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RESEARCH ARTICLE | *Higher Neural Functions and Behavior*

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

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Waselius T, Wikgren J, Halkola H, Penttonen M, Nokia MS. Learning by heart: cardiac cycle reveals an effective time window for learning. *J Neurophysiol* 120: 830–838, 2018. First published May 9, 2018; doi:10.1152/jn.00128.2018.—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, 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 contingently 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 on the basis of 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. In this study, 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.

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 1884). James's philosophy has the fundamental idea of the consciousness being an inseparable stream of bodily and mental states. Since the 1880s, 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) suggested 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 and these memories "become crystalized," they are no longer 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 heartbeat 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 (Quelhas Martins et al. 2014) and emotional appraisals of facial expressions (Gray et al. 2012). To our knowledge, whether baroreceptor activity could influence hippocampus dependent associative learning has not been tested.

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.

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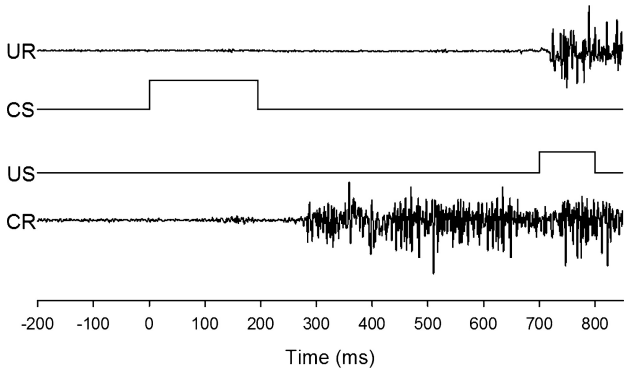

Fig. 1. Trace eyeblink conditioning. Rabbits (n = 25) and humans (n = 29) were trained in trace eyeblink conditioning with tone as a conditioned stimulus (CS) and an air puff aimed toward the eye as an unconditioned stimulus (US). The trace period was 500 ms for the rabbits and 600 ms for the human participants.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

Theta (~4–8 Hz being peak frequency, depending on the species) is the most prominent oscillation in the hippocampus (e.g., Buzsáki 1989). Theta oscillation 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 activity in declarative learning is supported by a multitude of experimental findings (Berry and Seager 2001; Berry and Thompson 1978; 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 heartbeat (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 such as mammalian sniffing (Macrides et al. 1982) and rats whisking with their snout hairs (Grion et al. 2016).

In the present study, 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 (Fig. 1), a hippocampus-dependent task considered to model declarative learning in both animals and humans (Holland and Bouton 1999; Solomon et al. 1986). The onset of the conditioned stimulus (CS) was timed to either the systolic or the diastolic phase of the cardiac cycle (Fig. 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. Furthermore, 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 rabbits. However, we had no presumption concerning which phase, diastole or systole, would be optimal for learning.

