Learning by heart: Cardiac cycle reveals an effective time window for learning

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ABSTRACT

Cardiac cycle phase is known to modulate processing of simple sensory information. This effect of the heartbeat on brain function is likely exerted via baroreceptors, the neurons sensitive for changes in blood pressure. From baroreceptors the signal is conveyed all the way to the forebrain and the medial prefrontal cortex. In the two experiments reported here, we examined whether learning, as a more complex form of cognition, can be modulated by the cardiac cycle phase. Human participants (Experiment 1) and rabbits (Experiment 2) were trained in trace eyeblink conditioning, while neural activity was recorded. The conditioned stimulus was presented in contingency with either the systolic or diastolic phase of the cycle. The tone used as the conditioned stimulus evoked amplified responses in both humans (electroencephalogram from 'vertex' (Cz)) and rabbits (hippocampal CA1 local-field potential) when its onset was timed at systole. In humans, the cardiac cycle phase did not affect learning, but rabbits trained at diastole learned significantly better than those trained at a random phase of the cardiac cycle. In summary, our results suggest that neural processing of external stimuli and also learning can be affected by targeting stimuli based on the cardiac cycle phase. These findings might be useful in applications aimed at maximizing or minimizing the effects of external stimulation.

NEW & NOTEWORTHY

It has been shown, that rapid changes in bodily states modulate neural processing of external stimulus in brain. Here, we show that modulation of neural processing of external stimulus and learning about it depends on the phase of the cardiac cycle. This is a novel finding that can be applied to optimize associative learning.

Keywords: baroreceptor, classical conditioning, hippocampus, theta oscillation

INTRODUCTION

In 1884, William James reminded “brain physiologists”, as he called them, that bodily states affect how we experience the world. James’ philosophy has the fundamental idea of the consciousness being an inseparable stream of bodily and mental states. Since the 1880’s science has verified in many ways that bodily states do alter the way we perceive or experience the outer world through the inner world. Wilson (2002) suggests that episodic memory consists of embodied experiences of the world. In his view, new forming episodic memories are merged from contextual experiences of the environment as sensory information and information of the body in different states experiencing the world and itself within it. As the time passes on and these memories “become crystalized” they are not anymore modified by the bodily sensations. Therefore, if episodic memories are embodied, how is bodily information merged with sensory information?

Information about the state of internal organs travels to the brain through neural and humoral pathways (Critchley and Harrison 2013). Baroreceptors, stretch and pressure -sensitive sensory neurons found in blood vessels, activate during each heart beat as the vessel walls distort. Their function is crucial in maintaining suitable blood pressure and they convey information about the timing and strength of heartbeat to the nucleus of the solitary tract (NTS, Critchley and Harrison 2013; Jänig 2006). The NTS is connected to the hypothalamus, the parabrachial nucleus and the periaqueductal gray, which in turn are connected to forebrain regions such as the amygdala, insular cortex, cingulate cortex and orbitomedial prefrontal regions (Critchley and Harrison 2013). These anatomical and functional connections hint at the idea that the heartbeat via baroreceptor activity could affect cognition and behavior (Lacey and Lacey 1974, 1978).

Indeed, the reported detection of a visual stimulus can be enhanced by presenting the stimuli time-locked to a certain cardiac cycle phase (for T-wave, see Park et al. 2014; for P-wave, see Sandman et al. 1977). Likewise, visual evoked potentials are modulated as a function of cardiac cycle phase (Walker and Sandman 1982). Thus, baroreceptor activity affects cognition at least on the sensory level. There are indications that baroreceptor activity might affect even more complex cognitive processes such as short-term memory performance (Martins et al. 2014) and emotional appraisals of facial expressions (Gray et al. 2012). To our knowledge, it has not been tested whether baroreceptor activity could influence hippocampus dependent associative learning.

The hippocampus is the critical hub of complex learning and episodic memory in the mammalian brain (Squire 1992). Different frequencies of hippocampal electrophysiological oscillatory activity reliably index different behavioral states. Theta (~4–8 Hz being peak frequency, depending on the species) is the most prominent oscillation in the hippocampus (e.g., Buzsáki 1989). Theta is elicited by external stimuli and paced by cholinergic input from the medial septum and glutamatergic input from the entorhinal cortex (Buzsáki 2002). The critical role of the hippocampal theta in declarative learning is supported by a multitude of experimental findings (Berry and Thompson 1978; Berry and Seager 2001; Griffin et al. 2004; Nokia and Wikgren 2010, 2014; for conflicting findings, see Múnera et al., 2001). Overall, temporally robust hippocampal theta-band responses to the conditioned stimulus predict good learning (Nokia et al. 2015).

In addition to contributing to cognitive processes via its mutual connections with the neocortex (Buzsáki 1989), the hippocampus has connections with the hypothalamus including supramammillary nucleus and posterior nucleus (Abrahamson and Moore 2001; Cavdar et al. 2001; Pan and McNaughton 2004). The hypothalamus regulates the function of

the autonomic nervous system; therefore, for example, it affects blood pressure and heart beat (Guyenet 2006), both directly and indirectly (Fanselow and Dong 2010). A few studies actually propose that hippocampal theta oscillations are temporally aligned with the cardiac cycle (Komisaruk 1970; Pedemonte et al. 2003) and cycles of rhythmic behavior like mammalian sniffing (Macrides et al. 1982) and rats' whisking with their snout hairs (Grion et al. 2016).

Here, to elucidate the potential connection between different phases of the cardiac cycle, brain activity and associative learning, we subjected humans (Experiment 1) and rabbits (Experiment 2) to trace eyeblink conditioning (see Figure 1), a hippocampus-dependent task considered to model declarative learning both in animals and in humans (Solomon et al. 1986; Holland and Bouton 1999). The onset of the conditioned stimulus (CS) was timed to either the systolic or the diastolic phase of the cardiac cycle (see Figure 2). In rabbits, we also included a group that was trained irrespective of cardiac cycle phase. Brain activity during training was recorded from the scalp in humans (electroencephalogram, EEG) and directly from the hippocampus in rabbits. In rabbits, we expected to see strong phase synchrony between the ongoing hippocampal theta rhythm and the cardiac cycle. Further, we expected both neural responses to the conditioning stimuli and learning at the behavioral level to be different between the experimental groups in both humans and in rabbits. However, we had no presumption concerning which phase, diastole or systole, would be optimal for learning.

