Virus infection causes specific learning deficits in honeybee foragers

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In both mammals and invertebrates, virus infections can impair a broad spectrum of physiological functions including learning and memory formation. In contrast to the knowledge on the conserved mechanisms underlying learning, the effects of virus infection on different aspects of learning are barely known. We use the honeybee (Apis mellifera), a well-established model system for studying learning, to investigate the impact of deformed wing virus (DWV) on learning. Injection of DWV into the haemolymph of forager leads to a RT-PCR detectable DWV signal after 3 days. The detailed behavioural analysis of DWV-infected honeybees shows an increased responsiveness to water and low sucrose concentrations, an impaired associative learning and memory formation, but intact non-associative learning like sensitization and habituation. This contradicts all present studies in non-infected bees, where increased sucrose responsiveness is linked to improved associative learning and to changes in non-associative learning. Thus, DWV seems to interfere with molecular mechanism of learning by yet unknown processes that may include viral effects on the immune system and on gene expression.

Keywords: Apis mellifera; virus; learning; memory; responsiveness

1. INTRODUCTION

In vertebrates, viral infections can cause defects in the morphology and in the function of the nervous system including a wide range of impairments in cognitive and motor function, but also social behaviour (Tomonaga 2004; Beraki et al. 2005). In human brains, HIV infection selectively damages the cortex and affects brain regions that control language, sensory and motor functions (Thompson et al. 2005). Viral infection in mice causes neurobiological impairments and alterations in aggressive behaviour, cognitive ability, locomotor activity and deficits in spatial reference memory (Kamitani et al. 2003; Beraki et al. 2005). Even offspring of mice infected by the influenza virus show deficiencies in exploratory behaviour and social interaction (Shi et al. 2003). Observations from a variety of insect species also reveal evidence for the effects of viral infection on developmental processes, locomotor activity, feeding, mating and other behaviours (Platt et al. 1997; Burand et al. 2005; Kamita et al. 2005; Vasconcelos et al. 2005).

This—together with the high conservation of molecular processes underlying learning (Kandel 2001)—prompted us to study the impact of viral infection on different, well-characterized forms of learning in insects. The honeybee (Apis mellifera) provides an ideal model organism in this respect, since honeybees show a rich and well-investigated behavioural repertoire (Menzel & Müller 1996) and can be infected by more than 18 different viruses (Bailey & Ball 1991; Allen & Ball 1996).

Here, we study the impact of controlled infection with deformed wing virus (DWV) on sensory perception, non-associative and associative learning in adult honeybees. DWV infections are very abundant and can be detected in up to 90% of colonies (Tentcheva et al. 2004; Berenyi et al. 2006). While DWV infection in early development leads to deformation of wings, paralysis and mortality of the emerging bees (Lanzi et al. 2006), the infection of adult bees does not show such deformation. However, the DWV-infected colonies suffer from weakness, depopulation and sudden collapse (Berenyi et al. 2006).

In honeybees, the proboscis extension response (PER), which is elicited by an appetitive stimulus to antennae or proboscis, allows for testing sensory capabilities, non-associative as well as associative forms of learning (Menzel & Müller 1996). Our results show that controlled infection of honeybee foragers by DWV in the laboratory causes specific impairments in sensory responsiveness and associative learning, while non-associative learning remains intact.

2. MATERIAL AND METHODS

(a) Collection and screening of virus-infected honeybees

Adult bees were collected from hives owned by beekeepers near Saarbrücken, Germany (winter to spring 2006). At the collection site, bees were directly frozen and stored in liquid nitrogen until RNA extraction and analysis.

(b) RNA extraction

Five adult bees from each colony were pooled and homogenized in liquid nitrogen and 80–100 mg of the homogenized tissues was mixed with 1 ml Trizol reagent (Invitrogen, Germany) according to the manufacturer's instructions. To reduce the level of contamination with proteoglycans and polysaccharides, RNA was precipitated from the aqueous phase using 0.25 volume of isopropanol and 0.25 volume of buffer containing 1.2 M NaCl and 0.8 M sodium citrate (Chomczynski & Mackey 1995). After centrifugation, RNA pellets were resuspended in DEPC-treated water containing 0.1 mM

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EDTA (pH 6) and quantified by a spectrophotometer. RNA samples were stored at −80°C in the presence of ribonuclease inhibitor (Fermentas, Germany) for subsequent use.

(c) Oligonucleotide primers
For detecting DWV infection in the honeybees, DWV-specific primers (CCTGCTAATCAACAAGGACCTGG) and (CAGAACATGCTAAGCGTAAACCC) that lead to a fragment of 355 bp were used according to a previous work by Genersch (2005). Actin was used as a control and the specific primers were designed from available honeybee sequences (NC_007085) (National Center for Biotechnology Information, NCBI). The actin-specific primers, (CAGGACGCACTACAGCGAT) and (CAGCTCTGCGGTAAGTGGT), lead to a fragment with an expected length of 738 bp.

