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# Effects of the Muscarinic Antagonists Atropine and Pirenzepine on Olfactory Conditioning in the Honeybee

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CANO LOZANO, V. AND M. GAUTHIER. *Effects of the muscarinic antagonists atropine and pirenzepine on olfactory conditioning in the honeybee.* PHARMACOL BIOCHEM BEHAV **59**(4) 903–907, 1998.—One-trial conditioning of the proboscis extension reflex (PER) in honeybees was used to examine the qualitative effects of two muscarinic antagonists, atropine and pirenzepine, on the acquisition and retrieval of memory following intracranial injection. The main result of this study is that atropine, at a relatively high concentration of $10^{-2}$ M, impairs memory retrieval but not acquisition of memory after a single olfactory conditioning trial (at this concentration, there is no effect of atropine on the sensorimotor components of the PER). This result is in agreement with the effects of scopolamine, reported in a previously published article. Pirenzepine, at the same concentration as atropine, had no effect on either acquisition or retrieval of memory. These results suggest that blockade of muscarinic-like receptors, except those that bind to pirenzepine, induces solely an impairment of memory retrieval. © 1998 Elsevier Science Inc.

Honeybee Pavlovian conditioning Memory processes Muscarinic antagonists Atropine Pirenzepine

ACETYLCHOLINE is a widespread neurotransmitter in the central nervous system of a large range of invertebrates including insects (6,7,9,12,28).

In the honeybee brain, most of the elements required for the functioning of cholinergic synapses have been detected: acetylcholinesterase is widely distributed in the brain (3,22), nicotinic binding sites have been localized (22,29), pharmacological characteristics of cholinergic muscarinic binding sites have been described (1,19), and the anatomical location of these sites is partly known (11).

In spite of the fact that acetylcholine is largely distributed in the brain, the question of its involvement in complex central functions has not been considered in the honeybee until recently. For some years, we have been trying to answer this question in the field of learning and memory.

Using the well-known and well-described one-trial olfactory conditioning of the proboscis extension reflex (PER) (16, 25,26) we have already shown that intracranial injections of scopolamine, a muscarinic antagonist, induce an impairment of memory retrieval (15). We have also shown an impairment of acquisition and retrieval processes after intracranial injection of the nicotinic antagonist mecamylamine (10). Both antagonists injected into the α-lobes of the mushroom bodies induced a severe impairment of memory retrieval (unpublished results). In other respects, we have shown that a five-trial learning session exerts a modulatory effect on brain acetylcholinesterase activity (14).

To confirm our previous results obtained with scopolamine, we examined the effects of two other muscarinic antagonists on memory processes. The antagonists chosen were pirenzepine (a vertebrate $M_1$-selective antagonist) and the nonselective antagonist atropine. Both drugs were first tested on the PER, and then were injected at well-defined times before or after the one-trial learning session.

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PRELIMINARY EXPERIMENT: EFFECTS OF ANTAGONISTS ON PROBOSCIS EXTENSION REFLEX

These experiments were undertaken to test the drugs effects on the reflex response of proboscis extension and to define the lowest efficient doses of the drugs.

*Method*

*Animals.* Experiments were performed in summer time with foragers from the same colony. Worker honeybees (*Apis mellifera*) of about the same age were caught at the hive entrance and collected in small Plexiglas boxes (8 × 6 × 10 cm) where they were kept a couple of hours with water ad lib. Once their activity dropped, the honeybees were fixed in small tubes with a drop of wax–collophane mixture on the thorax allowing free movements of head and forelegs. They were fed to repletion with a sugar–water solution (2 M), delivered directly on to the proboscis with a syringe. To minimize a possible habituation of the PER to repetitive antennal sugar test stimulations, they were left on average 24 h without food, before the beginning of the experiments.

*Injection of solutions.* To allow dissection of the head and injection of the solutions, the head and the antennae were immobilized with a strip of tape. A small window was made in the head cuticle between the antennae and the median ocellus. The size of the window permitted the passage of a microsyringe. A 45-min interval was left between dissection and the beginning of experiments to allow the honeybees to recover from the operation.

Atropine sulfate salt or pirenzepine dihydrochloride from Sigma (St. Quentin Fallavier, France) were used for injections.

A volume of 0.4 μl of atropine, pirenzepine, or Ringer solution was deposited at the brain surface level in the hemolymph. No loss of hemolymph was noticed after injection. Consequently, the final concentration of the drugs was equivalent between individuals, but no experiment was avalaible to control how far the injected drugs were diffusing into the brain. Drugs were prepared in a bee Ringer solution containing KCl ($2.68\ 10^{-3}$ M), $CaCl_2$ ($1.8\ 10^{-3}$ M), NaCl ($1.54\ 10^{-1}$ M), and sucrose ($1.17\ 10^{-2}$ M) in one liter of distilled water. All these chemicals were from Prolabo (Gradignan, France).

