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Immune response inhibits associative learning in insects

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In vertebrates, it is well established that there are many intricate interactions between the immune system and the nervous system, and vice versa. Regarding insects, until now little has been known about the link between these two systems. Here, we present behavioural evidence indicating a link between the immune system and the nervous system in insects. We show that otherwise non-infected honeybees whose immune systems are challenged by a non-pathogenic immunogenic elicitor lipopolysaccharide (LPS) have reduced abilities to associate an odour with sugar reward in a classical conditioning paradigm. The cost of an immune response therefore not only affects survival of the host, as previously shown, but also everyday behaviour and memory formation.

Keywords: *Apis mellifera*; trade-offs; immunity; associative learning; proboscis extension

1. INTRODUCTION

Recent experimental work in animal learning has begun to document marked effects of parasitic infections on the ability of individuals to learn and remember certain tasks (Kavaliers & Colwell 1995; Sheldon & Verhulst 1996). There is the possibility that these effects are due not to any pathogenic actions of the parasite but rather to the immune response of the host itself. Great interest has been shown in these and other connections between the nervous system and the immune system (Ader *et al.* 1991; Maier *et al.* 1994; Dunn 2001). All of this work has, thus far, concentrated on vertebrates. Here, we show the suitability of the honeybee *Apis mellifera* for these kinds of study.

Associative learning and memory formation, in the honeybee, *A. mellifera*, can be readily studied and quantified by exploiting the proboscis extension reaction (Bitterman *et al.* 1983; Hammer & Menzel 1995; Menzel & Müller 1996; Hammer 1997; Faber *et al.* 1999; Menzel & Giurfa 2001). In this experimental paradigm, when their antennae are stimulated by sugar water (the unconditioned response (US)) honeybees extend their proboscis. If prior to this sugar stimulation an odour (the conditioned stimulus (CS)) is presented, the honeybee will associate this odour with the sugar stimulus. Learning in this paradigm occurs very fast such that even after only one learning trial many bees will be conditioned to respond by extending their proboscis to the odour alone.

Honeybees are potential hosts to a large and varied fauna of parasites (Morse & Flottum 1997). As with all insects, they possess a highly developed immune system with which to defend themselves (Gillespie *et al.* 1997), comprising both cellular and humoral arms. Previous studies have shown that this immune response can be elicited by non-pathogenic means (Moret & Schmid-Hempel 2000). Lipopolysaccharide (LPS), a component of gram-negative bacteria cell walls, is one such non-pathogenic immune elicitor. We therefore propose that honeybees, with both their well-known neurophysiology and behaviour (Menzel & Mercer 1987) and the ease of manipulation of their immune response, are ideally suited to answer questions on the connections between the immune and neural systems. We begin by using the proboscis extension reaction to assess the effect of an immune response on the ability of a host to learn.

2. METHODS

(a) *Injection of treatments*

We challenged the bee's immune system by injecting, into the haemolymph, a dose of 2 µl of Ringer solution of 4% LPS (Sigma L-2755) (0.5 mg ml^{-1}), a highly immunogenic but non-pathogenic elicitor of the immune response (Moret & Schmid-Hempel 2000). In all, an experimental group of $n = 63$ bees was injected with LPS. Two control groups were used: (i) bees ($n = 59$) were injected with 2 µl of Ringer solution alone to control for the effect of the injection; and (ii) bees ($n = 60$) were sham-manipulated with no injection occurring to control for the effects of handling. The bees originated from three colonies and were assigned so as to balance colony origin across all treatments. After experimental manipulation, each group was housed for 3 days in plastic containers (17 cm × 13.5 cm × 9.5 cm) and fed *ad libitum* before testing began. This time allows an immune response to be expressed (P. Korner, personal communication).

(b) *Sugar response threshold test*

The night before they were to be tested, bees were harnessed in small metal tubes. They were fed *ad libitum* until fully engorged and then left overnight. Before the conditioning experiment, bees were first tested to determine whether treatment had an effect on their sugar response threshold. Each bee was offered at, 10 min intervals, either water or sucrose solution in concentrations of 0.1%, 0.3%, 1%, 3%, 10% or 30%. Whether or not they responded was recorded. Bees that did not respond were not included in the memory consolidation test.