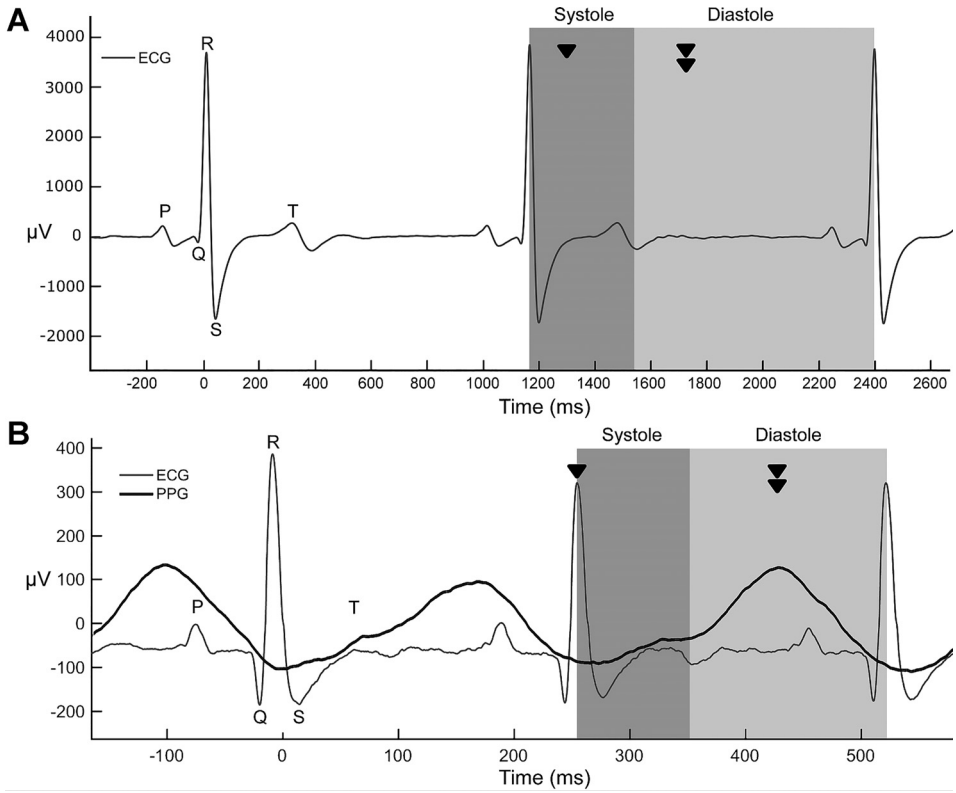

Fig. 2. In both human participants (*A*; *experiment 1*) and rabbits (*B*; *experiment 2*), the conditioned stimulus (CS) was timed to either the diastole or the systole. The cardiac cycle can be divided into 2 phases, systole and diastole. During systole, the heart contracts and pumps blood to arteries. 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 the ECG, which reflects atrial depolarization that 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).

MATERIALS AND METHODS

Experiment 1: Human Eyeblink Conditioning and Event-Related Potentials

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 right-handed adults (23 women, 7 men, age 18–32 yr) 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. Because of technical difficulties, electrophysiological data from five participants were not analyzed.

Experimental procedure. Before the experiment, participants filled out a modified BIS/BAS (behavior inhibition system/behavior activation system) personality inventory and answered background questions about age, sex, 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 5 min of resting data were recorded, 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 two-thirds of the peak amplitude of the R peak. The participants were randomized into systole ($n = 15$) and diastole ($n = 14$) groups. In the systole group, the trial onset was delayed by 100 ms from the rising slope of the R peak, whereas in the diastole group, the trial onset was delayed by 500 ms. The conditioned stimulus (CS) 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 air puff (0.4 bar source pressure, 100 ms) targeted to the corner of the left eye and was delivered via a plastic tube attached to modified safety goggles.

Before the actual conditioning phase, four air puffs alone were delivered at 5-s intervals to accustom the participant to it. The conditioning procedure consisted of 80 trials. The first (unpaired) and last (extinction) 10 trials were CS-alone trials. The intertrial interval (ITI) varied randomly between 9 and 19 s.

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, 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 were recorded using a 64-channel EEG cap (64 BrainCap with Multitrodes; EASYCAP, Woerthsee-Etterschlag, Germany). Resting state data were recorded for 5 min before and after the actual experiment.

EYEBLINKS. The EMG signal was high-pass filtered (>60 Hz), rectified, and then low-pass filtered (<20 Hz) offline using Brain Vision Analyzer software. One of the authors (J. Wikgren) blind to the experimental group visually assessed all trials for conditioned responses. An eyeblink was considered a conditioned response (CR) if it occurred within a period of 500 ms before the US onset. The exclusion criterion was subjectively rated as excessive EMG activity during the 500-ms 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 analysis of variance (ANOVA).