*Procedure.* Six groups of 15 honeybees were used in the atropine experiments. One control group received a Ringer injection. In the other groups, the drug was injected at concentrations varying from $10^{-1}$ M to $10^{-3}$ M, depending on the group. Three groups of 15 honeybees received an injection of pirenzepine (respectively, $10^{-1}$ M, $10^{-2}$ M, $10^{-3}$ M) made in parallel with a Ringer injection to a control group. In each group, extension of the proboscis to antennal sucrose (2 M) stimulation (unconditioned response, UR) was tested 1 min before and then 10, 20, 30, 45, and 60 min after injection.

*Results*

Before drug injection, the antennal sugar stimulation induced the PER in all the honeybees. Injection of Ringer did not affect the reflex response level (100% of UR) evaluated after 10, 20, 30, 45, and 60 min (Fig. 1). Atropine injections with concentrations higher than $2.5\ 10^{-2}$ M induced a decrease in the percentage of unconditioned responses (Fig. 1). This effect was time dependent and reversible. This preliminary experiment led us to use a concentration of $10^{-2}$ M of atropine, which did not affect the PER.

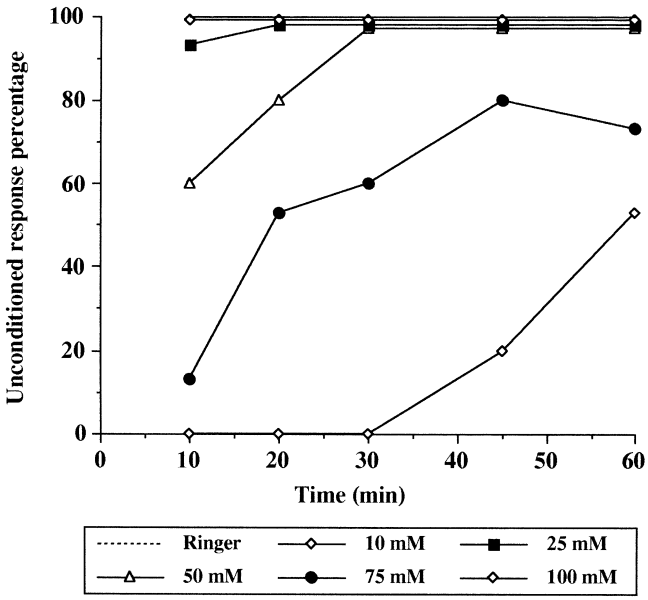


FIG. 1. Effects of different concentrations of atropine on PER (unconditioned response percentage) tested 10, 20, 30, 45, and 60 min after the injection ($n$ = 15 bees in each group).

Pirenzepine had no effect on the PER, whatever the dose of the drug and the delay between injection and the test. This result showed that neither the sensory afferent pathways nor the motor efferent pathways involved in this reflex were affected by pirenzepine. A concentration of $10^{-2}$ M of pirenzepine was used for behavioral experiments.

CONDITIONING EXPERIMENTS

These experiments were designed to evaluate drug effects on memory processes, using one-trial olfactory conditioning of the PER.

The one-trial learning paradigm has already allowed sequential memory processing to be demonstrated in the honeybee [for review, see (26,27)]. It has been shown that the conditioned response rate is at a maximum immediately after a single learning trial and drops to a minimum during the following 2 to 4 min. A high, 1-h stable performance level is reached after a period of 15 to 20 min, which subsequently fade according to a classic forgetting curve. This U-shaped curve of performance during the 30 min after a single learning trial could reflect a transient unavailability of the memory trace. This could correspond to transition of information from a short-term memory to a medium-term memory and to the so-called consolidation phase. In the context of these data, the principle of drug injection was as follows: pretrial injections performed 10 min before the learning session allowed us to test drug effects on acquisition processes; injections performed 20 min after learning, outside the estimated consolidation phase, served to test the effect of the drugs on retrieval processes. Considering that an amnestic effect induced by the pretrial injection could also lead to an impairment of retrieval processes, we first established the duration of a putative effect of the antagonist on the retrieval processes with the 20-min delayed injection experiment.

*Method*

*Animals and intracranial drugs injections.* Animals were caught from the hive and fitted out for experiments. The head of each animal was dissected to allow drug injection. All these points have been previously described in the Method section for the preliminary experiment. Learning experiments began 45 min after dissection of the head.

