Effects of morphine on associative memory and locomotor activity in the honeybee (*Apis mellifera*)

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ABSTRACT

Morphine can modulate the processes underlying memory in vertebrates. However, studies have shown various modulations by morphine: positive, negative and even neutral. The honeybee is a potential platform for evaluating the effects of drugs, especially addictive drugs, on the nervous system. However, the involvement of morphine in learning and memory in insects or other invertebrates is poorly understood. The current work evaluated whether morphine affects memory acquisition, consolidation and retrieval in honeybees, using the proboscis extension response (PER) paradigm. We demonstrated that morphine treatment (5 µg/bee) before training decreased the percentage of correct PERs and the response latency related to aversive rather than rewarding odors when tested after 1 or 24 h. Morphine treatment after training also caused a decrease in this latency when tested after 24 h. Meanwhile, morphine treatment reduced the ambulation distance when tested after 30 min. Our findings suggest that morphine impairs the acquisition of short- and long-term associative memory and slightly disrupts the consolidation of long-term memory in honeybees. These negative effects cannot be explained by reduced locomotion but by impaired memory associated with aversion.

Keywords: morphine; memory; locomotor activity; honeybee

INTRODUCTION

In vertebrates, increasing evidence has demonstrated that morphine modulates the acquisition, consolidation and retrieval of memory. This modulation is positive, negative, or even neutral in different animal models. For example, in rodents, most studies show that morphine administration before training inhibits the acquisition of memory in paradigms such as active or passive avoidance tasks^[1–3] and a wide variety of maze tasks^[4–6]. Other studies report that morphine administration after training impairs memory retrieval in step-down or step-through inhibitory avoidance tasks^[7–9]. However, some studies show that morphine administration after training or before testing does not alter performance in the step-down inhibitory avoidance test^[1], and administration before testing even facilitates memory retrieval in the passive avoidance task^[10]. Thus, further investigations are required to clarify the effect of morphine on memory processes.

The honeybee has attracted much attention as its genome has been sequenced^[11], and behavioral testing can easily be performed in this species. The proboscis extension response (PER) establishes the honeybee as an acceptable subject for controlled training and testing. In this paradigm, harnessed honeybees are trained to associate an odor with a sugar reward delivered to their antennae that elicits the PER. The association, based on Pavlovian theory, results in long-lasting conditioning of the honeybee to the odor stimulus, which is then able to elicit a PER when presented by itself^[12,13]. With this PER, olfactory

associative learning and memory can be reliably tested in this species^[12,14-19].

In addition, previous studies have shown that the honeybee is a potential platform for evaluating the effects of drugs, especially addictive drugs, on the nervous system. For example, caffeine improves both motivation and cognitive performance of free-flying honeybees in complex learning tasks^[20], and cocaine at low doses increases the likelihood and rate of honeybees dancing after foraging^[21]. Moreover, octopamine injection into the mushroom body calyces and antennal lobes of honeybees induces an associative memory enhancement in a PER paradigm^[22].

In this study, we sought to determine whether morphine affects memory in insects. While the effects of morphine on memory processes remain to be fully elucidated, its impairing effects on memory retention are well-established in vertebrates. In contrast, the involvement of morphine in learning and memory in insects and other invertebrates is poorly understood. The present study therefore investigated whether morphine affects the processes underlying memory in insects. By using PER differential conditioning, morphine was administrated 30 min prior to training, immediately following training or 30 min prior to testing, to assess its effects on the acquisition, consolidation and retrieval of memory in honeybees^[23,24]. In addition, locomotor activity was tested 30 min following morphine administration.

MATERIALS AND METHODS

Bees

Honeybees (*Apis mellifera*) from breeding colonies in experimental hive boxes were purchased from Yunnan Agricultural University (Kunming, China). Individual frames of brood comb were removed from the boxes and placed in an incubator. The temperature in the incubator was maintained at 32–33°C. Newly-emerged honeybees from the previous night were collected daily, ensuring that the experiment was performed only on honeybees of a known age. The experiments were conducted in accordance with the Guidelines for the National Care and Use of Animals and were approved by the National Animal Research Authority.