BRAIN RESPONSES. Valid EEG data were gathered from 24 participants (systole, $n = 12$; diastole, $n = 12$). BrainVision Analyzer 2.1 (Brain Products, Gilching, Germany) was used to remove bad channels and to low-pass filter (<30 Hz) the raw data. Independent component analysis (ICA) was run on the data, and components related to eyeblink, eye movement, and heartbeat artifacts were removed. The heartbeat itself is an event that induces a stereotypical activity pattern in the EEG called heartbeat-evoked potential. It is found in recordings over the somatosensory cortex (Kern et al. 2013) as well as frontocortical (Schandry and Montoya 1996) and frontotemporal areas (Montoya et al. 1993). Therefore, an ECG channel was included in the ICA 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 before CS onset. The baseline-corrected epochs were then averaged. For each participant, an average peak for N1 (minimum amplitude within 90–130 ms post-CS onset) and P2 (maximum amplitude within 150–230 ms post-CS onset) were calculated from the paired trials ($n = 60$).

Experiment 2: Rabbit Eyeblink Conditioning and Hippocampal Field Responses

Subjects. The subjects were 25 adult female New Zealand White rabbits (Lidköpings Kaninfarm, Vinninga, 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-h light-dark cycle, with lights on at 8:00 AM. 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 of an anti-inflammatory drug [50 mg/ml carprofen (Rimadyl vet; Pfizer 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 of ketamine-xylazine cocktail [7.8 ml of 50 mg/ml Ketaminol vet (Intervet International) mixed with 2.8 ml of 20 mg/ml Narcoxyl vet (Intervet International)]. A 0.8 ml/kg dose of the cocktail was injected intramuscularly before surgery. During surgery, additional doses of either the cocktail or ketamine alone were injected subcutaneously approximately every 20–30 min or as needed. Before the surgery, the rabbit's fur was shaved from the top of its head. The rabbit was then 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)] were injected subcutaneously in the area of surgery before the opening incision was made.

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 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 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 (catalog no. 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 5 mg/ml Primperan (Sanofi Winthrop Industrie)] was administered subcutaneously, 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] 4 h after surgery and then every 8 h for the next 44 h.

Experimental procedure. The experimental procedure is illustrated in Fig. 1. After 1 wk 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 min before, during, and 1 min after each session. ITI always varied randomly between 30 and 60 s. LabVIEW (National Instruments) was used to monitor the cardiac cycle and blinking online, to execute the experimental procedures, and to present stimuli. After the ITI was expired, trial presentation was always delayed for 1 s 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).

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 a 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) 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 (model 2100; A-M Systems), amplified 1,000 times, and bandpass filtered from 100 to 500 Hz. The EMG signal was high-pass filtered offline (>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 100 ms before US onset were counted as CRs.

HIPPOCAMPAL LOCAL FIELD POTENTIALS. For neural recordings of monopolar electrodes, a tenfold amplification was performed with a preamplifier [model MPA8I; MultiChannel Systems (MCS)] attached to the electrode connector in the rabbit's head. The signal was then bandpass filtered (1–5,000 Hz) with a 64-channel filter amplifier (MCS). Last, the signal was further low-pass filtered (500 Hz) and digitized at a rate of 2 kHz with an MCS USB-ME64 system (MC_Rack software). SPSS (IBM) and MATLAB (The MathWorks) were used for offline data analysis. Rabbits implanted with the 32-channel probes (Atlas Neuroengineering) were recorded with the use of a wireless data acquisition system (W2100-HS32-headstage; 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 thus is 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 bandpass filtered between 4 and 8 Hz. A Hilbert transform was then 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 (1 session) and taking the absolute value of the mean. The PLV varies between 0 and 1, with 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. 2005a) of the hippocampal theta (bandpass filtered between 4 and 8 Hz) and PPG (bandpass filtered between 3 and 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. The phase difference of the two signals was then calculated by multiplying the first signal by 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 Fig. 6*B*) 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 ANOVA, with training sessions (or blocks of 2 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 by using the session from the last four training sessions for each individual animal where they achieved their best performance in CRs.

