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Animal behaviour

The short neuropeptide F (sNPF) promotes the formation of appetitive visual memories in honey bees

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Motivation can critically influence learning and memory. Multiple neural mechanisms regulate motivational states, among which signalling via specific neuropeptides, such as NPY in vertebrates and NPF and its short variant sNPF in invertebrates, plays an essential role. The honey bee (*Apis mellifera*) is a privileged model for the study of appetitive learning and memory. Bees learn and memorize sensory cues associated with nectar reward while foraging, and their learning is affected by their feeding state. However, the neural underpinnings of their motivational states remain poorly known. Here we focused on the short neuropeptide F (sNPF) and studied if it modulates the acquisition and formation of colour memories. Artificially increasing sNPF levels in partially fed foragers with a reduced motivation to learn colours resulted in significant colour learning and memory above the levels exhibited by starved foragers. Our results thus identify sNPF as a critical component of motivational processes involved in foraging and in the cognitive processes associated with this activity in honey bees.

1. Introduction

A crucial requisite for appetitive learning is the presence of the appropriate motivation to respond to appetitive reinforcements [1]. Thus, characterizing the neural mechanisms that regulate motivational states and in consequence affect learning and memory constitutes an important goal of cognitive neuroscience [2].

Honey bees, *Apis mellifera*, constitute a privileged model for the study of animal cognition owing to their remarkable learning capacities and the tractability of their nervous system for multiple invasive procedures [3,4]. Bee foragers associate multiple sensory cues with appetitive nectar, which in the laboratory can be replaced by a drop of sucrose solution [3]. The motivational state of bees, controllable via their feeding state, determines their willingness to either learn or express a learned response [5,6]. Bees offer, therefore, an opportunity to characterize the neural mechanisms mediating appetitive motivation and influencing cognitive processes.

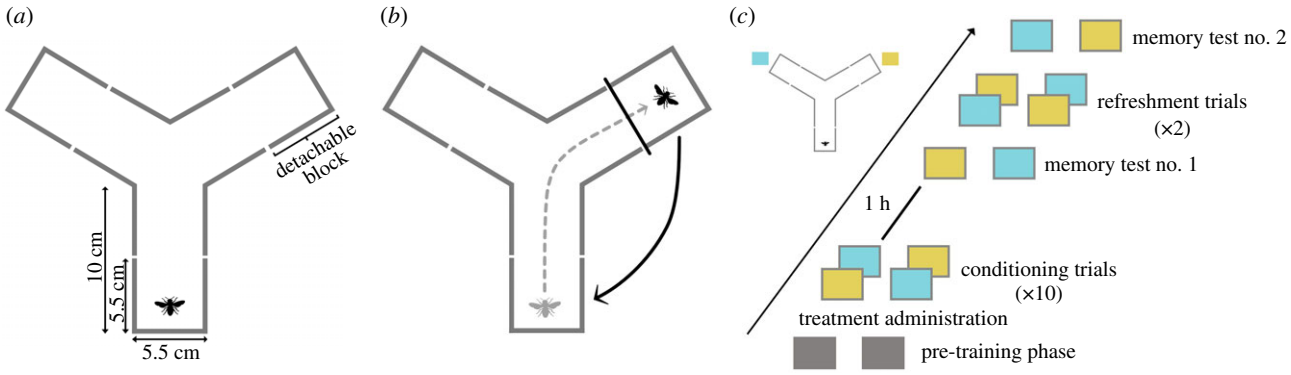

Figure 1. Experimental set-up and colour-conditioning procedure. Marked bees were (*a*) trained in a miniature Y-maze whose end compartments could be closed by a sliding door after each choice, and (*b*) translocated to the maze entrance for another trial. (*c*) Each bee was trained, over the course of 10 consecutive trials, to discriminate a yellow from a blue target. One colour was paired with sucrose and the other with water. One hour after conditioning, bees were tested twice for memory retention in the absence of reinforcement, with two refreshment trials interspersed between tests.

Two key molecules regulating food-related behaviours in invertebrates are the neuropeptide F (NPF) and its short variant (sNPF) [7,8]. In honey bees, the genes *npf* and *snpf* and their corresponding peptides NPF and sNPF have been identified, but only a receptor gene for sNPF (*snpfR*) was found [9,10]. Increasing sNPF levels in forager bees enhances their food ingestion and reponsiveness to appetitive stimuli [11]. If and how sNPF modulates appetitive learning in bees is presently unknown. Evidence from other insect species is contradictory: while in the fruit fly *Drosophila melanogaster* downregulation of sNPF levels or knockdown of sNPF receptors in the brain impaired olfactory memory [12], in the desert locust (*Schistocerca gregaria*), knocking down sNPF had no influence on appetitive visual learning [13].

