Cardiac cycle and respiration phase affect responses to the conditioned stimulus in young adults trained in trace eyeblink conditioning.
Cardiac cycle and respiration phase affect responses to the conditioned stimulus in young adults trained in trace eyeblink conditioning

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Abstract

Rhythms of breathing and heartbeat are linked to each other as well as to rhythms of the brain. Our recent studies suggest that presenting conditioned stimulus during expiration or during the diastolic phase of the cardiac cycle facilitates neural processing of that stimulus and improves learning in a conditioning task. To date, it has not been examined whether utilizing information from both respiration and cardiac cycle phases simultaneously allows even more efficient modulation of learning. Here we studied whether the timing of the conditioned stimulus to different cardiorespiratory rhythm phase combinations affects learning in a conditioning task in healthy young adults. The results were consistent with previous reports: Timing the conditioned stimulus to diastole during expiration was more beneficial for learning than timing it to systole during inspiration. Cardiac cycle phase seemed to explain most of this variation in learning at the behavioral level. Brain evoked potentials (N1) elicited by the conditioned stimulus and recorded using electroencephalogram were larger when the conditioned stimulus was presented to diastole during expiration than when it was presented to systole during inspiration. Breathing phase explained the variation in the N1 amplitude. To conclude, our findings suggest that non-invasive monitoring of bodily rhythms combined with closed-loop control of stimulation can be used to promote learning in humans. The next step will be to test if performance can also be improved in humans with compromised cognitive ability, such as in older people with memory impairments.

Keywords

breathing, heartbeat, event-related potential, learning
36 **New & Noteworthy**

37 We report for the first time that the rhythms of breathing and the beating of the heart have a
38 phase combination that is indicative of a neural state beneficial for cognition. This suggests
39 that bodily rhythms not only modulate cognition but that this phenomenon can be non-
40 invasively harnessed to improve learning in humans.
Introduction

Bodily rhythms like cardiac cycle and respiration usually vary at their own pace without much conscious thought put into them. When we are in a relaxed psychophysiological state, breathing and heartbeats synchronize [1–3]. The beat-to-beat intervals of the heart are longest at the end of expiration [4] and shorter during inspiration [5]. This is called respiratory sinus arrhythmia (RSA). In addition, the last heartbeat of each expiration delays the onset of the following inspiration, the “working phase” of breathing, so that the cardiac “working phase”, systole, precedes inspiration onset by 150–500 ms [6,7]. This phenomenon is termed cardioventilatory coupling. Especially RSA is thought to increase efficiency and stability in pulmonary gas exchange [2] and to decrease energy use caused by heartbeats [8]. Nevertheless, the root cause and outcome of cardiorespiratory synchrony is somewhat unclear [6].

Interestingly, cardiac cycle and respiration also synchronize with electrophysiological rhythms of the brain [9], which in turn affect neural processing of external information. Temporal correlations exist, for example, between cardiac cycle and the hippocampal theta oscillation (3–12 Hz) in rodents [10,11]. Theta oscillation is crucially involved in memory formation during spatial [12] and non-spatial tasks [13,14] as it, for example, regulates the firing of hippocampal principal cells. In addition to cardiac cycle, also the respiration rhythm couples with brain oscillations [15]. For example, electrophysiological oscillations in the limbic system are entrained by nasal breathing in humans at the delta (0.5–4 Hz), theta (4–8 Hz) and beta (13–30 Hz) frequency bands [16]. In addition, hippocampal sharp-wave ripples (SWRs, 100–200 Hz), crucial for memory consolidation [17], are entrained by respiration in mice [18].
Further and most importantly, brain responses to external stimuli and consequent behavior such as startle eyeblinks and premotor reaction times [19,20] and even associative learning [21] are modulated by the cardiac cycle phase. Regarding respiration, associative learning is enhanced when the significant stimuli are presented during expiration [22]. However, to our knowledge, there are no studies considering the combined effect of cardiac cycle and respiration phases on brain responses nor behavior. It is possible that utilizing combined information from these bodily rhythms might allow even more efficient modulation of behavior to the desired direction. Hence, we investigated the combined effect of cardiac cycle and respiration phase on learning in an associative task called trace eyeblink conditioning (TEBC). Participants were trained while watching a documentary film, using a tone as a conditioned stimulus (CS) and an air puff towards the corner of the right eye as an unconditioned stimulus. The presentation of the CS was fixed to a certain phase of the cardiac cycle (systole, SYS or diastole, DIA) and respiration (expiration, EXP or inspiration, INS) for each participant. In addition to conditioned responses also electroencephalogram (EEG) was recorded. Our previous data [21] showed mixed effects of cardiac cycle phase on CS-evoked brain responses in humans and in rabbits while we have not examined the effect of respiration phase. Based on our previous behavioral results [21, 22], we hypothesized that timing the CS to systole during inspiration would be less than optimal for learning TEBC whereas presentation of the CS to the diastole during expiration would be most favorable for learning (see Figure 1B).
Participants were recruited via student email lists. All participants gave informed written consent to this study and were free to discontinue participation in the experiment at any point. All participants received a reward (a movie ticket or a gift card) even if they discontinued the experiment at some point (however, no one did). The study was approved by the University of Jyväskylä Ethical Committee. A total of 59 young adults (12 males; aged 20–30 years: mean 23.4 years, standard error of mean 0.4 years) took part in the study. All participants were healthy with no history of psychiatric or neurological illnesses. They were not taking medication affecting the central nervous system, and they had no disabilities in hearing or vision.

