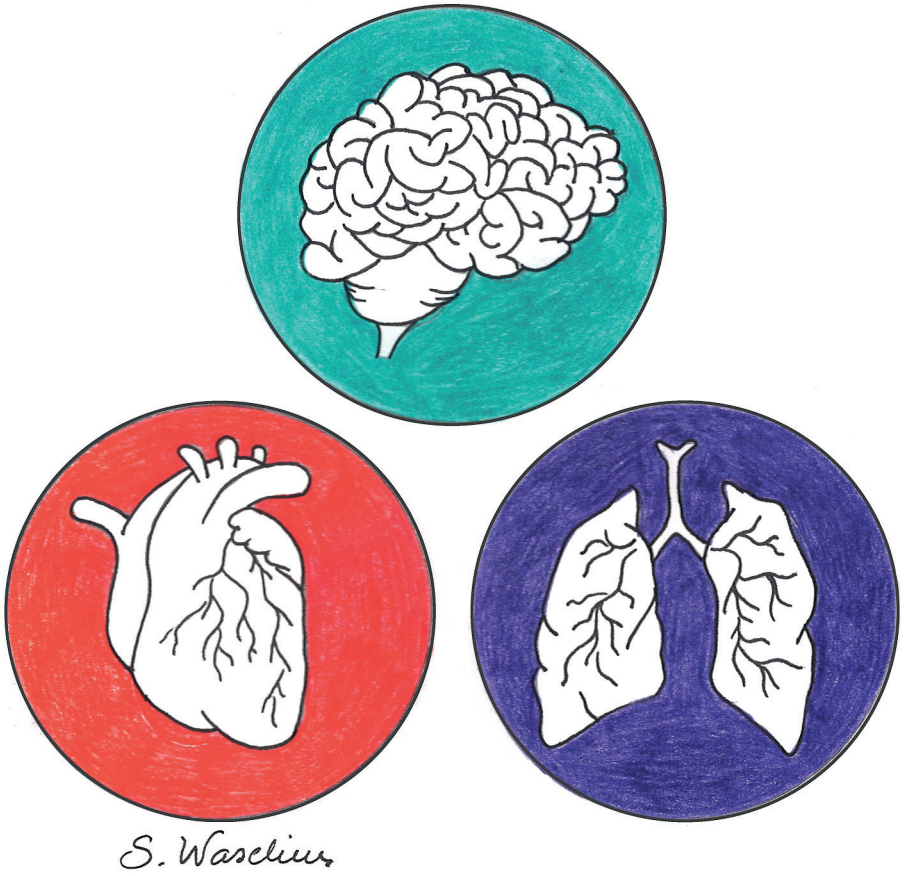
JYU DISSERTATIONS 32

Tomi Waselius

Rapid Changes in Bodily and Neural States Affect Learning



UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF EDUCATION AND PSYCHOLOGY

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Rapid Changes in Bodily and Neural States Affect Learning

Esitetään Jyväskylän yliopiston kasvatustieteiden ja psykologian tiedekunnan suostumuksella
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ABSTRACT

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Declarative memories consist of the past events and factual information that can be recalled. One of the most popular experimental paradigms used in studying long-term memory formation is classical conditioning. Trace conditioning is a hippocampus-dependent task where two separate sensory stimuli are presented in contingency but with a temporal gap between. In trace eyeblink conditioning a conditioned stimulus, usually a tone, is presented with a short silent period followed by an airpuff to the eye. In the beginning of the learning process the behavioral response to the airpuff is naturally a blink of an eye. After repeated trials the subject learns to shut the eyelid after perceiving the tone but timing it before the noxious airpuff. These are called the conditioned responses. Neural oscillations occurring in the hippocampus are connected to learning. In addition, hippocampal oscillations and some rapidly changing bodily states, such as the cardiac cycle and respiration pattern, have rhythmical coupling. In the studies reported here, rabbits and humans were trained in trace eyeblink conditioning in contingency with specific states of hippocampal theta oscillation, cardiac cycle and respiration. The neural processing of the external stimuli and behavioral learning was affected when the conditioned stimulus was presented during different phases of hippocampal theta oscillation. Next, we showed that processing of responses evoked by an external stimulus was modulated differently in the cortex and in the hippocampus when the conditioned stimulus was presented either at the diastolic or systolic phase of the cardiac cycle. Learning was enhanced if the conditioned stimulus was presented during the diastolic phase. Finally, we showed that timing the whole conditioning trial to the expiration phase was optimal for learning. The findings of these studies are novel and suggest that not only the rapid changes in neural states but also bodily states are connected and have an impact on learning and the neural processing of the perceived external world. The aforementioned effects of bi-directional coupling of bodily states and the limbic system and, thus, the modulation of stimulus processing should be considered in electrophysiological measurements and experimental psychology. Furthermore, timing the presentation of significant stimulus to noninvasively monitored specific bodily states could be used to facilitate learning in cognitively demanding tasks.

Keywords: classical conditioning, hippocampus, theta, learning, memory, cardiac cycle, respiration

