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Alpha oscillations govern interhemispheric spike timing coordination in the honey bee brain

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In 1929 Hans Berger discovered the alpha oscillations: prominent, ongoing oscillations around 10 Hz in the electroencephalogram of the human brain. These alpha oscillations are among the most widely studied brain signals, related to cognitive phenomena such as attention, memory and consciousness. However, the mechanisms by which alpha oscillations affect human cognition await demonstration. Here, we suggest the honey bee brain as an experimentally more accessible model system for investigating the functional role of alpha oscillations. We found a prominent spontaneous oscillation around 18 Hz that is reduced in amplitude upon olfactory stimulation. Similar to alpha oscillations in primates, the phase of this oscillation biased both timing of neuronal spikes and amplitude of high-frequency gamma activity (40–450 Hz). These results suggest a common role of alpha oscillations across phyla and provide an unprecedented new venue for causal studies on the relationship between neuronal spikes, brain oscillations and cognition.

1. Introduction

The brain encodes information by the rate and by the timing of action potentials (spikes) across neurons. The importance of timing has already been stressed in 1929 by Hans Berger who discovered the alpha oscillations: the strongest rhythmic oscillation measurable from the human scalp electroencephalogram (EEG) at a frequency of 10 Hz [1]. In 1934 Adrian & Matthews confirmed this observation and concluded that the alpha oscillation arises 'from an area in the occipital lobes connected with vision, and not from the whole cortex' [2, p. 384, 3]. Today, alpha oscillations are among the most widely studied psychophysiological signals. They have been linked to cognitive functions such as attention and memory in humans and other vertebrates [4–7]. Amplitude modulation of alpha oscillations is hypothesized to regulate neuronal excitability throughout the cortex [4,6,8], and the phase of alpha oscillations biases the rate of neuronal spiking within [9] and across cortical areas [10]. On a larger scale, neural processing is functionally organized in feed-forward (bottom up) and feedback (top down) streams [11]. Feedback streams have been linked to alpha oscillations and feed-forward streams to higher-frequency oscillations above 30 Hz (gamma oscillations) [12–14]. However, the mechanisms by which alpha and gamma oscillations affect neural processing and manifests in behaviour still await demonstration. Progress in revealing the mechanisms and functions of oscillatory brain activity is hampered by the difficulty to relate oscillatory brain activity to the spiking activity of identified neurons in behaving animals.

In recent years insects have become model systems for studying the relationship between the spiking activity of identified neurons and the animal's behaviour. For example, the perceived quality of an odour can be predicted from the ensemble of co-active olfactory neurons, each being identified by its

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specific pattern of afferent and lateral inputs [15–19], and the mechanistic understanding of odour learning is unparalleled both in regard to molecular pathways and the identity of neuronal circuits [20–22]. Similarly as in vertebrates, insect brains generate oscillatory activity patterns (e.g. spontaneous 10–20 Hz oscillations in water beetles [23] and honey bees [24,25], sleep state-dependent 10 Hz oscillations in fruit flies [26], odour-induced 20–100 Hz oscillations in locusts [27], moths [28,29] and bees [30–32] and visual stimulus-induced 20–30 oscillations in fruit flies [33,34]). However, albeit ubiquitous, most studies on insect brain function ignore spontaneous oscillatory activity and rather focus on stimulus-driven changes in spike rates.

To investigate the temporal relationship between spikes and spontaneous oscillatory activity within and across brain hemispheres, we performed paired recordings of local field potentials (LFP) and spikes in both mushroom bodies of the honey bee brain. The mushroom bodies are involved in olfactory learning [22,35–39], and they integrate unilaterally learned odour-reward associations across hemispheres [40]. We found an 18 Hz oscillation that exhibits properties of alpha oscillations in humans and non-human primates. First, it was spontaneously generated, second, it reduced amplitude upon stimulus presentation and third, it biased the timing of spikes and high-frequency neuronal activity, effectively regulating the functional connectivity within and between brain hemispheres. In addition, fluctuations in the power of the alpha oscillation affected the spike rate, which increased with increasing alpha power. Our data demonstrate that both spontaneous and odour-induced spikes in the honey bee mushroom body are timed by a network-generated oscillatory clock. This oscillatory spike synchronization within and across hemispheres could play a role in object recognition as has been proposed for vertebrates [41–43].