Here, we focused on visual learning and memory of honey bees [14] and analysed if and how sNPF levels modulate their capacity to learn and memorize colours. We artificially increased sNPF levels in partially fed, free-flying honey bees with a reduced motivation to learn and memorize colours, and trained them to discriminate colours within a miniature Y-maze. We asked if high levels of sNPF lead to a significant improvement of learning and memory in these bees, matching or even exceeding the performance of starved foragers. In this way, we aimed at identifying a specific neural mechanism underlying appetitive motivation and playing a significant role in insect cognition.

2. Material and methods

(a) Pre-training and set-up

Free-flying honey bee foragers from a colony located at a distance of 50 m from the laboratory were trained to visit a pneumatic feeder containing a 30% (w/w) sucrose solution. Foragers captured upon landing at the feeder were brought to the laboratory and released within a miniature PVC Y-maze [15] covered with a transparent plastic cover (figure 1a). The maze was placed on a table adjacent to a window sill and had a unique entrance facing the laboratory window. Bees learned to collect a 50% (w/w) sucrose solution provided alternately between the left and right arms of the maze by means of 1 µl Eppendorf tips inserted in the middle of the back walls. Each arm measured 10 × 5.5 × 4 cm and had a detachable end section (5.5 × 4 cm) that allowed relocation of the focal bee to the start of the maze after each choice. The back walls displayed a grey HKS-92N colour paper. A bee was selected for further use if it had visited at least twice both the left and the right arm. It was then marked with a colour spot on the thorax to allow its identification.

(b) Experimental groups and pharmacological treatments

Upon return to the maze, the selected bee was moved to a small compartment (5.5 × 5.5 × 4 cm) offering an Eppendorf tip that could be filled either with distilled water (*starved bees*) or with 5 µl of a mixture of honey, pollen, sucrose and water and then with 15 µl of a 1.5 M sucrose solution (*partially fed bees;* henceforth P-fed bees) [11,16]. The volume of food provided corresponded to a third of a bee's crop capacity [17] so that bees might decide to continue foraging. After feeding, or after 5 min in the case of the starved bees, each bee was placed in a container with ice for 5 min to immobilize it and then either left unhandled or, in the case of P-fed bees, topically exposed to 1 µl of one of two doses of sNPF applied on the thorax: 1 µg µl^{-1} (hence 'P-fed sNPF1') or 10 µg µl^{-1} (hence 'P-fed sNPF10'). Topical application of neuropeptides and neurotransmitters and their antagonists has been repeatedly used in honey bees [11,16,18,19]. Another group of P-fed bees received 1 µl of the solvent used to dissolve sNPF (hence 'P-fed solvent'), which was a mixture of 20% dimethyl sulfoxide and 80% acetone. Each bee was then placed in an individual small cage where it recovered for 30 min. As sNPF is supposed to enhance appetitive responsiveness, it was not delivered to starved bees, which were presumed to be at a ceiling level regarding this trait. Starved bees constituted a control to establish whether sNPF improved learning and memory of P-fed bees. Overall, five groups of bees were established (P-fed', 'P-fed sNPF1', 'P-fed sNPF10', 'P-fed solvent' and 'starved). Sucrose and solvents were purchased from Sigma-Aldrich (Steinheim, Germany), while honey bee (*Apis mellifera*) sNPF was purchased from NovoPro (Shanghai, China; sequence: SDPHLSILSKPMSAIPSYKFDD [20]).

(c) Differential visual conditioning

After recovery, the bee was released in a maze identical to the one in which it had been pre-trained (figure 1*a*,*b*). Only one bee was present in the maze at a time. Each bee was conditioned during ten trials to discriminate a blue (B, HKS-47N) from a yellow (Y, HKS-2N) card displayed on the back walls. Colours (electronic supplementary material, figure S1*a*) were easily distinguishable from each other but had different chromatic contrasts against the grey HKS-92N background (electronic supplementary material, figure S1*b*,*c*), with yellow (Y) being more salient than blue (B). The visual angle subtended by each card to the decision point of the maze was 31°, which ensured that bees were guided by the chromatic properties

of the stimuli [21]. A 1 µl Eppendorf tip was inserted in the middle of each colour card. One of them offered a 50% (w/w) sucrose solution and the other distilled water.