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Deklaratiivinen muisti koostuu tapahtumamuistista ja yleistietomuistista. Trace-ehdollistamista on käytetty jo vuosikymmeniä assosiatiivisen oppimisen tutkimiseen, jonka ajatellaan olevan deklaratiivisesta muistista riippuvainen oppimisen muoto. Trace-silmäniskuehdollistamisessa ehdollinen ärsyke on yleensä ääni, jonka esittämisen jälkeen tulee lyhyt ärsykkeetön tauko, jota seuraa silmään kohdistuva ilmapuhallus. Oppimisen alussa ilmapuhallukseen reagoidaan spontaanisti räpäyttämällä silmää. Ärsykeparia esitetään toistuvasti ja lopulta silmää opitaan räpäyttämään äänimerkin jälkeen ennen silmään tulevaa ärsyttävää ilmapuhallusta. Tällöin ärsykkeiden välinen assosiaatio aivoissa on vahvistunut ja tuotetaan opittuja käyttäytymisvasteita. Merkittävin aivojen alue trace-ehdollistamisen oppimisen kannalta on hippokampus. Hippokampuksen hidasaaltovärähtelyiden väitetään olevan olennaisesti yhteydessä muistijälkien syntymiseen. Lisäksi, kehon ja aivojen nopeiden tilavaihteluiden väitetään olevan yhteydessä toisiinsa. Väitöskirjan tutkimuksissa selvitettiin hippokampuksen hidasaaltovärähtelyjen rytmisten vaiheiden, sydämen toimintavaiheiden ja hengityksen vaiheiden vaikutusta oppimiseen ja hermostollisten vasteiden muokkautumista. Aluksi selvitettiin hidasaaltovärähtelyjen vaiheiden vaikutusta oppimiseen. Hermostolliset vasteet ulkoisiin ärsykkeisiin organisoituivat hippokampuksessa eri tavoin riippuen hidasaaltovärähtelyn vaiheesta. Tällä oli vaikutusta myös oppimiseen. Seuraavaksi tutkittiin sydämen eri toimintavaiheiden, systolisen ja diastolisen, vaikutusta oppimiseen ja ulkoisen ärsytyksen aiheuttamien hermostollisten vasteiden muokkautuvuuteen. Hermostolliset vasteet hippokampuksessa ja aivokuorella muokkautuivat eri tavoin diastolisessa ja systolisessa vaiheessa. Lisäksi, oppiminen tehostui kun ehdollinen ärsyke esitettiin sydämen toiminnan diastolisessa vaiheessa. Lopuksi todistettiin, että myös hengityksen vaiheella on merkitystä oppimisen kannalta. Väitöskirjan tutkimukset osoittavat, että hermoston ja kehon nopeat tilavaihtelut ovat yhteydessä toisiinsa ja että niiden tilavaihteluilla on merkittävä vaikutus oppimiseen ja ulkoisen aistiärsykeinformaation prosessointiin. Tulokset tulisi ottaa huomioon kokeellisessa tutkimuksessa, jossa mitataan aivojen sähköisen toiminnan muutoksia. Oppimista voitaisiin tehostaa haastavissa oppimistilanteissa esimerkiksi käyttämällä kehon tilavaihteluja seuraavaa laitetta.

Avainsanat: klassinen ehdollistaminen, hippokampus, theta, oppiminen, muisti, sydämen toimintavaiheet, hengityksen vaiheet

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Tomi Waselius
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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I Nokia, M. S., Waselius, T., Mikkonen, J. E., Wikgren, J., & Penttonen, M. (2015). Phase matters: responding to and learning about peripheral stimuli depends on hippocampal θ phase at stimulus onset. *Learning & Memory*, 22, 307-317.

II Waselius, T., Pöllänen, E., Wikgren, J., Penttonen, M., & Nokia, M. S. (2018). Hippocampal theta phase–contingent memory retrieval in delay and trace eyeblink conditioning. *Behavioural Brain Research*, 337, 264-270.

III Waselius, T.*, Wikgren, J. *, Halkola, H., Penttonen, M., & Nokia, M. S. (2018). Learning by heart: Cardiac cycle reveals an effective time window for learning. *Journal of Neuropshysiology*, 120, 830-838.

IV Waselius, T., Wikgren, J., Penttonen, M., & Nokia, M. S. (2018). Breathe out and learn: expiration -contingent stimulus presentation facilitates associative learning. Submitted manuscript.

The author took part in collecting data and writing the report in Study I. Taking into account the instructions given and comments made by the coauthors, the author of the thesis collected the data, conducted the analyses, and wrote the reports of the three publications of studies II, III, and IV. He also took part in designing the experiments in studies II, III and especially in Study IV.

*In Study III, the author and Jan Wikgren equally contributed to collecting data, conducting analyses and writing the report.

FIGURES

TABLE

CONTENTS

LIST OF ABBREVIATIONS

CS	Conditioned stimulus
CR	Conditioned response
ECG	Electrocardiography
EEG	Electroencephalography
EMG	Electromyography
ERP	Event-related potential
LFP	Local-field potential
PPG	Photoplethysmography
RSA	Respiratory sinus arrhythmia
SPW-R	Sharp wave-ripple
TEBC	Trace eyeblink conditioning
UR	Unconditioned response
US	Unconditioned stimulus

1 INTRODUCTION

"There is no learning without remembering."
–Socrates

Memory forms the neural basis for processing new experiences and learning about them. Learning is manifested as cognitive and behavioral changes in animals. Most of the external stimuli that an animal perceives seem to be irrelevant in the perspective of survival and the memories fade quickly as a function of time. In contrast, the relevant information of experiences needs to be memorized. In modern life we need some capacity to memorize a few items, such as a new series of numbers or the name of a person that has been introduced to you, just for a short time. This is called *working memory* (Baddeley & Hitch, 1974). The working memory of a human has the capacity to preserve information about approximately seven items for a while in the prefrontal cortex of the brain (Jensen & Lisman, 1996; Miller, 1956). These items have to soon be stored by either making a physical note somewhere or it has to be memorized, that is, encoded into the brain, in the *long-term memory* (Atkinson & Shiffrin, 1968). Otherwise some or all of the information is lost. There is no functional or pragmatic reason for the brain to store every piece of information we perceive about the external world. However, the relevant information has to be encoded somehow and somewhere so that it can be retrieved from the memory (Buzsáki, 2002; Hasselmo, Bodelón, & Wyble, 2002).

Long-term memory has usually been divided into *declarative* and *nondeclarative* memory (Squire & Zola-Morgan, 1988). Declarative memory refers to things that can be consciously recalled, such as your perceptual experiences of the past and the things you consider as facts, for example who has been and who is the current president of Finland. Nondeclarative memory holds your physical and motor skills (e.g., walking), perceptual learning (e.g., immediately without thought separating different tones in voices or red wine from white wine on the basis of their taste) and habits (e.g., pinching your nose). The division of the declarative and nondeclarative memory is not just abstract. It is based on classical neuroanatomical case studies like H.M. (Scoville

& Milner, 1957). Decades of experimental psychology, neurobiology and other research continue on their quest to connect different types of memory-systems to processes in different brain areas.