Physiological recordings

Recording electrodes were attached after participants had signed the written consent. Respiration was recorded and monitored during the experiment with a reusable fabric belt (RESPA00000, Spes Medica, Italy), which was fastened on top of the clothes on the lower chest area. Heart rate was recorded using three electrocardiogram (ECG) electrodes (Kendall, H92SG); one electrode was placed on top of the right clavicle, one on the left lower ribs, and the grounding electrode on the back of the neck. Electromyography (EMG) to determine eyeblinks was recorded using two electrodes (70010-K/12, Ambu, Ballerup, Denmark) that were attached on top of the participant’s right eye muscles (orbicularis oculi). EEG was recorded using a 128-channel EGI Sensor Net (Electrical Geodesics Inc., Hydrogel GSN 128, 1.0). All signals were high-pass filtered (0.16 Hz) and low-pass filtered (250 Hz) online and
recorded with NeurOne Tesla (with Analog Out Option, Bittium Biosignals Ltd., Finland) at a 1-kHz sampling rate.

Experimental procedure

We chose to use a between-subjects design with four groups (INS-SYS, INS-DIA, EXP-SYS, and EXP-DIA) to keep the paradigm simple and use just one CS and one US. We do acknowledge that a within-subjects design would be more powerful as there is quite a lot of inter-individual variability in TEBC, and this is something that should be addressed in follow-up studies.

The outline of the experimental procedure is presented in Figure 1A. The participants sat in a chair in front of a TV screen (Asus VG236 series H, 23”; distance: approximately 100 cm). They were informed that the aim of the study was to record physiological responses to different types of stimuli while their attention was to be directed at a silent film depicting landscapes and animals. The participants were instructed to pay attention to the film and told that there would be questions considering the content of the footage after the recording session. They were also instructed to sit comfortably in the chair and not pay attention to the disturbing stimuli. In other words, the participants were led to believe that the idea was to study the disturbance caused by beeping sounds and air puffs on their attention towards the film.

Trace eyeblink conditioning

The conditioned stimulus (CS) was a 200-ms, 440-Hz, 66-dB tone delivered via a loudspeaker situated in the lower right-hand corner of the room. The unconditioned stimulus (US) was an air puff (0.2 bar source pressure, 100 ms) targeted at the right eye and it was delivered via a plastic tube attached to modified safety goggles. Note that the air pressure was
low and none of the participants reported that the air puff hitting the eye was unbearable.

During conditioning trials, a 600-ms trace interval separated the tone-CS offset and the airpuff-US onset. The presentation of the stimuli used for conditioning was controlled by custom software running on an Arduino-based device (ABD).

First, five US-alone trials with an inter-trial interval (ITI) of 5 s were presented to make sure that the participants felt comfortable enough to proceed with the experiment. After this, 5 minutes of resting data were recorded, followed by five CS-alone trials to determine baseline eyeblink rate. Then, 50 CS+US classical conditioning trials were presented either at inspiration-systole, inspiration-diastole, expiration-systole or at expiration-diastole. Last, five CS-alone trials were presented as an extinction training block. A random ITI of 20-40 s was applied throughout the experiment.