EXPERIMENT 1: HUMAN EYEBLINK CONDITIONING AND EVENT RELATED POTENTIALS

MATERIALS AND METHODS

Participants

Participants, recruited mainly via e-mail lists, gave an informed written consent to this study and were free to discontinue participation at any point. The study was approved by the University of Jyväskylä Ethical Committee. Thirty (23 female and 7 male, aged 18–32 years) right-handed adults took part in the study. All participants were healthy with no history of psychiatric or neurological illnesses or medication affecting brain function. One participant had to be excluded from all analyses because of a software malfunction. Due to technical difficulties electrophysiological data from five participants were not analyzed.

Experimental procedure

Before the experiment, participants filled out a modified BIS/BAS personality inventory and answered background questions about age, gender, height, weight, and schooling. In addition, their blood pressure was measured before (and after) the experiment.

Participants were informed that the aim of the study was to record physiological and neural responses to different types of stimuli. After recording 5 minutes of resting data, the trace eyeblink conditioning procedure was started. The experiment was controlled by custom-written software running on an Arduino-based device that received input signal from the electrocardiogram (ECG) -recording device whenever the signal exceeded the threshold set roughly at 2/3 of the peak amplitude of the R-peak. The participants were randomized into systole (n = 15) and diastole (n = 14) groups. In systole group, the trial onset was delayed by a 100 ms from the rising slope of the R-peak, whereas in diastole group the trial onset was delayed by 500 ms. The conditioned stimulus was a 200-ms, 440-Hz tone delivered via a loudspeaker situated to the lower right-hand corner of the room. A 600-ms trace interval separated the tone offset and the unconditioned stimulus (US) onset. The US was an airpuff

(0.4 bar source pressure, 100 ms) targeted to the corner of the left eye and it was delivered via a plastic tube attached to modified safety goggles.

Before the actual conditioning phase, 4 air puffs alone were delivered at 5-second intervals to accustom the participant to it. The conditioning procedure consisted of 80 trials. The first (unpaired) and last (extinction) ten trials were CS-alone trials. The inter trial interval (ITI) varied randomly between 9 to 19 seconds.

Recordings and data analysis

During the experiment, heart rate, eyeblinks and brain activity were recorded. The participants were in a seated position during the experiment. Heart rate was recorded using three ECG electrodes: one placed near the sternum and one over the right ribs, and the grounding electrode over the left flank. Eyeblinks were recorded using two electromyography (EMG) electrodes, which were placed underneath the participant's left eye. EEG data was recorded using a 64-channel EEG-cap (64 BrainCap with Multitrodes, EASYCAP GmbH, Woerthsee-Etterschlag, Germany). Resting state data was recorded for 5 minutes before and after the actual experiment.

Eyeblinks

The EMG signal was high-pass filtered (> 60 Hz), rectified, and then low-pass filtered (< 20 Hz) off-line using Brain Vision Analyzer software. One of the authors (JW) blind to the experimental group visually assessed all trials for conditioned responses. An eye-blink was considered a CR if it occurred within a period of 500ms before the US onset. The exclusion criterion was subjectively rated as excessive EMG activity during the 500ms time period before the CS onset. The trials were grouped into 8 blocks of 10 for the sake of analysis and the percentage of CRs per block was calculated. The learning curves of the systole and diastole groups were compared using repeated measures ANOVA.

Brain responses

Valid EEG data was gathered from 24 participants (Systole: n = 12; Diastole: n = 12). BrainVision Analyzer 2.1 (Brain Products GmbH, Gilching, Germany) was used to remove bad channels and low-pass filter (< 30 Hz) the raw data. Independent component analysis (ICA) was run on the data and components related to eye blink, eye movement and heart beat artifacts were removed. The heartbeat itself is an event which induces a stereotypical activity pattern in the electroencephalogram, called heartbeat evoked potential (HEP). It is found in recordings over the somatosensory cortex (Kern et al. 2013) as well as fronto-cortical (Schandry and Montoya 1996) and fronto-temporal (Montoya et al. 1993) areas. Therefore, including an ECG channel in the ICA was used to remove a potential confounder from the EEG data.

Event-related potentials (ERPs) recorded from the Cz channel were used for further analysis. ERPs were calculated from a 500-ms time window at -100 to 400 ms in relation to the CS onset. These epochs were first baseline-corrected by subtracting the average amplitude during the 100-ms time window prior to CS onset. Then the baseline-corrected epochs were averaged. For each participant, an average peak for N1 (minimum amplitude within 90 to 130 ms post-CS onset) and P2 (maximum amplitude within 150 to 230 ms post-CS onset) were calculated from the paired trials (n = 60).

RESULTS

CS evoked a larger N1 response when presented to the systole

An independent samples t-test on ERPs showed that the N1 responses were larger in the systole group compared to the diastole group ($t(22) = 3.14$, $p < 0.01$, Cohen's $d = 1.28$; see

Figure 3). The P2 responses were also larger in amplitude in the systole group, but the difference did not reach statistical significance ($t(22) = 1.23$, $p = 0.23$, Cohen's $d = 0.52$).

Due to potential confounding effect of heart-beats on ERPs, the ICA was used to remove heart-related artefacts. Figure 4 depicts the effects of ICA on the EEG recorded at the Cz electrode. As seen, there is a small (~1 μv) deflection at about 350-400 ms after the R-peak in the signal before the ICA. However, the corrected EEG signal is virtually flat. Thus, it can be concluded that neither the heart beat related evoked potentials nor artifacts related to cardiac cycle contribute to CS-evoked ERP amplitudes.

Cardiac cycle phase did not modulate learning in humans

Repeated measures ANOVA on the effects of Block (1–7, extinction block excluded) and Group (systole vs. diastole) on CR percentage revealed a significant main effect of Block ($F[6, 162] = 39.89$, $p < 0.001$, partialEta2 = 0.57), indicating that the amount of conditioned responses increased as a function of training. Neither the main effect of group ($F[1, 27] = 0.03$, $p = 0.864$, partialEta2 = 0.001) nor the interaction between Group and Block ($F[6, 162] = 0.585$, $p = 0.742$, partialEta2 = 0.021) reached significance, indicating that timing the CS onset based on different phases of the cardiac cycle did not have an effect on learning trace eyeblink conditioning (see Figure 5).