(d) Reverse transcription and PCR amplification
A two-step reverse transcription polymerase chain reaction (RT-PCR) protocol was used for the diagnosis of DWV from extracted RNA. Reverse transcription was carried out with an average of 1 µg RNA, random hexamer primer and MuLV reverse transcriptase (Fermentas, Germany) according to the manufacturer’s protocol. PCR amplification was performed with 5 µl cDNA, appropriate specific primers, Taq polymerase and 2 mM MgCl₂ (Fermentas, Germany) in 20 µl total reaction mixture. The mixture was heated at 95°C for 10 min, followed by 35 amplification cycles under following conditions: 95°C for 30 s, 58°C for 1 min and 72°C for 1 min followed by 72°C for 10 min to complete the polymerization. A negative control containing water instead of RNA and a DWV-positive control (provided by Reinhold Siede, Bieneninstitut Kirchhain, Germany) was included in each RT-PCR experiment. PCR products were analysed by 1% agarose gel electrophoresis.

(e) Animal collection for behaviour and controlled infection with DWV
Adult honeybees were caught from a hive that had been tested negative for viral infections. After immobilization on ice, the bees were mounted in small metal tubes. The animals were fed (1 M sucrose) each evening to satiation and kept in darkness at a relative humidity of 70% and at 20–25°C until used for controlled infection and behavioural tests (Müller & Hildebrandt 2002). For controlled infection, the DWV lysate and control lysate were diluted by 35 amplification cycles under following conditions: 95°C for 30 s, 58°C for 1 min followed by 72°C for 10 min to complete the polymerization. A negative control containing water instead of RNA and a DWV-positive control (provided by Reinhold Siede, Bieneninstitut Kirchhain, Germany) was included in each RT-PCR experiment. PCR products were analysed by 1% agarose gel electrophoresis.

(f) Responsiveness to appetitive stimuli
Responsiveness of the bees to appetitive stimuli was tested by using the proboscis extension response (PER). An antenna was stimulated by a series (inter-trial interval, 2 min) of defined stimuli with gradually increasing sucrose concentrations (0 M, 30 mM, 100 mM, 300 mM and 1 M). For each sucrose stimulus, the PER was monitored and used as measurement of sucrose responsiveness for each bee (Friedrich et al. 2004; Scheiner 2004).

(g) Non-associative learning tests
(i) Sensitization
Two minutes after testing the initial responsiveness to an odour stimulus, the honeybee was sensitized by antennal stimulation with sucrose (1 M). After 20 s, the second odour stimulus was presented to test for sensitization.

(ii) Habituation
Habituation of PER was tested by repeated stimulation of an antennae (1 M sucrose) at an inter-stimulus interval of 1 s. The number of PER occurring before five consecutive response failures defines the habituation criterion (Müller & Hildebrandt 2002).

(h) Associative olfactory learning
Associative conditioning was performed as described earlier (Müller 2002). A conditioning trial comprises pairing an odour stimulus (carnation) (conditioned stimulus, CS) with a sucrose reward (1 M) (unconditioned stimulus, US). After the animals received three successive conditioning trials at an inter-trial interval of 2 min, memory tests were performed 2 and 24 h after training.

3. RESULTS
(a) Detection and characterization of DWV infection
The RT-PCR-based screening revealed strong DWV signals in bees collected from colonies that died off during the winter (figure 1a). In these bees, we found no evidence for infections by other viruses (acute bee paralysis virus,
sacbrood bee virus, Kashmir bee virus and black queen cell virus) as tested by RT-PCR. Despite the strong DWV infection, there were no symptoms for wing deformation, emphasizing the importance of PCR-based molecular diagnosis to verify viral infections in honeybees. Repeated RT-PCR experiments show that the DWV signal is high in the abdomen and gradually decreases in the thorax and head (figure 1b).

To test for a possible transfer of DWV via food, groups of bees were fed with sucrose alone or sucrose contaminated with control or DWV lysate. DWV infection in the animals was tested at different times after infection using RT-PCR. As shown in figure 1c, these feeding experiments did not lead to a detectable infection by DWV within the tested time window of 7 days. Thus, oral application does not lead to a DWV infection at all, or it takes much longer to reach the threshold level for DWV infection.

Injection of DWV lysate directly into the haemolymph of bees causes a strong RT-PCR signal when compared with the control groups injected with control lysate or PBS. A signal is visible 3 days after injection and the signal increases gradually from 3 to 5 days (figure 1d). Thus, injection is suited for a controlled DWV infection of bees for the behavioural experiments. Although there is no difference in the survival rate between both groups, the DWV lysate-injected bees show a slight decrease in motor activity (e.g. slow extension of the proboscis) after sucrose stimulation.

(b) DWV infection affects sucrose responsiveness
Honeybees provide the opportunity to study non-associative learning—such as sensitization and habituation—as well as associative olfactory learning with single animals. Since these learning paradigms are based on the proboscis extension response (PER) elicited by appetitive stimuli like sucrose, it is necessary to test whether processing of this appetitive stimuli is affected by DWV infection.

