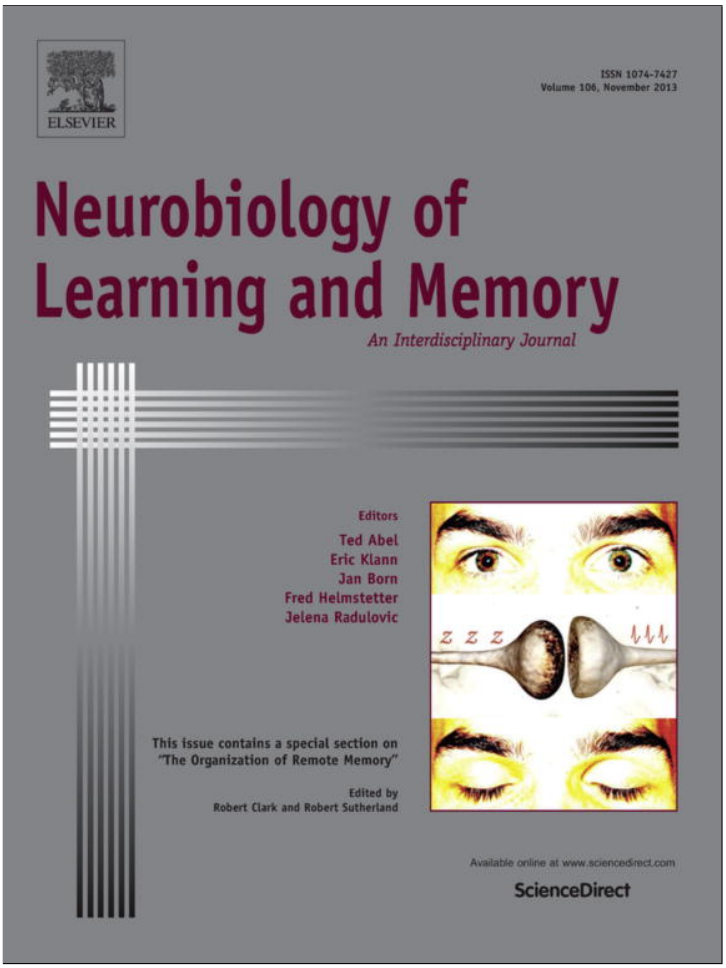

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Dopamine interferes with appetitive long-term memory formation in honey bees

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ABSTRACT

Studies in vertebrates and invertebrates have proved the instructive role that different biogenic amines play in the neural representation of rewards and punishments during associative learning. Results from diverse arthropods and using different learning paradigms initially agreed that dopamine (DA) is needed for aversive learning and octopamine (OA) is needed for appetitive learning. However, the notion that both amines constitute separate pathways for appetitive and aversive learning is changing. Here, we asked whether DA, so far only involved in aversive memory formation in honey bees, does also modulate appetitive memory. Using the well characterized appetitive olfactory conditioning of the proboscis extension reflex (PER), we show that DA impairs appetitive memory consolidation. In addition, we found that blocking DA receptors enhances appetitive memory. These results are consistent with the view that aversive and appetitive components interact during learning and memory formation to ensure adaptive behavior.

1. Introduction

The cellular and molecular mechanisms underlying associative learning are a major focus of research in neurobiology. Studies in vertebrates and invertebrates agree in the instructive role that different biogenic amines play in the processing of rewards and punishments during associative learning. Dopamine (DA) has been shown to be involved in reward seeking behavior and processing of appetitive reinforcement from humans to nematodes (Barron, Søvik, & Cornish, 2010; Schultz, 1997). Arthropods seem to be an exception in which DA has been mostly related with aversive learning, and instead the biogenic amine octopamine (OA) is considered the main neurotransmitter involved in reward processing and appetitive learning (Barron et al., 2010). Several studies based on genetically manipulated *Drosophila* indicated that DA and OA are necessary for aversive and appetitive learning (Aso et al., 2012; Claridge-Chang et al., 2009; Honjo & Furukubo-Tokunaga, 2009; Riemensperger et al., 2011; Schroll et al., 2006; Schwaerzel et al., 2003) and similar results were obtained in crickets *Gryllus bimaculatus* using agonists and antagonists of the OA- and DA-receptors (Unoki, Matsumoto, & Mizunami, 2005, 2006). Furthermore, we found in the crab *Chasmagnathus granulatus* that OA and DA are required for appetitive and aversive memory formation respectively (Kaczer & Maldonado, 2009; Klappenbach, Maldonado, Locatelli, & Kaczer, 2012).