To time the classical conditioning trials, the respiration, cardiac cycle, and EMG signals were conveyed to a custom script running in LabVIEW (National Instruments). Signals were sampled at 1 kHz. At each time point, the last second of respiration, ECG and EMG signals were analyzed. EMG was evaluated for spontaneous eyeblinks, that is, the signal had to stay below a set amplitude threshold to proceed with presenting the conditioning trial. The respiration signal was analyzed in two consecutive 500-ms windows. To trigger a trial, the signal amplitude during the latter 500-ms time window had to cross a set absolute threshold value (peak for inspiration, trough for expiration) and the signal had to either rise (inspiration) or fall (expiration) at a certain rate between the two consecutive time windows. In addition, R-peaks were detected from the ECG and used for timing the trial either at systole (immediately) or diastole (delayed from R-peak). Note that the threshold values for the EMG and for the respiration peak and rise (inspiration) and for the trough and fall (expiration) were set individually for each participant during the 5-minute baseline recording
prior to conditioning. As a result, when the participant was not spontaneously blinking, and respiration and cardiac cycle were at desired phases, LabVIEW sent a TTL pulse to the ABD, which then presented the actual conditioning stimuli. In addition, whenever voluntary movement of the participant was visible either in the monitoring video or in the breathing signal, the trial presentation was manually halted. Also, any changes in the overall breathing baseline were taken into account and ABD controlling LabView parameters were adjusted accordingly.

Two minutes of spontaneous breathing and ECG without any external stimuli were recorded after the conditioning session to visually confirm online that the experimental manipulation had not changed the respiration pattern overall and that the respiration belt signal quality had remained similar to that recorded before experimental manipulations. The whole procedure lasted about 40 minutes depending on the random ITI.

**Questionnaire**

After the experiment, participants answered background questions about age, sex, and handedness and five questions concerning the silent film (e.g., “What equipment did the man in the film use for travelling in the snow?”) and an open question about the disruptive stimuli. Questions about the film were asked to find out if participants had been concentrating on the film because attention has a serious impact on learning in classical conditioning [23]. Participants also answered seven true/false questions about the occurrence of the disruptive stimuli (e.g., air puffs occurred immediately after beeping sounds). These questions were asked to find out how conscious the participants became of the CS-US association. For the complete questionnaire (translated into English for reporting purposes), please see Appendix 1.
Data analysis

Conditioned responses

The conditioned responses (CR) performed by each participant were analyzed offline using MATLAB (The MathWorks Inc.). First, the EMG signal was low pass filtered (40 Hz) and the absolute value of the signal was derived. Then the mean amplitude of the rectified EMG signal during a 500-ms pre-US period (MEANpre) was calculated. In addition, the mean of the standard deviation of the signal amplitude during the 500-ms pre-CS period (SDpre) was determined. Learned responses were detected from a 200-ms time window immediately preceding the US. To qualify as a learned response, the rectified EMG signal amplitude had to exceed the following threshold: MEANpre + 2 * SDpre. For statistical analysis, trials were grouped into blocks of five trials and the proportion (%) of conditioned responses per block was calculated. These measures were used as dependent variables when analyzing learning. Further, to create a simple measure of the outcome of TEBC, we determined the highest proportion (%) of conditioned responses during any given 10-trial block (50 trials, 5 blocks). This measure is referred to as the best performance in the TEBC task.

Event-related potentials (ERPs)

EEG data were analyzed using the MNE python [24]. First, EEG channels were visually inspected and bad channels were interpolated using spherical spline interpolation method [25]. Then fast independent component analysis (ICA) was applied to remove any eyeblink and cardiac artifacts related components [26]. Our previous study has shown that after applying ICA to remove cardiac related components, sometimes also referred to as heart-evoked potentials, the cardiac-related signal in EEG is virtually flat (see Figure 4 in Waselius et al. 2018). Then a band-pass filter of 0.1–30 Hz (zero phase finite impulse response filter with a Hamming-window) was applied to the continuous EEG recordings. After filtering, the
EEG signal was re-referenced to the common average. Then the EEG data were segmented into epochs spanning from −100 to 500 ms relative to the onset of the CS. The EEG epochs were manually checked to exclude any trials that were contaminated by movement-related artifacts or other high-amplitude noise. EEG epochs exceeding 100 µV peak-to-peak amplitudes were excluded from further analysis. Finally, the event-related potentials (ERP) were obtained by averaging EEG epochs around the CS over all paired conditioning trials.