Based on the results derived from RT-PCR measurement, we tested the responsiveness of bees at two time points during DWV infection: the first and the fourth day after injection (figure 1d). At these time points, we verified the level of DWV infection with RT-PCR and tested whether an antennal stimulation with sucrose elicits the PER. On the first day after injection, the responsiveness of DWV-infected bees does not differ from that of the control group (figure 2a). However, on the fourth day after injection, the responsiveness of the DWV-infected group to water and low sucrose concentration is strongly increased when compared with the control group (figure 2b). Since there are no differences in the responsiveness to high sucrose concentrations between DWV-infected and control bees, we use 1 M sucrose as appetitive stimuli in the following experiments.

(c) Sensitization and habituation of the PER
Sensitization is the increased responsiveness to a neutral sensory stimulus (odour) shortly after application of a stimulus (sucrose) that arouses the animal. DWV-infected bees do not differ from control bees. In both groups, the first odour stimulus elicits PER only in a few bees (less than 10%), while the arousing stimuli (1 M sucrose) leads to high levels of PER (more than 90%). Odour stimulation immediately after arousal also triggers PER in only a few animals (less than 20%).

(d) Associative olfactory learning
The well-established associative olfactory conditioning paradigm consists of the pairing of an odour stimulus (CS) with a sucrose reward (US). Figure 3 shows that DWV-infected bees show a significantly reduced acquisition when compared with control bees. Moreover, memory retention as tested 2 and 24 h after conditioning is also low in DWV-infected bees. Thus, DWV infection seems to have specific effects on neuronal signalling processes because DWV infection only impairs associative learning without affecting non-associative processes.

4. DISCUSSION
In our behavioural analysis, we demonstrate that DWV infection of adult forager bees leads to specific impairments in sucrose responsiveness and associative olfactory
learning but does not affect non-associative forms of learning like sensitization and habituation. Only direct haemolymph infection by DWV is effective and leads to a strong replication of the virus, while oral application is ineffective, which is in agreement with other studies (Bailey & Ball 1991). However, since we tested only up to 7 days of continuous oral application, we cannot exclude that a long-lasting oral uptake of DWV-contaminated food during hibernation may be a source of DWV infection. Thus, under natural conditions, haemolymph infection via parasites like Varroa destructor as proposed by Bailey & Ball (1991) is the most probable scenario for DWV transmission. Although the transmitted material (virus, bacteria, etc.) has not been specified, a recent report demonstrates that foragers infested by Varroa destructor are affected in non-associative forms of learning (Kralj et al. 2007).

In agreement with results on queens and drones (Chen et al. 2006), our RT-PCR measurements of DWV-infected foragers showed a maximal signal in the abdomen, followed by the thorax and head of the adult honeybee. Although viral infection in the head is quite weak, it may interfere with molecular mechanisms underlying learning at different levels. So, it is feasible that a viral infection interferes with signalling cascades underlying learning by triggering the immune system. This is supported by the observation that injection of lipopolysaccharide (LPS), which triggers immune response, affects olfactory conditioning but not sensitization in honeybees (Mallon et al. 2003; Riddell & Mallon 2006). This impairment in associative learning is observed approximately 3 days after LPS injection, which is in agreement with our study. Interestingly, LPS injection and viral infections also cause learning deficits in mammals (Weed & Gold 2001; Sparkman et al. 2005). Our study however shows that, based on the present knowledge, a simple explanation of the potential molecular targets affected by DWV is yet not possible.

The unique combination of enhanced sucrose responsiveness, normal non-associative learning and defects in associative learning caused by DWV infection has not yet been observed. All previous reports show that elevated sucrose responsiveness is always combined with an improved associative learning performance, which is in clear contrast to our observations. The genotype, the role of the bees in foraging, the satiation level and many other parameters affect sucrose responsiveness and—in parallel—very defined features of associative learning (Scheiner et al. 2001; Friedrich et al. 2004; Scheiner 2004). These strongly linked physiological processes depend to a great extent on molecular processes mediated by the biogenic amine octopamine. While injection of octopamine elevates sucrose responsiveness and enhances acquisition, inhibition of octopaminergic transmission decreases responsiveness and impairs acquisition (Hammer & Menzel 1998; Menzel et al. 1999; Scheiner et al. 2002). All this is in contrast to our demonstrated effects of DWV infection on sucrose responsiveness and acquisition and points to yet unknown processes affected by DWV. Our finding that DWV infection does not interfere with habituation supports this notion. Processes that affect sucrose responsiveness and associative learning also affect habituation (Braun & Bicker 1992; Müller & Hildebrandt 2002; Scheiner 2004). Thus, the discrepancy between the current knowledge about the interaction between sucrose responsiveness, non-associative and associative learning in bees and the behavioural effects after DWV infection demands a search for new explanations. Since the effects on behaviour are observed days after infection, it is feasible that viral infections (possibly also LPS injections) lead to changes in the gene expression pattern, which has been reported in mice (Linneaa et al. 2005). So, future studies have to show whether gene expression patterns that change during development and caste differentiation of bees (Whitfield et al. 2003) are targets of viral infections.

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