INTERIM DISCUSSION

As even relatively complex cognitive processes have been shown to be modulated by baroreceptor activity, we assumed that learning hippocampus-dependent trace eyeblink conditioning would differ between the humans trained at systole vs. diastole. Contrary to this expectation, both groups learned the task equally well. However, the systole group showed larger evoked responses (N1) to the conditioned stimulus. This suggests that cardiac cycle

197 phase affects sensory processing of external stimuli but these effects do not directly carry

198 over to learning at the behavioral level.

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EXPERIMENT 2: RABBIT EYEBLINK CONDITIONING AND HIPPOCAMPAL FIELD RESPONSES

MATERIALS AND METHODS

Subjects

The subjects were 25 adult female New Zealand White rabbits (Lidköpings Kaninfarm, Sweden) weighing ~2.8 kg at the time of surgery. The rabbits were housed in individual cages in the laboratory center of the University of Jyväskylä. Food and water were freely available, and room temperature and humidity were controlled. The rabbits were maintained on a 12/12-hour light/dark cycle, with lights on at 8:00 a.m. All experiments were carried out during the light period. All experimental procedures, caretaking and handling were executed in accordance with Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes. Animal handling was done only by trained personnel and rabbits were introduced to human contact and handling for a sufficient amount of time before the surgery.

Surgery

Before the surgery, rabbits were treated with subcutaneous injections (s.c.) of an anti-inflammatory drug (50 mg/ml carprofen [Rimadyl vet, Pfizer Inc. Animal Health], dose: 0.1 ml/kg) and with 2 ml of an analgesic drug (0.3 mg/ml buprenorphine [Temgesic, Schering-Plough Europe] diluted with 0.9 ml of 0.9% NaCl) to moderate acute pain after surgery. The rabbits were anesthetized with an intramuscular injection (i.m.) of ketamine-xylazine cocktail (7.8 mL of 50 mg/ml Ketaminol vet [Intervet International B.V.] mixed with 2.8 ml of 20 mg/ml Narcoxyl vet [Intervet International B.V.]). A dose of 0.8 ml/kg of the cocktail was injected i.m. before surgery. During surgery, additional doses of either the cocktail or

ketamine alone were injected s.c. approximately every 20–30 min. or as needed. Before the surgery, the rabbit's fur was shaved from the top of its head. Then, the rabbit was positioned in a stereotaxic instrument (Kopf Instruments) with the bregma 1.5 mm higher than the lambda. Eye gel was inserted into the rabbit's eyes. At this point, 2.0 ml of lidocaine (10 mg/ml Lidocain [Orion Pharma]) was injected s.c. in the area of surgery before making the opening incision.

A longitudinal incision was made on the scalp and local anesthetic (2 g of lidocaine-hydrochloride Xylocain [AstraZeneca]) was administered to the wound. The skull was drilled with holes for electrodes and four holes for the anchoring screws (5 mm anterior and 5 mm lateral to the bregma; 13 mm posterior and 5 mm lateral to the bregma). Two of the screws were connected together, and they were used as a reference. The other two served as the ground for the electrophysiological recordings. For eight rabbits, eight monopolar recording electrodes (Formvar-insulated nichrome wire; 0.05 mm bare [A-M Systems]) were chronically implanted in the left dorsal hippocampus, with four electrodes aiming at the CA1 (4 mm posterior, 3.5–6.5 mm laterally and from the bregma; electrode tip depth from the bregma 6–8 mm) and four above the hippocampal fissure (5 mm posterior, 4–7 mm laterally from the bregma; electrode tip depth 6.2–8.5 mm below the bregma). For nine rabbits, eight monopolar electrodes were implanted in both hippocampi (see coordinates above). For four animals, a 32-channel (E32B-20-S04-L10.0-200, ATLAS Neuroengineering) adjustable four-shank probe was chronically implanted in the left dorsal hippocampus (5 mm posterior, 4 mm laterally from the bregma) with a microdrive (nDrive xL, NeuroNexus). Wires, skull screws, a preamplifier interface, one mounting screw for an air puff mount and the incision area were cemented with dental acrylic. To prevent nausea after surgery, metoclopramide (0.1 ml/kg, concentration 5mg/ml; Primperan [Sanofi Winthrop Industrie]) was administered s.c. and the rabbit was returned to its home cage wrapped in a towel. Recovery was monitored and the

rabbits were medicated with analgesic (buprenorphine [Temgesic, Schering-Plough Europe] diluted with 0.9 ml of 0.9% NaCl) four hours after surgery and then every eight hours for the next 44 hours.

Experimental procedure

The experimental procedure is illustrated in Figure 1. After one week of recovery from surgery, animals were accustomed to a Plexiglas restraining box without restraining and their overall behavior was monitored. Local field potentials (LFPs) and EMG from the right eye were recorded 5 minutes prior, during and 1 minute after each session. ITI always varied randomly between 30 and 60 s. LabVIEW (National Instruments) was used to monitor the cardiac cycle and blinking on-line, to execute the experimental procedures and to present stimuli. After the ITI was expired, trial presentation was always delayed for one second every time the rabbit was spontaneously blinking. The percentage of learned responses performed by each animal was analyzed after every session using MATLAB (The MathWorks Inc.).

During the first training session (CS-Alone), 60 tone-alone (200-ms, 5-kHz, 75-dB tone) trials were presented regardless of cardiac cycle phase. In addition to hippocampal LFPs, EMG from the right eye was also recorded to determine the frequency of spontaneous eyeblinks elicited by the tone later used as a CS.

Trace eyeblink conditioning was carried out with the tone specified above as the CS and a 100-ms air puff (0.35 bar source pressure) to the right eye as an unconditioned stimulus (US). A trace period of 500 ms was used. A total of 60 training trials were presented during each session, regardless of neural state and in the absence of spontaneous blinking. The trials were timed so that the CS started either at the systolic or the diastolic phase of the cardiac cycle, or irrespective of cardiac cycle phase. A total of 14 sessions were conducted.

