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1	Cardiac cycle and respiration phase affect responses to the conditioned
2	stimulus in young adults trained in trace eyeblink conditioning
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12

13 Abstract

14 Rhythms of breathing and heartbeat are linked to each other as well as to rhythms of the 15 brain. Our recent studies suggest that presenting conditioned stimulus during expiration or 16 during the diastolic phase of the cardiac cycle facilitates neural processing of that stimulus 17 and improves learning in a conditioning task. To date, it has not been examined whether 18 utilizing information from both respiration and cardiac cycle phases simultaneously allows 19 even more efficient modulation of learning. Here we studied whether the timing of the 20 conditioned stimulus to different cardiorespiratory rhythm phase combinations affects 21 learning in a conditioning task in healthy young adults. The results were consistent with 22 previous reports: Timing the conditioned stimulus to diastole during expiration was more 23 beneficial for learning than timing it to systole during inspiration. Cardiac cycle phase 24 seemed to explain most of this variation in learning at the behavioral level. Brain evoked 25 potentials (N1) elicited by the conditioned stimulus and recorded using electroencephalogram 26 were larger when the conditioned stimulus was presented to diastole during expiration than 27 when it was presented to systole during inspiration. Breathing phase explained the variation 28 in the N1 amplitude. To conclude, our findings suggest that non-invasive monitoring of 29 bodily rhythms combined with closed-loop control of stimulation can be used to promote 30 learning in humans. The next step will be to test if performance can also be improved in 31 humans with compromised cognitive ability, such as in older people with memory 32 impairments.

33 Keywords

34 breathing, heartbeat, event-related potential, learning

35

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.

42 Introduction

43 Bodily rhythms like cardiac cycle and respiration usually vary at their own pace without 44 much conscious thought put into them. When we are in a relaxed psychophysiological state, 45 breathing and heartbeats synchronize [1-3]. The beat-to-beat intervals of the heart are longest 46 at the end of expiration [4] and shorter during inspiration [5]. This is called respiratory sinus 47 arrhythmia (RSA). In addition, the last heartbeat of each expiration delays the onset of the 48 following inspiration, the "working phase" of breathing, so that the cardiac "working phase", 49 systole, precedes inspiration onset by 150-500 ms [6,7]. This phenomenon is termed 50 cardioventilatory coupling. Especially RSA is thought to increase efficiency and stability in 51 pulmonary gas exchange [2] and to decrease energy use caused by heartbeats [8]. 52 Nevertheless, the root cause and outcome of cardiorespiratory synchrony is somewhat unclear 53 [6]. 54 Interestingly, cardiac cycle and respiration also synchronize with electrophysiological 55 rhythms of the brain [9], which in turn affect neural processing of external information. 56 Temporal correlations exist, for example, between cardiac cycle and the hippocampal theta 57 oscillation (3-12 Hz) in rodents [10,11]. Theta oscillation is crucially involved in memory 58 formation during spatial [12] and non-spatial tasks [13,14] as it, for example, regulates the 59 firing of hippocampal principal cells. In addition to cardiac cycle, also the respiration rhythm 60 couples with brain oscillations [15]. For example, electrophysiological oscillations in the 61 limbic system are entrained by nasal breathing in humans at the delta (0.5-4 Hz), theta (4-8 Hz)62 Hz) and beta (13–30 Hz) frequency bands [16]. In addition, hippocampal sharp-wave ripples 63 (SWRs, 100–200 Hz), crucial for memory consolidation [17], are entrained by respiration in 64 mice [18].