2. Material and methods

(a) Animals

We used nine female forager honey bees (*Apis mellifera*) in an *in vivo* preparation. The head capsule, thorax and abdomen were fixated with dental wax (Dr Böhme & Schöps Dental GmbH, Deiberit 502) in a metal tube. The bases of the antennae were immobilized with eicosan (Sigma–Aldrich). The cuticle of the head capsule between antennae and eyes was removed to get access to the brain. The glands in the head were removed and the head capsule was rinsed with artificial haemolymph (in mM: NaCl 130, KCl 6, glucose 25, $MgCl_2$ 2, $CaCl_2$ 7, sucrose 160, 10 HEPES, pH 6,7, 500 mOsmol). To avoid recording muscular activity we used Philanthotoxin-343 to paralyze the muscles in the head (50 µl, 10^{-4} Mol in artificial haemolymph; donated by P.N.R. Usherwood). Trachea between the antennal lobes and above the vertical lobes of the mushroom bodies were removed.

(b) Olfactory stimuli and experimental protocol

Olfactory stimuli were applied with a computer-controlled stimulator [44]. The following odorants were used: essential oils of clove, peppermint, orange (all from Apotheke Dahlem-Dorf), and geraniol, citral, isoamyl acetate and 1-heptanol (all from Sigma–Aldrich). Four microlitres of pure odorants were applied on 1 cm^2 filter paper in a 1 ml syringe. Residual odorants were removed via an exhaust tube (5 cm inner diameter) positioned 3 cm behind the bee. The different odorants were applied alternatingly with an interstimulus interval of 10–15 s. Each bee received between 16 and 108 odorant stimuli (mean = 57, s.d. = 28 stimuli) during a 3–37 min long recording session (mean = 19, s.d. = 11 min).

(c) Data acquisition

LFP and extracellular neuronal spikes were recorded in the centre of the mushroom body vertical lobes (depth: 20–150 µm) where olfactory Kenyon cells converge onto mushroom body output neurons [45]. Recordings were performed with artificial haemolymph-filled glass microelectrodes (1/0.58 mm outer/inner diameter) that were pulled with a micropipette puller (P-2000, Sutter Instrument Co) to get a tip resistance of 5–10 MΩ and which were then broken to get a tip resistance of 1.3–3.5 MΩ. Spikes could only be recorded with electrodes that had a tip resistance between 2.4 and 3.5 MΩ (which was the case in 5 out of the 9 recorded bees). The reference electrode was a chloridized silver wire (0.2 mm diameter). The recording chain consisted of a 10×-amplifier (Simmonds-Amplifier, Cambridge, UK), a 1000×-amplifier and 0.1–3.000 Hz band-pass filter (AM503, Tektronix) and an analogue–digital converter (1401+, Science Park Cambridge, UK). Experiments were performed at room temperature under daylight.

(d) Data analysis

Data analysis was performed with the MATLAB FieldTrip toolbox [46]. Spectral analysis was computed for each trial using a fast Fourier transformation using a multi-taper approach with orthogonal Slepian tapers [47]. For frequencies below 40 Hz, three orthogonal Slepian tapers were used, resulting in frequency smoothing of ±2 Hz. Spectrally resolved Granger causality (GC) analysis [48–50] was used to identify the directionality (so-called feed-forward versus feedback influences) of information flow between cortical areas (figure 2*c*). The first 0.5 s post-stimulus onset was omitted in order to avoid transient responses biasing Granger estimates. A bivariate nonparametric spectral matrix factorization approach [50] was applied in order to estimate GC. This algorithm estimates the spectral density matrix on the basis of Fourier coefficients that were computed using multitapers as described above (three orthogonal Slepian tapers) with frequency smoothening of ±3 Hz for frequencies of 0–100 Hz with 1 Hz spectral resolution. On the basis of the spectral density matrix (i.e. applying spectral factorization), the noise covariance matrix and the transfer function were obtained [50].

Neuronal spikes were identified from the raw LFP recordings using the peak detection algorithm implemented in MATLAB-*findpeaks.m*. Spikes were identified as local maxima exceeding 0.1 mV as illustrated in the electronic supplementary material, figure S2. Subsequently, the indices of these local maxima were used as triggers in order to re-segment the data ± 1 s around each spike (figure 2*a*,*b*).

Effects of spontaneous oscillatory power on spike rate (figure 2*d*) were evaluated by comparing high versus low power data segments in a bee × latency (spontaneous versus odour stimulation or high versus low spontaneous power) analysis of variance (ANOVA). Significant main effects and interactions were decomposed by simple effects ANOVA or *t*-test.