Recordings and data analysis

Cardiac cycle

The cardiac cycle was monitored with a pulse oximeter (Shimmer Optical Pulse Sensor, Realtime Technologies Ltd) attached to the rabbit's shaved right earlobe. Photoplethysmography (PPG) is a robust measure for monitoring the cardiac cycle (see Wisely and Cook 2001). The temporal relation between ECG and PPG in rabbits was confirmed during surgery with an anesthetized rabbit by recording the ECG with two needles positioned on both sides of the animal, leaving the heart in between.

Eyeblinks

Bipolar EMG from the trained eye was recorded using stainless steel wire-hooks placed around the right upper and lower eyelids for the duration of the training sessions. The raw EMG signal was conveyed to a filter-amplifier (A-M Systems Model 2100), amplified 1000x and band-pass filtered from 100 to 500 Hz. The EMG signal was high-pass filtered off-line (>100 Hz) and Hilbert-transformed. An envelope curve following the peaks of the signal was calculated. Baseline EMG activity was defined for each animal and session as the mean of the peak EMG amplitude during a 250-ms pre-CS period (MEANpre). The mean of the standard deviation of the EMG activity during the 500-ms pre-CS period (SDpre) was also determined. Eyeblinks were defined as EMG activity exceeding a threshold of [MEANpre + 4 × SDpre] for at least 10 ms. Trials with eyeblinks during the 100-ms period immediately preceding CS onset were rejected. Eyeblinks 100ms prior to US onset were counted as conditioned responses.

Hippocampal local-field potentials

For neural recordings of monopolar electrodes, a tenfold amplification was performed with a preamplifier (MPA8I, MultiChannel Systems [MCS]) attached to the electrode connector in the rabbit's head. Then the signal was band-pass filtered (1–5000 Hz) with a 64-channel filter amplifier (MCS). Lastly, the signal was further low-pass filtered (500 Hz) and digitized at a rate of 2 kHz with a MCS USB-ME64 System (MC_Rack software). SPSS (IBM) and MATLAB (MathWorks) were used for offline data analysis. Rabbits implanted with the 32-channel probes (Atlas Neuroengineering) were recorded with a wireless data acquisition system (W2100-HS32-headstage, MultiChannel Systems [MCS]) with a 20 kHz sampling rate.

To assess the temporal accuracy of the theta-band responses to the conditioning stimuli, a phase-locking value (PLV) was calculated (Palva et al. 2005b). The PLV is based on amplitude-normalized phase information and is thus resistant to changes or differences in signal amplitude. This allows comparable measurements to be obtained from data recorded over time in multiple subjects. The hippocampal LFP data were first band-pass filtered between 4 and 8 Hz. Then, a Hilbert transform was run on the signal to obtain the phase information, and the amplitude of the transformed signal was normalized to 1 by dividing each data point by its absolute value. Finally, the PLV was obtained by averaging over 60 trials (one session) and taking the absolute value of the mean. The PLV varies between 0 and 1, 0 indicating no phase locking and 1 indicating perfect phase locking. For statistical analyses, the mean of the PLV during the CS and subsequent trace-period (700 ms) was derived and averaged over one session for each subject.

The phase synchrony (PS, Palva et al. 2005b) of the hippocampal theta (band-pass filtered between 4–8 Hz) and PPG (band-pass filtered between 3–6 Hz) was analyzed next. The LFP and PPG sweeps were selected randomly from occasions where theta ratio was high (>80%)

and the PPG signal quality was good. Both signals were Hilbert-transformed and their amplitudes normalized to 1, as explained above. Then, the phase difference of the two signals was calculated by multiplying the first signal with the complex conjugate of the second signal (each data point of each sweep). Finally, the PS was derived by averaging the phase difference matrix over sweeps, taking the absolute value.

Neural responses evoked by the CS in hippocampal CA1 were averaged within each session per animal. Negative peak amplitudes of these event-related potentials (ERPs) were analyzed from 25 to 60 ms after CS onset (see Figure 6B) and normalized to CS-Alone session amplitudes [({session ERP amplitude – CS-Alone ERP amplitude} / CS-Alone ERP amplitude) * 100]. The placement of the electrodes in CA1 was confirmed with histology and in addition by inspecting sharp-wave ripples.

Statistical analyses

Repeated-measures analysis of variance (ANOVA), with training sessions (or blocks of two sessions) as a within-subjects factor and group as a between-subjects factor, was used to analyze changes across training and differences between experimental groups. For post hoc comparisons, Bonferroni-corrected p-values are reported. One-way ANOVA or an independent samples t-test was used for comparisons between groups one dependent variable at a time. One-way ANOVA was used to test the difference between the groups using the session from the last four training sessions for each individual animal where they achieved their best performance in conditioned responses.

Histology

Rabbits were anesthetized with an i.m. injection of ketamine-xylazine cocktail and then overdosed with an i.v. pentobarbital (Mebunat vet, Orion-Yhtyma Oyj) injection. Then, the brain was perfused with physiological saline followed by 9% formalin solution through the

ascending aorta. The locations of the electrode tips were marked by passing a DC current (200 mA, 10 s) through them. The brain was then removed and stored in formalin for several days. The brain was coronally sectioned with a vibratome into 60-µm-thick slices. The slices were attached to gelatinized slides, dried, and stained with Prussian blue and cresyl violet. The electrode locations were determined with the help of a microscope.

RESULTS

Hippocampal theta phase was not in synchrony with cardiac cycle phase

Phase synchrony between hippocampal fissure LFP and the PPG signal reflecting the cardiac cycle was analyzed from periods of spontaneously occurring theta oscillations (theta ratio > 80% during ITI). The average phase synchrony from all sessions and all rabbits was 0.17 (SD = 0.07), on a scale of 0 to 1, with 1 indicating perfect phase synchrony. That is, no phase synchrony between theta and the cardiac cycle was detected. The mean heart rate of the rabbits during the sessions was ~180 beats per minute (bpm), which is within normal variation (130 bpm to 325 bpm; see Pritchett-Corning et al. 2011).

CS evoked larger hippocampal responses when presented to the systole

Histological examinations confirmed that recording electrodes were in or near the hippocampal CA1, as intended, in 19 animals (see Figure 6A). Note that four of the rabbits had been implanted with multisite silicon probes that were adjusted constantly during the experiment. Therefore they are not included in this analysis.