65 Further and most importantly, brain responses to external stimuli and consequent behavior 66 such as startle eyeblinks and premotor reaction times [19,20] and even associative learning 67 [21] are modulated by the cardiac cycle phase. Regarding respiration, associative learning is 68 enhanced when the significant stimuli are presented during expiration [22]. However, to our 69 knowledge, there are no studies considering the combined effect of cardiac cycle and 70 respiration phases on brain responses nor behavior. It is possible that utilizing combined 71 information from these bodily rhythms might allow even more efficient modulation of 72 behavior to the desired direction. Hence, we investigated the combined effect of cardiac cycle 73 and respiration phase on learning in an associative task called trace eyeblink conditioning 74 (TEBC). Participants were trained while watching a documentary film, using a tone as a 75 conditioned stimulus (CS) and an air puff towards the corner of the right eye as an 76 unconditioned stimulus. The presentation of the CS was fixed to a certain phase of the cardiac 77 cycle (systole, SYS or diastole, DIA) and respiration (expiration, EXP or inspiration, INS) for 78 each participant. In addition to conditioned responses also electroencephalogram (EEG) was 79 recorded. Our previous data [21] showed mixed effects of cardiac cycle phase on CS-evoked 80 brain responses in humans and in rabbits while we have not examined the effect of respiration 81 phase. Based on our previous behavioral results [21, 22], we hypothesized that timing the CS 82 to systole during inspiration would be less than optimal for learning TEBC whereas 83 presentation of the CS to the diastole during expiration would be most favorable for learning 84 (see Figure 1B).

86 Materials and Methods

87 Participants

88 Participants were recruited via student email lists. All participants gave informed written 89 consent to this study and were free to discontinue participation in the experiment at any point. 90 All participants received a reward (a movie ticket or a gift card) even if they discontinued the 91 experiment at some point (however, no one did). The study was approved by the University 92 of Jyväskylä Ethical Committee. A total of 59 young adults (12 males; aged 20-30 years: 93 mean 23.4 years, standard error of mean 0.4 years) took part in the study. All participants 94 were healthy with no history of psychiatric or neurological illnesses. They were not taking 95 medication affecting the central nervous system, and they had no disabilities in hearing or 96 vision.

97 Physiological recordings

98 Recording electrodes were attached after participants had signed the written consent. 99 Respiration was recorded and monitored during the experiment with a reusable fabric belt 100 (RESPA00000, Spes Medica, Italy), which was fastened on top of the clothes on the lower 101 chest area. Heart rate was recorded using three electrocardiogram (ECG) electrodes (Kendall, 102 H92SG); one electrode was placed on top of the right clavicle, one on the left lower ribs, and 103 the grounding electrode on the back of the neck. Electromyography (EMG) to determine 104 eyeblinks was recorded using two electrodes (70010-K/12, Ambu, Ballerup, Denmark) that 105 were attached on top of the participant's right eye muscles (orbicularis oculi). EEG was 106 recorded using a 128-channel EGI Sensor Net (Electrical Geodesics Inc., Hydrogel GSN 128, 107 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) ata 1-kHz sampling rate.

110 Experimental procedure

111 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

114 inter-individual variability in TEBC, and this is something that should be addressed in

115 follow-up studies.

116 The outline of the experimental procedure is presented in Figure 1A. The participants sat in a

117 chair in front of a TV screen (Asus VG236 series H, 23"; distance: approximately 100 cm).

118 They were informed that the aim of the study was to record physiological responses to

119 different types of stimuli while their attention was to be directed at a silent film depicting

120 landscapes and animals. The participants were instructed to pay attention to the film and told

121 that there would be questions considering the content of the footage after the recording

122 session. They were also instructed to sit comfortably in the chair and not pay attention to the

123 disturbing stimuli. In other words, the participants were led to believe that the idea was to

124 study the disturbance caused by beeping sounds and air puffs on their attention towards the

125 film.

126 Trace eyeblink conditioning

127 The conditioned stimulus (CS) was a 200-ms, 440-Hz, 66-dB tone delivered via a

128 loudspeaker situated in the lower right-hand corner of the room. The unconditioned stimulus

- 129 (US) was an air puff (0.2 bar source pressure, 100 ms) targeted at the right eye and it was
- 130 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).

135 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

137 minutes of resting data were recorded, followed by five CS-alone trials to determine baseline

138 eyeblink rate. Then, 50 CS+US classical conditioning trials were presented either at

139 inspiration-systole, inspiration-diastole, expiration-systole or at expiration-diastole. Last, five

140 CS-alone trials were presented as an extinction training block. A random ITI of 20-40 s was

141 applied throughout the experiment.