The honey bee *Apis mellifera* is a well used model for studying the neural basis of appetitive and aversive learning. Two olfactory learning paradigms are commonly used to study appetitive and aversive olfactory learning and memory. In the appetitive paradigm, an olfactory stimulus predicts a sucrose reward applied to receptors on antennae or proboscis (Takeda, 1961). Associative learning becomes evident when the conditioned odor elicits the extension of the proboscis. A multiple-trials training protocol induces the formation of a long term-memory that lasts for several days (Menzel, 2001). Different experimental approaches (Farooqui, Robinson, Vaessin, & Smith, 2003; Hammer, 1997; Hammer & Menzel, 1998) coincide about the key role of OA in mediating the appetitive reinforcement during appetitive learning. In the aversive olfactory conditioning paradigm the odor is paired with an electric shock. After learning, the conditioned odor elicits a defensive response evident by the sting extension (Vergoz, Roussel, Sandoz, & Giurfa, 2007). The use of biogenic amines receptor antagonists revealed that this form of aversive learning depends on DA receptors while OA receptor antagonists showed no effect (Vergoz et al., 2007).

Recent studies have started to change the view that each amine is exclusively involved in only appetitive or aversive learning. The possibility to get precise control of the action of specific subsets of DAergic or OAergic neurons in Drosophila, revealed a specific subset of dopaminergic neurons that modulate persistence (Berry, Cervantes-Sandoval, Nicholas, & Davis, 2012) or retrieval of appetitive memory (Krashes et al., 2009). Furthermore, it was determined

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that certain dopaminergic neurons are also necessary for appetitive memory (Kim, Lee, & Han, 2007; Liu et al., 2012; Selcho, Pauls, Han, Stocker, & Thum, 2009) and that octopaminergic neurons synapse into a specific set of dopaminergic neurons which in turn mediate appetitive reinforcement necessary for learning (Burke et al., 2012). Using systemic drug administration we demonstrated in crabs, that in addition to its role in aversive learning, DA interferes with memory formation of a concurrent appetitive experience (Klappenbach et al., 2012), and that OA, only presumed to mediate appetitive learning, impairs long-term aversive memory formation (Kaczer & Maldonado, 2009). A similar interaction was recently reported in honey bees. OA, so far involved in appetitive learning in bees, has a detrimental effect on learning performance of aversive place conditioning (Agarwal et al., 2011) and DA was shown to mediate conditioned food aversion (Wright et al., 2010). These results suggest that octopaminergic and dopaminergic pathways interact during learning and memory formation.

In the present study, we evaluate if DA, so far involved in aversive learning in bees, also modulates appetitive memory formation. We use the well characterized appetitive olfactory conditioning of the proboscis extension reflex (PER) (Bitterman, Menzel, Fietz, & Schäfer, 1983). Using systemic administration of DA, a DA receptors agonist and a DA receptor antagonist we found that DA negatively modulates formation of olfactory appetitive memory. These findings shed light on the interaction of aversive and appetitive pathways during learning and memory formation.

2. Materials and methods

2.1. Animals

Honey bee (*A. mellifera*) pollen-foragers were collected at the entrance of two regular hives situated at the Campus of the University of Buenos Aires (34°32′S; 58°6′W). Bees were immobilized by shortly cooling them on ice and restrained in individual harnesses that allow movements of antennae and proboscis. After recovery from cooling, bees were fed 5 μl of 1.0 M sucrose solution and remained undisturbed until the evening when they were fed *ad libitum*. At the laboratory, bees were kept in a humid box at room temperature (20–24 °C) on a 12:12 h light:dark cycle. All experiments lasted 5 days including from capture to the test session 72 h after training and bees were fed to satiation every evening with 1 M sucrose solution. All training and testing sessions were carried out between 10:00 AM and 2:00 PM. Thirty minutes before training all animals passed a selection test that consisted in touching the antennae with 2.0 M sucrose solution. Only animals that showed a rapid and conspicuous extension of the proboscis were used in the experiments. In the experiment performed to measure ingestion volume, feeding was done using a micropipette that offered drops of 2 M sucrose solution onto the tip of the extended proboscis. Each animal was weighed using a precision microbalance immediately before and after feeding to determine the ingested volume.

2.2. Olfactory conditioning

The odor used for conditioning was 2-octanone diluted 1/100 in mineral oil (both from Sigma–Aldrich). A small strip of filter paper embedded with 10 μl of the odor dilution was inserted into a 20 ml cylindrical cartridge. One end of the cartridge was connected to a valve that controlled air flow into the cartridge. The output of the cartridge was positioned 3 cm in front of the bee's head and provided the odor-laden air stream that was used as conditioned stimulus. A gentle air exhaust placed 10 cm behind the bee continuously removed odors from the training arena.

Honey bees were subjected to olfactory conditioning of the proboscis extension reflex (Bitterman et al., 1983). During each training trial an animal was positioned in the training arena facing toward the odor delivery device. Twenty seconds later the odor started and lasted 4 s. Three seconds after odor onset the antennae were touched with a 2.0 M sucrose-solution which elicited the proboscis extension. When the proboscis was extended the sucrose solution was rapidly moved towards it and the bee was allowed to lick. Twenty seconds after the end of the reward the bee was returned to the rest position until the next trial. Two types of training protocols were used: a strong training that consisted of 4 trials separated by 5 min of inter-trial interval (Fig. 1a), and a weak training protocol that consisted of only one trial (Fig. 2a). The test sessions consisted of 4 s of odor presentation without reward. During training and test trials the response of each subject was recorded as positive if the subject extended its proboscis beyond a virtual line between the open mandibles during the stimulation with the odor and before stimulation with sucrose. Percentage of response was calculated as the number of bees that extend the proboscis over total number of bees assayed. Statistical comparison of performance during trainings with 4 trials was based on 2 factors repeated measures ANOVA (Friedrich, Thomas, & Müller, 2004), with drug/vehicle as one factor and trials 2, 3 and 4 as the repeated factor. Further statistical analysis performed to compare level of conditioned response at individual trials was based on least squares test for pair-wise comparisons (Mustard, Dews, Brugato, Dey, & Wright, 2012; Wright et al., 2010).