Event-related potentials to the CS recorded from the CA1 had a mean latency of 41 ms (SD = 4.80 ms) from CS onset. The amplitude of this response was moderated by the phase of the cardiac cycle so that the amplitudes were higher in the Systole group (n = 6) compared to the Diastole group (n = 7) (repeated measures ANOVA, interaction of group and session: F [12,

96] = 0.28, p = 0.99; main effect of session: F [6, 96] = 0.47, p = 0.83; main effect of group F[2, 16] = 4.44, p < 0.05; Systole vs. Diastole, Bonferroni-corrected post-hoc comparison: p = 0.027).see Figure 6C).

Phase-locked hippocampal theta-band responses to the CS were not different between the Diastole, the Systole, and the Random group during trace conditioning (repeated measures ANOVA, interaction of group and session: F [12, 108] = 1.23, p = 0.27; main effect of session: F[6, 108] = 1.29, p = 0.267; main effect of group F [2, 18] = 0.24, p = 0.79).

Rabbits trained at diastole learned better than those trained irrespective of cardiac cycle phase

Twenty-one out of the 25 animals learned trace eyeblink conditioning. Learning differed between the Diastole (n = 10), Systole (n = 8) and the Random (n = 7) groups (repeated measures ANOVA, interaction of group and session: F [12, 132] = 0.79, p = 0.66; main effect of session: F [6, 132] = 11.08, p < 0.0001; main effect of group F [2, 22] = 3.94, p < 0.05; see Figure 7A). Specifically, learning was better, when the CS onset was timed to the diastole phase of the cardiac cycle compared to when it was presented in a random phase (Diastole vs. Random, Bonferroni-corrected post-hoc comparison: p = 0.036).

There was also a significant difference between the groups in the best performance (CR %) they reached during the last four sessions of conditioning (one-way ANOVA: F [2, 22] = 4.38, p = 0.025). Post hoc comparisons indicated that the best performance in the Diastole group (M = 82.36, SD = 11.93) was significantly higher than that in the Random group (M = 52.33, SD = 32.54) (Bonferroni-corrected p = 0.022). However, the Systole group (M = 68.64, SD = 15.67) did not significantly differ from the other two groups (Bonferroni-corrected post-hoc comparisons: Systole vs. Diastole: p = 0.524; Systole vs. Random: p = 0.422; see Figure 7B).

388 *INTERIM DISCUSSION*

389 As anticipated, the cardiac cycle phase affected both neural responses as well as behavior
390 during trace eyeblink conditioning in rabbits. Namely, hippocampal responses evoked by the
391 conditioned stimulus were larger in amplitude in the Systole group compared to the Diastole
392 group. Further, rabbits learned trace eyeblink conditioning better when the CS onset was
393 timed to the diastole phase of the cardiac cycle. In fact, almost all (90%) of the animals in the
394 Diastole group reached a limit of 80% CRs per session, whereas only half in the Systole
395 group and less than third in the Random group reached this limit during the 14 sessions of
396 trace eyeblink conditioning. The animals in the Diastole group also learned exceptionally
397 well when compared to previous results from our lab using the same paradigm for trace
398 eyeblink conditioning (Nokia and Wikgren 2014; Nokia et al. 2015; Waselius et al. 2017).

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DISCUSSION

Neural responses, as well as simple sensory phenomena have been shown to vary depending on the timing of the stimuli in relation to the phase of the cardiac cycle. Here, both human participants and rabbits were subjected to trace eyeblink conditioning where the onset of the conditioning trial was timed either to the systolic or diastolic phase of the cardiac cycle. This task revealed that neural responses (scalp EEG in humans and LFPs from hippocampal CA1 in rabbits) to the tone-CS differed between the Systole and Diastole groups. Namely, the responses to the tone-CS were enhanced when targeted to the systolic phase. On the contrary, an enhancement of the learning rate was evident in the Diastolic group in rabbits. No effect of cardiac cycle phase on learning rate was found in humans.

Earlier studies show, that behavioral (Sandman et al. 1977; Gray et al. 2012; Martins et al. 2014; Park et al. 2014) and neural (Walker and Sandman 1982) responses in humans can be modulated by presenting stimuli time-locked to the cardiac cycle phase. In Experiment 1 neural responses to the tone used as a conditioned stimulus measured with EEG differed between participants trained at diastolic vs. systolic phase of the cardiac cycle. However, both of the groups learned the task at the same pace. It is to be noted that single-cue trace eyeblink conditioning is a relatively easy task for humans and learning occurs rapidly. Factors known to affect the learning rate include, for example, awareness (Manns et al. 2000), and cholinergic blockade by scopolamine (Solomon et al. 1993). While cardiac cycle might have some effect on the way a stimulus is processed (as indicated by previously reported ERP and sensory threshold studies), the effect might be too subtle to manifest in associative learning which is a process governed by a multitude of top-down and bottom-up factors. It might be that making the task a bit more demanding (e.g., increasing the trace period or lowering the amplitude of the conditioned stimulus near to the detection threshold) might yield differences

also at a behavioral level. Running Experiment 1 again using the same parameters but with elderly adults could also reveal differences in learning between groups, as it is known that aging has a deteriorating effect on the ability to learn trace eyeblink conditioning (for example, see Woodruff-Pak et al. 2001). In the future, it would be important to use a more demanding task that should make the initial learning rate slower but eventually result in progressing to a better overall performance of conditioned responses.

In Experiment 2, we utilized the same set-up as in Experiment 1, but conducted the study in rabbits with chronically implanted recording electrodes in the hippocampus. First we tested whether there is a temporal correlation between the cardiac cycle and hippocampal theta oscillation (see, Komisaruk 1970; Pedemonte et al. 2003). Much to our surprise, there was no phase synchrony between theta and the cardiac cycle. Next, we examined hippocampal responses to the conditioned stimulus. Our previous studies indicate that hippocampal responses at the theta-band (4–8 Hz) are generally better time-locked to the CS-onset in subjects that learn well (Nokia et al. 2015). Like in human participants in Experiment 1, neural responses evoked by the tone-CS were also modulated by the cardiac phase in rabbits. That is, the hippocampal CA1-evoked potentials were larger in the Systole group compared to the Diastole group. However, the phase-locking of CA1 theta-band responses evoked by the CS did not differ between groups. This is perhaps a consequence of the lack of synchrony between theta and the cardiac cycle. Last but not least, rabbits trained at diastole learned trace eyeblink conditioning better than those trained at systole.