Next, the ERP data were grand averaged across all participants. Two major ERP components were evident: An auditory N1, which peaked around 112 ms, and auditory P2, which peaked around 189 ms after the onset of the CS. In addition, the center of activities for both N1 and P2 peaks were around the channels number 6, 7 and 106 (128-channel EGI Sensor Net), which are located around the center of the head (see Figure 3A). This pattern (vertex negative-positive potentials) is consistent with our previous study [21] and other studies [27, 28]. Based on this, auditory N1 and P2 mean amplitudes were extracted from each participant for further statistical analysis from a 30-ms time window around the grand average N1 (112 ms) and P2 (189 ms) peaks from channels number 6, 7 and 106.

Statistics

One way analysis of variance (ANOVA) and independent samples t-test were used to examine differences between groups in single variables. Repeated-measures (rm) ANOVA was used to analyze changes across training and differences between the groups in conditioned responding: Five-trial averages (blocks, 10) were used as a within-subjects factor and respiration phase (2) and cardiac cycle phase (2) as between-subjects factors. Univariate ANOVA was used to examine the effects of respiration phase (2) and cardiac cycle phase (2) on single variables: best performance, N1 and P2 amplitude. Cohen’s d or partial eta squared ($\eta^2$) are reported for statistically significant differences.
Results

Participants concentrated on watching the documentary film

Of the 59 participants, 56 answered correctly to all the questions about the film content (questions 1–5 of Appendix 1) and the rest of them had only one missing answer. Respectively, only 27 of 59 participants answered correctly to the questions about how the disruptive stimuli were presented (questions 7–13 of Appendix 1). This indicates that the participants were generally well concentrated on watching the film and not on the conditioning stimuli.

Participants trained during expiration-diastole made more conditioned responses than those trained during inspiration-systole

Fifty-one out of 52 participants made conditioned responses at some point during the TEBC and were included in the analyses (EXP-DIA: n = 13, EXP-SYS: n = 13, INS-DIA: n = 12, INS-SYS: n = 13) (see Figure 2). Participants in all groups responded (i.e., blinked their eye) at an equal rate (mean ± standard error of mean: 10 % ± 2 percentage units) to the CS during the CS-alone trials (one way ANOVA: F [3, 47] = 1.146, p = 0.340). To test the effects of breathing (EXP: n = 26 vs. INS: n = 25) and cardiac cycle phase (DIA: n = 25 vs. SYS: n = 26) on TEBC, we analyzed the conditioned response data with rm ANOVA using respiration phase (2) and cardiac cycle phase (2) as between-subjects factors and block (10) as the within-subjects factor. In addition to the statistically significant main effect of block (F [9, 423] = 8.051, p < 0.001, η²_p = 0.146) a statistically significant main effect of cardiac cycle phase (F [1, 47] = 6.109, p = 0.017, η²_p = 0.115) was detected. Interactions were not statistically significant (within-subjects: F [9, 423] = 0.434–0.963, p = 0.461–0.889; between subjects: F [1, 47] = 0.471, p = 0.496) nor was the main effect of breathing phase (F [1, 47] =
0.852, p = 0.361). Next, to test our hypothesis directly, an independent samples t-test was used to analyze the difference in conditioned responses to CS during all blocks (10, average) between EXP-DIA and INS-SYS. Conditioned responding was higher in the EXP-DIA (63% ± 18 percentage units) than in the INS-SYS group (44% ± 19 percentage units), $t(24) = 2.588, p = 0.016$, Cohen's d 1.015.