*Conditioning.* We used one-trial Pavlovian conditioning of the proboscis extension reflex. The conditioned stimulus (CS) was a scent of vanillin delivered to one antenna for 6 s. After the first 3 s of this olfactory stimulation, the unconditioned stimulus (US) consisting of a drop of sugar water (2 M), was presented to the same antenna, and this elicited the extension of the proboscis. Then, the bee was allowed to feed on a drop of sugar water for 3 s. In subsequent retention testing, the olfactory stimulus alone was presented to the honeybee. A bee that has learned to associate the CS and the US will show proboscis extension in response to the conditioned stimulus, this characterizing a conditioned response (CR).

Less than 1% of honeybees were discarded because they responded spontaneously to the vanillin odour. All the animals responded with a PER to antennal sucrose stimulation.

*Procedure.* In retrieval experiments, 0.4 $\mu$l of drugs ($10^{-2}$ M) or saline were injected 20 min after the conditioning trial. Six pirenzepine-injected groups and six saline-injected groups were constituted, each group comprising 30 honeybees. The conditioned response was tested immediately before and 5, 10, 20, 30, 45, and 60 min after injection. The same procedure was used for atropine injections. Five atropine groups and five saline groups were tested immediately before and 10, 20, 30, 45, and 60 min after injection. Each animal received an injection whether it responded or not to the odor test performed immediately before injection. Conditioning rates before and after injection were determined and compared in each group.

To test the effects of the drugs on acquisition processes, atropine ($10^{-2}$ M) or pirenzepine ($10^{-2}$ M) were injected 10 min before the conditioning trial in two groups of 30 honeybees. Each of these experimental groups was associated with a control group of 30 animals receiving a saline injection in the same conditions. The retention test was performed 45 min after conditioning for the atropine group (i.e., 55 min after injection, as a previous experiment had revealed that at this time, atropine was no longer active). For the pirenzepine group, performance was evaluated 30 min after conditioning (40 min after injection, as the previous experiment had shown that there was no observable effect of the drug on information retrieval).

*Statistical analysis.* In experiments with a 20-min delayed drug injection, each animal was its own control. Performance evaluated before injection was compared to that observed after injection with a Fisher $\chi^2$ test.

In pretrial injection experiments, the conditioned response rate of the experimental and control groups were compared with a Fisher $\chi^2$ test.

*Results*

*Twenty-minute posttrial injections.* In the control groups, the Ringer injection did not impair retention performance even at the shortest time after injection (Fig. 2a). Atropine produced a significant decrease ($p < 0.005$) of retrieval performance in groups tested 10 and 20 min after injection (Fig. 2b). No difference was observed in the other groups between pre- and postinjection performance, indicating that after 20 min, atropine was no longer active on retrieval processes.