We admit, that the timing of the US in the Experiment 2 was incoherent compared to the timing of the CS, since the heart rate varied greatly in rabbits. This could have affected learning in trace eyeblink conditioning. At the same time, we emphasize that varying the trace interval between the CS and US could have affected the learning even more and the

448 results of the experiment would have been hard to interpret. If we could have managed to
449 come around with a solution, where trace interval would have been stable and timing of CS
450 and US would have been in the same phase of the cardiac cycle, the results could have been
451 different i.e. learning rates in Systole group would have been lower. Also, we recorded neural
452 responses to CS only in the CA1 region which is in the end of the trisynaptic circuit of the
453 hippocampus. Neural responses in the CA3 and the dentate gyrus could have been modulated
454 differently i.e. responses to the CS during the diastolic phase could have been larger than
455 those elicited in the systolic phase.

456 Taken together our results suggest that the effects that the cardiac cycle phase has on neural
457 responses to a conditioned stimulus, or learning at the behavioral level, cannot be explained
458 by the connection between hippocampal theta and learning (Nokia et al. 2015; Waselius et al.
459 2017; see also Hasselmo et al. 2002). Based on our current results it would seem, that the
460 neural state affecting learning fluctuates also according to baroreceptor signaling based on the
461 pressure in arteries. This signal is conveyed to the brain via the NTS but which brain regions
462 and what mechanisms are affected by the fluctuating signal remains unclear and should be
463 studied further. It is known that input from sensory terminals arrives to the hippocampus
464 through two primary, connected pathways: the non-lemniscal (via the medial septal nucleus)
465 and the lemniscal (through the primary auditory cortex and the entorhinal cortex) (see
466 Bickford et al. 2002). The function of the non-lemniscal pathway is reflected in hippocampal
467 theta activity when cholinergic input from the medial septum to the hippocampus is strong
468 (Buzsáki 2002). At the same time, the pulsatile activity of baroreceptors is constantly
469 projected to the hippocampus through the lemniscal pathway, via the neocortex.
470 Hippocampal responses to external stimuli are modulated by the functioning of these two
471 pathways and possibly by some other mechanisms as yet unknown. In the future we should
472 run a cardiac cycle phase -contingent experiment and record i.e. the activity of ventral portion

of the medial prefrontal cortex (vMPFC) which has inputs from baroreceptors (see Resstel et al. 2004). Also, we could study peripheral sensitivity (see Edwards et al. 2009) of auditory organs during different phases of the cardiac cycle.

CONCLUSIONS

We found that the phase of the cardiac cycle at stimulus onset affects neural responses to a behaviorally relevant external stimulus in humans and in rabbits. Furthermore, learned behavioral responding to the stimulus was modulated in rabbits. That is, very rapid changes in bodily state can affect learning. Monitoring cardiac cycle and timing of the stimulus in contingency with it might be used to optimize the effect of external stimulation and learning.

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REFERENCES

Abrahamson EE, **Moore RY**. The posterior hypothalamic area: chemoarchitecture and afferent connections. *Brain Res* 889: 1–22, 2001.

Berry SD, **Seager MA**. Hippocampal Theta Oscillations and Classical Conditioning. *Neurobiol Learn Mem* 76: 298–313, 2001.

Berry SD, **Thompson RF**. Prediction of learning rate from the hippocampal electroencephalogram. [Online]. *Science* 200: 1298–300, 1978. http://www.ncbi.nlm.nih.gov/pubmed/663612 [20 Feb. 2018].

Bickford PC, **Miller CL**, **Moxon K**, **Luntz-Leybman V**, **Stevens K**, **Adams C**, **Freedman R**, **Rose G**, **Adler L**. Auditory Sensory Gating in the Hippocampus. In: *Memory and Emotion*. World Scientific, p. 130–137.

Buzsáki G. Two-stage model of memory trace formation: a role for "noisy" brain states. [Online]. *Neuroscience* 31: 551–70, 1989. http://www.ncbi.nlm.nih.gov/pubmed/2687720 [20 Feb. 2018].

Buzsáki G. Theta oscillations in the hippocampus. [Online]. *Neuron* 33: 325–40, 2002. http://www.ncbi.nlm.nih.gov/pubmed/11832222 [20 Feb. 2018].

Cavdar S, **Onat F**, **Aker R**, **Sehirli U**, **San T**, **Raci Yananli H**. The afferent connections of the posterior hypothalamic nucleus in the rat using horseradish peroxidase. *J Anat* 198: 463–472, 2001.

Critchley HD, **Harrison NA**. Visceral Influences on Brain and Behavior. *Neuron* 77: 624–638, 2013.

Edwards L, Ring C, McIntyre D, Winer JB, Martin U. Sensory detection thresholds are modulated across the cardiac cycle: evidence that cutaneous sensibility is greatest for systolic stimulation. *Psychophysiology* 46: 252–6, 2009.

Fanselow MS, **Dong H-W**. Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures? *Neuron* 65: 7–19, 2010.

Gray MA, **Beacher FD**, **Minati L**, **Nagai Y**, **Kemp AH**, **Harrison NA**, **Critchley HD**. Emotional appraisal is influenced by cardiac afferent information. *Emotion* 12: 180–191, 2012.

Griffin AL, **Asaka Y**, **Darling RD**, **Berry SD**. Theta-contingent trial presentation accelerates learning rate and enhances hippocampal plasticity during trace eyeblink conditioning. *Behav Neurosci* 118: 403–11, 2004.

Grion N, **Akrami A**, **Zuo Y**, **Stella F**, **Diamond ME**. Coherence between Rat Sensorimotor System and Hippocampus Is Enhanced during Tactile Discrimination. *PLoS Biol* 14: e1002384, 2016.

Guyenet PG. The sympathetic control of blood pressure. *Nat Rev Neurosci* 7: 335–346, 2006.