To further analyze the outcome of TEBC we determined a measure of best performance for each participant as the highest proportion (%) of conditioned responses during any given 10-trial block. Univariate ANOVA indicated a significant difference in best performance between participants trained at systole vs. diastole (cardiac cycle phase: $F[1, 47] = 7.667, p = 0.008, \eta^2_p = 0.140$; respiration phase, $F[1, 47] = 0.357, p = 0.553$; interaction, $F[1, 47] = 0.008, p = 0.930$). To directly test our hypothesis, we performed a comparison between just the EXP-DIA (82% ± 17 percentage units) and the INS-SYS (62% ± 22 percentage units) groups using independent samples t-test which indicated a significant difference: $t(24) = 2.734, p = 0.012$, Cohen’s d = 1.073.

To summarize, participants in all groups readily acquired the conditioned response and those trained during diastole made more conditioned responses than those trained at systole. Specifically, and in accordance with our hypothesis, participants trained during expiration-diastole made more conditioned responses than those trained during inspiration-systole.

The conditioned stimulus evoked a larger N1 response in participants trained during expiration than in those trained during inspiration.

High-quality EEG data were recorded from 40 participants with valid behavioral data (10 in each group, see Figure 3). In analyzing the EEG data, we followed the same logic as for the conditioned responses: Univariate ANOVA revealed a significant effect of respiration phase
on the N1 amplitude (F [3, 36] = 12.219, p = 0.001, η²_p = 0.253; cardiac cycle phase: F [3, 36] = 0.632, p = 0.432; interaction: F [3, 36] = 0.737, p = 0.396) but not on the P2 amplitude (F [3, 36] = 0.567, p = 0.456; cardiac cycle phase: F [3, 36] = 3.144, p = 0.085; interaction: F [3, 36] = 0.698, p = 0.409). To follow up on our direct hypothesis of better learning in the expiration-diastole group compared to inspiration-diastole group, we performed independent samples t-test on the N1 and P2 amplitudes. The N1 amplitude was larger in the EXP-DIA compared to the INS-SYS group (t [18] = 2.766, p = 0.013, Cohen’s d 0.135) but there was no difference in P2 amplitude (t [18] = 1.626, p = 0.121). To conclude, N1 responses were largest in the EXP-DIA group and overall larger N1 responses were evoked when the CS was presented during expiration rather than during inspiration.
Discussion

Respiration rhythm and cardiac cycle are known to synchronize to each other [6], to modulate brain activity [11, 15] and to affect, for example, perception and learning [29, 30]. However, it is unknown whether combinations of respiration and cardiac cycle phases modulate learning. Here, healthy young adults were trained in trace eyeblink classical conditioning, timing the conditioned stimulus based on four combinations of respiration and cardiac cycle phases (inspiration-systole, inspiration-diastole, expiration-systole, expiration-diastole; see Figure 1). Based on results of our previous studies [21, 22], we assumed that the diastolic phase during expiration would be a beneficial phase for stimulus presentation when learning, whereas systolic phase during inspiration would be less beneficial for learning.

As expected, timing the CS onset to diastole during expiration resulted in more frequent conditioned responding compared to timing the CS onset to systole during inspiration. Further, conditioned responding was overall more frequent if the CS was timed to diastole than to systole. Parallel differences were also observed in electrophysiological brain responses evoked by the CS: The N1 response was larger in amplitude when the CS occurred during expiration, and especially when it occurred during expiration and diastole. Together these results support our main assumption and our previous findings [21, 22] that bodily rhythms can be used to facilitate learning in humans.

Most importantly, our current study indicates that learned behavior can be modulated by the combinatory phases of breathing and the cardiac cycle. Overall, participants in our study acquired the conditioned eyeblink very fast, within the first few training blocks. As hypothesized, in our participants trained exclusively during the “resting states” of the heart and respiratory muscles (expiration-diastole), performance of a learned motor response was more likely compared to that in participants trained in the “working phase” of these organs.
Further, the phase of the cardiac cycle was the main factor explaining this difference. This result is in contrast with our earlier finding indicating no effect of cardiac cycle phase on learning in humans [21]. However, this could be explained by the further development of the conditioning paradigm in terms of triggering the trials to systole or diastole, which was more accurate in the current experiment. Namely, the delay from the R-peak was individually adjusted to suit each participant’s heart rate instead of using a set delay for all participants. We also did not detect a main effect of breathing phase on conditioned responding, again in contrast with our earlier finding [22]. However, it could be that as in the current experiment the timing of the CS hinged on the R-peak, the phases of the respiration (EXP vs. INS) are not directly comparable to those in our earlier study. Namely, in our current study, the onset of the CS was delayed until the next heartbeat within the expiration or the inspiration phase of breathing while in the Waselius et al. 2019 study a CS was triggered immediately as the desired breathing phase was detected. In any case, putting all evidence together, it seems that the neural state during diastole and expiration might be most favorable for acquiring a auditory CS–somatosensory US association and then performing a learned motor response. This conclusion is in line with all our findings, current and previous [21, 22]. It is also in line with a report of faster reactions to and higher saliency evaluations of auditory startle stimuli when presented during expiration rather than during inspiration [31]. Further support comes from studies reporting greater startle eyeblink responses to auditory stimuli presented at diastole than systole [19, 20].