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(c) *Olfactory conditioning*

The olfactory conditioned stimulus used was citronella mixed with mineral oil. Each bee was given one learning trial, i.e. one US/CS pairing. For this, each bee was first exposed to a continuous air stream for 45 s. Then the citronella odour (US) was pulsed into the continuous air stream for 2 s. Directly upon the offset of the odour pulse, the bee was rewarded for 3 s with 30% sucrose water (CS). Either 1 min or 12 min after this learning trial, the bees were tested by offering them the odour alone (45 s in the continuous air stream and 2 s of odour stimulation), and their proboscis extension response was observed (Menzel 1990; Pelz *et al.* 1997).

(d) *Measurement of zone of inhibition*

We wished to confirm the previously found increase in antibacterial activity (immune response) found in LPS-injected bees (Moret & Schmid-Hempel 2000). One of the simplest techniques to measure the antibacterial activity is to perform a zone of inhibition assay. This assay is based on the ability of immune proteins to inhibit bacterial growth when placed onto an agar plate seeded with bacteria (*Arthrobacteur globiformis*; 105 bacteria ml^{-1} of agar). All workers used from one of the colonies were sacrificed after the memory assay and stored at -20 °C for later analysis. Each thorax was homogenized in 300 µl of sodium cacodylate solution and 2 µl of the supernatant from the centrifuged solution (1300 g, 10 min, 4 °C) were pipetted into a hole on the agar plate. This was incubated overnight (at 28 °C). The resulting zones of inhibition (in mm^2) were measured by the computer program ImageJ.

3. RESULTS

(a) *Increased antibacterial activity*

We confirmed the increased expression of antibacterial activity in the haemolymph of LPS-challenged bees (Moret & Schmid-Hempel 2000) (zones of inhibition; LPS: median ± interquartile range 18.39 ± 22.7 mm^2, $n = 11$; Ringer-injected: 7.42 ± 5.19, $n = 13$; sham-manipulated 5.46 ± 7.46, $n = 23$; Kruskal–Wallis $H = 8.781$, d.f. = 2, $p = 0.012$. Mann–Whitney comparing LPS with Ringer-group: $U = 30$, $p = 0.016$).

(b) *Sugar response threshold test*

The treatments (sham, Ringer and LPS) did not affect the response thresholds (Page *et al.* 1998; Scheiner *et al.* 2001*a*,*b*). In a binary logistic regression, treatment was found to have no effect (Wald $\chi^2 = 3.322$, d.f. = 2, $p = 0.190$).

(c) *Effects on associative learning*

We found that there was no difference in the response between the three groups when tested at 1 min ($\chi^2 = 0.429$, d.f. = 2, $p = 0.81$; figure 1). However, at 12 min, activating the immune system led to a significantly lower response of LPS-treated bees ($\chi^2 = 8.81$, d.f. = 2, $p = 0.012$; figure 1). In particular, in a pairwise test, LPS-treated bees responded significantly less than the Ringer control ($\chi^2 = 5.11$, d.f. = 1, $p = 0.024$).

