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Involvement of NO-synthase and nicotinic receptors in learning in the honey bee

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ABSTRACT

Restrained worker honey bees (*Apis mellifera*) are one of the main models for the comparative study of learning and memory processes. Bees easily learn to associate a sucrose reward to antennal tactile scanning of a small metal plate (associative learning). Their proboscis extension response can also be habituated through repeated sucrose stimulations (non-associative learning). We studied the role of nitric oxide synthase and nicotinic acetylcholine receptors in these two forms of learning.

The nicotinic antagonist MLA or the nitric oxide synthase inhibitor L-NAME impaired the formation of tactile associative long-term memory that specifically occurs during multiple-trial training; however these drugs had no effect on single-trial training. None of the drugs affected retrieval processes. These pharmacological results are consistent with data previously obtained with olfactory conditioning and indicate that MLA-sensitive nicotinic receptors and NO-synthase are specifically involved in long-term memory. MLA and L-NAME both reduced the number of trials required for habituation to occur. This result suggests that a reduction of cholinergic nicotinic neurotransmission promotes PER habituation in the honey bee.

1. Introduction

Restrained worker honey bees provide a valuable model for studying the neurobiology of learning as they combine complex learning capabilities with a readily accessible nervous system [1–5]. A common learning paradigm involves olfactory conditioning of the proboscis extension reflex (PER); the proboscis is the mouthpart of the honey bee. This conditioning paradigm consists of blowing an odor across the antennae which are then touched by a drop of sucrose solution. The sucrose stimulation elicits a PER, and the bee is then allowed to consume the sucrose solution. Subsequent presentations of the odor alone are sufficient to induce the PER, revealing a new associative link between the odor and the sucrose and/or the PER [6]. The PER can also be conditioned to an antennal tactile stimulus. In this case, blind restrained honey bees learn to associate the sucrose reward with the antennal tactile scanning of a small metal plate, so that subsequent antennal scanning of the plate elicits the PER [7]. These protocols include features of both classical and operant conditioning [6,7], though the olfactory conditioning is mainly classical.

In invertebrates, the description of the different memory phases is somewhat different than in vertebrates [8–14]. In the case of honey bee conditioning, acquisition (i.e. training) leads to formation of a memory trace the duration of which depends upon acquisition strength. Single-trial acquisition induces the formation of medium-term memory (MTM, [2]), which underlies the performance up to 24 h after acquisition [15]. Multiple-trial acquisition produces both the initial MTM and a long-term memory (LTM, [2]), that underlies the performance from 24 h onwards. MTM underlies the performance during retrieval 3 h after acquisition for both single- and multiple-trial acquisition. This kind of distinction between LTM and MTM is an important topic in honey bee learning studies (for a review, see [2]). Synthesis of nitric oxide (NO) is required to form LTM in olfactory conditioning. Inhibiting the NO-synthase enzyme with Nω-nitro-L-arginine-methyl-ester (L-NAME) during acquisition specifically impairs LTM formation and leaves MTM intact [16], and releasing or activating second messengers of NO (cGMP, protein kinases) elicits LTM even after single-trial acquisition [17–20].

Several studies have demonstrated the presence of nicotinic acetylcholine receptors in the honey bee brain [25–41]. Various antagonists [e.g. mecamylamine, α-bungarotoxin, dihydro-β-erythroïdine, methyllycaconitine (MLA)] can be used to block nicotinic receptors. In vertebrates these drugs are known to target different types of nicotinic receptors: mecamylamine (at low dose) and dihydro-β-erythroïdine block α-bungarotoxin insensitive receptors, whereas MLA blocks α-bungarotoxin sensitive receptors (see [42] for a review). Similarly, the existence of two types of nicotinic receptors

Abbreviations: D-NAME, Nω-nitro-D-arginine-methyl-ester; L-NAME, Nω-nitro-L-arginine-methyl-ester; LTM, long-term memory; MLA, methyllycaconitine; MTM, medium-term memory; NO, nitric oxide; PER, proboscis extension reflex; PKA, protein kinase A.

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was reported in adult bees [35,43] and is also described in other insects [44–57]. Previous studies have shown that like NO-synthase inhibitors [16], α-bungarotoxin and MLA specifically impaired the formation of LTM during multiple-trial acquisition in olfactory conditioning, whereas mecamylamine blocked retrieval processes and single-trial olfactory acquisition [58–61]. These results have been partly reproduced with tactile conditioning, using α-bungarotoxin and mecamylamine injections [62] but the effects of MLA and NO-synthase inhibitors on this form of learning are not known. Furthermore, as NO and its second messengers are involved in chemosensory perception [18,21–24] they may be specifically linked to olfactory conditioning, rather than being involved in common learning processes.

As all the experiments involving nicotinic drugs have had consistent results between olfactory and tactile conditioning paradigms, we can make the hypothesis that MLA will have the same effect in tactile conditioning as in olfactory conditioning, i.e. specifically blocking LTM. Similarly, we expect that NO plays the same role in tactile learning as in olfactory learning. We will test this hypothesis (hypothesis 1) by injecting MLA (nicotinic antagonist) and L-NAME (NO-synthase inhibitor) during antennal tactile conditioning. We will particularly focus on the effects of these drugs on LTM, as we expect to see a specific inhibition of LTM while MTM should remain unaffected. It is also worth noting that verifying hypothesis 1 would be an indication that the formation of olfactory and tactile long-term memory could share common neurobiological processes.