Hasselmo ME, **Bodelón C**, **Wyble BP**. A Proposed Function for Hippocampal Theta Rhythm: Separate Phases of Encoding and Retrieval Enhance Reversal of Prior Learning. *Neural Comput* 14: 793–817, 2002.

Holland PC, **Bouton ME**. Hippocampus and context in classical conditioning. [Online]. *Curr Opin Neurobiol* 9: 195–202, 1999. http://www.ncbi.nlm.nih.gov/pubmed/10322181 [20 Feb. 2018].

James W. What is an Emotion? *Mind* 9: 188–205, 1884.

Jänig W. Regulation of organ systems by the lower brain stem 2006 jänig. In: *Integrative Action of the Autonomic Nervous System: Neurobiology of Homeostasis*. 2006.

Kern M, **Aertsen A**, **Schulze-Bonhage A**, **Ball T**. Heart cycle-related effects on event-related potentials, spectral power changes, and connectivity patterns in the human ECoG. *Neuroimage* 81: 178–190, 2013.

Komisaruk BR. Synchrony between limbic system theta activity and rhythmical behavior in rats. [Online]. *J Comp Physiol Psychol* 70: 482–92, 1970. http://www.ncbi.nlm.nih.gov/pubmed/5418472 [20 Feb. 2018].

Lacey BC, **Lacey JI**. Two-way communication between the heart and the brain. Significance of time within the cardiac cycle. [Online]. *Am Psychol* 33: 99–113, 1978. http://www.ncbi.nlm.nih.gov/pubmed/637402 [20 Feb. 2018].

Lacey JI, **Lacey BC**. On heart rate responses and behavior: a reply to Elliott. [Online]. *J Pers Soc Psychol* 30: 1–18, 1974. http://www.ncbi.nlm.nih.gov/pubmed/4427211 [20 Feb. 2018].

Macrides F, **Eichenbaum HB**, **Forbes WB**. Temporal relationship between sniffing and the limbic theta rhythm during odor discrimination reversal learning. [Online]. *J Neurosci* 2: 1705–17, 1982. http://www.ncbi.nlm.nih.gov/pubmed/7143047 [20 Feb. 2018].

Manns JR, **Clark RE**, **Squire LR**. Awareness predicts the magnitude of single-cue trace eyeblink conditioning. *Hippocampus* 10: 181–6, 2000.

Martins QA, **McIntyre D**, **Ring C**. Effects of baroreceptor stimulation on performance of the Sternberg short-term memory task: a cardiac cycle time study. *Biol Psychol* 103: 262–6, 2014.

Múnera A, Gruart A, Muñoz MD, Fernández-Mas R, Delgado-García JM. Hippocampal Pyramidal Cell Activity Encodes Conditioned Stimulus Predictive Value During Classical Conditioning in Alert Cats. *J Neurophysiol* 86: 2571–2582, 2001.

Montoya P, **Schandry R**, **Müller A**. Heartbeat evoked potentials (HEP): topography and influence of cardiac awareness and focus of attention. [Online]. *Electroencephalogr Clin Neurophysiol* 88: 163–72, 1993. http://www.ncbi.nlm.nih.gov/pubmed/7684965 [20 Feb. 2018].

Nokia MS, **Waselius T**, **Mikkonen JE**, **Wikgren J**, **Penttonen M**. Phase matters: responding to and learning about peripheral stimuli depends on hippocampal θ phase at stimulus onset. *Learn Mem* 22: 307–317, 2015.

Nokia MS, **Wikgren J**. Hippocampal theta activity is selectively associated with contingency detection but not discrimination in rabbit discrimination-reversal eyeblink conditioning. *Hippocampus* 20: 457–60, 2010.

Nokia MS, **Wikgren J**. Effects of Hippocampal State-Contingent Trial Presentation on Hippocampus-Dependent Nonspatial Classical Conditioning and Extinction. *J Neurosci* 34: 6003–6010, 2014.

Palva JM, **Palva S**, **Kaila K**. Phase Synchrony among Neuronal Oscillations in the Human Cortex. *J Neurosci* 25: 3962–3972, 2005a.

Palva S, **Linkenkaer-Hansen K**, **Näätänen R**, **Palva JM**. Early Neural Correlates of Conscious Somatosensory Perception. *J Neurosci* 25: 5248–5258, 2005b.

Pan W-X, **McNaughton N**. The supramammillary area: its organization, functions and relationship to the hippocampus. *Prog Neurobiol* 74: 127–166, 2004.

Park H-D, **Correia S**, **Ducorps A**, **Tallon-Baudry C**. Spontaneous fluctuations in neural responses to heartbeats predict visual detection. *Nat Neurosci* 17: 612–618, 2014.

Pedemonte M, **Goldstein-Daruech N**, **Velluti RA**. Temporal correlations between heart rate, medullary units and hippocampal theta rhythm in anesthetized, sleeping and awake guinea pigs. *Auton Neurosci* 107: 99–104, 2003.

Pritchett-Corning K, **Girod A**, **Avellaneda G**, **Fritz P**, **Chou S**, **Brown M**. *Handbook of Clinical Signs in Rodents & Rabbits*. 2nd ed. Charles River, 2011.

Resstel LB, Fernandes KB, Corrêa FM. Medial prefrontal cortex modulation of the baroreflex parasympathetic component in the rat. *Brain Res* 1015: 136–144, 2004.

Sandman CA, **McCanne TR**, **Kaiser DN**, **Diamond B**. Heart rate and cardiac phase influences on visual perception. [Online]. *J Comp Physiol Psychol* 91: 189–202, 1977. http://www.ncbi.nlm.nih.gov/pubmed/838914 [20 Feb. 2018].

Schandry R, **Montoya P**. Event-related brain potentials and the processing of cardiac activity. [Online]. *Biol Psychol* 42: 75–85, 1996. http://www.ncbi.nlm.nih.gov/pubmed/8770371 [20 Feb. 2018].

Solomon PR, **Groccia-Ellison ME**, **Flynn D**, **Mirak J**, **Edwards KR**, **Dunehew A**, **Stanton ME**. Disruption of human eyeblink conditioning after central cholinergic blockade with scopolamine. [Online]. *Behav Neurosci* 107: 271–9, 1993. http://www.ncbi.nlm.nih.gov/pubmed/8387313 [21 Feb. 2018].