2.3. Drugs and injection procedure

In the present work, all animals in all experiments were injected either drug or vehicle. Injections were performed 15 min before or 15 min after the training session in the case of the 4-trials training protocols (Fig. 1a) and 15 min before training in the case one trial protocol (Fig. 2a). Injections consisted of 1 μl of drug dilution or vehicle injected into the thorax using a tabulated microcapillary (Felsenberg, Gehring, Antemann, & Eisenhardt, 2011). Drugs used were cis-(Z)-flupentixol dihydrochloride and dopamine hydrochloride, both from Sigma–Aldrich, and 6,7 ADTN hydrobromide from Santa Cruz Biotechnology. All drugs were fresh diluted before every experiment in injection buffer (5 mM KCl, 10 mM NaH_2PO_4, pH 7.8) (Mustard, Pham, & Smith, 2010) and kept on ice until injection.

3. Results

3.1. Dopamine interferes with appetitive long-term memory formation

Previous works on the role of DA in appetitive learning in honey bees reported that DA impairs appetitive conditioned response but not acquisition (Mercer & Menzel, 1982; Michelsen, 1988). More recent studies performed in bees have only focused their analysis on the role of DA on aversive learning (Agarwal et al., 2011; Vergoz et al., 2007; Wright et al., 2010). The aim of this work was to evaluate the role of DA in appetitive memory formation. First, we performed an experiment containing a group of bees injected with vehicle and three groups injected with different doses of DA. Fifteen minutes after injection all groups underwent a four-trials training protocol that is normally used to induce robust appetitive learning and memory retention over days (Menzel, 2001) As shown in Fig. 1b, similar acquisition curves were obtained during training for vehicle and DA injected bees (repeated measures ANOVA; group factor: $F_{3,141} = 0.43$, $p = 0.856$; trial factor: $F_{2,282} = 59.20$, $p < 0.001$; interaction: $F_{6,282} = 0.12$, $p = 0.945$). It is here to remark, that during training, no effect of DA was observed in regards to the