Histology. Rabbits were anesthetized with an intramuscular injection of ketamine-xylazine cocktail and then overdosed with an intravenous pentobarbital sodium (Mebunat vet; Orion-Yhtyma Oyj) injection. The brain was then perfused with physiological saline followed by 9% formalin solution through the ascending aorta. The locations of the electrode tips were marked by passing direct 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

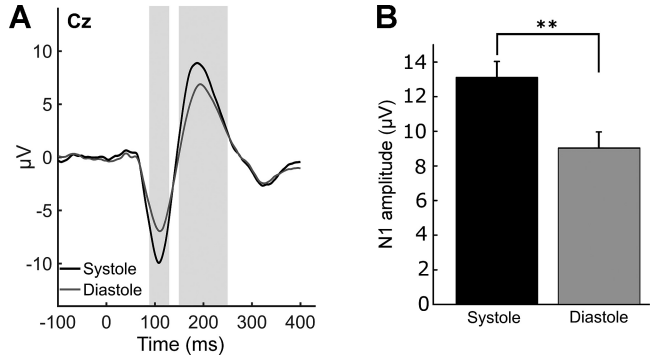

Fig. 3. Topographies of the event-related potentials to the tone-conditioned stimulus (CS; *A*) and maximum amplitudes of N1 and P2 responses measured at the Cz electrode (*B*) in human participants in *experiment 1*. The N1 response was significantly larger when the tone onset was contingent with the systolic phase ($n = 12$) compared with the diastolic phase ($n = 12$). The P2 response was also larger in amplitude but did not reach statistical significance. In *B*, error bars are SE. $^{**}P < 0.01$.

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 use of a microscope.

RESULTS

Experiment 1: Human Eyeblink Conditioning and Event-Related Potentials

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 with the diastole group [$t(22) = 3.14$, $P < 0.01$, Cohen's $d = 1.28$; Fig. 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 $d = 0.52$].

Because of a potential confounding effect of heartbeats on ERPs, the ICA was used to remove heart-related artifacts. Figure 4 depicts the effects of ICA on the EEG recorded at the Cz electrode. As shown, there is a small (~1 μV) deflection at ~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 heartbeat-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 (1st 7 blocks, extinction block excluded) and group (systole vs. diastole) on CR percentage revealed a significant main effect of block [$F(6, 162) = 39.89$, $P < 0.001$, partial $\eta^2 = 0.57$], indicating that the number of CRs increased as a function of training. Neither the main effect of group [$F(1, 27) = 0.03$, $P = 0.864$, partial $\eta^2 = 0.001$] nor the interaction between group and block [$F(6, 162) = 0.585$, $P = 0.742$, partial $\eta^2 = 0.021$] reached significance, indicating that basing the timing of the CS onset on different phases of the cardiac cycle did not have an effect on learning trace eyeblink conditioning (Fig. 5).

Interim discussion. Because 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 CS. This suggests that cardiac cycle phase affects sensory processing of external stimuli but that these effects do not directly carry over to learning at the behavioral level.

Experiment 2: Rabbit Eyeblink Conditioning and Hippocampal Field Responses

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 (0.07) [mean (SD)] 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/min, which is within normal variation (130 to 325 beats/min; Pritchett-Corning et al. 2011).

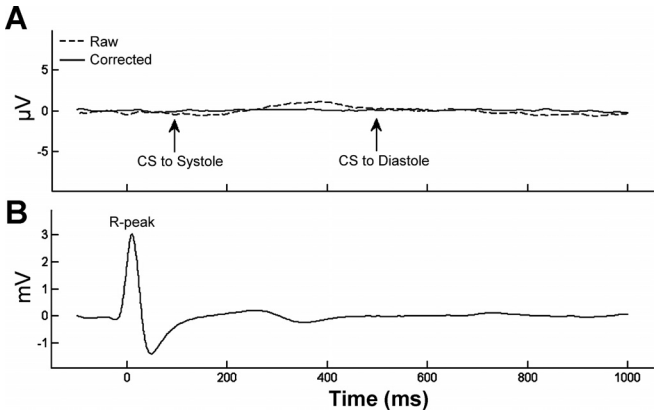

Fig. 4. *A*: effect of independent component analysis (ICA)-based artifact correction on EEG recorded at the Cz electrode. The EEG traces are grand-average responses to the heartbeat. Arrows mark the onsets of conditioned stimulus (CS) in the systole and diastole groups. There are minor deflections related 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 timescale.