For simple nondeclarative learning, brain structures like striatum (physical skills), neocortex (perceptual learning), cerebellum (delay eyeblink conditioning) and amygdala (emotional learning) are essential (Gilbert, Li, & Piech, 2009; LeDoux, 1995; Mccormick et al., 1981; Mishkin, Malamut, & Bachevalier, 1984). The declarative memory and complex learning like trace eyeblink conditioning (TEBC) is based mainly on the working of the hippocampus and brain structures adjacent to it (Milner, 1972). It has been proposed that hippocampus is needed only in the early stages of learning (Takehara, Kawahara, Takatsuki, & Kirino, 2002) and that the actual representations (or engrams) are encoded in the neocortex (Zola-Morgan & Squire, 1991). The details of the memory formation and theories considering the location of the memory engrams are still very vague. Nonetheless, there is compelling evidence that the hippocampus and slow hippocampal oscillations seem to play a crucial part in the memory formation of declarative memories (Buzsáki, 1989; Hasselmo et al., 2002; Kim, Clark, & Thompson, 1995).

It has been suggested that different neural oscillations (e.g., theta, beta, delta and gamma) are not just agreed classifications for frequency bands, but that they actually serve different functions in the brain and that there is harmonic coupling in these oscillations (Penttonen & Buzsáki, 2003). Second, not only does the brain have harmonic frequencies but so does the body and the brain (Klimesch, 2013). A critically overlooked point is that there is some evidence of rhythmical coupling between the hippocampal oscillations, cardiac cycle (Komisaruk, 1970; Pedemonte, Goldstein-Daruech, & Velluti, 2003) and respiratory phases (Chi et al., 2016; Liu, McAfee, & Heck, 2017; Tort, Ponsel, et al., 2018; Zelano et al., 2016). On a behavioral level, there are a few studies that have found a connection between phases of the cardiac cycle and learning in a simple working memory task but not in a more complex task like TEBC. For example, in one study negative emotions were observed more intense during systolic phase of the cardiac cycle (Gray et al., 2012). Also processing of memory retrieval was slowed during diastolic phase while participating in a short-term memory task (Martins, McIntyre, & Ring, 2014). Interestingly, respiration has rhythmic coupling with the cardiac cycle (Bregher & Hubay, 1955) as well as the neural states of the limbic system (Chi et al., 2016; Tort, Brankačk, & Draguhn, 2018) but there is no general idea nor studies that would combine these findings.

The aim of the research was first to test if rhythmically altering phase of hippocampal theta oscillation would modulate neural responses to external stimuli and affect encoding and retrieval of memories in classical conditioning. The next step was to test if bodily states modulate neural processing of external stimuli in learning. The last aim was to explore if behavioral learning is affected by timing the conditioning trials to rapidly changing bodily states, namely the cardiac cycle and respiratory pattern, which should have a parallel coupling to neural states in the brain.

1.1 Classical conditioning

In 1901 Russian psychologist Ivan Pavlov (and his assistant) learned that when a sound was played prior to presenting food to a dog repeatedly and subsequently, the dog would develop a *conditioned reflex*. Initially the dog would salivate whenever receiving food but after the conditioning the salivation would be initiated by just hearing the sound. From those days to now, a mass of philosophical and psychological theories has been formulated and many studies have been conducted concerning classical conditioning with numerous variations. During the last decade one of the dominant paradigms in behavioral research has been classical conditioning. Rabbits (Gormezano, Schneiderman, Deaux, & Fuentes, 1962; Nowak, Kehoe, Macrae, & Gormezano, 1999) as well as humans are capable of learning classical conditioning (Prokasy, 1965; Rescorla, 1967), a task that has been widely used in studying learning and memory formation.

1.1.1 Trace eyeblink conditioning

In trace eyeblink conditioning (see Figure 1) two different types of stimuli are presented with a temporal gap between. First, a neutral *conditioned stimulus* (CS) is presented. This can be, for example, a tone (as in in Pavlov's dog experiment) or some visual stimulus such as a bright light. After the CS is presented, there is a stimulus-free period called the *trace period*. An unconditioned stimulus (US) is presented after the trace period. This is a natural and potent stimulus that is usually an airpuff to the corner of the eye. The airpuff causes an unconditioned response (UR), which in this case is the blinking of the eye. The learning process here is to associate these two stimuli so that the eyelid is shut well before the slightly noxious air puff hits the eye. This is called the conditioned response (CR). It takes only a few conditioning trials in humans to learn this association and to acquire CR but in, for example, rabbits it takes considerably longer. The learning rate depends highly on the properties of the CS and US, the length of the trace period and the intertrial interval (ITI).

The hippocampus is the most crucial brain structure when learning trace eyeblink conditioning (Holland & Bouton, 1999; Solomon, Vander Schaaf, Thompson, & Weisz, 1986). To support this view, there are pharmacological (Asaka, Seager, Griffin, & Berry, 2000) and lesion studies (Moyer, Deyo, & Disterhoft, 2015; Zola-Morgan & Squire, 1991) that indicate this. Interestingly, forebrain regions of the brain are also needed to associate the silent temporal gap between the CS and US (Connor & Gould, 2016; Woodruff-Pak & Disterhoft, 2008). It has been stated that learning trace eyeblink conditioning needs working memory, declarative memory and some level of awareness about the stimuli to form an association between them (Clark, Manns, & Squire, 2001; Clark & Squire, 1998; Connor & Gould, 2016).

1.1.2 Delay eyeblink conditioning

The delay eyeblink conditioning (see Figure 1) is quite similar to trace eyeblink conditioning explained above. The difference is that there is no stimulus-free gap between the CS and US. The CS is so long that it overlaps the US and then they both co-terminate. For example, an 800 ms tone overlaps with an 100 ms airpuff 700 ms after CS onset. The main difference to trace eyeblink conditioning is that delay eyeblink conditioning is not a hippocampus-dependent learning task (Berger & Orr, 1983; Schmaltz & Theios, 1972; Solomon & Moore, 1975) but a highly cerebellum dependent task (Steinmetz, Lavond, Ivkovich, Logan, & Thompson, 1992) and can be learned without conscious awareness of the conditioning stimuli (Clark & Squire, 1998).

