Appetitive olfactory learning and memory in the honeybee depend on sugar reward identity

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ABSTRACT

One of the most important tasks of the brain is to learn and remember information associated with food. Studies in mice and Drosophila have shown that sugar rewards must be metabolisable to form lasting memories, but few other animals have been studied. Here, we trained adult, worker honeybees (*Apis mellifera*) in two olfactory tasks (massed and spaced conditioning) known to affect memory formation to test how the schedule of reinforcement and the nature of a sugar reward affected learning and memory. The antennae and mouthparts of honeybees were most sensitive to sucrose but glucose and fructose were equally phagostimulatory. Whether or not bees could learn the tasks depended on sugar identity and concentration. However, only bees rewarded with glucose or sucrose formed robust long-term memory. This was true for bees trained in both the massed and spaced conditioning tasks. Honeybees fed with glucose or fructose exhibited a surge in haemolymph sugar of greater than 120 mM within 30 s that remained elevated for as long as 20 min after a single feeding event. For bees fed with sucrose, this change in haemolymph glucose and fructose occurred with a 30 s delay. Our data showed that olfactory learning in honeybees was affected by sugar identity and concentration, but that olfactory memory was most strongly affected by sugar identity. Taken together, these data suggest that the neural mechanisms involved in memory formation sense rapid changes in haemolymph glucose that occur during and after conditioning.

1. Introduction

The brain has been shaped by natural selection to learn to associate cues that predict the occurrence of nutritiously valuable food. Sensory input is organized to produce memory traces for food that are stored for retrieval when animals are hungry, so that animals can identify signals associated with nutritional rewards and avoid signals that are irrelevant or that are associated with intoxication. An important mechanism for assessing food value and forming lasting memories of sensory cues is through post-ingestive signalling. This was first studied in the context of aversion learning; within one trial, animals can learn to associate tastes and smells with the post-ingestive consequences of ingesting toxins in foods (Bernays and Lee, 1988; Garcia et al., 1955; Wright et al., 2010). More recently, experiments with mice and fruit flies have shown that post-ingestive signals are important for assessing the nutritional value of food; memories last longer when foods have metabolic value (Burke and Waddell, 2011; de Araujo et al., 2008; Dus et al., 2011; Fujita and Tanimura, 2011; Sclafani and Ackroff, 2016). For example, insects trained in an olfactory learning task with a non-metabolisable sugar such as arabinose can learn to associate an odour with the taste of this sugar, but they do not form long-lasting memories of the odour (Burke and Waddell, 2011).

Memories of food should reflect food value: learning should happen faster and memories should be stronger and longer lasting for high valence rewards (Pavlov, 1927). Few studies have tested how reward quality affects learning and memory, and whether all metabolisable sugars are equally rewarding to animals. Mice are more likely to learn and remember when they are rewarded with sugars metabolised into glucose-units but not when rewarded with fructose (Matsumura et al., 2010; Sclafani and Ackroff, 2016). In contrast, studies in Drosophila indicate that flies form lasting memories for several metabolisable sugars including fructose and glucose (Burke and Waddell, 2011; Dus et al., 2013, 2011; Miyamoto et al., 2012; Musso et al., 2015; Perisse et al., 2013). This could indicate that the mechanisms of post-ingestive nutrient detection or memory formation in insects and mammals are different.

The honeybee learns to associate floral signals with reward very quickly, and is an important insect model for studying learning and memory (Bitterman et al., 1983; Eisenhardt, 2014; Stollhoff et al., 2008). Our previous work indicated that like Drosophila, honeybees also require a metabolic reward to form a lasting olfactory memory of odours associated with food (Wright et al., 2007). Specifically, we

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found that the taste of the reward was not sufficient for long-term memory: only honeybees that had been fed with a sucrose reward exhibited memories that lasted longer than 10 min. This work implied that to form olfactory memories, the bee brain also requires a post-ingestive, metabolic reward, but this has not been explicitly shown.

Previous work in honeybees also showed that time interval between conditioning trials also affects the formation of long-term memory (Menzel et al., 2001). When bees are trained in a 'massed' conditioning task (i.e. inter-trial interval of 30 s) and rewarded with sucrose, they are less likely to remember the conditioned odour than bees trained in a 'spaced' conditioning task (i.e. inter-trial interval of 3–10 min). Furthermore, single conditioning trial where an odour stimulus lasts ∼4 s and is paired with an equally brief, but metabolisable food reward does not produce a lasting memory in bees (Stollhoff et al., 2008). Instead, several trials with inter-trial intervals of > 1 min are necessary (Menzel et al., 2001; Stollhoff et al., 2008). This suggests that the neurons encoding long-term olfactory memory must receive sensory input on a time scale that overlaps or occurs soon after a period of flux in haemolymph nutrients. The fact that bees form lasting memories when they receive several conditioning trials with long inter-trial intervals could indicate that memory formation depends on the timing of post-ingestive reinforcement relative to sensory input but this has not yet been tested in any animal.

Here, we tested whether long-term olfactory memory in honeybees depends upon the nature of the metabolisable sugar, its value/concentration, and the inter-trial interval. Bees were conditioned to associate an odour stimulus with a food reward in a spaced (5 min intertrial interval) or massed (30 s intertrial interval) task for conditioned proboscis extension response (PER). After training, all bees were tested for their short-term (10 min) and long-term (24 h) olfactory memory with the conditioned odour and a novel odour (NO). With the aim of identifying how haemolymph sugar flux could influence learning and memory, we also measured the amount of time necessary for post-ingestive changes in haemolymph sugars to occur.