Drug Administration

Morphine hydrochloride (10 mg/mL) was purchased from

Shenyang Pharmaceutical Factory, Shenyang, China. Saline especially for honeybees contained 5 mmol/L KCl (Huazhen Specialty Chemical Factory, Tianjin, China) and 10 mmol/L NaH₂PO₄ (Beijing Chemical Factory, Beijing, China) at pH 7.8 in double-distilled water.

To facilitate drug administration, as previously reported^[12,24], individual honeybees were placed on ice for anesthetization and secured in thin-walled straws (5 mm in diameter). The honeybee was mounted in the tube with the head and antennae free to move and with the dorsum of the thorax exposed. Honeybees were fed 1 mol/L sugar solution *via* a syringe without a needle twice per day. During feeding, a drop of solution was applied to one of the honeybee's antennae, causing a PER, and it was then allowed to suck up the solution. The honeybees were fed until the proboscis retracted and no longer showed a rapid and reliable PER when their antennae touched the solution (up to ~0.25 mL per bee). The honeybees that had been mounted in the tube were arranged on a perforated wood board and then placed in an incubator overnight (29°C).

For injections, previous studies have shown that drugs injected into the thorax of the honeybee pass to the brain^[25]. So, this type of injection was adopted with minor modifications. Briefly, the honeybees in straws were placed in modeling clay under an anatomical lens ($\times 10$), and a small hole was made in the left side of the thorax. Injection of 0.5 μ L morphine (5 μ g/bee) or saline was carried out using a 1- μ L micro-syringe (Ningbo City Zhenhai Glass Instrument Factory, Ningbo, China). Since most previous studies reported that the injection doses of drugs in the honeybees varied from 0.5 to 2 μ L for each insect^[20,22,24-28], we chose a low dose for the morphine injection in this study.

PER Conditioning

In the PER differential conditioning^[23,24], honeybees learn to respond to the rewarding odor stimulus associated with a sugar solution and to avoid responding to the aversive odor stimulus associated with NaCl solution. This paradigm eliminates association with the researchers' movements when administering the sugar reward stimulus. Honeybees were trained to associate the odor of limonene with the sugar reward (CS+) and the odor of menthene with aversive NaCl (CS-). Limonene in the 1 mol/L sugar solution and menthene in the NaCl solution were both diluted to 1:200, which is close to that reported in the literature^[23-25]. Then the solution

was drawn into a 1-mL syringe. This paradigm consisted of one learning session involving the paired presentations of two odors, one closely following the other. First, a drop of sugar solution with limonene (CS+) was hung in front of the honeybee for 6 s. The sugar solution was then applied to one antenna, causing a PER, and the honeybee was allowed to drink the sugar for 1 s. Then, after ~2 min, the salt solution with menthene (CS-) was hung in front of the honeybee for 6 s, and then the solution was applied to one antenna. Honeybees were given the salt solution for 1 s if they extended the proboscis (~90% of the honeybees extended the proboscis when the menthene was presented, similar to that in the presence of the odor of limonene; however, all honeybees retracted the proboscis when the antennae touched the salt solution). The above two steps represent a conditioning trial.

PER Testing on Short-Term Olfactory Memory

Eighty-five honeybees were divided into three groups, control ($n = 26$), acquisition ($n = 33$), and retrieval ($n = 26$); all were 6 days old when mounted and 7 days old when tested. In the acquisition group, morphine was administered 30 min prior to training; in the retrieval group, morphine was administered 30 min prior to testing; and in the control group, saline was administered 30 min prior to testing. The time schedule of drug administration was designed according to the half-life of morphine in the brain (up to ~1 h)^[29,30] and previous studies of morphine in rodents^[4]. Besides, in our preliminary studies, the honeybees were treated with saline (1 μ L/bee) 30 min prior to training, immediately after training, and 30 min prior to testing, and their PER responses were 75.9% (22 of 29), 79.3% (23 of 29), and 64.5% (20 of 31), respectively. We supposed that the honeybees would be more sensitive to the treatment prior to testing and therefore administered saline to the control group prior to testing to measure against the other two groups. Such an experimental design reduced the use of honeybees and drugs.

In this study, only one conditioning trial was used because previous studies have shown that honeybees can consolidate (short-term memories) following a single PER conditioning trial^[31].