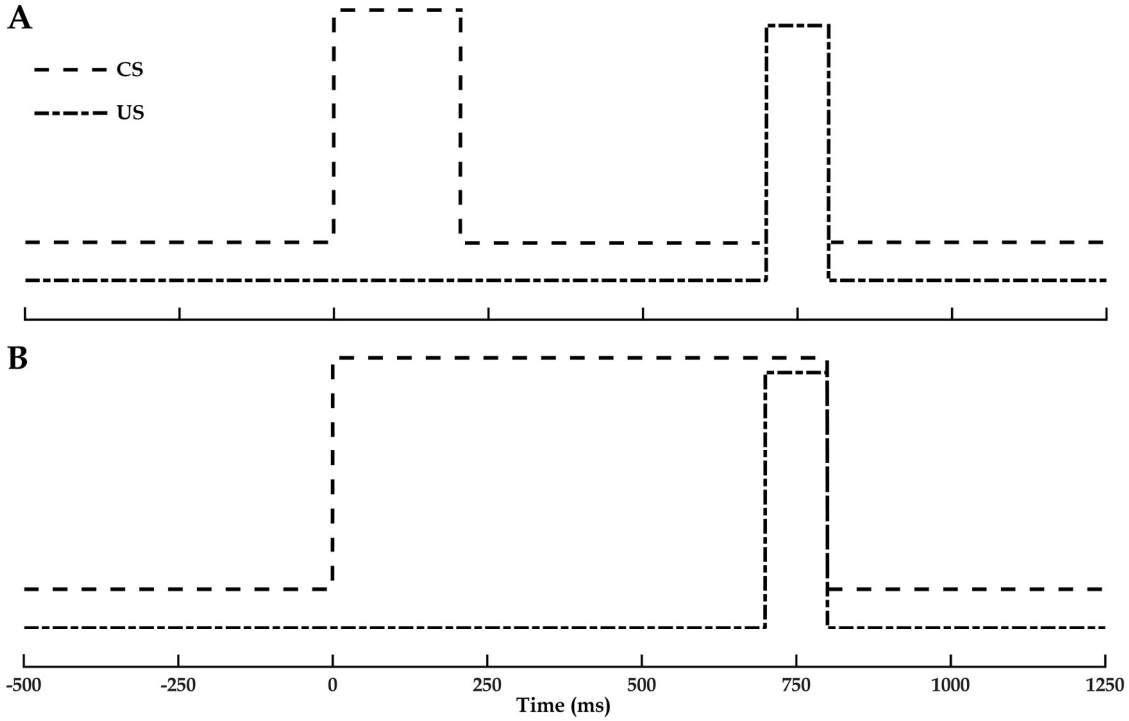

FIGURE 1 Trace eyeblink conditioning and delay conditioning. A) An example of stimulus organization in trace eyeblink conditioning, where a 200 ms long CS and the unconditioned stimulus is 100ms long are separated by a 500 ms trace period (as in Study I). B) An example of delay eyeblink conditioning, where the co-terminating CS is 800 ms long and unconditioned stimulus is 100 ms long (as in Study II, Experiment 2).

1.2 Hippocampal theta and learning

Local-field potentials (LFP) are extracellular electrophysiological events that represent the accumulated activity of neurons (Buzsáki, Anastassiou, & Koch, 2012; Einevoll, Kayser, Logothetis, & Panzeri, 2013). Theta oscillation (3-12Hz) is manifested as sinusoidal-shaped rhythmic slow wave activity in raw LFPs recorded from the hippocampus (see Figure 2). It has been suggested that the pacing of hippocampal theta is driven or mediated via fornix by the septum (Buzsáki, Lai-Wo S., & Vanderwolf, 1983). The frequency of theta depends on the species but in awake rabbits it is approximately 6 Hz (Nokia & Wikgren, 2010). Theta oscillations are actually the most dominant frequency band observed in the hippocampus. Buzsáki's (1989) two-stage model states that hippocampal theta-band activity rises when an animal is experiencing the external world whereas theta activity decreases when the attention is projected into the internal world, for example when resting. Hippocampal theta has been detected in humans during several cognitive behaviors like verbal behavior (Arnolds, Lopes Da Silva, Aitink, Kamp, & Boeijinga, 1980), resting eyes open (Meador et al., 1991), and working memory task (Raghavachari et al., 2001; Tesche, Karhu, Chabot, Asaka, & Berry, 2000) (for a review, see Kahana et al., 2001). In fact, it has been stated and shown that learning trace eyeblink conditioning is enhanced during spontaneous theta activity in the hippocampus (Berry & Thompson, 1978; Griffin, Asaka, Darling, & Berry, 2004; Seager, Johnson, Chabot, Asaka, & Berry, 2002). It should be noted that contradictory results have also been reported (Nokia & Wikgren, 2014).

Hippocampal theta oscillation has a phase reversal between the striatum of the CA1 region and the fissure (Buzsáki, 2002). This has to be taken into account when doing theta phase–related research. Furthermore, one sinusoidal-shaped theta cycle has two phases: a *peak* and a *trough*. During the trough phase of hippocampal theta in the fissure, the hippocampus has strong synaptic input from the entorhinal cortex (EC) and long-term potentiation (LTP) from the CA3 region is strong, whereas in the peak phase of the theta the input from the EC is weak and synaptic input from the CA3 is strong (Hasselmo et al., 2002). According to the computational model of Hasselmo and his colleagues (2002), during the trough phase of the theta, the hippocampus is in the most favorable neural state to encode a new memory while the peak phase is effective for memory retrieval. This means, for example, the following when behaviorally important stimulus is presented during the theta trough: (a) it is encoded in memory more effectively and (b) neural responses to the stimulus should be temporally more uniform, that is, phase-locked to the theta oscillation (S. Palva, Linkenkaer-Hansen, Näätänen, & Palva, 2005).