2. Methods

Animals: Worker honey bees (*Apis mellifera* var carnica or *Apis mellifera* var buckfast) were captured during April-August 2011 and 2012 from a hive located at Newcastle University (UK) as they returned from foraging. A plastic blockade was placed over the hive entrance to ensure only returning foragers were captured. Each bee was collected in a plastic vial and restrained in a harness as described in Wright et al. (2007). Bees were used either for the gustatory assays, haemolymph collection or for olfactory conditioning; each bee was fed to satiety with 1.0 M sucrose and left for 18–24 h at room temperature (RT) in a humidified plastic box.

2.1. Gustatory assays

Bees from this experiment were captured during April-May 2011. Antennal assay: The antennae of each honeybee was stimulated with an ascending concentration series (0.3, 0.6, 1.0, 1.3, 1.6 and 2.0 M) of sucrose, fructose, or glucose to elicit the PER. Between each stimulation, each bee was tested for its response to water as described in (Page et al., 1998). Stimuli were applied such that an interval of 3–5 min occurred between each stimulus to avoid producing habituation to the test stimuli. All bees were tested with each series of each sugar. A total of 140 bees were tested; 50 of them did not respond to any of the stimuli. Mouthparts assay: Each bee was tested with a water stimulus and one concentration of each sugar as using the assay for proboscis sensitivity previously described in Wright et al. (2010). We tested individual bees with one concentration of each sugar; this was done to avoid alterations to motivation state that could confound the experiments when the bees ate the solutions. (Note: motivation state to respond to the solution is not altered in bees who have had their antennae

touched with the solution only, as in the antennal assay). To accomplish the application of the solution to the mouthparts, the antennae were first stimulated with the test solution to elicit proboscis extension. The test stimulus was then applied to the mouthparts. Whether or not the bee consumed the solution was recorded as a binary variable. Bees that did not respond to antennal stimulation were not used in the experiment. Between 0 and 50% of the subjects did not respond during this assay, depending on the stimulus used as the test stimulus (total N/treatment = 20, only data for bees that responded to antennal stimulation is plotted).

2.2. Olfactory conditioning

Bees from this experiment were captured during June-August 2012. After 24 h, each bee was trained in a protocol for olfactory conditioning of the PER (Bitterman et al., 1983). Methods for odour stimulus delivery are described in Wright et al. (2007). Only subjects that responded with PER to antennal stimulation with 1.0 M sucrose were selected for conditioning. Bees were conditioned for 6 trials with an inter-trial interval (ITI) of 30 s (massed conditioning) or 5 min (spaced conditioning). The conditioned stimulus (CS) was 1-hexanol (Sigma-Aldrich) and was presented for 4 s. The unconditioned stimulus (food reward, US) presented on each trial was a 0.4 μl droplet of reagent-grade fructose, glucose or sucrose delivered using a Gilmont syringe (Cole Parmer). We also tested 3 concentrations of each sugar: 0.3 M, 1.0 M, and 2.0 M. Any bee that responded with a conditioned response on the first trial was removed from the experiment during the experiment. Two unreinforced olfactory memory tests were administered 10 min and 24 h after olfactory conditioning: one with the CS odour and one with a novel odour (2-octanone, Sigma-Aldrich). The order of presentation of the test odours was randomized across subjects. Each treatment group was randomized across the course of the study; on any given day, at least 3 treatment groups were trained and tested.

2.3. Haemolymph analysis

Honeybees were individually harnessed as described above and a small incision was made above the median ocellus using a 1.1 mm × 40 mm needle (BD Microlance). Honeybees were split into one of four experimental groups and fed: 5 μl of 1.0 M sucrose, 1.0 M glucose, or 1.0 M fructose or fed to satiety with 1.0 M sucrose. (Note: for the bees fed to satiety, the time taken for each bee to feed to satiety was recorded in order to gauge the change in sugar levels from the initiation and termination of feeding). At a specific time point post-feeding, haemolymph was collected using a 10 μl capillary tube (Hirschmann) from the incision above the median ocellus. The haemolymph was sampled at one of the following time points: 30 s, 1 min, 3 min, 5 min, 10 min and 20 min post-feeding. Each capillary tube was placed in the head capsule for a total of 2 min after the specified time point. Haemolymph was also collected from a subset of bees prior to feeding (time point zero). A minimum of 1 μl of haemolymph was collected for each bee and immediately added to 1 μl 0.1 M perchloric acid; any volume greater than 1 μl was matched with an equal volume of 0.1 M perchloric acid and subsequently stored at −20 °C until further processing. Samples less than 1 μl were discarded, as was any haemolymph available after the 2 min collection time in order to standardise all samples. Haemolymph samples were taken from 10 bees per treatment group and analysed using HPLC.

Haemolymph samples were centrifuged for 10 min at 14,000 rpm (Eppendorf model no. 5424), and 1 μl of the haemolymph supernatant was removed and diluted 1:200 with nanopure water (Fisher Scientific). Diluted samples were filtered through a syringe filter (Puradisc sample preparation nylon 0.45 μm pore, 4 mm diameter, Whatman). High performance liquid chromatography (HPLC) was used to measure concentrations of specific sugars (glucose, fructose, sucrose and trehalose) in each sample. HPLC analysis was conducted by injecting 20 μl of