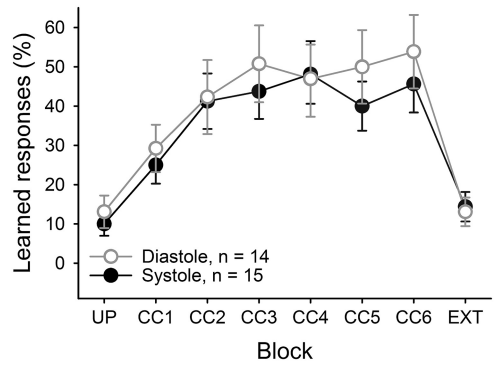

Fig. 5. Human participants in *experiment 1* learned trace eyeblink conditioning at the same rate regardless of group (systole vs. diastole). Error bars are SE. UP, unpaired; CC, conditioning; EXT, extinction.

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 (Fig. 6*A*). 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.

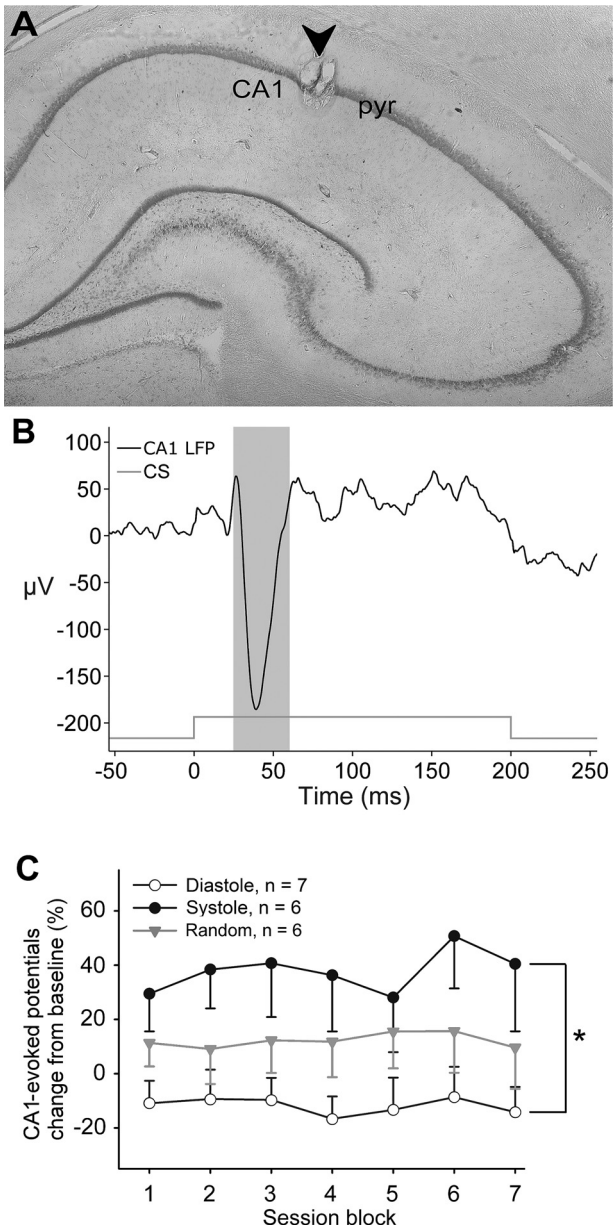

Fig. 6. In *experiment 2*, relative amplitudes of event-related potentials (ERPs) to the conditioned stimulus (CS) recorded from the rabbit hippocampal CA1 were larger in the systole group compared with the diastole group. *A*: a 60-μm cresyl violet-stained slice of a rabbit left dorsal hippocampus with the locations of CA1 and pyramidal cell layer (pyr) indicated. The electrode tip location was marked by passing direct current through the electrode (arrowhead). *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 shaded bar). LFP, local field potential. *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 MATERIALS AND 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 are SE.

Event-related potentials to the CS recorded from the CA1 had a mean peak latency of 41 (4.80) ms from CS onset (Fig. 6*B*). 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 with 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: $P = 0.027$; Fig. 6*C*].