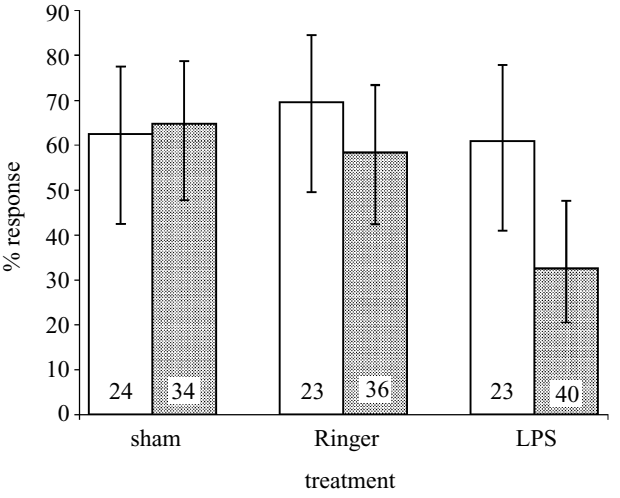

Figure 1. Percentage of bees responding to the odour stimulus when tested after 1 min (open bars) or 12 min (shaded bars). The numbers within the bars are the respective sample sizes. Responses did not differ after 1 min ($\chi^2 = 0.429$, d.f. = 2, $p = 0.81$) but were different after 12 min ($\chi^2 = 8.81$, d.f. = 2, $p = 0.012$). In particular, in a pairwise test, LPS-treated bees responded significantly less than the Ringer control ($\chi^2 = 5.11$, d.f. = 1, $p = 0.024$). Error bars show the 95% confidence limits.

4. DISCUSSION

We have shown that LPS-injected bees have a reduced ability to associate an odour with a sugar reward when compared with control bees. As LPS has no pathogenic effects, this strongly suggests that the immune response, triggered by the LPS, somehow interfered with learning and/or memory formation during the experiment.

The time-course of memory formation in honeybees is highly stereotyped (Menzel 1999). Less than 1 min after a single learning trial, the bee is highly sensitized to respond to any appropriate olfactory stimulus. Over the next 2 min this non-associative, sensitization effect disappears. Associative memory develops over several minutes. In the first minutes after the conditioning trial, the associative memory is still very fragile and liable to disruption. At *ca.* 10 min after one conditioning trial the response level is at a similar level as the previous non-associative sensitization response at 1 min (Menzel 1999).

In our results, the similar high response level in all treatment groups at 1 min after conditioning demonstrates that activating the immune response with LPS has no effect on the general ability of bees to perceive and respond to an olfactory stimulus and has no effect on the sensitization effect of the sugar water reward. The decrease in proboscis response of LPS-treated bees at 12 min shows that the immune response does, however, have an effect on the bees' ability to associate an odour with sugar reward.

Why then should immune function affect associative learning? Both processes rely on intercellular communication via biogenic amines and other messenger molecules and upregulation of enzyme activity and gene expression via second messenger cascades. It is reasonable to assume that a trade-off in substrates could exist.

The specific effect found here, i.e. a reduction in associative learning but not in sensitization, is particularly intriguing. It tentatively suggests that some specific substrate that would have normally been required for associative learning and/or memory formation may be used up in the immune response. There is strong evidence in

honeybees that associative learning depends on octopamine release (Müller 2000). Octopamine could be a possible candidate for trade-off substrate, as it has been found to be important in immune response (Wiesner *et al.* 1996).

A different hypothesis is that there is signalling between the immune system and the nervous system in insects. Recent studies in vertebrates have demonstrated a two-way communication between the nervous system and the immune system (Ader *et al.* 1991; Pugh *et al.* 2001). For example, it has been found that the pro-inflammatory cytokine interleukin-1-β, released as part of the immune response, affects the hippocampus and so leads to a reduction in memory consolidation (Pugh *et al.* 2001). A similar communication in insects is possible. Eicosanoids (Stanley 2000), oxygenated metabolites of certain polysaturated fatty acids (arachidonic acid), act as mediators of insects' responses to bacterial infection (as simulated here by the LPS challenge) (Park & Kim 2000; Stanley & Howard 2001; Dean *et al.* 2002) and they have also been shown to modulate invertebrate neural and synaptic physiology, which is important for learning processes (Piomelli 1994).

In vertebrates, the emerging field of psychoneuroimmunology (Ader *et al.* 1991) studies the ever-increasing number of connections between neurobiology and immunology. Regarding insects, the two latter fields are still separated, although in each system similar substances have been found to be important in signalling pathways, e.g. octopamine, eicosanoids and nitric oxide. Our results are the first, to our knowledge to suggest a connection between the immune and nervous systems in insects. This adds yet another layer of similarity between insects and vertebrates.

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