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Enhanced cholinergic transmission promotes recall in honeybees

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ABSTRACT

The involvement of the cholinergic system in learning and memory in honeybees has been well established using olfactory conditioning. We examined the effect of Methyl Parathion (MeP), an acetylcholinesterase inhibitor of the organo-phosphate family, on the learning and recall of visual and olfactory discrimination tasks in honeybees. One of our expectations was to observe the effects induced by both the nicotinic and muscarinic systems, as the blocking of acetylcholinesterase should induce an increase in the activity of both systems. We were also interested in knowing whether the type of tasks could influence the results. The visual tasks involved learning to discriminate the orientation of gratings in a Y-maze; the olfactory task involved learning to discriminate odours in a proboscis extension reflex (PER) paradigm. The results indicate that MeP treatment enhances recall of learned tasks in the visual and olfactory domains, but it does not affect the acquisition phase in either domain. Surprisingly, MeP treatment led to muscarinic-like effects but failed to mimic the nicotinic-like effects already described in relation to learning phases in honeybees. Implications for the role of cholinergic pathways in learning and memory and the nature of their involvement are discussed, and a hypothesis relating to the organisation of the cholinergic system and the relationship between the nicotinic and muscarinic systems in honeybees is proposed. The results are also discussed in terms of their ecotoxicological consequences.

1. Introduction

The mechanisms underlying learning and memory have long interested researchers, and there has been considerable interest in unraveling the participation of a number of different neuromessenger systems in these processes (e.g. for review in the honeybee see Bicker (1999); Weinberger (2006); Farooqui (2007)).

In the mammalian cholinergic system, the nicotinic (Levin and Simon, 1998) and muscarinic (Fibiger et al., 1991) pathways have been linked to learning and memory. In mammals, for example, treatment with nicotinic agonists are known to improve performance on a variety of memory tasks, whereas nicotinic antagonists can impair memory function (Levin and Simon, 1998). In the honeybee, a popular invertebrate model in the study of learning and memory, work over the past 10 years has demonstrated the involvement of the cholinergic and aminergic systems in learning and memory (Lambin et al., 2001; Cano Lozano et al., 1996, 2001; Gauthier et al., 1994, 2006; Cano Lozano and Gauthier, 1998; Hammer and Menzel, 1998; Guez et al., 2001, 2003). Most of these studies have used the well-known olfactory conditioning paradigm of the proboscis extension reflex (PER) (Kuwabara, 1957) or its habituation (Braun and Bicker, 1992). Braun and Bicker (1992) used this paradigm to show that eserine, an acetylcholinesterase blocker, significantly altered the performance of bees in the habituation of the proboscis extension reflex (PER) by increasing the number of trials before the onset of habituation.

It has also been shown that nicotinic agonists such as Imidacloprid (a neonicotinoid) have a significant impact on the habituation of the PER in young bees (Guez et al., 2001, 2003). In 7-day-old bees, treatments with Imidacloprid led to an increase in the number of trials before habituation. In comparison with 8-day-old bees, the same treatments led to a decrease in the number of trials before habituation 15 min and 1 h after treatment, and an increase in the number of trials 4 h after treatment. In the case of older bees (of foraging age), Imidacloprid treatments led to a decrease in the number of trials before habituation 1 h after treatment (Lambin et al., 2001). In addition, evidence obtained by using antagonists of the nicotinic system (Cano Lozano et al., 1996, 2001) and the muscarinic system (Cano Lozano and Gauthier, 1998; Cano Lozano et al., 2001) suggests the participation of the nicotinic system in acquisition and retrieval. Treatments with nicotinic antagonists compromise acquisition as well as recall (Cano Lozano et al., 1996, 2001). On the other hand, treatments with muscarinic antagonists compromise only the recall process,

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suggesting that the muscarinic system is involved solely in the recall process (Gauthier et al., 1994; Cano Lozano et al., 1996, 2001).

In the present study we investigated the role of the cholinergic system on learning and memory in the honeybee. We used Methyl Parathion (MeP), an insecticide of the organo-phosphate family, which is known to affect the insect cholinergic pathway by blocking acetylcholinesterase (e.g. Haynes, 1988; Fukuto, 1990). We examined the effect of MeP on acquisition and retention of learning by using two well-known associative learning paradigms. One paradigm involved training free flying bees to choose between two visual stimuli in a Y-maze by associating one of them with a food reward (revs. Wehner (1981); Srinivasan (1994)). The second paradigm involved the well-known olfactory conditioning of the PER (Kuwabara, 1957).