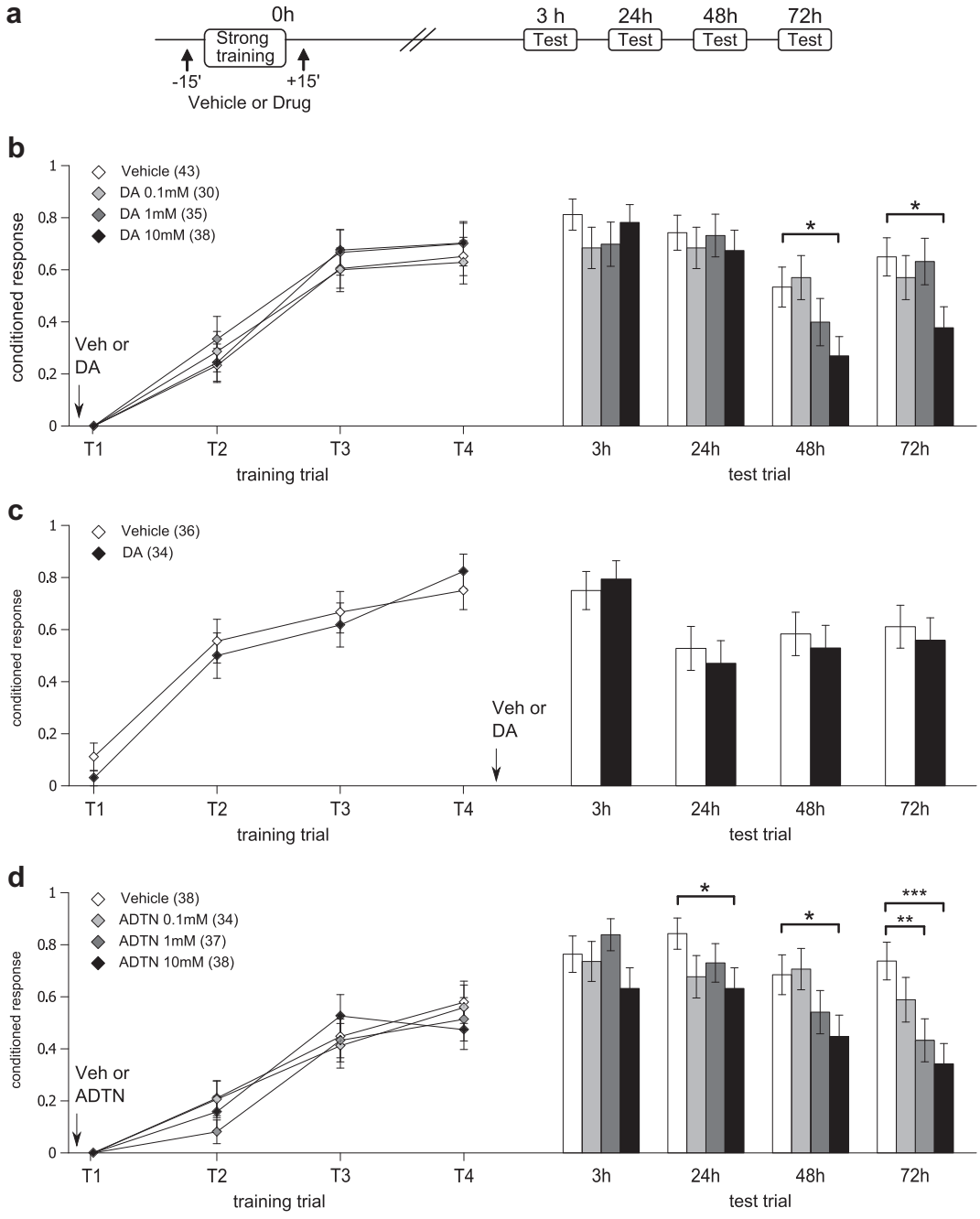

Fig. 1. Dopamine impairs appetitive memory. (a) Experimental procedure. All animals underwent a training session that consisted of 4 conditioning trials separated by 5 min inter-trials interval. Injections of vehicle or drugs were performed 15 min before (b and d) or 15 min after training (c). All animals were tested at the indicated time intervals counted from the end of the training session. (b) DA injected before training impairs long-term memory. Values represent the proportion of bees that extended the proboscis to the odor. White symbols and bars correspond to the vehicle injected bees, light grey to 0.1 mM DA, dark grey to 1 mM DA and black to 10 mM DA. Numbers within brackets indicate the number of animals in each group. (c) DA injected after training does not impair appetitive memory. Colors and symbols as in b. (d) 6,7 ADTN injected before training impairs long-term memory. White symbols and white bars correspond to the vehicle injected bees, light grey to 0.1 mM 6,7 ADTN, dark grey to 1 mM 6,7 ADTN, and black to 10 mM 6,7 ADTNs. In all graphs, (*) $p < 0.05$, (**) $p < 0.01$ and (***) for $p < 0.001$ in Least Squares Comparisons between the indicated and the vehicle injected group from the same session. When there is no indication between a column and its respective vehicle group it means that the comparison was not statistically significant.

unconditioned proboscis extension elicited by stimulation with sucrose. Indeed 100% of bees in all groups responded to sucrose extending the proboscis and ingesting the reward. Subsequently, the conditioned response was evaluated at successive time intervals after training (Fig. 1b). Least squares comparisons were performed to compare DA injected groups with the vehicle injected group. A significant decrease of the conditioned response was found in 10 mM DA injected bees in test sessions at 48 and 72 h, but not 3 and 24 h after training (3 h: $F_{1,141} = 0.10$ $p = 0.757$; 24 h: $F_{1,141} = 0.44$, $p = 0.507$; 48 h: $F_{1,141} = 5.81$, $p < 0.05$; 72 h: $F_{1,141} = 6.14$, $p < 0.05$). Injection of DA at concentrations 0.1 mM and 1.0 mM revealed no significant differences with the vehicle group ($p > 0.05$ for all comparisons). These results suggest that administration of DA shortly before training interferes with mechanisms involved in the formation of a late memory phase. In the next experiment, vehicle or 10 mM DA were injected 15 min after the end of the training session. The performance during training and all test sessions are shown in Fig. 1c. Repeated Measures

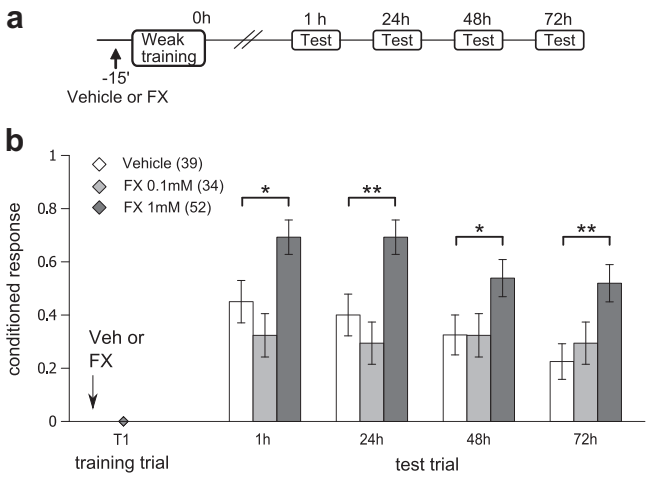

Fig. 2. DA receptor antagonist flupentixol facilitates appetitive memory. (a) Experimental procedure. All animals underwent a training session that consisted of one conditioning trial. Injections of vehicle or drug were performed 15 min before training. All animals were tested at the indicated intervals counted from the end of training. (b) Flupentixol enhances appetitive memory. White symbols and bars correspond to the vehicle injected bees, light grey to 0.1 mM FX and dark grey to 1 mM FX. (*) $p < 0.05$; (**) $p < 0.01$ in Least Square Comparisons between the indicated and the control group.