Phase-locked hippocampal theta-band responses to the CS were not different between the diastole, systole, and random groups 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 of the 25 animals learned trace eyeblink conditioning. Learning differed between the diastole ($n = 10$), systole ($n = 8$), and 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: $F(2, 22) = 3.94$, $P < 0.05$; Fig. 7*A*). Specifically, learning was better when the CS onset was timed to the diastole phase of the cardiac cycle compared with 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 [82.36 (11.93)%] was significantly higher than that in the random group [52.33 (32.54)%; Bonferroni-corrected $P = 0.022$]. However, the systole group [68.64 (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, $P = 0.422$; Fig. 7*B*).

Interim discussion. As anticipated, the cardiac cycle phase affected both neural responses as well as behavior during trace eyeblink conditioning in rabbits. Namely, hippocampal responses evoked by the conditioned stimulus were larger in amplitude in the systole group compared with the diastole group. Furthermore, rabbits learned trace eyeblink conditioning better when the CS onset was timed to the diastole phase of the cardiac cycle. In fact, almost all (90%) of the animals in the diastole group reached a limit of 80% CRs per session, whereas only one-half the animals in the systole group and less than one-third of those in the random group reached this limit during the 14 sessions of trace eyeblink conditioning. The animals in the diastole group also learned exceptionally well compared with previous results from our laboratory using the same paradigm for trace eyeblink conditioning (Nokia and Wikgren 2014; Nokia et al. 2015; Waselius et al. 2018).

DISCUSSION

Neural responses as well as simple sensory phenomena have been shown to vary depending on the timing of the stimuli in