142 To time the classical conditioning trials, the respiration, cardiac cycle, and EMG signals were 143 conveyed to a custom script running in LabVIEW (National Instruments). Signals were 144 sampled at 1 kHz. At each time point, the last second of respiration, ECG and EMG signals 145 were analyzed. EMG was evaluated for spontaneous eyeblinks, that is, the signal had to stay 146 below a set amplitude threshold to proceed with presenting the conditioning trial. The 147 respiration signal was analyzed in two consecutive 500-ms windows. To trigger a trial, the 148 signal amplitude during the latter 500-ms time window had to cross a set absolute threshold 149 value (peak for inspiration, trough for expiration) and the signal had to either rise 150 (inspiration) or fall (expiration) at a certain rate between the two consecutive time windows. 151 In addition, R-peaks were detected from the ECG and used for timing the trial either at 152 systole (immediately) or diastole (delayed from R-peak). Note that the threshold values for 153 the EMG and for the respiration peak and rise (inspiration) and for the trough and fall 154 (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.

162 Two minutes of spontaneous breathing and ECG without any external stimuli were recorded 163 after the conditioning session to visually confirm online that the experimental manipulation 164 had not changed the respiration pattern overall and that the respiration belt signal quality had 165 remained similar to that recorded before experimental manipulations. The whole procedure 166 lasted about 40 minutes depending on the random ITI.

167 <u>Questionnaire</u>

168 After the experiment, participants answered background questions about age, sex, and 169 handedness and five questions concerning the silent film (e.g., "What equipment did the man 170 in the film use for travelling in the snow?") and an open question about the disruptive stimuli. 171 Questions about the film were asked to find out if participants had been concentrating on the 172 film because attention has a serious impact on learning in classical conditioning [23]. 173 Participants also answered seven true/false questions about the occurrence of the disruptive 174 stimuli (e.g., air puffs occurred immediately after beeping sounds). These questions were 175 asked to find out how conscious the participants became of the CS-US association. For the 176 complete questionnaire (translated into English for reporting purposes), please see Appendix 177 1.

178 Data analysis

179 <u>Conditioned responses</u>

180 The conditioned responses (CR) performed by each participant were analyzed offline using 181 MATLAB (The MathWorks Inc.). First, the EMG signal was low pass filtered (40 Hz) and 182 the absolute value of the signal was derived. Then the mean amplitude of the rectified EMG 183 signal during a 500-ms pre-US period (MEANpre) was calculated. In addition, the mean of 184 the standard deviation of the signal amplitude during the 500-ms pre-CS period (SDpre) was 185 determined. Learned responses were detected from a 200-ms time window immediately 186 preceding the US. To qualify as a learned response, the rectified EMG signal amplitude had 187 to exceed the following threshold: MEANpre + 2 * SDpre. For statistical analysis, trials were 188 grouped into blocks of five trials and the proportion (%) of conditioned responses per block 189 was calculated. These measures were used as dependent variables when analyzing learning. 190 Further, to create a simple measure of the outcome of TEBC, we determined the highest 191 proportion (%) of conditioned responses during any given 10-trial block (50 trials, 5 blocks). 192 This measure is referred to as the best performance in the TEBC task.

193 Event-related potentials (ERPs)

194 EEG data were analyzed using the MNE python [24]. First, EEG channels were visually

195 inspected and bad channels were interpolated using spherical spline interpolation method

- 196 [25]. Then fast independent component analysis (ICA) was applied to remove any eyeblink
- 197 and cardiac artifacts related components [26]. Our previous study has shown that after
- 198 applying ICA to remove cardiac related components, sometimes also referred to as heart-
- 199 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
- 201 with a Hamming-window) was applied to the continuous EEG recordings. After filtering, the