ANOVA yielded significant effect only for training trials but not among groups (group factor: $F_{1,68} = 0.01$, $p = 0.915$; trial factor: $F_{2,136} = 13.94$, $p < 0.001$; interaction: $F_{2,136} = 0.12$, $p = 0.338$). In contrast with the injection before training, no difference was disclosed between groups at any of the analyzed time intervals (3 h: $F_{1,68} = 0.188$, $p = 0.67$; 24 h: $F_{1,68} = 0.222$, $p = 0.64$; 48 h: $F_{1,68} = 0.200$, $p = 0.66$; 72 h: $F_{1,68} = 0.192$, $p = 0.66$), suggesting that the time window during which DA interferes with the formation of appetitive memory is restricted to the time of training, presumably while the consolidation of long-term memory is triggered.

In the next experiment we evaluated the effect of the DA receptor agonist 6,7 ADTN (Mustard et al., 2003). Bees were divided in four groups and were injected with vehicle or one of three concentrations of the agonist: 0.1, 1 and 10 mM. Fifteen minutes after the injection all bees underwent a 4 trials training protocol. No difference was observed between groups during the training session (Fig. 1d) (Repeated measures ANOVA; group factor: $F_{3,143} = 0.24$, $p = 0.870$; trial factor: $F_{2,282} = 45.96$, $p < 0.001$; interaction: $F_{6,286} = 0.88$, $p = 0.508$). As with DA, the effect of 6,7 ADTN became evident in the test sessions during the subsequent days. The highest dose of the agonist showed a slight but significant impairment of memory 24 h after training ($F_{1,143} = 4.21$, $p < 0.05$) and a clear amnesic effect emerged 48 and 72 h after training (48 h: $F_{1,143} = 4.50$, $p < 0.05$; 72 h: $F_{1,143} = 12.74$, $p < 0.001$). The mid dose of the agonist showed a less pronounced effect on memory that was delayed relative to the highest dose and was statistically significant 72 h after training ($F_{1,143} = 7.47$, $p < 0.01$). Finally, the lowest dose did not reveal any effect on memory ($p > 0.05$ for all comparisons). This time- and dose dependent effect of 6,7 ADTN is consistent with a memory trace that is modulated by DA during training and becomes gradually evident when short- term memory decays.

A tentative explanation for the decline in long-term memory is that DA treated animals felt satiated or did not ingest the reward. It is established that any of these two parameters affect long-term memory formation (Friedrich et al., 2004; Wright, Mustard, Kottcamp, & Smith, 2007). Considering these possibilities, we have controlled that all animals responded to the stimulation with sucrose and ingested the reward during the training trials. However, it could happen that the same amount of sucrose ingested during trials has a different subjective value depending on how much the bee would eat. Thus, we performed an experiment to control if sucrose intake under *ad libitum* feeding conditions is different in DA injected animals. Two groups of bees were injected 1 µl of vehicle or 1 µl of 10 mM DA. Fifteen minutes after injection they were fed with 2.0 M sucrose solution using a micropipette until they did not ingest any more. The ingested amount of solution was calculated with the micropipette used for feeding and corroborated weighing the bees before and after feeding. No difference was observed between vehicle and DA injected bees (Vehicle, $n = 20$: 28.15 ± 2.89 mg; DA, $n = 20$: 28.05 ± 3.34 mg; two tailed t-test: $p = 0.98$). Thus, the effect of DA on long term memory does not seem to be consequence of a lower appetite for sucrose or a lower amount of reward ingested during conditioning.

3.2. Dopamine receptor antagonist enhances appetitive memory

The observation that DA impairs appetitive long-term memory together with previous studies that proved the role of DA signaling aversive stimuli (Vergoz et al., 2007; Wright et al., 2010) suggest that appetitive and aversive pathways may interact to modulate memory formation. We hypothesized that if DA inhibits appetitive memory formation, then a DA receptor antagonist that blocks aversive learning could remove such inhibitory action and in turn facilitate appetitive learning and memory. Cis-Z-flupentixol was selected for this experiment because it has been proved to interfere with different forms of aversive learning in honey bees (Vergoz et al., 2007; Wright et al., 2010). We performed an experiment containing three groups of bees; one group was injected with vehicle and 2 other groups with different doses of flupentixol. Fifteen minutes after injection the bees were trained using a weak training protocol that consists of a single conditioning trial (Fig. 2a). This protocol is normally insufficient to generate a stable long-term memory and it is used to evaluate the facilitatory effect of different treatments (Locatelli, Bundrock, & Müller, 2005; Müller, 2000). No effect of the drug was observed in the training trial in regards to the unconditioned response to sucrose or in spontaneous response to the odor. The results of the test sessions carried out 1, 24, 48 or 72 h after training are shown in Fig. 2b. The vehicle injected group shows 1 h after the weak training protocol a learning performance of 40% and a decay of memory to circa 20% after three days which is consistent with previous observations (Friedrich et al., 2004, p. 2002020; Müller, 2000). Least squares comparisons revealed significant differences between 1 mM FX and vehicle groups in all test sessions (1 h: $F_{1,123} = 6.14$, $p < 0.05$; 24 h: $F_{1,123} = 8.57$, $p < 0.01$; 48 h: $F_{1,123} = 4.35$, $p < 0.05$; 72 h: $F_{1,123} = 8.91$, $p < 0.01$) but not between 0.1 mM and vehicle groups ($p > 0.05$ for all comparisons). This dose dependent effect of the DA receptor antagonist on appetitive memory suggests a tonic dopaminergic control of appetitive memory formation. Furthermore, the fact that a DA antagonist shows an effect that is opposite to the one induced by DA argues in favor of the specificity of the treatments.