Both olfactory and antennal tactile conditioning are associative processes. They involve the formation of an association between the PER (and/or the sucrose reward) and either the odor or the antennal scanning of the metal plate, respectively. The PER is also a suitable response for studying non-associative learning such as habituation. In this paradigm, repeated sucrose stimulations of the antennae progressively lead to a decrease and finally a disappearance of the PER occurrence [63–66]. The best-studied model to explain the neurobiological basis of habituation has been the *Aplysia* gill withdrawal reflex, which relies on homosynaptic depression of primary afferent terminals. The decrease of neurotransmitter release induced by repeated non-noxious stimulation of sensory neurons of the head or the tail of the animal leads to a gradual decline of the gill withdrawal [8]. In the honey bee, the PER is released by antennal sugar stimulation and gustatory information from the antennae ends in the dorsal lobe of the deutocerebrum [67] and probably reaches the motor neurons located in the suboesophageal ganglion driving the mouthpart movements [68]. These primary gustatory afferents are probably cholinergic as α-bungarotoxin binding sites have been found in the dorsal lobes [29,30]. In addition, several different nicotinic α subunits are expressed in the dorsal lobe [26,27]. We previously observed a facilitation of PER habituation induced by the insecticide imidacloprid [69,70] (see also [43]), which displays both nicotinic agonist and antagonist properties [32]. This suggests that nicotinic receptors are involved in habituation (see also [66]), but the complex effect of imidacloprid prevented a clear interpretation. Thus, we were interested in investigating the role of the cholinergic pathways in habituation of the PER in the honey bee.

Repeated sucrose stimulation applied to the antenna leads to a gradual increase in protein kinase A (PKA) activity mediated by the NO/cGMP system in the antennal lobes, a part of the deutocerebrum [21–23]. Müller and Hildebrandt [21] reported that NO-synthase inhibition in the antennal lobe impairs PER habituation. However, their experiments were performed on satiated animals. Here, we would like to investigate habituation using hungry bees, as are bees used for associative conditioning. Our aim was to have similar satiation levels for both associative learning and non-associative plasticity protocols. Owing to the importance of satiation level on habituation [63–66], our hypothesis (hypothesis 2) is that performing habituation with hungry bees may lead to a different result compared with previous papers [21,66] that used satiated bees. Indeed, the role of nicotinic receptors and NO-synthase could be different in each case. To test hypothesis 2, we injected L-NAME before a PER habituation session and compared its effect to the effect of MLA on habituation. Finally, as the two hypotheses involve studying the effect of L-NAME and MLA on associative and non-associative learning using hungry bees, we will be able to compare the effects of both drugs on both learning situations. We expect that they will be the same in each protocol as both drugs have the same effect on olfactory learning.

2. Materials and methods

2.1. Animals

Honey bees were caught from within the hive and placed on ice until they stopped moving, then they were restrained in small tubes and fed ad libitum with sucrose solution (1.17 M, see [62] for details). Bees were left undisturbed and unfed overnight. The median ocellus lens (at the top of the head) and a small area of cuticle around it were removed. This operation was made in order to perform the drug injection (see below), so that it would affect the whole brain. Furthermore, animals that were used in antennal tactile conditioning had their eyes painted black with water diluted black acrylic paint to prevent them from using visual cues.

2.2. Drug and injection

The nicotinic antagonist MLA (2 μM) was used [36,38,42]. Concentration of this drug was chosen according to preliminary data [71] to make sure that the treatment did not affect sucrose sensitivity (measured on the antennae following established procedures [72,73]) or the PER. The NO-synthase inhibitor L-NAME (200 μM) was used at a concentration that was already demonstrated to completely block NO-synthase and to impair olfactory LTM without any effect on sucrose sensitivity or on the PER [16] (the injection was made in the thorax in [16] while we injected in the head, but in both cases this is a global injection affecting the entire brain). The inactive enantiomere (D-NAME, 200 μM) was used as control for L-NAME injected animals. D-NAME leaves honey bees' behavior intact and animals treated with this product did not differ from those treated with saline solution or from untreated animals, so it is routinely used as a control for L-NAME treatment [16]. All drugs were purchased from Sigma and were dissolved in bee saline solution (pH 7.8, 609 mOsmol l^{-1}: 2.7 mM KCl, 154.0 mM NaCl, 1.8 mM $CaCl_2$, 11.7 mM sucrose, 80.5 mM Na_2HPO_4, 18,5 mM NaH_2PO_4). Saline was used as control for MLA experiments. Solutions were injected into the hemolymph using a 1 μl microsyringe (Hamilton). The tip of the needle was gently introduced into the hole previously made in the head by removing the median ocellus lens. The needle did not enter the brain. The volume of the injection was 0.5 μl.

2.3. Habituation (test of hypothesis 2)

MLA (using saline as control) or L-NAME (using D-NAME as control) was injected in the hemolymph of the head 15 min before starting the habituation protocol. During habituation, both antennae were regularly stimulated with a sucrose solution (29 mM) every 10 s. The concentration and the inter-trial interval values were chosen to keep antennal sensilla sensitive to sucrose and to prevent sensory adaptation [65]. This point is important, as sensory adaptation could be misinterpreted as habituation. As stated above, the bees were food-deprived since the previous day. The sucrose stimulation was delivered onto the antennae, as stimulating the proboscis would allow the bees to drink the sucrose solution (which would prevent habituation and modify their satiation level). The criterion for habituation was 5