The testing session was carried out 1 h after the training session (conditioning). The honeybee was first presented with the aversive stimulus (menthene) and then

with the rewarding stimulus (limonene), and the presence or absence of a PER was recorded. The interval between the presentation of the stimulus and the PER was also recorded and defined as the latency. If the honeybee did not extend the proboscis within 6 s, a retracted proboscis was noted, and the latency was recorded as 6 s. Honeybees that extended the proboscis in the presence of the rewarding stimulus, but not in the presence of the aversive stimulus were scored as having responded correctly. Honeybees that responded to the aversive stimulus or to both stimuli were defined as having responded incorrectly. Meanwhile, honeybees not responding to either stimulus and unable to extend the proboscis when stimulated with sugar were excluded from the subsequent analysis because their learning status was erratic. The proportion of honeybees responding to either stimulus or both stimuli was defined as the response rate.

During the experiment, an exhaust fan worked behind the experimental honeybee, ensuring elimination of any olfactory stimuli. The injections of the pharmacological agents and the training and testing were performed in a double-blind design.

PER Testing on Long-Term Olfactory Memory

One hundred and seventy-nine honeybees were used and divided into four groups: control ($n = 41$), acquisition ($n = 50$), consolidation ($n = 48$) and retrieval ($n = 40$). The procedure was the same as that used above with four exceptions: (1) all were 6 days old when mounted, 7 days old when trained, and 8 days old when tested; (2) an additional group was used (consolidation group), in which morphine was administered into the thorax immediately after a training session; (3) during training, three conditioning trials were used at intervals of 6 min; and (4) the testing session was performed 24 h after the conditioning.

Locomotor Activity Testing

In this study, 42 honeybees were used and divided into two groups, control ($n = 21$) and morphine ($n = 21$). As described above, to facilitate solution administration, individual honeybees were mounted in tubes, arranged on a perforated wood board and placed in an incubator overnight. All were 6 days old when mounted and 7 days old when tested.

Thirty minutes before locomotor activity testing, morphine or saline was injected into the thorax. Locomotor

activity testing was carried out in 12 Petri dishes (8.5 cm in diameter) in a 3 × 4 array, each of which accommodated one honeybee. Before testing, the strips that secured the honeybees were slightly cut, and the honeybees were randomly assigned to the separate dishes. The behaviors of the honeybees were monitored with a ceiling-mounted CCD camera. The video signals were then displayed and saved by video-recording software. Using an in-house-developed video analysis system, the recordings were then analyzed, and the ambulation distances (cm) of the honeybees were measured. In addition, pharmacological agent injections and testing were performed in a double-blind design.

Data Analysis and Statistics

Data are expressed as mean ± SEM and analyzed by the χ^2 test, one-way analysis of variance (ANOVA) or paired *t*-tests, where appropriate. $P \leq 0.05$ was considered as statistically significant.

RESULTS

PER Study on Short-Term Olfactory Associative Memory

The short-term memory of the honeybees was assessed with a testing session 1 h after the training session. Compared

with the control, the percentage of correct PERs decreased in the acquisition group ($\chi^2 = 4.90$, $P < 0.05$) but not in the retrieval group ($\chi^2 = 0.03$, $P = 0.86$) (Table 1; Fig. 1A).

The latencies to the rewarding stimulus were lower than those to the aversive stimulus in all groups ($P < 0.001$; *t*-tests) (Table 1). Only the acquisition group showed a reduced latency to the aversive stimulus compared with that of the control [$F(1, 50) = 5.41$, $P < 0.05$] (Fig. 2A).

In addition, the response rate showed no difference between the control and the morphine injection groups (acquisition: $\chi^2 = 1.37$, $P = 0.24$; retrieval: $\chi^2 = 0.75$, $P = 0.39$) (Table 1).

PER Study on Long-Term Olfactory Associative Memory

Long-term memory was assessed with a testing session 24 h after the training session. Compared with control, the percentage of correct PERs decreased in the acquisition group ($\chi^2 = 3.98$, $P < 0.05$) but not in the consolidation ($\chi^2 = 2.19$, $P = 0.14$) or retrieval group ($\chi^2 = 0.65$, $P = 0.42$) (Table 1; Fig. 1B).