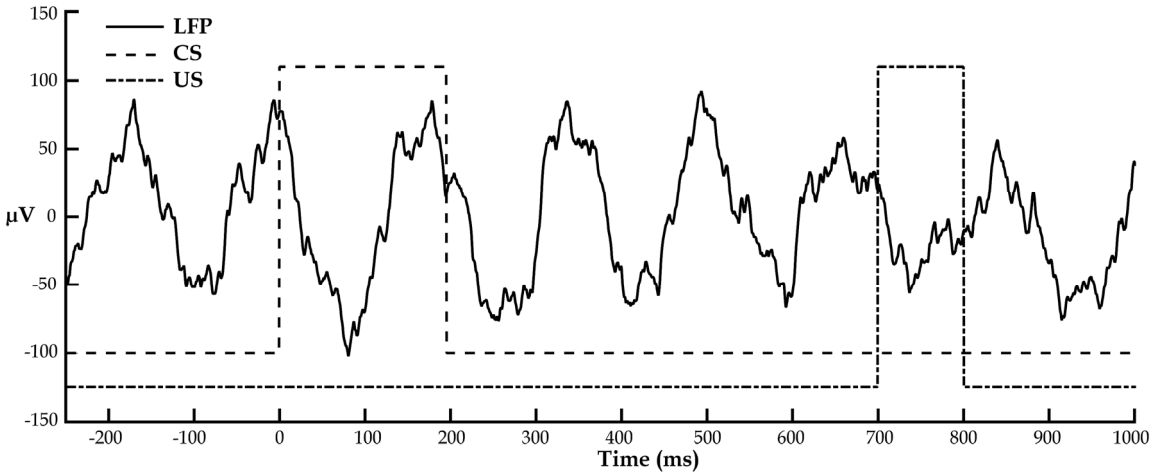

FIGURE 2 Trace eyeblink conditioning and hippocampal theta oscillation. Raw LFP signal showing spontaneous theta (~6Hz) recorded from the hippocampal fissure of a rabbit. An example of stimulus presentation in trace eyeblink conditioning: conditioned stimulus (CS: 200 ms), a 500 ms stimulus-free trace period and unconditioned stimulus (US: 200 ms). Here, CS onset is timed to the peak of hippocampal theta.

1.3 The cardiac cycle and hippocampal theta

In simple terms, the heart has two ventricles and two atria. During the *diastolic* phase, the ventricles of the heart fills with blood coming from the atria and during the *systolic* phase the heart pumps blood to the arteries (Tortora & Derrickson, 2016). According to studies (Komisaruk, 1970; Pedemonte et al., 2003), the hippocampus has rhythmic coupling with the cardiac cycle. In addition, some forms of visual learning are modulated by timing the stimulus in certain phases of the cardiac cycle (Park, Correia, Ducorps, & Tallon-Baudry, 2014; Walker & Sandman, 1982). The main idea behind the effects of the cardiac cycle on brain states and learning (B. C. Lacey & Lacey, 1978; J. I. Lacey & Lacey, 1974) is that baroreceptors located in the walls of blood vessels have an effect on neural states in other brain areas through the nucleus of the solitary tract (Critchley & Harrison, 2013; Jänig, 2006). The cardiac cycle is driven mostly by the pons of the brainstem (Baekey, Dick, & Paton, 2008; Farmer, Dutschmann, Paton, Pickering, & McAllen, 2016), which pace the cardiac cycle in a physically and emotionally stable condition. The inputs from the baroreceptors have a role, but they are actually just stretch-sensitive neurons that give feedback to the brainstem about blood pressure. Interestingly, the whole feedback mechanism between the brainstem and the heart remains something of a mystery, because the regulating system is also controlled by the autonomic nervous system.

1.4 Respiration, respiratory sinus arrhythmia and hippocampal oscillations

During inspiration several muscles in the chest area activate, the diaphragm slides lower and the ribs elevate, and during expiration the muscles that were active, relax (Del Negro, Funk, & Feldman, 2018). It has long been known that respiratory phases have a certain rhythmic coupling with the phases of the cardiac cycle (Bregher & Hubay, 1955). The temporal interval between subsequent systolic phases is shorter during inspiration (Hirsch & Bishop, 1981; Katona & Jih, 1975). The evolutionary explanation is that this mechanism has developed to optimize the balance of oxygen and carbon dioxide in the body (Yasuma & Hayano, 2004). The view is not complete without realizing that this auto-regulatory mechanism yields when the animal is facing serious behavioral and psychological demands (Grossman & Taylor, 2006).

Interestingly, rhythmical electrical activity (Biskamp, Bartos, & Sauer, 2017; Lockmann, Laplagne, Leão, & Tort, 2016; Nguyen Chi et al., 2016; Yanovsky, Ciatipis, Draguhn, Tort, & Brankačk, 2014) and sharp wave-ripples (SPW-Rs) (Liu et al., 2017) in the limbic system is entrained by the respiration but there is only a little evidence on the effects considering performance in behavioral tasks. It has been reported that memory retrieval is more effective during inspiration (Zelano et al., 2016).

1.5 Aims of the research

The main objective of this dissertation was to study behavioral and neural events and outcomes during the learning process when significant stimuli are timed in rapidly changing states of the body and the brain. Specifically, the timing was targeted to different phases of the cardiac cycle, respiration and hippocampal theta. The hippocampus is an essential hub for memory formation in declarative learning (Milner, 1972; Moyer et al., 2015; Scoville & Milner, 1957; Squire, 1992; Squire & Zola-Morgan, 1988). Moreover, hippocampal theta is connected to the neural modulation of responses to external stimuli and behavioral learning about those stimuli (Berry & Thompson, 1978; Buzsáki, 2002; Nokia & Wikgren, 2010, 2014). Furthermore, the phase of hippocampal theta should have an effect on memory encoding and retrieval (Hasselmo et al., 2002). Studies I and II were conducted to test the effects of the hippocampal theta phase on encoding and retrieval on recently associated memories. To our knowledge, Study I is the first of its kind to explore directly if the timing of the significant stimulus to a certain phase of hippocampal theta would modulate learning and neural responses. Study II was the first to test if the retrieval of a learned behavioral response would be most effective during the peak phase of the hippocampal theta oscillation.