Our current results suggest that respiration and cardiac cycle phases affect learning itself and not just the performance of the conditioned response, as 1) there is no difference between groups during the CS-alone treatment or the very first conditioning trials and 2) there is a clear distinction in the probability of a conditioned eyeblink once it reaches a plateau (see...
According to Prokasy’s theory (1984), during eyeblink conditioning the participants first learn an association between the conditioned stimulus and the unconditioned stimulus. Then they learn to shut their eye before the irritating air puff, that is, they learn to perform the motor conditioned response. Over time, with extended training, the conditioned eyeblink is adjusted temporally so that it optimally protects the eye from the flow of air [32]. Considering this, it seems that in our current experiment the effects of the neural state indicated by the phases of the bodily rhythms center on the acquisition of the CS-US contingency and the motor conditioned response taking place early in training and not so much on the later phases of the process when the CR is further adjusted. Learning the CS-US association during trace eyeblink conditioning is considered to be hippocampus-dependent [33–35] because of the gap between the CS-offset and the US onset while the simpler version of the task where the two stimuli partially overlap relies solely on the cerebellum responsible for motor learning [36, 37]. Thus, it is possible that the neural state indicated by diastole during expiration is related to a more efficient acquisition of the CS-US association, perhaps involving the hippocampus, and to a more reliable execution of the conditioned motor response governed by the cerebellum.

As anticipated based on the behavioral results, electrophysiological brain responses evoked by the conditioned stimulus also differed between the experimental groups in our study. Specifically, the N1 component of the ERP responses was largest in the participants trained in diastole during expiration and an overall larger N1 was evoked when the CS was presented during expiration rather than during inspiration (Figure 3). This suggests that the CS evoked more synchronous neural activity and was possibly perceived as more surprising or salient [38] during expiration (and diastole). It should be noted that earlier studies have not addressed how respiration might modulate auditory ERPs, but it has been suggested that...
auditory startle stimuli are subjectively rated more intense if presented to mid-expiration [31].

Regarding the effects of cardiac cycle phase, in our earlier study [21] the N1 of the ERP was in fact higher in amplitude during systole than diastole. However, Schulz and colleagues (2020) found the N1 to be higher in amplitude during diastole than systole when studying the effects of the cardiac cycle phase on responses to auditory startle stimuli [20]. Clearly, the modulation of neural responses by bodily rhythms should be explored in more detail and considered in data analysis. One might claim, for example, that a fixed inter-stimulus interval could result in the stimulus being presented repeatedly in the same phase of respiration and/or cardiac cycle, depending on the rate. This might then affect the amplitude of the different ERP components.

When it comes to the mechanism behind the link between bodily rhythm phases, brain function and behavior, not a lot is known. It does seem clear that the spontaneous rhythms of the brain relate to the rhythms of breathing and heartbeat [9], but a deeper mechanistic explanation of the anatomical and functional connections is missing. One of the most studied phenomena is the link between respiration, olfaction, and related brain activity. Several studies report that respiration rhythm and phase are connected to brain oscillatory activity [15,16,18,39–41]. In most studies, the connection seems to be limited to nasal respiration [16] and to crucially depend on the function of the olfactory bulb neurons [18]. Some of the respiration-driven brain rhythms might even be separate from the traditional brain rhythms, such as theta paced by subcortical structures [14]. Specifically, sniffing rodents are reported to display hippocampal oscillations that occur at the theta-frequency and couple with respiration but are not theta [39]. Since oscillations are the foundation of information transfer in the brain [42–44], it is obvious that rodents largely dependent on their olfactory senses could benefit from this coupling of respiration and brain oscillations [45]. As an
evolutionarily close relative to rodents, humans might also possess this characteristic.