Cross-frequency coupling was computed according to the procedures of Tort *et al.* [51]. Modulating frequencies from 2 to 40 Hz in steps of 2 Hz and modulated frequencies from 10 to 450 Hz in steps of 10 Hz were analysed. A time-domain, zero phase lag, finite impulse response filter served to band-pass filter the data in the frequencies of interest. The filter order was frequency-dependent: number of cycles × sampling frequency/frequency of interest. Phase and amplitude estimates were derived from a Hilbert transform of the filtered data corresponding to angle and magnitude, respectively. Three cycles were used for estimation of the modulating and the modulated frequency.

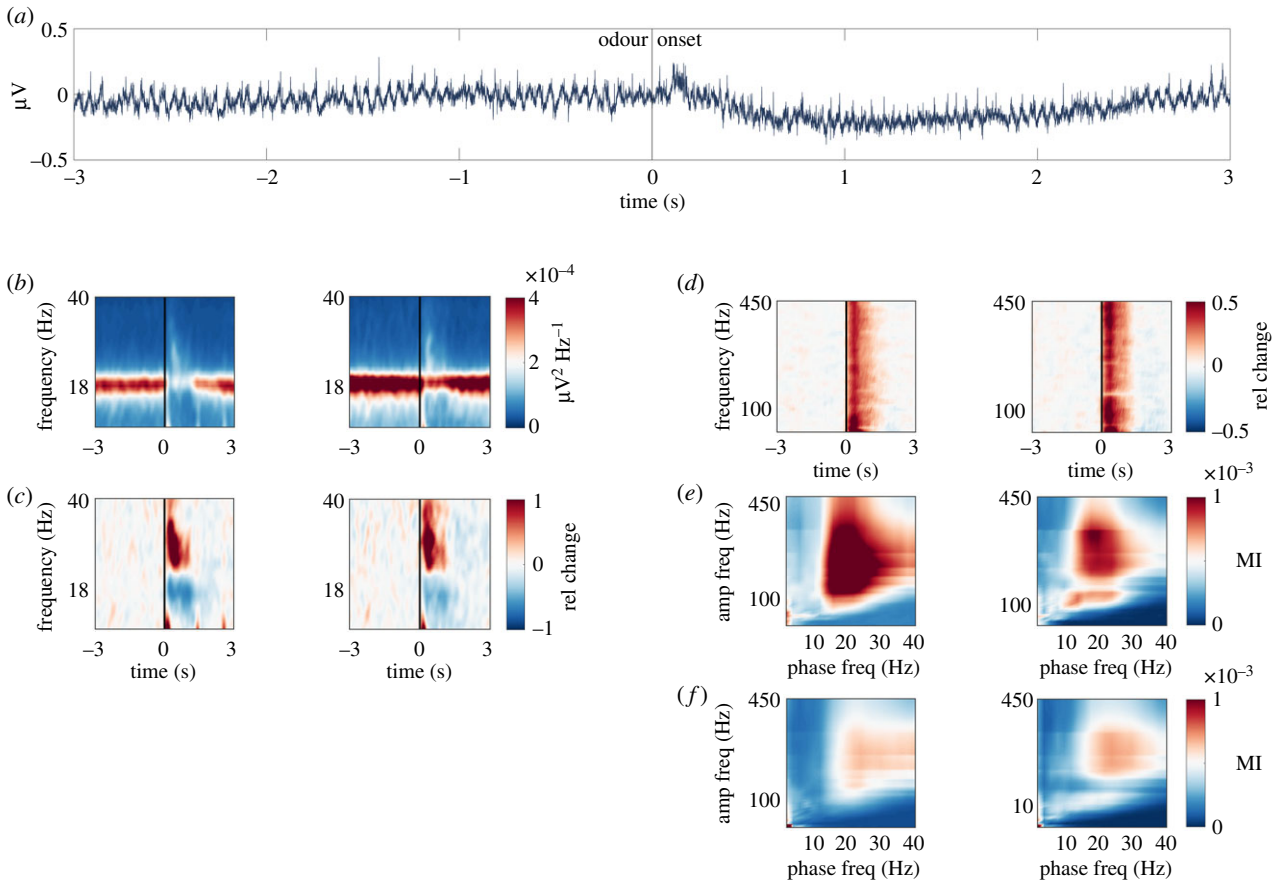

Figure 1. Spontaneous alpha and odour-induced gamma oscillations in the mushroom bodies. (*a*) Raw trace of a single trial in a representative bee. Odour is presented at 0 s. (*b*) Time-frequency (5–40 Hz, alpha and low gamma) representation of raw power (colour-coded, $\mu V^2\ Hz^{-1}$) recorded in the left (left) and right hemisphere (right), respectively. A 1 s long odour stimulus was presented at 0 s. $n = 9$ bees (stimulations per bee mean/s.d. 53/29). (*c*) The same as in (*b*) baseline-corrected. Warm colours indicate increase and cold colours decrease in oscillatory power expressed as relative change from pre-stimulus baseline. (*d*) Same as in (*c*) but for higher frequencies (40–450 Hz, high gamma). (*e*) Cross-frequency phase-to-amplitude coupling. Bispectra illustrating cross-frequency relationships in the left and right hemisphere, respectively. The phase-providing frequency is depicted on the *x*-axis and amplitude-providing frequency on the *y*-axis. Colour code shows the modulation index (MI). (*f*) Same as (*e*) but during odour stimulation. (Online version in colour.)

The modulation index was calculated for each electrode, each low-frequency phase and each high-frequency amplitude estimate. This measure quantifies cross-frequency coupling as the divergence of the observed amplitude distribution for each phase bin from a uniform distribution.

3. Results