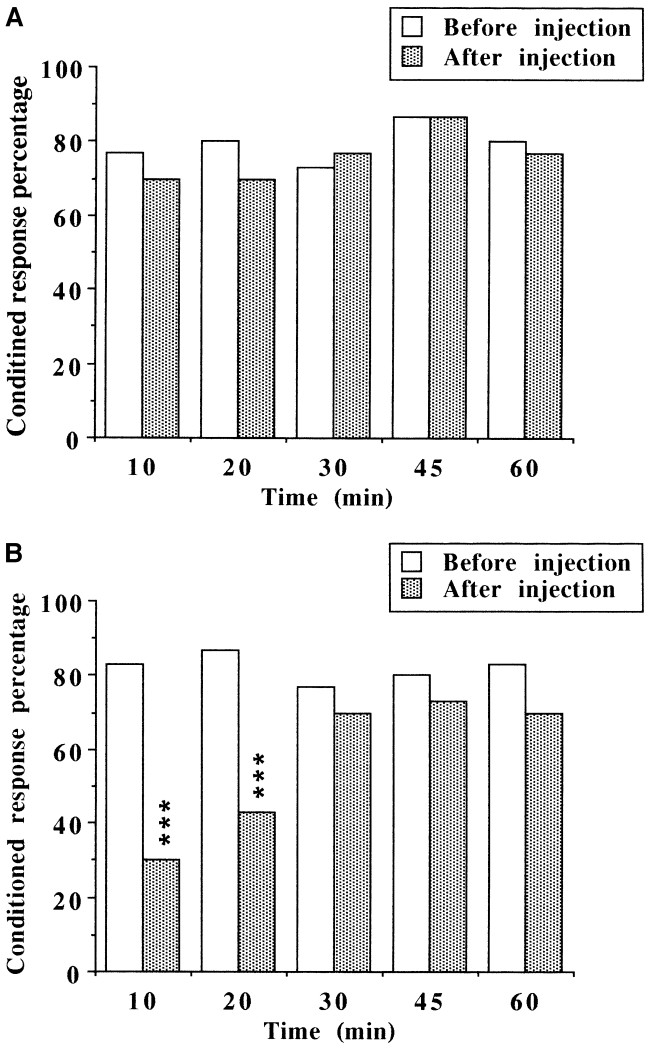


FIG. 2. (A) Retention performance (conditioned response percentage) measured in five groups ($n = 30$) tested immediately before and 10, 20, 30, 45, or 60 min after a 20-min posttrial Ringer injection. (B) Retention performance (conditioned response percentage) measured in five groups ($n = 30$) tested immediately before and 10, 20, 30, 45, or 60 min after a 20-min posttrial atropine injection. ***$p < .005$.

Pirenzepine, at the concentration of $10^{-2}$ M, had no effect on retrieval of the olfactory conditioned stimulus; neither did the Ringer injection. We first tested honeybees from 10 to 60 min after pirenzepine injection and no effect was detectable on retrieval performance. To make sure that we had not missed an earlier effect, we added one more group tested 5 min after injection. Even at this time, there was no effect of pirenzepine on retrieval performance (Fig. 3a and b).

*Pretrial injections.* The conditioned response rates of the control and atropine groups that received an injection 10 min before the conditioning trial were not statistically different (respectively, 83 and 77%). This indicated an absence of effect of atropine on the acquisition and consolidation processes.

In the group receiving a pretrial pirenzepine injection, the conditioned response rate was 73% and was not different from that of the control group (77%) that received a saline in-

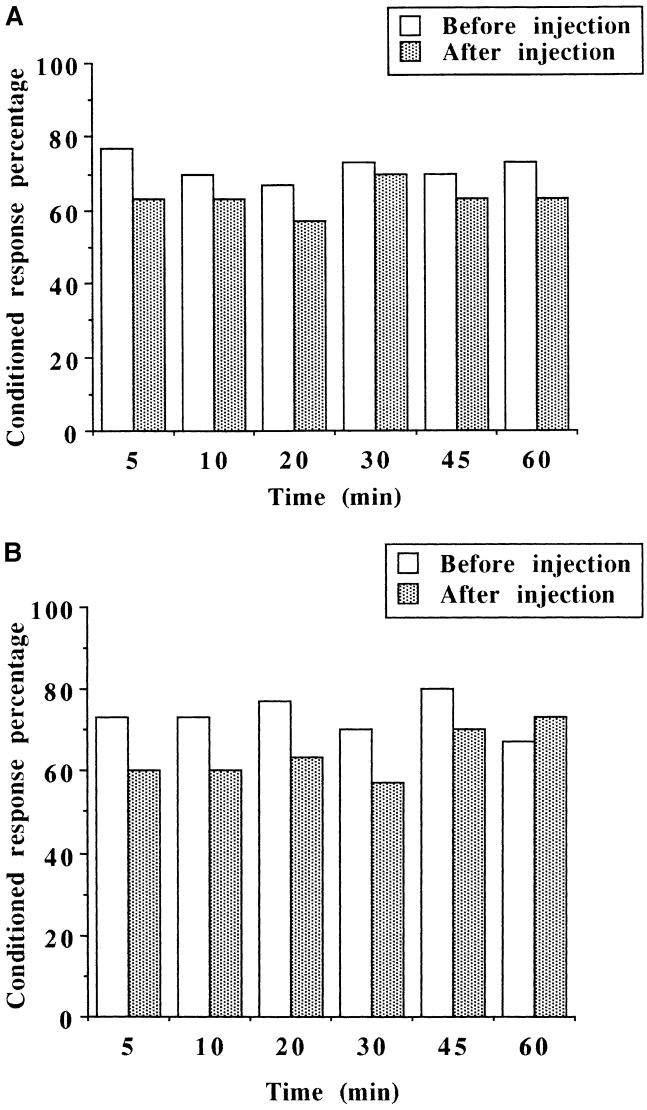


FIG. 3. (A) Retention performance (conditioned response percentage) measured in six groups ($n$ = 30) tested immediately before and 5, 10, 20, 30, 45, or 60 min after a 20-min posttrial Ringer injection. (B) Retention performance (conditioned response percentage) measured in six groups ($n$ = 30) tested immediately before and 5, 10, 20, 30, 45, or 60 min after a 20-min posttrial pirenzepine injection.

jection in the same conditions. So we can conclude that pirenzepine, like atropine, has no effect on acquisition and consolidation processes.