For each treatment, two subgroups with reversed contingencies (Y+ versus B− and Y− versus B+; '+': sucrose reward, '−': distilled water) were conditioned. Colours were swapped pseudo-randomly between the arms of the maze (figure 1*c*). A choice was recorded when the bee entered one of the two arms and contacted the tip in the middle of the colour stimulus. The bee was then translocated to the entrance compartment by means of the detachable end block of the arm maze. A sliding door allowed this compartment to be closed and the bee to be moved to the entrance of the maze, thus allowing the presentation of a new choice (figure 1*b*). The latency of each choice was also recorded. If it was longer than 1 min (less than 1% of 1610 choices recorded), a criterion value of 60 s was assigned. Each trained bee remained in the maze for the entire training sequence as returning to the hive and unloading the food gathered would change the crop contents, the motivational state and possibly sNPF levels. After completing the 10 choices, the focal bee was captured and kept in a dark box for 1 h. Thereafter, it was released in the maze again in order to be tested for memory retention in the absence of reward in a first memory test. Longer retention intervals were not tested as they may change the feeding state and thus the motivational state of bees. Two refreshment trials were performed after this retention test to counteract possible extinction induced by the first test. During these refreshment trials, colours were presented on the back walls of the maze and reinforcements were provided on the appropriate colours. The amount provided (1 µl) and the inter-trial interval (a maximum of 60 s) were identical to those used during training. Two refreshment trials were necessary to balance the presentation side (left/right) of the training colours. Afterwards, a second memory test with the colours swapped between arms with respect to the first test was performed.

(d) Data analysis

Data were analysed using R software [22]. Learning curves are shown in the electronic supplementary material, figure S2. Responses in the last trial were analysed with a generalized linear model (GLM). Choice latency during learning trials was analysed using a linear mixed model (LMM) (R package *nlme*) followed by ANOVA (R package *car*) and Tukey *post hoc* tests. Latency served as a quantitative variable, while treatments, trials and colour served as fixed effects. Bee's identity was entered as a random effect.

Cumulative learning scores (see electronic supplementary material, figure S2) were calculated for each bee by attributing a score of +1 upon each correct choice and −1 upon each incorrect choice. The cumulative score of each bee was obtained by summing the scores of all trials (see electronic supplementary material, information). Their distribution was analysed using pairwise Kolmogorov–Smirnov tests with Bonferroni correction for *p*-values. The proportion of correct choices in the last trial and in both memory tests was compared with a theoretical value of 50% using a Wilcoxon signed-rank test.

3. Results

To evaluate learning success in each group, we focused on the last conditioning trial because successful learning should be visible through correct discrimination at the end of training (figure 2, first bar in each panel). Neither the treatment nor the colour conditioned had any effect on colour discrimination in that trial (treatment, GLM, $\chi^2 = 3.93$, d.f. = 4, $p = 0.42$; colour conditioned, GLM, $\chi^2 = 0.53$, d.f. = 1, $p = 0.47$). Only P-fed sNPF10 bees showed a proportion of correct choices that