LFP recordings in the vertical lobes of the honey bee mushroom bodies revealed a spontaneous approximately 18 Hz oscillation in both brain hemispheres (figure 1*a*). The power of the 18 Hz oscillation decreased during odour stimulation and returned back to baseline shortly after (figure 1*b*). This odour-induced power reduction was accompanied by a power increase in the 20–40 Hz band (low gamma oscillation) (figure 1*c*) and above 40 Hz (high gamma activity [52]) (figure 1*d*). Increases in high gamma power are thought to reflect the increase in the spiking activity of multiple neurons [53,54]. Both the spontaneous alpha oscillation and the odour-induced low and high gamma activity required an intact antenna ipsilateral to the recording site (electronic supplementary material, figure S1). This suggests that both alpha and gamma activity are generated in or require input from the ipsilateral antennal lobe (primary olfactory neuropil and functional analogue of the vertebrate olfactory bulb).

The cross-frequency coupling of high gamma activity to the phase of the spontaneous alpha oscillation is considered a mechanism of neural communication in cortical circuits [51,55–63]. Analysis of the cross-frequency coupling between high gamma activity and alpha oscillations confirms that the high gamma amplitude is modulated by the phase of the spontaneous alpha oscillation around 18 Hz (figure 1*e*). Moreover, there was also a cross-frequency coupling between high gamma activity and odour-induced low gamma oscillations (figure 1*f*).

In human EEG recordings, such cross-frequency coupling between high gamma activity and alpha oscillations is typically interpreted as an indicator of stimulus- or task-induced changes in neuronal spiking that is biased by the phase of the spontaneous alpha rhythm [9]. Unlike in human EEG, in honey bees, the accesses to both LFP and neuronal spikes, allows the empirical test of this hypothesis. An affirmative confirmation of the coupling between spikes and alpha oscillations is illustrated by the spike-field coherence analysis in figure 2. A reliable 18 Hz peak in the coherence spectrum was visible in both hemispheres, suggesting that the timing of spikes is coupled to the phase of the spontaneous 18 Hz oscillation. The timing of spikes in the right hemisphere was phase-coupled to the 18 Hz oscillation in the left hemisphere (figure 2*a*). By contrast, the