The next step chosen was to test if rapid bodily states that have rhythmical coupling with neural activity in the hippocampus would modulate learning in classical conditioning. The main idea was to explore if bodily states that are known to have a rhythmical coupling with neural activity in the hippocampus would modulate learning in trace eyeblink conditioning. In Study III, the first task was to confirm Komisaruk's (1970) findings of the rhythmic coupling of hippocampal theta and the cardiac cycle. The second task was to explore if neural responses to external stimuli would be modulated differently during the systolic and diastolic phases of the cardiac cycle. Third, we assumed that behavioral learning would be different when CS is timed at the systolic or diastolic phase of the cardiac cycle. After this, there were two major reasons to conduct Study IV. First, respiratory-driven oscillations have been detected in the hippocampus (Liu et al., 2017; Zelano et al., 2016). Second, there is rhythmic coupling between respiration and the cardiac cycle (Yasuma & Hayano, 2004). The cardiorespiratory mechanism is physiologically intertwined in the brainstem (Dergacheva, Griffioen, Neff, & Mendelowitz, 2010), and it could be that the neurons that drive the cardiac cycle and respiration have similar effects on the overall neural state of the limbic system and, hence, on learning.

2 METHODS

2.1 Materials and methods for animal experiments

A more detailed description of the methods can be found in the original papers (studies I, II, III and IV).

2.1.1 Subjects

The subjects were a total of 71 New Zealand White female rabbits weighing over 2.5 kg at the time of the surgery in studies I, II and III. All experimental procedures were carried out in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes. Animal experiments were authorized by the Finnish Animal Experiment Board.

2.1.2 Surgery and post-surgery

Rabbits were anesthetized with the same method in studies I, II and III: an intramuscular (i.m.) injection of ketamine–xylazine cocktail. Additional ketamine and cocktail doses were injected subcutaneously (s.c.) every 30 minutes. The state of anesthesia was followed by pinching the skin between the toes every now and then, and also by following the breathing rate. An electrical warming cushion was always placed under the rabbit during the surgeries.

In Study I, the tip of the linear probe was aimed at the left dorsal hippocampal dentate gyrus. This enabled the possibility to have recording sites linearly from the CA1 region to the fissure. In Study II, eight monopolar electrodes were implanted in the left dorsal hippocampus: four aimed at the CA1 and four at the fissure. In Study III, electrodes were implanted in eight rabbits as in Study II. In addition, eight monopolar electrodes were implanted in nine rabbits in both hippocampi with the same coordinates.

The rabbits recovered from the surgeries quickly. They were usually awake one to two hours after the surgery. Analgesic was injected s.c. for the next 48 hours at intervals of 8 and 10 hours. If the animal was not drinking enough water after the surgery, saline was injected subcutaneously until the animal had recovered enough to drink on their own.

2.1.3 Conditioning procedures

The trace eyeblink conditioning was used in studies I–IV. In Study I, rabbits were randomized into three groups: Trough, Peak and Random. This means, that CS was timed either at the trough or peak phase of the theta monitored in the hippocampal fissure. In the Random group the CS was timed irrespective of the neural state. Delay conditioning was used in Study II (Experiment 2) to train rabbits during dominant hippocampal theta or in the absence of theta. In Study III (Experiment 2) three separate groups (Systole, Diastole and Random) were trained in trace eyeblink conditioning. The CS was timed either at the systolic, diastolic or random phase of the cardiac cycle. During the conditioning the rabbits were immobilized in a Plexiglas box (head not fixed) situated in a dimly lit cabinet. For detailed information about the properties of the experiments, see Table 1.

TABLE 1 Summary of conditioning parameters in Studies I, II, III and IV

Study	Paradigm*	Conditioned Stimulus (CS)	Trace Period	Unconditioned Stimulus (US)	Inter Trial Interval (ITI)
I	TEBC	Tone: 200 ms, 4 kHz, 80 dB	500 ms	Air Puff: 100 ms, 0.35 bar	> 35 s
II	TEBC	Tone: 40 ms, 5 kHz, 75 dB	660 ms	Air Puff: 100 ms, 0.35 bar	> 30 s
II	DELAY	Tone: 800 ms, 5 kHz, 75 dB	NA	Air Puff: 100 ms, 0.35 bar	> 30 s
III Human	TEBC	Tone: 200 ms, 440 Hz, 66 dB	600 ms	Air Puff: 100 ms, 0.4 bar	9-19 s
III Rabbit	TEBC	Tone: 200 ms, 5 kHz, 75 dB	500 ms	Air Puff: 100 ms, 0.35 bar	30-60 s
IV	TEBC	Tone: 200 ms, 440 Hz, 66 dB	600 ms	Air Puff: 100 ms, 0.2 bar	20-40 s

* TEBC = Trace eyeblink conditioning; DELAY = Delay eyeblink conditioning

2.1.4 Recordings

In studies I–III, electromyogram (EMG) was recorded for eyelid responses. This was done by placing self-made stainless steel wire hooks (blunt) around the upper eyelid and the lower eyelid of the right eye. The cardiac cycle was recorded with photoplethysmography, PPG (Wisely & Cook, 2001) from the right earlobe in Study III. All signals (LFPs, EMG and PPG) were recorded with a 20 kHz sampling rate with Mc_Rack software (Multichannel Systems).

2.1.5 Histology

After the experiments, the rabbits were anesthetized with an i.m. injection of ketamine-xylazine cocktail and then overdosed with an intravenous (i.v.) injection of pentobarbital. The brain was perfused and the electrode tip locations were marked by passing a DC current trough the electrode. The brain

was then removed and stored in formalin for several days. The brain was coronally sectioned with a vibratome; the slices were attached to gelatinized slides, dried, and stained with Prussian blue and cresyl violet. The electrode locations were determined with the help of a microscope and rabbit brain atlas.