4. Discussion

In the present work we show that DA or the DA receptors agonist 6,7 ADTN applied shortly before training have a detrimental effect on appetitive long-term memory. This action takes place in a narrow time window close to or during the time of training. We have controlled that this effect is not due to reduced ingestion of the reward or less appetite for sucrose during training. Finally, we found that the administration of flupentixol, a DA receptors antagonist, enhances appetitive memory induced by a weak training protocol. The results suggest the existence of a dopaminergic pathway that modulates appetitive memory formation in honey bees.

4.1. Methodological considerations

In the present study we injected DA, 6,7 ADTN or flupentixol into the hemolymph in the thoracic cavity of honey bees (Felsenberg et al., 2011). This method produces minimal damage and stress in comparison to brain injections and it has been successfully used to target the central nervous system and cellular mechanisms underlying learning and memory (Friedrich et al., 2004; Wüstenberg, Gerber, & Menzel, 1998). Minimizing physical damage was crucial for the present study; otherwise measurement of memory up to three days after training would not be possible. The time of injection in the present work was set to 15 min before or 15 min after training. According to results based on the distribution of radiolabelled glutamate injected into the thorax, drugs are expected to reach the brain 3 min after injection (Maleszka, Helliwell, & Kucharski, 2000). In the case of octopamine, which is chemically related to DA, the injection into the thorax has shown to provide effective concentration in the brain 15 min after injection and decays to its half 60 min after injection (Barron, Maleszka, Vander Meer, Robinson, & Maleszka, 2007). Notice that in the case of the four-trials protocol that we used, the whole training last 15 min. Thus, we chose a time of injection that is separated enough from training to avoid the stress of the injection to disrupt learning, but close enough to ensure high drug concentration during the whole training. After systemic injection, multiple factors as dilution, oxidation and uptake may reduce the concentration of drugs reaching the CNS. This might have been the reason for the apparently high doses that were required to observe effects on memory. We have controlled eventual effects on sensory (Dacks, Riffell, Martin, Gage, & Nighorn, 2012) and motor functions (Mustard et al., 2010) that could be misinterpreted as changes in memory performance. The observation that unconditioned response, sucrose ingestion and learning performance were normal in bees treated with the drugs suggests that odor and sucrose perception were not affected during conditioning. Finally, memory retention was measured at time intervals that were distant from drug injection. Thus, all changes detected in memory performance cannot be interpreted as an acute action of the drugs on perception of the odor or on motor responses during testing.

4.2. DA interferes with appetitive memory

It is well established that DA signaling is required in insects for aversive learning and that DA can replace the unconditioned stimulus in aversive learning (Claridge-Chang et al., 2009; Schwaerzel et al., 2003; Unoki et al., 2005; Vergoz et al., 2007). Thus, if DA internally represents an aversive stimulus, it is reasonable to predict that it may counteract a memory process elicited by a concurrent appetitive conditioning. Accordingly, we found that DA or the DA receptor agonist 6,7 ADTN injected 15 min before training have amnesic effect over appetitive memory and this effect becomes progressively evident during the subsequent days after training (Fig. 1). This effect is consistent with our previous report in crabs and suggests that the inhibitory role of DA on appetitive memory formation might be conserved across several arthropod species (Klappenbach et al., 2012). It is not surprising that the effect of DA and 6,7 ADTN emerges during a late memory phase and not during short-term memory. In this regard, several works have proved that different signaling pathways and cellular mechanisms are differentially involved in the induction and consolidation of different memory traces whose formation are triggered early during training but expressed in different and sequential time windows (Friedrich et al., 2004; Grünbaum & Müller, 1998; Locatelli & Romano, 2005; Mustard et al., 2012; Schwärzel & Müller, 2006; Trannoy, Redt-Clouet, Dura, & Preat, 2011; Yu, Akalal, & Davis, 2006). We found that the emergence of the effect of DA coincides with the emergence of a long–term memory trace in bees that depends on PKA activation during training (Müller, 2000) and mRNA synthesis (Friedrich et al., 2004; Lefer, Perisse, Hourcade, Sandoz, & Devaud, 2012). DA showed no effect when it was injected immediately after training. This result suggests that the action of DA on appetitive memory is restricted to the time of training and makes it unlikely that the amnesic effect is due to delayed effect acting during the test sessions. In that case we should have seen the same effect when the injection of DA was done 15 min after training (Fig. 1c).

