keleş, Lu, Zolotova, & Frye, 2015; Koenig, Wolf, & Heisenberg, 2016). In the bee brain, a neuronal cluster (C4) has been recently identified in the lobula (Tedjakumala

et al., 2014; Tedjakumala et al., 2017), which may provide attentional control of visual processing. However, the fact that no effect of DA or flupentixol on phototactic responses was observed suggests that these drugs acted rather on neural sucrose representation and/or on color-sucrose association.

4.3. The role of 5HT in visual associative learning and retention

Anatomical studies of the bee brain have described a large network of serotonergic fibers and a high density of serotonin binding sites in the three neuropils – lobula, medulla, lamina – of the optic lobes as well as in the mushroom bodies and central complex (Schürmann & Klemm, 1984; Brüning et al., 1987; Blenau & Thamm, 2011). In addition, localized brain injections of 5-HT decrease the bees' responsiveness to light (Thamm, Balfanz, Scheiner, Baumann, & Blenau, 2010), the activity of motion-sensitive neurons in the lobula (Kloppenburg & Erber, 1995) as well as the antennal movement reflex to moving visual cues (Erber & Kloppenburg, 1995). All these studies suggest an important role of 5-HT neurons on visual processing.

Accordingly, both enhancing and inhibiting serotonergic neurotransmission impaired visual-discrimination learning and retention (Fig. 4). Contrary to OA and DA, which participated in these tasks because they signal the appetitive US and/or mediate CS − US associativity, 5-HT seems to intervene as a neurotransmitter of visual circuits. Thus, the general impairment of visual learning observed upon injection of both agonists and antagonists of 5-HT receptors was probably due to deficits in visual (CS) processing as an impairment of phototaxis was induced by both kinds of drugs (Fig. 5), which on the contrary left intact sucrose responsiveness (Fig. 6).

5. Conclusion

Our study represents the first attempt to couple neuropharmacological treatments with a visual conditioning protocol in harnessed bees. The discrimination task that bees had to learn was elemental as it consisted in learning that a color was rewarded with sucrose and that a different color was not rewarded. The method used to deliver drugs yielded clear results with the advantage of not damaging the bees, which under harnessing conditions do not always exhibit robust visual learning performances. Given the exceptional visual cognitive abilities of the honey bee (Avarguès-Weber, Deisig, & Giurfa, 2011; Avarguès-Weber & Giurfa, 2013), it is worth exploring if and how aminergic signaling contributes to non-elemental forms of learning that could be implemented in the harnessing preparation. Visual variants of non-linear discriminations, in which individuals have to learn that a compound is different from the sum of its constitutive elements (Giurfa, 2003), can be studied, as these learning forms require specific forms of inhibitory neurotransmission in the olfactory domain (Devaud et al., 2015). Our work constitutes a first step towards the study of the neural substrates of higher-order visual abilities, which have historically contributed to the reputation of the honey bee as an attractive model for studies on cognition.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.nlm.2018.05.014.

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