The latency to the rewarding stimulus was lower than that to the aversive stimulus in all groups ($P < 0.001$ for all; *t*-tests) (Table 1). Meanwhile, both the acquisition and consolidation groups exhibited a lower latency to the aversive

Table 1. Descriptive statistics for indices associated with short- and long-term memory and locomotor activity in control and morphine injection groups

	Control group	Morphine injection groups		
		Acquisition	Consolidation	Retrieval
<i>Short-term memory</i>				
PER	75% (18/24)	44% (12/27)		73% (16/22)
Latency-R (s)	2.54 ± 0.37	2.11 ± 0.29		2.32 ± 0.30
Latency-A (s)	5.46 ± 0.30	4.37 ± 0.35		5.00 ± 0.41
Response rate	92% (24/26)	82% (27/33)		85% (22/26)
<i>Long-term memory</i>				
PER	76% (25/33)	53% (23/43)	60% (25/42)	84% (26/31)
Latency-R (s)	2.94 ± 0.31	2.70 ± 0.27	2.50 ± 0.31	2.84 ± 0.30
Latency-A (s)	5.64 ± 0.14	4.44 ± 0.30	4.81 ± 0.26	5.52 ± 0.21
Response rate	80% (33/41)	86% (43/50)	88% (42/48)	78% (31/40)
<i>Locomotor activity</i>				
Ambulation distance (cm)	833.24 ± 42.09		690.81 ± 48.58	

Latency-R, latency to rewarding stimulus; latency-A, latency to aversive stimulus.

Fig. 1. Effect of morphine (5 μ g/bee) on the percentage of correct responses for the associative olfactory task tested 1 h (A) and 24 h (B) after honeybee training. The percentage of correct responses was evaluated by the proboscis extension reflex (PER). The labels under the x axis indicate morphine treatment conditions. Morphine was injected 30 min prior to training (Acquisition), immediately after training (Consolidation), and 30 min prior to testing (Retrieval). The control (Ctrl) in all cases was injected with saline 30 min before testing. The numbers on the bars give the number of honeybees tested in each condition. * $P < 0.05$ (χ^2 test).

Fig. 2. Effect of morphine on the latency of honeybee proboscis extension in response to rewarding and aversive stimuli in the PER paradigm tested 1 h (A) and 24 h (B) after training. The labels under the x axis indicate morphine treatment conditions. Morphine was injected 30 min prior to training (Acquisition), immediately after training (Consolidation), and 30 min prior to testing (Retrieval). The control (Ctrl) in all cases was injected with saline 30 min before testing. ^{AA} $P < 0.01$ between rewarding and aversive stimuli (t -test). * $P < 0.05$ and ** $P < 0.01$ compared with control (one-way ANOVA).

stimulus than the control [acquisition: $F(1, 75) = 10.94$, $P < 0.01$; consolidation: $F(1, 74) = 6.68$, $P < 0.05$] (Fig. 2B).

In addition, the response rate showed no difference between the control and the morphine injection groups (acquisition: $\chi^2 = 0.50$, $P = 0.48$; consolidation: $\chi^2 = 0.82$, $P = 0.37$; retrieval: $\chi^2 = 0.11$, $P = 0.74$) (Table 1).

Locomotor Activity

Thirty minutes after morphine administration, the locomotor activity was measured and compared with that of the control group. The 5-min ambulation distance was lower

in honeybees receiving morphine treatment than in the controls [$F(1, 41) = 4.91$, $P < 0.05$] (Table 1; Figs. 3 and 4).

DISCUSSION

Our study demonstrates that acute morphine treatment impairs the acquisition of both short- and long-term memory and slightly disrupts long-term memory consolidation in honeybees. And this impairment mainly results from the impaired memory associated with aversion rather than reward. Furthermore, morphine decreases the locomotor

Fig. 3. Effects of morphine on ambulation paths of honeybees. Saline (A, B) or morphine (C, D) was injected 30 min prior to the test. The heading of each figure is composed of the honeybee group number and the number of the Petri dish where the honeybee was tested. Ctrl, control; Mor, morphine.

activity.

To test the effects of morphine on the acquisition of memory, morphine was injected 30 min prior to training, and the retention test was conducted 1 or 24 h later. The honeybees showed a lower proportion of correct PERs and a lower latency to the aversive stimulus than those of the control. The impairment of memory acquisition induced by morphine is consistent with the results of previous studies in rats and mice^[1-6]. However, it is possible that morphine injection 30 min prior to training affected not only the acquisition but also the consolidation and retrieval of memory. We supposed that this injection mainly affected the acquisition of memory, because the half-life of morphine in the brain is up to ~1 h^[29,30].