2. Materials and methods

2.1. Y-maze experiments

All experiments were performed in the all weather bee flight facility at the Australian National University. Forager Bees (*Apis mellifera*) were first trained to enter the Y-maze by visiting a feeder placed in the maze. The feeder was progressively placed further into the maze and its placement was alternated in both branches of the maze. The feeder was then placed in the reward box at the back of the apparatus (see Fig. 1), and the bees were trained to enter the reward box in order to gain access to the feeder. The feeder position alternated in each reward box (grey targets were present at the front of each reward box and therefore could not be used to discriminate between the non-reward and the reward box). Getting the bees to enter the reward box was the longest part of the pre-training. Bees who were accessed the feeder at this stage were individually marked using non-toxic paint, and were trained to discriminate between two visual stimuli, each presented in one arm of a Y-maze (Fig. 1). The stimuli differed either in orientation (as in Fig. 3) or in colour (as in Fig. 6). In each experiment, one stimulus (termed the positive stimulus, +) was associated with a reward of sugar solution provided by a feeder placed in a box behind the stimulus. The other stimulus (termed the negative stimulus, −) carried no reward. Bees could access the reward by entering the box via a tube passing through the centre of the stimulus. During training, the positions of the two stimuli were regularly interchanged. This ensured that the bees learned to associate the reward with the positive stimulus, and not with a particular arm of the Y-maze.

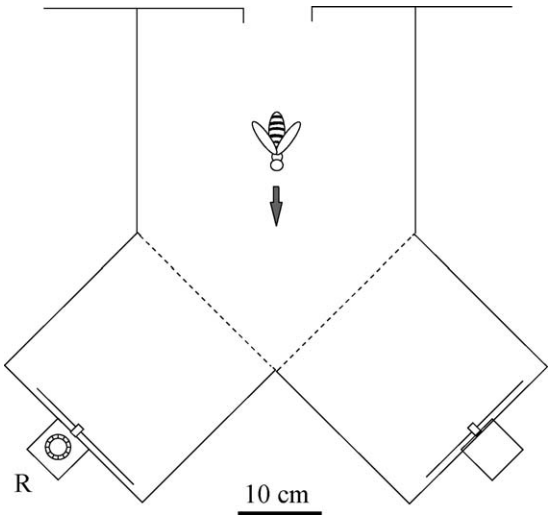

Fig. 1. Plan view of Y-maze (transparent plexiglass top, sides and bottom). Each stimulus is presented in the vertical plane on the end wall of one arm. The feeder is placed in the reward box, R, behind the positive stimulus. A similar box, carrying no reward, is placed behind the negative stimulus.

Although we were using indoor facilities, the bees were also given access to the outside environment and so hive activity was still greatly influenced by outdoor weather conditions. However, it was not possible to run the control and treatment sets that were needed in order to account for hive and weather variations (etc.) if bee were trained individually, so bees were trained simultaneously. For this reason, a training block was deemed to be complete when each stimulus had occupied each arm of the Y-maze once and not after a specified number of trials for each individual bee. The total duration of the first training block was 30 min, with each stimulus remaining in a given arm for 15 min. The duration of each subsequent training block was 20 min, with each stimulus remaining in a given arm for 10 min. The choice performance of each marked bee was continuously monitored, starting from the commencement of the training. The performance was scored by recording the first choice of each bee after it entered the Y-maze. If a bee entered the arm containing the positive stimulus, it was regarded as a correct choice (such a visit invariably resulted in the entry of the reward box and reinforcement). An entry into the arm containing the negative stimulus was regarded as an incorrect choice. The choice frequency in favour of the positive stimulus was calculated separately for each block as $(n_1/n_1 + n_2) \times 100$, where n_1 and n_2 denote the number of correct and incorrect choices, respectively. Control tests, carried out by temporarily removing the feeder from its usual location behind the positive stimulus, assured us that the bees were not choosing the correct stimulus on the basis of pheromone scents deposited on the feeder. Further details of training and testing procedures for Y-mazes are given in van Hateren et al. (1990).

Each training/testing experiment was carried out at least three times using a fresh set of bees each time. The figures show the choice frequencies in favour of the positive stimulus for each training block, obtained by pooling choices across bees and across repeated experiments.

2.1.1. Treatment

Bees were treated by topical application of 1 μl of MeP (Sigma) dissolved in DMSO (50 mg/l). The DMSO (solvent) functioned as a transport medium for the drug by facilitating the transfer of the drug through the insect cuticle. The drug was applied on the dorsal side of the thorax while the bees visited and drank at the feeder during a 10-min period prior to acquisition (Fig. 3), prior to reacquisition (Figs. 4 and 5) or prior to recall (Fig. 6). Since preliminary experiments and previously published works showed that DMSO did not elicit any observable behavioural effects in free flying conditions (Guez et al., 2005), and given the experimental constraints that precluded the simultaneous use of a large number of bees in order to follow them accurately, controls with DMSO were not included. Pure DMSO has also been shown to have no effect on other aspects of honeybee behaviour, such as habituation (Guez et al., 2001, 2003). The only claim of an effect of DMSO on honeybee behaviour can be found in Lambin et al. (2001) on habituation, although this effect was obtained only when DMSO was mixed with a saline solution and not on its own (see also Guez et al. (2001, 2003).

Our choice of using topical application over injection was based upon two factors. Firstly, we were using honeybees in free flying condition which made the use of injections unpractical. As noted by Barron et al. (2007), topical application is technically easier and much less stressful than injection for the animal as it does not involve anaesthetic treatments. Secondly, injection and anaesthesia treatment in insects are known to cause an immune response