202 EEG signal was re-referenced to the common average. Then the EEG data were segmented 203 into epochs spanning from -100 to 500 ms relative to the onset of the CS. The EEG epochs 204 were manually checked to exclude any trials that were contaminated by movement-related 205 artifacts or other high-amplitude noise. EEG epochs exceeding 100 µV peak-to-peak 206 amplitudes were excluded from further analysis. Finally, the event-related potentials (ERP) 207 were obtained by averaging EEG epochs around the CS over all paired conditioning trials. 208 Next, the ERP data were grand averaged across all participants. Two major ERP components 209 were evident: An auditory N1, which peaked around 112 ms, and auditory P2, which peaked 210 around 189 ms after the onset of the CS. In addition, the center of activities for both N1 and 211 P2 peaks were around the channels number 6, 7 and 106 (128-channel EGI Sensor Net), 212 which are located around the center of the head (see Figure 3A). This pattern (vertex 213 negative-positive potentials) is consistent with our previous study [21] and other studies [27, 214 28]. Based on this, auditory N1 and P2 mean amplitudes were extracted from each participant 215 for further statistical analysis from a 30-ms time window around the grand average N1 (112 216 ms) and P2 (189 ms) peaks from channels number 6, 7 and 106. 217 Statistics 218 One way analysis of variance (ANOVA) and independent samples t-test were used to 219 examine differences between groups in single variables. Repeated-measures (rm) ANOVA 220 was used to analyze changes across training and differences between the groups in

221 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

225 $(\eta 2)$ are reported for statistically significant differences.

226 **Results**

227 Participants concentrated on watching the documentary film

228 Of the 59 participants, 56 answered correctly to all the questions about the film content

229 (questions 1–5 of Appendix 1) and the rest of them had only one missing answer.

- 230 Respectively, only 27 of 59 participants answered correctly to the questions about how the
- 231 disruptive stimuli were presented (questions 7–13 of Appendix 1). This indicates that the
- 232 participants were generally well concentrated on watching the film and not on the
- 233 conditioning stimuli.
- 234 Participants trained during expiration-diastole made more conditioned responses than those
- 235 trained during inspiration-systole

236 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,

- 238 INS-SYS: n = 13) (see Figure 2). Participants in all groups responded (i.e., blinked their eye)
- at an equal rate (mean \pm standard error of mean: 10 % \pm 2 percentage units) to the CS during

240 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 =

242 26) on TEBC, we analyzed the conditioned response data with rm ANOVA using respiration

243 phase (2) and cardiac cycle phase (2) as between-subjects factors and block (10) as the

244 within-subjects factor. In addition to the statistically significant main effect of block (F [9,

245 423] = 8.051, p < 0.001, η^2_{p} = 0.146) a statistically significant main effect of cardiac cycle

- 246 phase (F [1, 47] = 6.109, p = 0.017, η_{p}^{2} = 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] = 0.471)

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)

251 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) =

253 2.588, p = 0.016, Cohen's d 1.015.

254 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 =

258 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

260 the EXP-DIA (82 $\% \pm 17$ percentage units) and the INS-SYS (62 $\% \pm 22$ percentage units)

261 groups using independent samples t-test which indicated a significant difference: t (24) =

262 2.734, p = 0.012, Cohen's d = 1.073.

263 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.

265 Specifically, and in accordance with our hypothesis, participants trained during expiration-

266 diastole made more conditioned responses than those trained during inspiration-systole.

- 267 The conditioned stimulus evoked a larger N1 response in participants trained during
- 268 expiration than in those trained during inspiration
- 269 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
- 271 conditioned responses: Univariate ANOVA revealed a significant effect of respiration phase

- 272 on the N1 amplitude (F [3, 36] = 12.219, p = 0.001, $\eta_p^2 = 0.253$; cardiac cycle phase: F [3,
- 273 36] = 0.632, p = 0.432; interaction: F [3, 36] = 0.737, p = 0.396) but not on the P2 amplitude
- 274 (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
- 276 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
- 280 largest in the EXP-DIA group and overall larger N1 responses were evoked when the CS was
- 281 presented during expiration rather than during inspiration.

283 Discussion

284

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.

Respiration rhythm and cardiac cycle are known to synchronize to each other [6], to modulate

292 whereas systolic phase during inspiration would be less beneficial for learning.