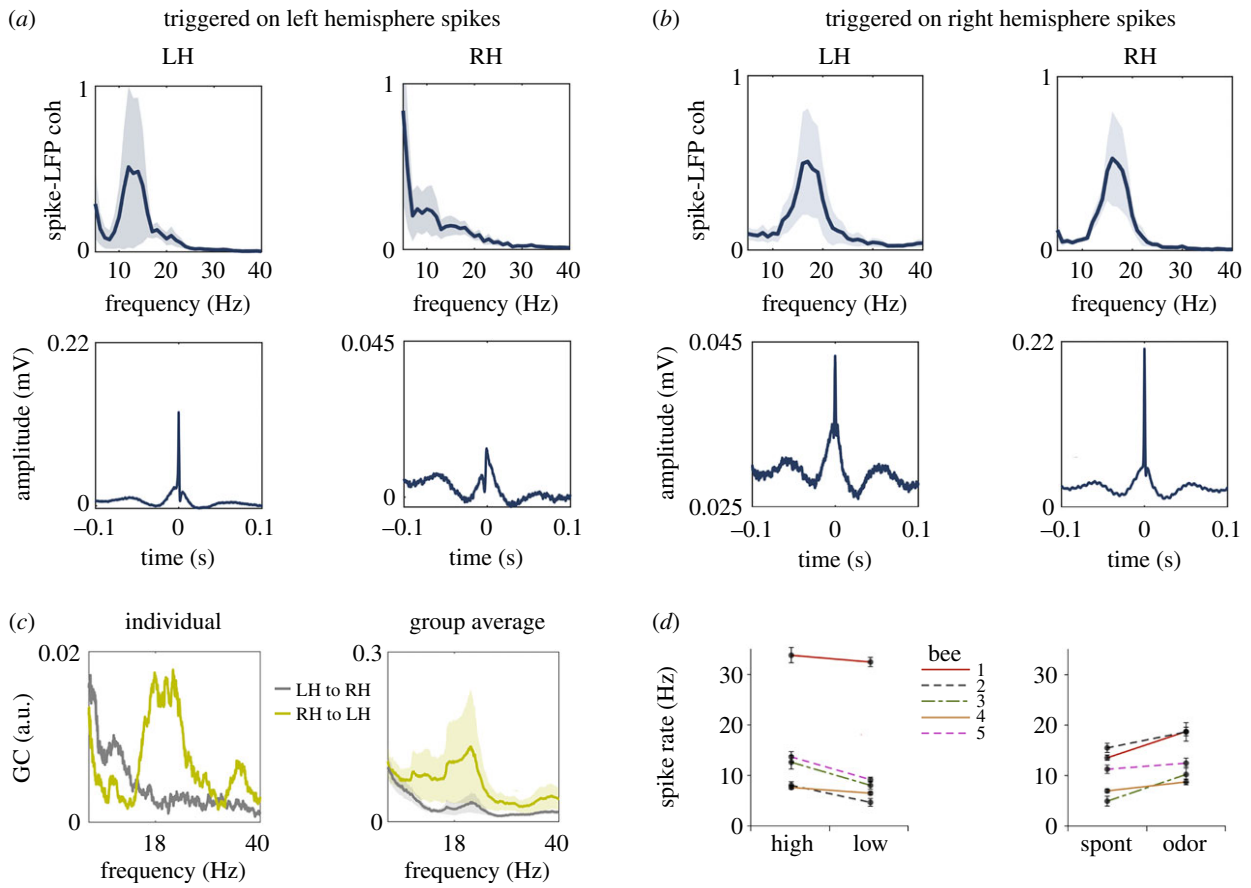

Figure 2. Neuronal spikes are phase-locked to the alpha oscillation. (*a*) Spike-triggered averages computed on left hemisphere spikes. Top: coherence between spikes and LFPs computed over the spike-triggered averages shows an approximately 18 Hz peak in the spectrum (mean and s.e.m., $n = 5$ bees). Bottom: spike-triggered averages for the left (LH) and right hemisphere (RH), respectively. Spike onset is at 0 s. $n = 5$ bees (spikes per bee mean/s.d. 4441/2415). (*b*) Same as (*a*) computed on right hemisphere spikes. (*c*) Granger causality spectra for an individual bee (left) and the group average (right) indicates stronger Granger causality (GC) influence from the right hemisphere (RH) to the left hemisphere (LH) ($n = 9$ bees, line represents the mean, the shaded area the s.e.m., right panel). (*d*) Left: spike rate per bee for high and low alpha power. Points and error bars show the mean and s.e.m. over trials. Values show the number of spikes per second. Right: spike rate per bee during spontaneous activity (during 2–1 s before odour onset) and odour-induced activity (during 0.1–1.1 s after odour onset). (Online version in colour.)

timing of spikes in the left hemisphere was not phase-coupled to the 18 Hz oscillation in the right hemisphere (figure 2*b*). In other words, the direction of functional connectivity quantified by spike transmission was stronger in the direction from the right to left hemisphere. This right-over-left dominance in interhemispheric functional connectivity was also visible in an analysis of GC [48,50] (figure 2*c*).

During the spontaneous activity, the spike rate depended on the power of the alpha oscillation and was higher in epochs of high alpha power (figure 2*d*, left) as confirmed by the main effect latency ($F_{1,341} = 18.74$, $p < 0.001$). There was a main effect for bee ($F_{4,341} = 32.27$, $p < 0.001$) and no bee × latency interaction. Moreover, the spike rate was higher during odour-induced activity than during spontaneous activity (figure 2*d*, right, $F_{1,341} = 32.05$, $p < 0.001$), corresponding to the period characterized by the strongest decrease in alpha power (figure 1*b*,*c*).