Interestingly, olfaction and spatial memory seem to be congruent in humans: A good sense of smell is linked to good navigating ability [46]. Curiously, here and in our previous studies the “sniffing phase”, meaning inspiration, has emerged as the less optimal phase for associative learning of a causal connection between an auditory cue and an aversive somatosensory stimulation [22]. This might be related to the observations that hippocampal sharp-wave ripples (SPW-Rs) known to be crucial for memory consolidation [47, 48] and reflect a state in which rabbits acquire eyeblink conditioning better [47, 48] are more likely to occur during expiration than inspiration in mice [18]. Whether hippocampal SPW-Rs are more likely to occur during expiration in the diastolic phase of the cardiac cycle in humans is not known and should be studied in the future. Further, it should be tested whether limiting breathing to the oral route would possibly abolish the link between cardioventilatory rhythms and conditioned responding [16]. In sum, we suggest that the findings of our current experiment could be in part explained by the link between bodily rhythms and brain oscillatory activity, especially by that between expiration and SPW-Rs.

Limitations

There are some limitations in this study that we want to point out. Most obvious is the somewhat small sample size and small effect sizes. Nevertheless, the effect sizes in the ERP and behavioral results were large in our previous studies with similar group sizes and experimental set-up. Thus, we were confident that a similar group size should be large enough to detect possible effects in our current study as well. In addition, one must remember that associative learning in TEBC is by no means representative of all learning. In fact, in our current follow-up studies we have employed a different type of associative memory task, more closely resembling real-life situations. Moreover, here bodily rhythms modulated
auditory stimulus processing in a way that might or [22] might not be similar to how these rhythms modulate processing of visual or somatosensory stimuli in different settings. Last, we have previously reported [22] that participants have high RSA during this kind of an experimental setup but we did not take into account if the cardioventilatory coupling was strong or not and whether it showed fluctuations during the experiment [4]. In future studies, it might be worth investigating if the level of cardioventilatory coupling has an additional effect on learning.

Conclusion

To summarize, this study is the first to demonstrate that both breathing, and heartbeat rhythms influence the brain processing of external stimuli and learning about those stimuli. Learned responding is more likely when an auditory conditioned stimulus is presented during the resting phase of the heartbeat when breathing out instead of during the working state of the heartbeat when breathing in. In addition, the N1 component of the auditory-evoked potential is larger when the stimulus is presented during expiration (and diastole) than when it occurs during inspiration (and systole). These findings suggest that non-invasive measurement of cardiorespiratory rhythms combined with closed-loop control of stimulation can be utilized to promote learning in humans. The next step will be to test if performance can also be improved in humans with compromised cognitive ability [49, 50]. Further, it will be interesting to test the effects of bodily rhythm phases on learning tasks more closely resembling real-life situations.


Figure captions

Figure 1. Experimental design and examples of breathing and ECG signals used for timing the conditioning trials. (A) After presenting five US-alone trials the participants started to watch a documentary film. Then there was a five-minute resting period followed by five CS-alone trials. Next, 50 pairs of CS+US trials were timed to a certain cardiorespiratory phase (Ins-Sys, Ins-Dia, Exp-Sys or Exp-Dia). At last, five CS-alone trials were presented as an extinction training (EXT) followed by a two-minute rest period before the experiment ended. (B) Breathing signal and ECG were recorded and followed online to time conditioning trials to a certain cardiorespiratory phase. During the systolic phase (Sys) of the cardiac cycle, the ECG shows the QRS complex, reflecting ventricular depolarization, and the T wave, reflecting ventricular repolarization. Between the end of the T-wave and next R-peak, is the diastolic phase (Dia). The diastolic phase during expiration is marked with grey bars for demonstrating the hypothesized optimal phases for stimulus presentation.