In the consolidation group, the proportion of correct PERs exhibited a decreasing trend. In addition, the PER latency to the aversive stimulus was significantly lower than that of the control. These data suggest that morphine treatment slightly impaired the consolidation of memory in

Fig. 4. Mean ambulation distance of honeybees injected with morphine (Mor) or saline (Ctrl) 30 min prior to the test. The numbers on the bars indicate the number of honeybees tested in each condition. * $P < 0.05$ (one-way ANOVA).

the honeybees. Our data are in agreement with the results of studies in rodents^[7-9].

In this study, the latency was defined as the interval between the presentation of the rewarding or aversive odor and the extension of the proboscis. We hypothesized that when honeybees learned the association between a certain odor and an aversive stimulus, they would avoid extending their proboscis when that odor was presented by itself during testing. Here, we demonstrated that both the acquisition and consolidation groups showed a reduced latency for the aversive odor, indicating memory impairment regarding the association between the odor and aversion. In contrast, similar reductions were not found for latency in the case of the rewarding odor, indicating that these honeybees still possessed a memory of the association between the odor and the reward.

Taking this into account, we may conclude that morphine treatment impairs olfactory associative memory in honeybees, and this mainly results from impaired memory of the association between the odor and the aversive stimulus. Previous studies have shown that dopamine mediates aversive learning in insects^[32-37], and blocking dopaminergic receptors suppresses aversive learning in honeybees^[37]. So, the effects of morphine on associative memory in the honeybees may be mediated by the

dopamine systems. However, morphine affects learning and memory in primates and rodents mainly *via* opioid systems, but there is still controversy over whether similar systems exist in insects. On one hand, there are reports that an endogenous opioid system may be present in honeybees based on the use of morphine, naloxone and some opioid peptides^[26,28]. On the other hand, the honeybee genome sequence has shown that they have no homologous sequences of opioid receptor genes similar to those reported in vertebrates. Thus, the neurotransmitters involved in the effects found here require further investigation.

Another finding in this study was that morphine administration decreased the locomotor activity of the honeybees. Previous studies, however, have found that morphine exposure increases the locomotor activity in rats and mice^[4,38,39]. For example, morphine treatment prior to training and testing increases the total number of open-arm visits in a two-trial Y-maze with mice^[4]. It has been suggested that the high locomotor response in rodents is linked to dopamine release in the striatum^[38,39]. There are also reports that biogenic amines, principally dopamine and octopamine, modulate motor control in insects^[40–42]. The difference between these findings and ours may depend on experimental variables, such as dose and state (e.g., in the addiction or withdrawal phase). Besides, it is possible that a neurotransmitter system, such as the dopamine system, may perform different functions in different species. For example, the dopamine systems in primates and rodents are mainly associated with appetitive learning and in insects with aversive learning^[32–37,43,44].

In addition, evolutionary biologists have suggested that many plant secondary metabolites, including alkaloids such as morphine, nicotine and cocaine, are potent neurotoxins that have evolved to prevent consumption by herbivores. For instance, cocaine critically disrupts insect motor systems and protects the coca plant from consumption^[41,45–47]. Although studies show that addictive drugs such as cocaine share rewarding and reinforcing effects in insects^[21], the natural concentration in plants is toxic to them, especially their motor systems. Therefore, in this work, that morphine reduced the locomotion in honeybees is reasonable and in line with previous reports.

However, an intriguing question is raised by our finding that morphine treatment reduces the locomotor activity in honeybees; that is, whether this drug affects proboscis

extension and leads to a decrease in PER performance. As described above, the PER latency of the morphine injection groups to the rewarding stimulus was not different from control. Therefore, the effects of morphine on locomotor activity may not have influenced the PER performance in this study.

Taken together, the results of the current study showed that morphine negatively affected both memory processes and locomotor activity in honeybees. However, some previous studies have reported that addictive drugs such as caffeine and cocaine at a very low dose have positive effects on motivation or cognition in honeybees^[20,21]. The varying doses of drugs may be an important factor in defining the positive and negative test outcomes. We supposed that the dose of morphine in our work was relatively higher than that of caffeine or cocaine in previous studies, because the locomotion of honeybees in these studies was not reported or significant.

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