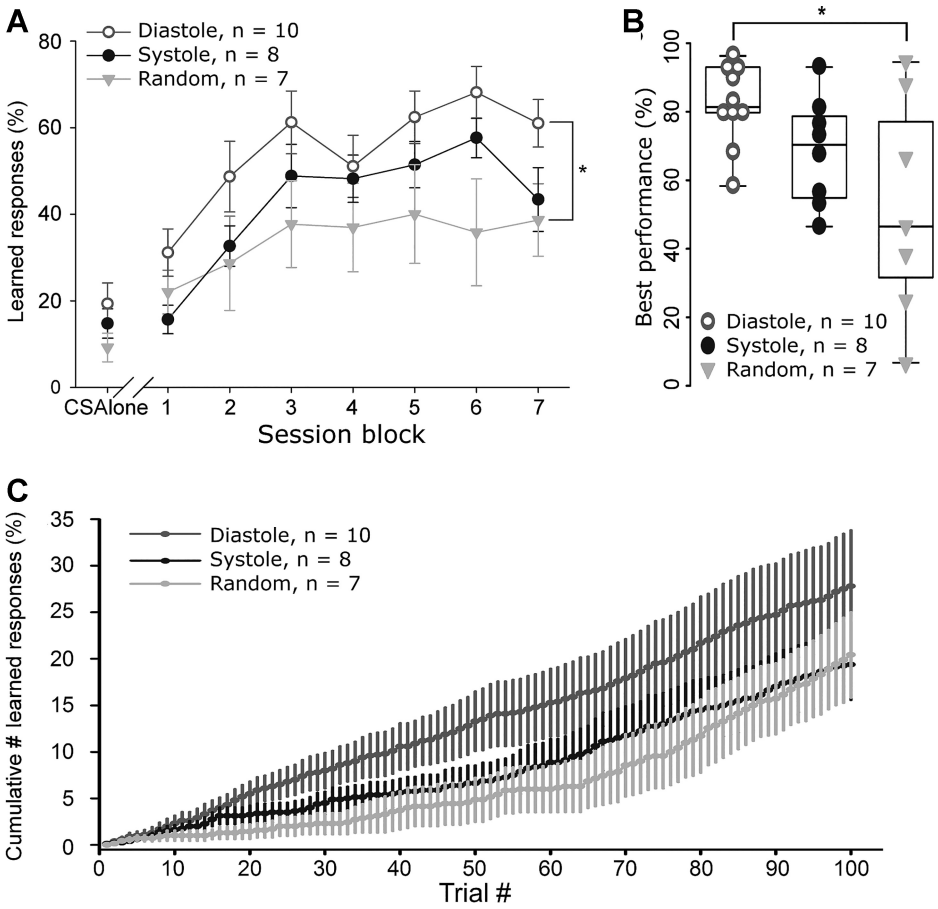

Fig. 7. In *experiment 2*, rabbits trained at diastole learned better than those trained irrespective of cardiac cycle phase. *A*: learning was faster and conditioned responding remained at a higher level in the diastole group throughout trace eyeblink conditioning. $*P < 0.05$, repeated-measures ANOVA indicating statistically significant difference (Bonferroni-corrected post hoc comparisons) between the diastole group and the random group. *B*: 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. $*P < 0.05$, significant difference between the diastole and the random groups. *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 are SE.

relation to the phase of the cardiac cycle. In the present study, both human participants and rabbits were subjected to trace eyeblink conditioning where the onset of the conditioning trial was timed to either 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 diastole group in rabbits. No effect of cardiac cycle phase on learning rate was found in humans.

Earlier studies have shown that behavioral (Gray et al. 2012; Park et al. 2014; Quelhas Martins et al. 2014; Sandman et al. 1977) 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 the 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). Although 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 may 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, because 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 will be important to use a more demanding task that should make the initial learning rate slower but eventually result in progression to a better overall performance of conditioned responses.

In *experiment 2*, we utilized the same setup 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). Similarly to those 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 com-

pared with 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 *experiment 2* was incoherent compared with the timing of the CS, because 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 results of the experiment would have been hard to interpret. If we could have managed to come up with a solution where the trace interval would have been stable and the timing of CS and US would have been in the same phase of the cardiac cycle, the results could have been different; i.e., learning rates in the systole group would have been lower. Also, we recorded neural responses to CS only in the CA1 region, which is in the end of the trisynaptic circuit of the hippocampus. Neural responses in the CA3 and the dentate gyrus could have been modulated differently; i.e., responses to the CS during the diastolic phase could have been larger than those elicited in the systolic phase.

Taken together, our results suggest that the effects that the cardiac cycle phase has on neural responses to a conditioned stimulus, or learning at the behavioral level, cannot be explained by the connection between hippocampal theta and learning (Nokia et al. 2015; Waselius et al. 2018; see also Hasselmo et al. 2002). On the basis of our current results, it would seem that the neural state affecting learning fluctuates also according to baroreceptor signaling based on the pressure in arteries. This signal is conveyed to the brain via the NTS, but which brain regions and what mechanisms are affected by the fluctuating signal remains unclear and should be studied further. It is known that input from sensory terminals arrives to the hippocampus through two primary, connected pathways: the nonlemniscal (via the medial septal nucleus) and the lemniscal (through the primary auditory cortex and the entorhinal cortex) (see Bickford et al. 2002). The function of the nonlemniscal pathway is reflected in hippocampal theta activity when cholinergic input from the medial septum to the hippocampus is strong (Buzsáki 2002). At the same time, the pulsatile activity of baroreceptors is constantly projected to the hippocampus through the lemniscal pathway, via the neocortex. Hippocampal responses to external stimuli are modulated by the functioning of these two pathways and possibly by some other mechanisms as yet unknown. In the future we should run a cardiac cycle phase-contingent experiment and record, for instance, the activity of the ventral portion of the medial prefrontal cortex, 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 contingent with it might be used to optimize the effect of external stimulation and learning.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.W., J.W., M.P., and M.S.N. conceived and designed research; T.W., J.W., and H.H. performed experiments; T.W., J.W., and M.S.N. analyzed data; T.W., J.W., and M.S.N. interpreted results of experiments; T.W. and J.W. prepared figures; T.W., J.W., and H.H. drafted manuscript; T.W., J.W., M.P., and M.S.N. edited and revised manuscript; T.W., J.W., H.H., M.P., and M.S.N. approved final version of manuscript.

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