2.1.6 Data analysis

All of the data analysis described below was executed with MATLAB (MathWorks) using self-written code. Eyeblinks were analyzed using a threshold calculated from the mean (MEANpre) amplitudes and the standard deviations (SDpre) of the EMG signal 500 ms before CS onset. These values were compared to the amplitude of the EMG signal 250 ms before US onset. For example, the threshold in Study I was MEANpre + 4 x SDpre.

The modulation effect of theta phase on neural responses to CS were analyzed with a phase-locking value (S. Palva et al., 2005) in studies I, II and III. In other words, band-pass filtered (4–8 Hz) LFP signals were normalized to values between 0 and 1, and Hilbert transformed to obtain the phase information. These values were then averaged between trials per session, giving a value between 0 and 1. In Study III, the phase synchrony (J. M. Palva, Palva, & Kaila, 2005) between hippocampal theta (band-pass filtered 4–8 Hz) LFPs and cardiac cycle PPG (band-pass filtered 3–6 Hz) was analyzed by first normalizing the amplitudes to 1 and then Hilbert transforming. The phase difference of the two signals was calculated by multiplying the PPG signal with the complex conjugate of the LFP signal. Finally, phase synchrony was obtained by averaging the phase difference matrix over sweeps, taking the absolute value. Neural responses to the CS in CA1 were averaged per training session and baselined by using the response amplitudes in CS-alone sessions as a reference in Study III.

2.2 Materials and methods for human experiments

A more detailed description of the methods can be found in the original papers (studies III and IV).

2.2.1 Participants

A total of 66 university students participated in studies III and IV. They were healthy and aged between 18 and 32 years. The studies were approved by the Ethical Committee of the University of Jyväskylä.

2.2.2 Conditioning procedures

Trace eyeblink conditioning was used in Study III (Experiment 1). Participants were randomized into two groups, Systole and Diastole. The CS was timed either at the systolic or diastolic phase of the cardiac cycle. During the conditioning participants were seated and looking at a mark on a wall. In Study IV, the participants were randomized into Inspire, Expire and Random groups. In the Inspire group the CS and US were both timed at the inspiration phase during trace eyeblink conditioning, whereas in Expiration group the CS and the US were timed at the expiration phase of the respiration. In the Random group, the stimuli were presented in a random state after ITI had expired and there was no spontaneous blinking of the eye. During the conditioning participants watched a silent film. For detailed information about the properties of the experiments, see TABLE 1.

2.2.3 Recordings

Electrocardiography (ECG), electromyography (EMG) and electroencephalography (EEG) were recorded in Study III (Experiment 1). The EEG data was recorded with a 64-channel EEG cap. The recording electrodes in ECG were placed on the participant's sternum, over the right ribs and over the left flank. Two electrodes were place under the left eye to record EMG. In Study IV respiration, ECG and eyeblinks were recorded. Respiration was recorded with a stretching belt placed on the lower chest area. Three electrodes were placed on top of the right collarbone, over the lowest rib on left and in the neck (grounding electrode) to record ECG. Eyeblinks were recorded with two electrodes. The minimum sampling rate of the recordings was set to 500 Hz.

2.2.4 Data analysis

The CS-evoked potentials in Study III were analyzed using Brain Vision Analyzer software. The eyeblinks were evaluated by one of the authors who did not have the information about the experimental group. In Study IV, all of the data analyses were executed with MATLAB (MathWorks) using self-written codes. Eyeblinks were analyzed by using a threshold calculated from the mean (MEANpre) amplitudes and the standard deviations (SDpre) of the eyeblinks during 500ms before the CS onset. These values were compared to the amplitude of the eyeblinks 300 ms before the US onset. The threshold in Study IV was MEANpre + 2 x SDpre. Respiratory sinus arrhythmia (RSA) was analyzed by comparing the mean intervals between R-peaks during inspiration vs. expiration.

3 OVERVIEW OF THE ORIGINAL STUDIES

3.1 Study I: Phase matters: responding to and learning about peripheral stimuli depends on hippocampal θ phase at stimulus onset

The computational model of Hasselmo et al. (2002) suggests that neural encoding and retrieval of memories is modulated by the phases of hippocampal theta. In simpler terms, encoding new memories should be more effective when timed at the trough of the hippocampal theta oscillation in the fissure. It is crucial to address the layer of the hippocampus in this context because there is a phase reversal between the CA1 region and the fissure. This is the phase when input from the EC is strongest and LTP from the hippocampal CA3 region is enhanced. In addition, retrieval of the encoded memories should be most effective during the theta peak. Study I was conducted to test the model by Hasselmo et al. (2002) on the encoding of newly associated memories on the neural and behavioral level. Rabbits in experimental groups were trained with TEBC, timing CS at the theta peak or the trough of the hippocampal theta oscillation while hippocampal theta activity in the fissure was monitored.

Neural responses to the CS were temporally more uniform when the CS onset was timed to the trough of the theta. Behavioral learning was deteriorated in when the CS was timed at the peak. However, learning was not enhanced when the CS onset was timed at the theta trough compared to the animals trained in theta peak. In fact, learning was best in animals whose CS was timed at the random neural state of the hippocampus. After the animals were trained for 300 trials, another 180 trials were trained in the absence of theta, that is, when the power of the theta band was low in hippocampus. The animals in the Trough and Peak groups achieved the same level of training during these trials but did not reach the level of learning of the control group. After this, memory retrieval was tested in the Trough and Peak groups by timing half of the CS+US trials at the peak or the trough of the theta. There was no difference in memory retrieval within subjects in well-trained animals.

3.2 Study II: Hippocampal theta phase–contingent memory retrieval in delay and trace eyeblink conditioning

As a continuation of Study I, Study II (Experiment 1) was conducted to study the retrieval of newly formed memories during hippocampal theta phases in a within-subject experiment in early, late and extinction training stages of learning in trace eyeblink conditioning. Hippocampal theta was recorded and monitored from the fissure. A test session of tone alone trials were run to each animal when they reached the criterion of over 30% and over 60% of CRs; test sessions were run also in extinction training (CS-alone sessions) when learned responses dropped under 60% and under 30 %. The test session consisted of 60 CS-alone test trials divided evenly and randomly, timed either at the trough or the peak of the hippocampal theta oscillation in the fissure during dominant theta (> 80%). The phase-locking (S. Palva et al., 2005) values of neural CS responses of the test session trials were also analyzed and compared within subjects.