Alternative explanations for the long-term memory decay in DA treated animals might involve altered perception of CS or US during training. Several observations argue against this possibility. The training curves do not differ among vehicle and DA treated animals indicating that odor and reward are normally perceived during training. In regards to the unconditioned stimulus, the response to sucrose was controlled in each animal 30 min before training (see methods: selection test) and again during the training trials which was 15 min after drug injection. No difference in proboscis extension response to stimulation with sucrose was detected between these two events. This observation seems to differ with a previous report by (Scheiner, Plückhahn, Oney, Blenau, & Erber, 2002) in which a decrease in gustatory responsiveness to sucrose was measured after DA injection. The discrepancy must be related with differences in the concentration of sucrose and the phenotype of the bees. While gustatory responsiveness is normally measured in a range from 0.1% to 30% sucrose, in the present work we used sucrose at 2 M concentration which is equivalent to ~68% w/v. This stimulation is probably beyond the range affected by DA. In regards to the phenotype of the bees, we used exclusively pollen foragers which are more responsive to sucrose than nectar foragers (Pankiw & Page, 1999). In addition, we made an experiment to evaluate if DA treated bees had less appetitive for sucrose and this could have modified the subjective value of the reward. We found that DA treated bees ate *ad libitum* the same amount of sucrose solution than bees injected with vehicle. Thus, on the basis of our observations of pollen foragers and using 2.0 M sucrose solution we conclude that differences obtained in appetitive memory are not due to an altered perception of the reward during training. In regards to the conditioned stimulus it could be argued that DA may alter odor perception in terms of its quality, and the reduction in memory could be consequence of a mismatch between the odor as it was perceived during acquisition and during testing (Macmillan & Mercer, 1987). Two observations make this possibility unlikely in the present study: (i) if there were an odor mismatch between training and testing, such mismatch should be evident in all testing sessions. (ii) If DA were amnesic because of distorting odor perception, then we should expect flupentixol to have no effect or to have the same effect as DA, but not to improve memory performance. In summary, the present results suggest that the administration of DA alters processes that are intrinsic to the formation of appetitive memory and not to the perception of the stimuli. Among the mentioned intrinsic processes it is plausible that DA modulates the internal representation of the reward at the site of association with the odor. A reduced internal representation of the reward at the sites of association could weaken the associative memory formation. Our current studies are now focused to test this hypothesis.

Our present results and interpretation seem to differ with a previous study (Mercer & Menzel, 1982) which reported that DA affects retrieval but not storage of appetitive memory. There are several differences among this work and ours that may account for the different outcomes. First, the way of administration and the doses were different: in the previous work DA was injected into the brain (~0.5 μl, 50 μM) while in the present work the injections were systemic (1 μl, 10 mM). These differences may lead to very different concentration and duration of the drug in the CNS.

Second, the previous work evaluated only short-term memory and reported that DA has no effect during appetitive conditioning. Indeed, we would have reached the same conclusion if we had tested memory only 3 h after training. However, the current knowledge about memory departs from the idea that memory is consolidated into its final form after several minutes. This prompted us to test memory during longer intervals after training. We found that induction of long-term memory, a memory phase not tested in the previous work, is modulated by DA during training.

4.3. Blockade of endogenous DA enhances appetitive memory

We showed that the administration of DA or the DA receptor agonist 6,7 ADTN interferes with appetitive long-term memory. The evidence that endogenous DA does exert a similar action is provided by the experiment with flupentixol, which shows that the DA receptor antagonist has a facilitatory effect on appetitive memory. Three distinct DA receptors, two D1-like receptors, AmDOP1 and AmDOP2 (Blenau, Erber, & Baumann, 1998; Humphries et al., 2003), and one D2-like receptor, AmDOP3 (Beggs, Hamilton, Kurshan, Mustard, & Mercer, 2005) are expressed in the honeybee brain. Cis-Z-flupentixol has been reported in insects to act as a general DA receptor antagonist with preferential effect on the D1-like receptor AmDOP2 (Beggs et al., 2005; Blenau et al., 1998; Hearn et al., 2002). This raises the possibility that the effect of DA on appetitive memory is mediated by AmDOP2 receptors. However, the pharmacology of invertebrate DA receptors is remarkably different from that of their vertebrate counterparts, and information in regards to selectivity of different antagonists for different kinds of DA receptor is still controversial (Beggs, Tyndall, & Mercer, 2011; Mustard, Beggs, & Mercer, 2005). Thus, attempts to identify the differential contribution of the distinct DA receptors types based on available antagonists would be still very uncertain. We selected Cis-Z-flupentixol because of its well proven effect on different forms of aversive learning in honey bees (Vergoz et al., 2007, p. 20; Wright et al., 2010). An asymmetrical action of flupentixol or DA in regards to the selectivity for different dopamine receptors involved in short and long-term memory could have been the reason for the temporal difference of the effect of flupentixol vs DA and 6,7 ADTN. This difference could be indicative of distinct dopaminergic components that are differentially involved in long- and short-term memory traces. However, in order to define the differential contribution of distinct DA receptors to the effect reported here on appetitive memory, more precise strategies, like for example knockingdown specific receptor by means of RNA interference will be required (Mustard et al., 2010).