4. Discussion

Here, we demonstrate that the honey bee brain generates oscillations (around 18 Hz) which share characteristics of alpha oscillations in the primate brain. We termed these 18 Hz oscillations 'alpha' based on their similarity with alpha oscillations in humans (around 10 Hz) and alpha/beta oscillations in non-human primates (10–20 Hz) [9,12,13,64]. Similar to primate's alpha/beta oscillations, honey bees' 18 Hz oscillations occurred spontaneously, decreased in power during sensory stimulation, and biased spike timing and higher frequency neuronal activity. We believe that it is adequate to term honey bees' 18 Hz oscillations 'alpha' rather than 'beta', because the distinction between human 'alpha' and monkey 'beta' oscillations is based on frequency bands rather than on function. However, human alpha and monkey alpha/beta oscillations carry out similar functions. In monkeys, for example, 10–20 Hz oscillations regulate communication between cortical modules in the visual system (e.g. V4–V1) [12,13]. This mechanism of top-down control of alpha/beta oscillations within an anatomically defined cortical hierarchy also occurs in the human visual cortex [14], strengthening the notion that alpha/beta oscillations serve similar functions across phyla. The present finding of asymmetric, alpha oscillation-mediated functional connectivity between the brain hemispheres (figure 2) is in line with this view. We, therefore, believe that it is reasonable to subsume honey bees' 18 Hz, monkeys' 10–20 Hz and humans 10 Hz oscillation under the term 'alpha'.

What is the origin of honey bees' brain oscillations? We recorded LFP oscillations and spikes in the output region of the mushroom bodies (vertical lobes). There, many thousand

mushroom body-intrinsic neurons (Kenyon cells) converge onto a few hundred output neurons [45,65]. The LFP is probably generated by the summation of excitatory postsynaptic potentials of mushroom body output neurons [66]. However, it is not possible to determine the number or the identity of output neurons that contributed to the LFP oscillations and to spikes. Previous studies showed that odour-induced 30 Hz oscillations in the input region of the mushroom body (calyx) reflect oscillatory spike synchronization across presynaptic neurons (projection neurons which connect the antennal lobe with the ipsilateral mushroom body [30,67]). The fact that our recorded odour-induced 30 Hz oscillations required input from the ipsilateral antenna, suggests that they also reflect oscillatory spike synchronization across projection neurons. Likewise, the spontaneous 18 Hz oscillation required an intact ipsilateral antenna, indicating that it is driven by spontaneous projection neuron activity. This hypothesis is supported by the fact that projection neurons are spontaneously active [68], and this spontaneous activity is reduced upon odour stimulation [69]. However, it is unknown whether the oscillatory spike synchronization is generated in the antennal lobes or in the mushroom bodies. The coupling of the spontaneous 18 Hz oscillations across hemispheres suggests that the oscillatory spike synchronization is generated by circuits that involve bilateral neurons, for example, neurons which connect both antennal lobes [70] or mushroom bodies [45,65].

What is the function of honey bees' alpha oscillation? Alpha oscillations could regulate the information transmission between brain regions. Both spike occurrence and GC analyses (figures 2*a–c*) indicate a right-over-left dominance in interhemispheric information flow during spontaneous activity. This right-over-left dominance in interhemispheric information flow could be related to honey bees' left–right-asymmetry of odour-reward learning [71] and of odour discrimination [72]. The interhemispheric coordination of spike timing could serve to bind bilateral olfactory information into coherent object representations [43], and it could underlie bees' ability to retrieve unilaterally learned odour-reward associations via both antennae [40,73]. It will be interesting to determine the alpha oscillation-mediated connectivity and hierarchical organization of the honey bee brain at higher spatial resolution. Given that honey bees show cognitive capacities (e.g. concept learning [74]; selective attention [75]; map-like spatial memories [76]), our results suggest the honey bee as model to examine the functional role of alpha oscillations in perception and cognitive function.

Data accessibility. The data and analysis scripts can be found here: https://osf.io/523tk/?view_only=bfa999c448014bd196d1f432fb3c593e.

Authors' contributions. P.S. performed the experiments. T.P. analysed the data. T.P. and P.S. wrote the manuscript.

Competing interests. The authors declare no competing interests.

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