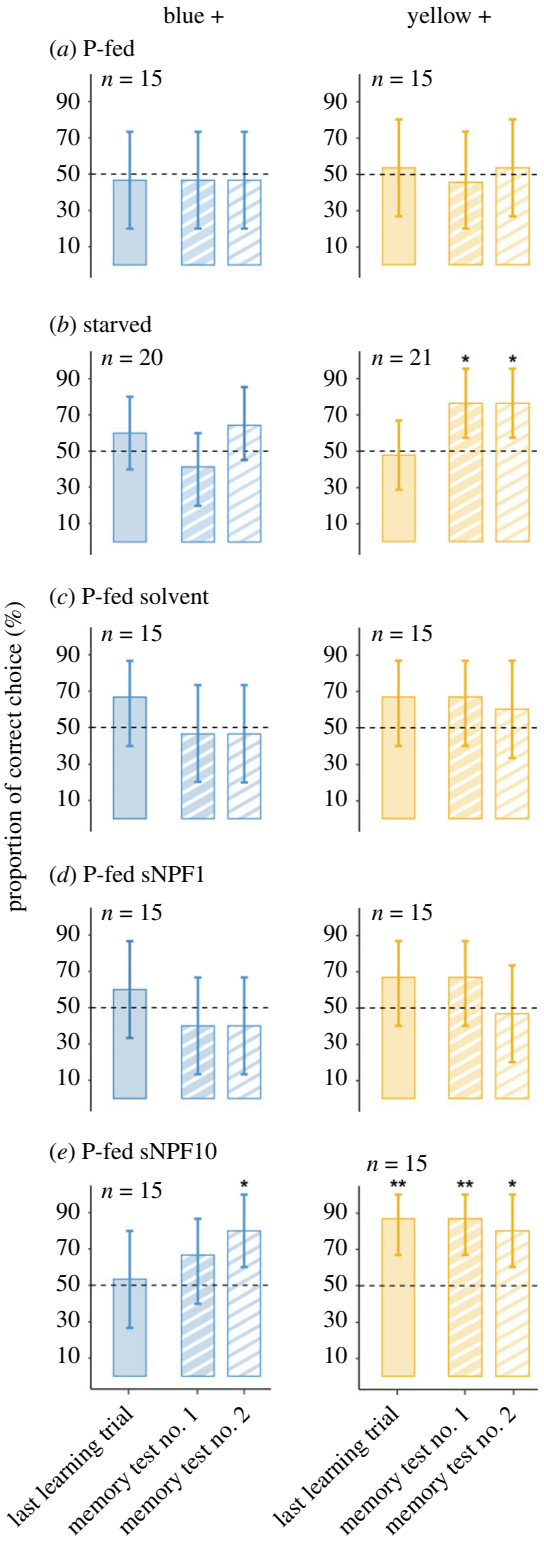

Figure 2. Learning and retention following colour conditioning. Proportion of correct choices in the last trial of colour conditioning (first, filled bar in each panel) and in the two retention tests (second and third, hatched bars in each panel). (*a*) P-fed, blue-trained: $n = 15$, yellow-trained: $n = 15$; (*b*) starved bees, blue-trained: $n = 20$, yellow-trained: $n = 21$; (*c*) P-fed solvent, blue-trained: $n = 15$, yellow-trained: $n = 15$; (*d*) P-fed sNPF1, blue-trained: $n = 15$, yellow-trained: $n = 15$; (*e*) P-fed sNPF10, blue-trained: $n = 15$, yellow-trained: $n = 15$. Error bars represent the 95% boostrapped confidence interval. ***p*-value < 0.01; **p*-value < 0.05.

differed significantly from chance level when rewarded on yellow (figure 2*e*; $V = 104$, $p = 4.98 \times 10^{-3}$). The learning curves of the different groups (electronic supplementary material, figure S2, *left*) did not show a consistent trend,

which may have been caused by the impossibility to return to the hive. It was thus difficult to decide if learning took place.

The choice latency of bees was not influenced by the colour conditioned (electronic supplementary material, figure S2 *right*: LMM, $\chi^2 = 0.08$, d.f. = 1, $p = 0.77$) but decreased significantly along with trials (electronic supplementary material, figure S2 *right*: LMM, $\chi^2 = 62.87$, d.f. = 9, $p = 2.2 \times 10^{-15}$) and varied with treatment (electronic supplementary material, figure S2 *right*: LMM, $\chi^2 = 14.33$, d.f. = 4, $p = 6.31 \times 10^{-3}$). Starved bees chose faster than P-fed sNPF1 bees ($p = 0.01$) and P-fed solvent bees ($p = 0.01$). The distribution of cumulative scores did not differ between treatments when bees were conditioned on yellow (electronic supplementary material, figure S3 *right*: all tests $p > 0.05$) or blue (electronic supplementary material, figure S3 *left*: all tests $p > 0.05$). Overall, these data did not provide clear evidence for learning, although P-fed sNPF10 bees trained on yellow exhibited a significant preference for yellow in the last conditioning trial (figure 2*e*; $V = 104$, $p = 4.98 \times 10^{-3}$) and choices became faster with conditioning (electronic supplementary material, figure S2 *right*: LMM, $\chi^2 = 70.32$, d.f. = 9, $p = 1.32 \times 10^{-11}$).

Figure 2 also shows the retention performances in the first and second memory tests performed 1 h after training (second and third bars in each panel). Starved bees exhibited a significant preference for yellow (figure 2*b*; test 1: $V = 176$, $p = 0.02$; test 2: $V = 176$, $p = 0.02$), thus showing that they had learned the colour–sucrose association at the end of the conditioning, even if they did not express it, and that the time elapsed since training rendered visible the information learned. In the case of blue training, starved bees exhibited a non-significant percentage of choices (40%) in the first test (figure 2b *left*; test 1: $V = 84$, $p = 0.38$), which increased in favour of the trained blue colour (65%) in the second test without reaching significance ($V = 136.5$, $p = 0.09$). Differences in performances between the starved animals trained on blue and yellow reflect the different chromatic salience of these colours.