## DISCUSSION

The results obtained in the present experiments support our earlier findings showing that the cholinergic system is involved in central information processing in the honeybee (10,11,14,15) and that scopolamine selectively impairs retrieval of an olfactory conditioning task (15).

Atropine, like scopolamine, is considered to be a competitive muscarinic antagonist (30). Current preliminary experiments show that atropine blocks the PER when its concentration is greater than 2.5 $10^{-2}$ M. This result is close to the one obtained with mecamylamine, a nicotinic antagonist, in similar experiments (10) but it differs from results obtained with scopolamine. This drug does not affect the PER, whatever the concentration used (unpublished results).

Atropine, injected during a time period beyond the consolidation phase, induces a time-dependent decrease of conditioned response rate in subsequent testing. These results show a limited impairment of retrieval processes for up to half an hour. This observation gives an indication on the duration of the drug action on central functions. When injected before the conditioning trial, atropine has no effect on the subsequent retention performance. Several conclusions can be drawn. First of all, there is no effect of atropine on learning acquisition and, consequently, we can assume that atropine exerts no disrupting effect on peripheral and central processes of olfaction. Then, atropine injected 10 min before the conditioning trial is still active during the consolidation phase. (It has previously been demonstrated that, for this kind of learning, information remains sensitive to disrupting agents for less than 10 min after the acquisition session (13)). So we can conclude that atropine does not interfere with consolidation of information. The same kind of results were obtained with scopolamine injected in the brain hemolymph (15) or in the α-lobes of muhroom bodies (unpublished results). The similar effects of these two antagonists permit us to assume that muscarinic-like receptors are involved in processes of information retrieval.

We observe that scopolamine induces a stronger impairment with a lower concentration compared to atropine. This may be due to the fact that scopolamine has a greater affinity than atropine for muscarinic-like binding sites, as was shown with binding experiments (19).

To better characterize the effects of muscarinic antagonists, we have tested pirenzepine, an $M_1$ selective antagonist in vertebrates. We previously used this antagonist linked to a fluorescent dye (Bodipy®-Fl) to stain muscarinic-like binding sites in the brain of the honeybee (11). In the present work, we show that pirenzepine, at a dose of $10^{-2}$ M, has no effect on the conditioning rate whatever the delay between drug injection and the acquisition session. Pharmacological experiments have shown that pirenzepine has a weak affinity for the muscarinic-like receptors (1). Moreover, pirenzepine is characterized by a poor penetration into the brain in vertebrates (8). Consequently, the lack of effect of pirenzepine on memory processes may be the result of too low a concentration. Using a higher concentration of pirenzepine ($10^{-1}$ M, i.e., 130 mg/kg) we observed no effect on the reflex response (preliminary experiments) or on retrieval processes (personal observations on a small number of honeybees). We can conclude that, unlike scopolamine and atropine, pirenzepine does not disturb memory processes and, particularly, retrieval processes. But we cannot exclude the hypothesis of the presence of different kinds of muscarinic receptors, as has been demonstrated in other insects (1,2,21) that could have different roles in synaptic transmission (4,5,20,23,24). The lack of effect of pirenzepine we observed in our experiments may suggest that muscarinic-like receptors other than those that bind to pirenzepine, are involved in learning and memory in the honeybee.

It is accepted that acetylcholine is the neurotransmitter involved in the transfer of sensory information in insects. However, it has been shown recently that octopamine mediates gustatory stimulations and is thus identified as the US pathway transmitter (17,18). Until now, there has been no direct proof of the assumption that acetylcholine could be the neurotransmitter of the CS. We have demonstrated several times

that cholinergic antagonists do not impair olfactory perception (10,15), and we suggest that acetylcholine rather acts as a neuromodulator. It can be hypothesized that acetylcholine stimulation of pre- or postsynaptic muscarinic receptors activates second-messenger systems that may help to regulate the excitability of neurons to other neurotransmitters or the release of neurotransmitters (including acetylcholine) through a variety of $Ca^{2+}$-dependent and -independent mechanisms. In vertebrates, for example, this seems to be the case in the hippocampus where acetylcholine facilitates glutamaergic transmission. However, the subtypes of muscarinic receptors involved in the olfactory conditioning in the honeybee cannot be derived from our results because scopolamine and atropine are nonspecific antagonists. Other kinds of experiments would be necessary to go further in the understanding of intracellular mechanisms linked to olfactory learning.

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