293 As expected, timing the CS onset to diastole during expiration resulted in more frequent

294 conditioned responding compared to timing the CS onset to systole during inspiration.

295 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

297 responses evoked by the CS: The N1 response was larger in amplitude when the CS occurred

298 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

300 rhythms can be used to facilitate learning in humans.

301 Most importantly, our current study indicates that learned behavior can be modulated by the

302 combinatory phases of breathing and the cardiac cycle. Overall, participants in our study

303 acquired the conditioned eyeblink very fast, within the first few training blocks. As

304 hypothesized, in our participants trained exclusively during the "resting states" of the heart

305 and respiratory muscles (expiration-diastole), performance of a learned motor response was

306 more likely compared to that in participants trained in the "working phase" of these organs

307 (inspiration-systole). Further, the phase of the cardiac cycle was the main factor explaining 308 this difference. This result is in contrast with our earlier finding indicating no effect of 309 cardiac cycle phase on learning in humans [21]. However, this could be explained by the 310 further development of the conditioning paradigm in terms of triggering the trials to systole 311 or diastole, which was more accurate in the current experiment. Namely, the delay from the 312 R-peak was individually adjusted to suit each participant's heart rate instead of using a set 313 delay for all participants. We also did not detect a main effect of breathing phase on 314 conditioned responding, again in contrast with our earlier finding [22]. However, it could be 315 that as in the current experiment the timing of the CS hinged on the R-peak, the phases of the 316 respiration (EXP vs. INS) are not directly comparable to those in our earlier study. Namely, 317 in our current study, the onset of the CS was delayed until the next heartbeat within the 318 expiration or the inspiration phase of breathing while in the Waselius et al. 2019 study a CS 319 was triggered immediately as the desired breathing phase was detected. In any case, putting 320 all evidence together, it seems that the neural state during diastole and expiration might be 321 most favorable for acquiring an auditory CS-somatosensory US association and then 322 performing a learned motor response. This conclusion is in line with all our findings, current 323 and previous [21, 22]. It is also in line with a report of faster reactions to and higher saliency 324 evaluations of auditory startle stimuli when presented during expiration rather than during 325 inspiration [31]. Further support comes from studies reporting greater startle eyeblink 326 responses to auditory stimuli presented at diastole than systole [19, 20]. 327 Our current results suggest that respiration and cardiac cycle phases affect learning itself and 328 not just the performance of the conditioned response, as 1) there is no difference between 329 groups during the CS-alone treatment or the very first conditioning trials and 2) there is a

330 clear distinction in the probability of a conditioned eyeblink once it reaches a plateau (see

331 Figure 2). According to Prokasy's theory (1984), during eyeblink conditioning the 332 participants first learn an association between the conditioned stimulus and the unconditioned 333 stimulus. Then they learn to shut their eye before the irritating air puff, that is, they learn to 334 perform the motor conditioned response. Over time, with extended training, the conditioned 335 eyeblink is adjusted temporally so that it optimally protects the eye from the flow of air [32]. 336 Considering this, it seems that in our current experiment the effects of the neural state 337 indicated by the phases of the bodily rhythms center on the acquisition of the CS-US 338 contingency and the motor conditioned response taking place early in training and not so 339 much on the later phases of the process when the CR is further adjusted. Learning the CS-US 340 association during trace eyeblink conditioning is considered to be hippocampus-dependent 341 [33–35] because of the gap between the CS-offset and the US onset while the simpler version 342 of the task where the two stimuli partially overlap relies solely on the cerebellum responsible 343 for motor learning [36, 37]. Thus, it is possible that the neural state indicated by diastole 344 during expiration is related to a more efficient acquisition of the CS-US association, perhaps 345 involving the hippocampus, and to a more reliable execution of the conditioned motor 346 response governed by the cerebellum.