Bees of the P-fed, P-fed solvent and P-fed sNPF1 groups, which exhibited no sign of learning during yellow and blue training, showed non-significant retention during the memory tests (figure 2*a–d*; all analyses: $p > 0.05$). Thus, the time elapsed between the last training trial and the memory tests did not improve *per se* the performance of P-fed bees. However, P-fed sNPF10 bees exhibited significant retention in both memory tests when the trained colour was yellow (test 1: $V = 104$, $p = 4.98 \times 10^{-3}$; test 2: $V = 96$, $p = 0.02$), in which case performance was similar to that of the starved group, and in the second memory test when it was blue ($V = 96$, $p = 0.02$), in which case performance was better than that of the starved group. Thus, P-fed bees treated with the higher dose of sNPF learned and memorized their respective colours, overcoming even the low chromatic salience of blue.

4. Discussion

Our results show that treating bees with the higher dose of sNPF promotes associative colour learning and the expression of colour memories during retention tests performed 1 h after training. Performance of P-fed sNPF10 bees was better than that of starved bees (compare figure 2*b*,*e*), thus showing the capacity of the higher dose of sNPF to improve cognitive processes. This improvement was affected by colour salience as it was clearer for the highly salient yellow colour than for the less conspicuous blue. While starved bees exhibited significant retention for yellow but not for blue, P-fed sNPF10 bees exhibited significant retention both for yellow and for blue. Remarkably, the memory performance of starved bees after blue training followed a similar tendency as in P-fed sNPF10 bees, with the difference that it did not reach significance in the second test (compare left panels of figure 2*b*,*e*). This difference reflects the well-known fact that the salience of a conditioned stimulus significantly affects learning and memory [23]. Low stimulus saliencies lead to reduced acquisition rates and thus to impaired memory as in the case of our blue colour. The fact that the higher dose of sNPF induced a significant memory for the less conspicuous blue colour (second retention test) shows that this neuropeptide can facilitate learning in conditions in which it is normally impaired.

Honey bees satiated before being subjected to olfactory conditioning exhibit impaired learning and memory, thus indicating that satiation interferes with the process of associative learning itself [5]. This was not the case in our visual conditioning experiments: although learning performances were not stable, P-fed sNPF10 bees exhibited significant memory performances 1 h after training. This suggests that the 1 h period between the last training trial and the retention tests contributed to consolidate the colour memories in these animals. The fact that the same lapse of time had no influence on memory expression in the other P-fed groups indicates that these animals did not learn the task and that their motivational state was inappropriate for learning the colour information. The highest dose of sNPF was able to reverse this state and enhanced appetitive motivation, thereby favouring learning and memory formation.

In our experiments, bees could not return to the hive after each colour trial as they were translocated to the maze entrance for additional choices. This procedure was important because returning to the hive and unloading the food gathered would change crop contents and eventually sNPF levels, which can vary according to the foraging phase [24]. This would render our experimental treatments useless. The fact that this impediment did not prevent P-fed sNPF10 bees from establishing colour memories confirms that sNPF critically regulates appetitive motivation and perceptual and attentional processes concerning food-related cues [11]. This is evident in the case of the blue colour, for which significant retention was only found under the higher dose of sNPF (2nd test), even above the level of starved bees.

The role of sNPF in insect visual learning has only been studied in the desert locust (*Schistocerca gregaria*) [13,25]. In this insect, starvation reduces the sNPF precursor transcript level in the optic lobes [26]. Yet, knocking down sNPF to mimic the starvation state had no influence on appetitive visual learning and memory [13]. This lack of effect contrasts with results obtained in odour-trained fruit flies, where starvation increases sNPF levels, and knockdown of the sNPF precursor to mimic a fed state leads to a reduction of olfactory memory [12]. Our results on honey bee visual learning and memory are in line with these findings on olfactory learning and memory in fruit flies and show that sNPF has the capacity to improve learning and memory via its effect on appetitive motivation. The identification of this neuropeptide as a crucial regulator of appetitive motivation should lead to further investigations of its role in learning tasks of different modalities and complexity.

Data accessibility. Raw data for this paper are available under. https://doi.org/10.6084/m9.figshare.16644922.v1.

Authors' contributions. L.B.: conceptualization, formal analysis, writing—original draft, and writing—review and editing; E.B.: investigation, and writing—review and editing; J.C.: investigation, and writing—review & editing; J.-C.S.: funding acquisition, and writing—review and editing; R.V.: investigation, and writing—review and editing; M.G.: conceptualization, formal analysis, investigation, project administration, supervision, writing—original draft, and writing—review and editing; M.G.B.S.: conceptualization, funding acquisition, project administration, supervision, validation, writing—original draft, and writing—review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed herein.

Competing interests. We declare we have no competing interests.

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