The facilitatory effect of flupentixol in the apparent absence of an aversive stimulus suggests the existence of a tonic modulation of appetitive memory by DA. We mention the absence of aversive stimuli only as apparent because it has to be considered that the whole experimental situation may have negative components that could affect the appetitive nature of the training and weaken appetitive learning and memory. For example the handling of the animal, the isolation from the colony and the restriction of body-movements are only some aspects from the experimental situation that may represent aversive stimuli and may compete with the reward learning. Previous evidence regarding stimuli that animals try to avoid, i.e. noxious stimuli (Agarwal et al., 2011; Vergoz et al., 2007), bad tasting solutions (Wright et al., 2010) and mated females (Keleman et al., 2012) are mediated in the nervous system by DA, with exception of post-ingestive feedback produced by toxins, which is mediated by serotonin (Wright et al., 2010). It is likely that the stressors that are inherent to the experimental situation are in part internally represented by DA that antagonizes the appetitive learning and memory formation elicited by the reward. Thus, it is plausible that the facilitatory effect of flupentixol might be due to removal of those negative elements. In addition, flupentixol may antagonize constitutively activated DA receptors (Mustard et al., 2003) and alter cAMP levels in way that favors appetitive learning and memory formation.

4.4. DA for aversive and appetitive learning

Recent works using precise neurogenetic tools in *Drosophila* revealed a subset of dopaminergic neurons that are located downstream of octopaminergic neurons and are necessary for appetitive learning (Burke et al., 2012; Kim et al., 2007; Liu et al., 2012; Selcho et al., 2009). These results contrast with the notion that held DA as neurochemical code for punishment in aversive learning and with the results presented in our work, which show that DA interferes with appetitive learning. It is reasonable that the distinct interpretations emerge from the differences between the experimental approaches, which in one case is manipulating specific dopaminergic neurons and in the other one activating or blocking dopamine receptors in the whole brain or areas of it. A dopaminergic relay in the pathway of the appetitive stimulus like the one found in *Drosophila* has not been reported yet in other insects including bees, and at first sight, it does not sound likely, because DA receptors antagonists do not impair appetitive learning (Unoki et al., 2005, 2006; Wright et al., 2010). Nevertheless, the results obtained from these pharmacological studies must be interpreted as the net effect of simultaneously targeting many different dopaminergic sites that might be involved in related, non-related or opposite processes. Under such scenario and assuming that the injected drugs reach all these sites, the present results would suggest that the action DA signaling aversive stimuli occludes its action on appetitive learning. More precise experimental tools will have to be used to tackle these kind of questions in honey bees as it was recently done in flies (Burke et al., 2012; Liu et al., 2012).

4.5. Interaction between appetitive and aversive pathways

Most of the works that have studied the role of amines in learning and memory in bees and insects in general were designed to provide training conditions that were clear appetitive or aversive learning. Only few studies have considered situations in which the conditioned stimulus predicts concurrent appetitive and aversive consequences (Smith, Abramson, & Tobin, 1991; Wright et al., 2010). This alternative represents a naturally relevant situation in which a stimulus anticipates a consequence that includes costs and benefits. We hypothesize that under these circumstances appetitive and aversive pathways should interact to avoid conflicting behavioral outputs. It is not clear yet whether such interaction exists and at which level of processing, from stimuli sensation to motor output, it takes place. A recent work in honey bees has shown that OA, the main neuromodulator involved in appetitive learning, reduces performance in an aversive learning paradigm (Agarwal et al., 2011). Here we present experiments revealing a similar relationship but between DA and appetitive memory. Thus OA and DA seem to have complementary roles in regards to appetitive and aversive learning. Their actions are not restricted to only one kind of learning, but they also modulate the opposite one. Evidence from different organisms among invertebrates support this idea; in crabs it was demonstrated that OA is required for appetitive memory while it interferes with aversive memory (Kaczer and Maldonado, 2009) and DA is necessary for aversive learning while it has a detrimental effect on appetitive memory (Klappenbach et al., 2012). In *Drosophila*, it was shown that activation of a specific subgroup of dopaminergic neurons prevents appetitive memory (Berry et al., 2012). Furthermore, it was found a subpopulation of

dopaminergic neurons that modulate retrieval of appetitive memory according to satiation levels (Krashes et al., 2009). Altogether, the present study and previous works across several species are providing evidence that DA and OA interact along appetitive and aversive learning in a much more intricate way than it was interpreted initially. Future studies have to take into account a more complex scheme of action of the neurotransmitters signaling unconditioned stimuli and instructing aversive and appetitive learning.

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