347 As anticipated based on the behavioral results, electrophysiological brain responses evoked 348 by the conditioned stimulus also differed between the experimental groups in our study. 349 Specifically, the N1 component of the ERP responses was largest in the participants trained 350 in diastole during expiration and an overall larger N1 was evoked when the CS was presented 351 during expiration rather than during inspiration (Figure 3). This suggests that the CS evoked 352 more synchronous neural activity and was possibly perceived as more surprising or salient 353 [38] during expiration (and diastole). It should be noted that earlier studies have not 354 addressed how respiration might modulate auditory ERPs, but it has been suggested that

355 auditory startle stimuli are subjectively rated more intense if presented to mid-expiration [31]. 356 Regarding the effects of cardiac cycle phase, in our earlier study [21] the N1 of the ERP was 357 in fact higher in amplitude during systole than diastole. However, Schulz and colleagues 358 (2020) found the N1 to be higher in amplitude during diastole than systole when studying the 359 effects of the cardiac cycle phase on responses to auditory startle stimuli [20]. Clearly, the 360 modulation of neural responses by bodily rhythms should be explored in more detail and 361 considered in data analysis. One might claim, for example, that a fixed inter-stimulus interval 362 could result in the stimulus being presented repeatedly in the same phase of respiration and/or 363 cardiac cycle, depending on the rate. This might then affect the amplitude of the different 364 ERP components.

365 When it comes to the mechanism behind the link between bodily rhythm phases, brain 366 function and behavior, not a lot is known. It does seem clear that the spontaneous rhythms of 367 the brain relate to the rhythms of breathing and heartbeat [9], but a deeper mechanistic 368 explanation of the anatomical and functional connections is missing. One of the most studied 369 phenomena is the link between respiration, olfaction, and related brain activity. Several 370 studies report that respiration rhythm and phase are connected to brain oscillatory activity 371 [15,16,18,39–41]. In most studies, the connection seems to be limited to nasal respiration [16] 372 and to crucially depend on the function of the olfactory bulb neurons [18]. Some of the 373 respiration-driven brain rhythms might even be separate from the traditional brain rhythms, 374 such as theta paced by subcortical structures [14]. Specifically, sniffing rodents are reported 375 to display hippocampal oscillations that occur at the theta-frequency and couple with 376 respiration but are not theta [39]. Since oscillations are the foundation of information transfer 377 in the brain [42-44], it is obvious that rodents largely dependent on their olfactory senses 378 could benefit from this coupling of respiration and brain oscillations [45]. As an

379 evolutionarily close relative to rodents, humans might also possess this characteristic.

380 Interestingly, olfaction and spatial memory seem to be congruent in humans: A good sense of 381 smell is linked to good navigating ability [46]. Curiously, here and in our previous studies the 382 "sniffing phase", meaning inspiration, has emerged as the *less* optimal phase for associative 383 learning of a causal connection between an auditory cue and an aversive somatosensory 384 stimulation [22]. This might be related to the observations that hippocampal sharp-wave 385 ripples (SPW-Rs) known to be crucial for memory consolidation [47, 48] and reflect a state in 386 which rabbits acquire eyeblink conditioning better [47, 48] are more likely to occur during 387 expiration than inspiration in mice [18]. Whether hippocampal SPW-Rs are more likely to 388 occur during expiration in the diastolic phase of the cardiac cycle in humans is not known and 389 should be studied in the future. Further, it should be tested whether limiting breathing to the 390 oral route would possibly abolish the link between cardioventilatory rhythms and conditioned 391 responding [16]. In sum, we suggest that the findings of our current experiment could be in 392 part explained by the link between bodily rhythms and brain oscillatory activity, especially by 393 that between expiration and SPW-Rs.

394 Limitations

395 There are some limitations in this study that we want to point out. Most obvious is the 396 somewhat small sample size and small effect sizes. Nevertheless, the effect sizes in the ERP 397 and behavioral results were large in our previous studies with similar group sizes and 398 experimental set-up. Thus, we were confident that a similar group size should be large 399 enough to detect possible effects in our current study as well. In addition, one must remember 400 that associative learning in TEBC is by no means representative of all learning. In fact, in our 401 current follow-up studies we have employed a different type of associative memory task, 402 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.