Figure 2. Participants trained during expiration-diastole (EXP-DIA) made more conditioned responses than those trained during inspiration-systole (INS-SYS). (A) The percentage of conditioned responses per 5-trial block was used as a measure of learned behavior. There was no difference in responding to the tone-CS prior to conditioning (CS alone, one way ANOVA). Participants in all groups learned the trace eyeblink conditioning task (TEBC, main effect of block) and participants trained in diastole made more conditioned responses than those trained in systole (rm ANOVA, main effect of cardiac cycle). Further, in accordance with our hypothesis, participants trained at EXP-DIA made more conditioned responses during TEBC than those trained at INS-SYS (independent samples t-test). (B) Conditioned responding at best was higher in participants trained at diastole than at systole (univariate ANOVA) and higher in participants trained at EXP-DIA than those trained at...
INS-SYS (independent samples t-test). Asterisks refer to statistical significance: * p < 0.050, ** p < 0.010, *** p ≤ 0.001. Vertical lines in panel A indicate standard error of mean. Horizontal lines in panel B refer to the mean.

**Figure 3. The conditioned stimulus evoked a larger N1 response in participants trained during expiration than in those trained during inspiration.** (A) Joint plot of the grand-average ERP waveform and topographic maps (depicted at the N1 and P2 peak) of all participants with valid data (n = 40). The butterfly plot of the ERP waveform is spatially colored by the channel locations. (B) Left: ERP waveform at region of interest (channels 6, 7 and 106) for the four groups separately (n = 10 in each group). Right: Topographic maps of two major components for the four groups separately: the auditory N1 peaks at around 112 ms and auditory P2 component peaks around 189 ms. N1 amplitude was larger in participants trained at EXP than at INS (univariate ANOVA) and larger in participants trained at EXP-DIA than at INS-SYS (independent samples t-test). Asterisks refer to statistically significant differences between groups in N1 amplitude: * p < 0.050, *** p ≤ 0.001.
Acknowledgements

We thank Jan Wikgren, Lauri Viljanto, Viki-Veikko Elomaa and Petri Kinnunen for technical help in building the recording systems. Special thanks to Jan Wikgren for helping with the manuscript.

Grants

The work was supported by the Academy of Finland grant number 321522 to MSN.

Disclosures

The authors declare no conflict of interest.
A

Watching a wildlife documentary

US-alone \( \rightarrow \) CS-alone

5 minutes of resting \( \rightarrow \) CS-alone

CS+US Ins-Sys

CS+US Ins-Dia

CS+US Exp-Sys

CS+US Exp-Dia

2 minutes of resting

B

Inspiration

Expiration

R-peak

T-wave

Sys

Dia

Breathing

ECG
APPENDIX 1: A FULL QUESTIONNAIRE FOR PARTICIPANTS

The researcher fills in: ID__________ Date__________ Time__________

Please fill in the missing information and answer the following questions.

Age:
Sex:
Handedness:

1) What equipment did the man in the film use for travelling in the snow?

2) Were there any light phenomena in the film?

3) Name two to three animals you saw in the film.

4) Did the fishermen catch any fish?

5) What large bird appeared at the beginning of the second film?

6) When did the air puff occur?

PLEASE TURN!
<table>
<thead>
<tr>
<th>Proposition</th>
<th>True</th>
<th>False</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The air puff occurred right <em>before</em> the beep.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. The air puff occurred right <em>after</em> the beep.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. The beep occurred right <em>before</em> the air puff.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. The beep occurred right <em>after</em> the air puff.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. The beep and the air puff always occurred very close to each other.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. The beep and the air puff occurred close to each other only occasionally.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. The beep predicted the air puff.</td>
<td></td>
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</tr>
</tbody>
</table>
Bodily rhythms affect conditioning

**CONCLUSION**
Breathing and heartbeat have a phase combination (diastole during expiration) that is indicative of a neural state beneficial for conditioning.

**METHODS**
Breathing (EXPiration vs. INSpiration) and cardiac cycle phase (DIAstole vs. SYStole) were monitored to trigger eyeblink conditioning trials in four different bodily states while participants were watching a wildlife documentary. Conditioned eyeblinks and electroencephalogram were recorded.

**OUTCOME**

![Graph showing the percentage of conditioned responding at best for different phases of respiration and cardiac cycle. There are significant differences indicated by asterisks.](image)