Solomon PR, **Vander Schaaf ER**, **Thompson RF**, **Weisz DJ**. Hippocampus and trace conditioning of the rabbit's classically conditioned nictitating membrane response. [Online]. *Behav Neurosci* 100: 729–44, 1986. http://www.ncbi.nlm.nih.gov/pubmed/3778636 [20 Feb. 2018].

Squire LR. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. [Online]. *Psychol Rev* 99: 195–231, 1992. http://www.ncbi.nlm.nih.gov/pubmed/1594723 [20 Feb. 2018].

Walker BB, **Sandman CA**. Visual evoked potentials change as heart rate and carotid pressure change. [Online]. *Psychophysiology* 19: 520–7, 1982. http://www.ncbi.nlm.nih.gov/pubmed/7134357 [20 Feb. 2018].

Waselius T, **Pöllänen E**, **Wikgren J**, **Penttonen M**, **Nokia MS**. Hippocampal theta phase-contingent memory retrieval in delay and trace eyeblink conditioning. *Behav. Brain Res.* (2017). doi: 10.1016/j.bbr.2017.09.001.

Wilson M. Six views of embodied cognition. [Online]. *Psychon Bull Rev* 9: 625–36, 2002. http://www.ncbi.nlm.nih.gov/pubmed/12613670 [20 Feb. 2018].

Wisely NA, **Cook LB**. Arterial flow waveforms from pulse oximetry compared with measured Doppler flow waveforms apparatus. [Online]. *Anaesthesia* 56: 556–61, 2001. http://www.ncbi.nlm.nih.gov/pubmed/11412162 [20 Feb. 2018].

Woodruff-Pak DS, **Vogel RW**, **Ewers M**, **Coffey J**, **Boyko OB**, **Lemieux SK**. MRI-assessed volume of cerebellum correlates with associative learning. *Neurobiol Learn Mem* 76: 342–57, 2001.

FIGURE CAPTIONS

Figure 1. Trace eyeblink conditioning.
Rabbits (n = 25) and humans (n = 29) were trained in trace eyeblink conditioning with tone as a conditioned stimulus (CS) and an air puff aimed toward the eye as an unconditioned stimulus (US). The trace period was 500 ms for the rabbits and 600ms for the human participants. UR = unconditioned response, CR = conditioned response.

Figure 2. In both human participants (A, Experiment 1) and rabbits (B, Experiment 2) the conditioned stimulus was timed either to the diastole or the systole.
The cardiac cycle can be divided into two phases, systole and diastole. During systole, the heart contracts and pumps blood to arteries. During this phase the electrocardiogram (ECG) shows the QRS-complex reflecting ventricular depolarization and the T-wave, reflecting ventricular repolarization. Diastole follows systole. During diastole, the heart relaxes and fills with blood and the P-wave is seen in ECG. It reflects atrial depolarization which occurs when the ventricles are almost full of blood. A) In human participants, the ECG was recorded and used for CS timing. B) In rabbits, the oxygen saturation signal (photoplethysmogram, PPG) was measured from the earlobe using a pulse oximeter. The PPG is at its lowest at the beginning of the systole phase. The highest peak in the PPG can be observed during the relaxation of the heart just before the atrial contraction (ECG P-wave), that is, during diastole. The CS was timed to start either at the systole (trough, arrowhead) or the diastole (peak, double arrowhead) or irrespective of cardiac cycle phase (Random, not shown).

Figure 3. Topographies of the event-related potentials to the tone-CS (A) and maximum amplitudes of N1 and P2 responses measured at Cz-electrode (B) in human participants in Experiment 1. The N1 response was significantly larger when the tone onset was contingent with the systolic phase (n = 12) compared to diastolic phase (n = 12). The P2 response was

also larger in amplitude but did not reach statistical significance. In B, error bars equal standard error of mean. Asterisks in B denote p-value of < 0.01.

Figure 4. (A) Effect of ICA-based artefact correction on EEG recorded at Cz electrode. The EEG traces in the upper panel are grand average responses to the heart beat. The arrows mark the onsets of CSs in the Systole and Diastole groups. As can be seen, there are minor deflections related to cardiac cycle in the signal before ICA correction (Raw) but after that (Corrected), the signal is virtually flat. (B) ECG topography in the same time scale is plotted in the lower panel.

Figure 5. Human participants in Experiment 1 learned trace eyeblink conditioning at the same rate regardless of group (Systole vs. Diastole). Error bars equal standard error of mean. UP = unpaired, CC = conditioning, EXT = extinction.

Figure 6. In Experiment 2, relative amplitudes of event-related potentials to the conditioned stimulus (CS) recorded from the rabbit hippocampal CA1 were larger in Systole group compared to the Diastole group.

A) A 60-micron cresyl violet–stained slice of a rabbit left dorsal hippocampus with the locations of CA1 and pyramidal cell layer (pyr) indicated. The electrode tip location was marked by passing direct current through the electrode (arrow). B) Example of a representative CS-evoked ERP (average of 60 trials) in hippocampal CA1. Negative peak amplitudes of ERPs were analyzed from 25 to 60 ms after CS onset (vertical gray bar). C) ERPs in CA1 were averaged per session, per animal. The value from the CS-Alone session was used as a baseline to calculate the relative change (%) in amplitude of the ERP during subsequent conditioning sessions (see Methods). Throughout the 7 session blocks of trace eyeblink conditioning the responses to CS were amplified in the Systole group and attenuated in the Diastole group ($p < 0.05$). Error bars equal standard error of mean.

Figure 7. In Experiment 2, rabbits trained at diastole learned better than those trained irrespective of cardiac cycle phase.

A) Learning was faster and conditioned responding remained at a higher level in the Diastole group throughout trace eyeblink conditioning. Asterisk refers to repeated-measures ANOVA indicating statistically significant difference (Bonferroni-corrected post-hoc comparisons, $p < 0.05$) between the Diastole group and the Random group. B) B) The best performance was determined as the highest achieved performance in learned responses during one session from the last four training sessions for each individual animal. There was a significant difference between the Diastole and the Random groups ($p < 0.05$). C) The cumulative number of learned responses plotted as a function of trial number in the Diastole, Systole and Random groups indicates no initial difference in responding. Error bars equal standard error of mean.