410 Conclusion

411 To summarize, this study is the first to demonstrate that both breathing, and heartbeat 412 rhythms influence the brain processing of external stimuli and learning about those stimuli. 413 Learned responding is more likely when an auditory conditioned stimulus is presented during 414 the resting phase of the heartbeat when breathing out instead of during the working state of 415 the heartbeat when breathing in. In addition, the N1 component of the auditory-evoked 416 potential is larger when the stimulus is presented during expiration (and diastole) than when it 417 occurs during inspiration (and systole). These findings suggest that non-invasive 418 measurement of cardiorespiratory rhythms combined with closed-loop control of stimulation 419 can be utilized to promote learning in humans. The next step will be to test if performance 420 can also be improved in humans with compromised cognitive ability [49, 50]. Further, it will 421 be interesting to test the effects of bodily rhythm phases on learning tasks more closely 422 resembling real-life situations.

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541 Figure captions

542 Figure 1. Experimental design and examples of breathing and ECG signals used for

543 **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

- 545 five CS-alone trials. Next, 50 pairs of CS+US trials were timed to a certain cardiorespiratory
- 546 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
- 548 ended. (B) Breathing signal and ECG were recorded and followed online to time conditioning
- 549 trials to a certain cardiorespiratory phase. During the systolic phase (Sys) of the cardiac cycle,
- 550 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

552 diastolic phase (Dia). The diastolic phase during expiration is marked with grey bars for

553 demonstrating the hypothesized optimal phases for stimulus presentation.

554 Figure 2. Participants trained during expiration-diastole (EXP-DIA) made more

555 conditioned responses than those trained during inspiration-systole (INS-SYS). (A) The

556 percentage of conditioned responses per 5-trial block was used as a measure of learned

- 557 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

560 responses than those trained in systole (rm ANOVA, main effect of cardiac cycle). Further, in

- 561 accordance with our hypothesis, participants trained at EXP-DIA made more conditioned
- 562 responses during TEBC than those trained at INS-SYS (independent samples t-test). (B)
- 563 Conditioned responding at best was higher in participants trained at diastole than at systole
- 564 (univariate ANOVA) and higher in participants trained at EXP-DIA than those trained at

565 INS-SYS (independent samples t-test). Asterisks refer to statistical significance: * p < 0.050,

566 ** p < 0.010, *** $p \le 0.001$. Vertical lines in panel A indicate standard error of mean.

567 Horizontal lines in panel B refer to the mean.

568 Figure 3. The conditioned stimulus evoked a larger N1 response in participants trained

569 during expiration than in those trained during inspiration. (A) Joint plot of the grand-

- 570 average ERP waveform and topographic maps (depicted at the N1 and P2 peak) of all
- 571 participants with valid data (n = 40). The butterfly plot of the ERP waveform is spatially
- 572 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
- 575 ms and auditory P2 component peaks around 189 ms. N1 amplitude was larger in participants
- 576 trained at EXP than at INS (univariate ANOVA) and larger in participants trained at EXP-
- 577 DIA than at INS-SYS (independent samples t-test). Asterisks refer to statistically significant
- 578 differences between groups in N1 amplitude: * p < 0.050, *** $p \le 0.001$.

579

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588 The authors declare no conflict of interest.

589







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!

Proposition	True	False
1. The air puff occurred right <i>before</i> the beep.		
2. The air puff occurred right <i>after</i> the beep.		
3. The beep occurred right <i>before</i> the air puff.		
4. The beep occurred right <i>after</i> the air puff.		
5. The beep and the air puff always occurred very close to each other.		
6. The beep and the air puff occurred close to each other only		
occasionarry.		
7. The beep predicted the air puff.		



Bodily rhythms affect 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.



EXP-DIA 20 % of conditioned responding at best EXP-SYS 100-**INS-DIA** INS-SYS 10 80 60. R 40--1020• *** DIA vs. SYS** -20 0-INS:DIA ETRISTS INS:SYS +P.DIA -0.10.1 0.2 0.3 0.0 0.4 Time (s)

OUTCOME

CONCLUSION

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