Indeed, phase-locking values (i.e., responses to the CS) were more temporally organized when they were timed at the trough of the theta. This confirmed the results of Study I. However, there was no difference in the retrieval of learned responses during the test sessions between trough and peak phases in within-subject tests.

Second, the modulation of neural responses to external stimuli was tested by theta-contingent delay eyeblink conditioning (Experiment 2). The idea was to confirm the findings of Seager et al. (2002). In detail, learning delay conditioning should be effective when trials are presented in the presence of dominant hippocampal theta. We trained a group of animals when the theta ratio was over 80% during 1 second before CS onset. The other group was trained irrespective of the neural state after ITI had expired and there was no spontaneous eye blinking. After the animals had reached a good level in learned responses (> 80% during two consecutive conditioning sessions) the animals were then evenly distributed into three groups and extinction trained by presenting CS alone trials either at the peak or trough of the theta, or in random neural state. In addition, hippocampal neural responses to the CS were analyzed in both groups during the delay conditioning and for the three groups in extinction training.

The results of Experiment 2 were somewhat contradictory to the theory. On a behavioral level, there was no difference in learned responses throughout the delay conditioning or extinction training. As in Experiment 1, neural responses to the CS were temporally more uniform when it was timed at the trough of the theta. Together with earlier findings, the results of Study II implied that at least retrieval of recently acquired memories is not hippocampus theta–phase modulated. The hippocampus might have a critical role only in the early stages of learning (Takehara et al., 2002).

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

It is clear that the formation of declarative memories is highly hippocampus dependent (Milner, 1972; Scoville & Milner, 1957; Squire, 1992), but the neural state of the hippocampus could also modulate learning in other ways than by the properties of hippocampal theta. After studies I and II we looked for bodily rhythms that are related or coupled to hippocampal oscillations.

According to Komisaruk (1970) and Pedemonte et al. (2003) there is a temporal correlation between hippocampal theta and phases of the cardiac cycle. In addition, neural responses (Walker & Sandman, 1982) and behavior (Gray et al., 2012; Martins et al., 2014; Park et al., 2014; Sandman, McCanne, Kaiser, & Diamond, 1977) are modulated by or during the cardiac cycle.

To study the neural and behavioral effects of the cardiac cycle on learning (TEBC) and to confirm the phase synchrony between hippocampal theta and the cardiac cycle, Study III was conducted with two experiments. In Experiment 1, humans were randomized into two groups: Systole and Diastole. In the Systole group the CS onset was timed 100 ms after R-peak (systolic phase) monitored in ECG where as in Diastole group CS onset was timed 500ms after the R-peak (diastolic phase). Event related potentials (ERPs) were recorded with EEG. At the same time Experiment 2 was run with rabbits. Animals were randomized into three groups (Systole, Diastole and Random). Monitoring the cardiac cycle from the PPG signal the CS onset was timed in trace eyeblink conditioning (TEBC) either at the systolic, diastolic or random phase of the cardiac cycle. LFPs from the hippocampus CA1 region were also recorded.

The ERPs in Experiment 1 were analyzed, taking into account the possible (insignificant) artefacts caused by the heartbeats, using independent component analysis (ICA). The N1 responses to CS on vertex were higher in amplitude in the Systole group compared to the Diastole group. In spite of the difference in neural responses there was no difference in learning rates between Systole and Diastole groups. The LFPs in CA1 (during training blocks) were standardized for each animal using the amplitudes of CS-alone evoked responses as a baseline. The relative amplitudes of LFPs were amplified in the Systole group whereas in the Diastole group the responses were attenuated throughout the conditioning process. Behavioral learning in TEBC was enhanced when the CS was timed at the diastolic phase of the cardiac cycle.

The results indicate that neural processing of external stimuli is modulated differently in the diastolic vs. systolic phase of the cardiac cycle in the hippocampus and the cortex. Thus, behavioral learning can also be noninvasively affected by timing the stimulus to the diastolic phase.

3.4 Study IV: Breathe out and learn: expiration-contingent stimulus presentation facilitates associative learning

Study III showed that rapid changes in bodily states, such as the cardiac cycle, can modulate the neural processing of external stimulus in the brain and thus affect learning. Study IV was conducted to further examine the effects of other bodily states on learning. The cardiac cycle and respiratory phases pattern has rhythmic coupling (Bregher & Hubay, 1955). This is called respiratory sinus arrhythmia (RSA). The mechanism has evolved to maintain the homeostasis of gasses and save energy in the body of vertebrate animals. There is some evidence that the neural state of the hippocampus differs between inspiration and expiration (Liu et al., 2017; Zelano et al., 2016). Interestingly, memory retrieval can be more effective during inspiration (Zelano et al., 2016).

Our first aim was to examine whether there is an effect on learning by timing the stimulus in different phases of respiration. Second, we wanted to confirm that there is a respiratory sinus arrhythmia. Human participants were randomized into three groups (Inspiration, Expiration and Random) and trained in TEBC according to the phase of respiration that was monitored online. The participants watched a silent film about which they were questioned afterwards. The experiment was designed so that CS and US were both timed to the same phase of respiration according to the experimental group.

The main result of the study was that humans trained in TEBC during expiration learned more effectively than those trained during the inspiration. Second, there was a respiratory sinus arrhythmia during the recordings. This hints that the autoregulatory state in the cardiorespiratory system was activated during the TEBC. Taking our results as a whole, we suggest that neural processing of stimuli and learning is modulated differently in inspiration vs. respiration. Again, the results show that learning can be noninvasively modulated by following rapidly changing bodily states.

4 DISCUSSION

All of the studies reported here showed that either the neural state of the hippocampus or the state of the body reflected in the brain affects neural processing of significant external stimuli. Studies I and II showed that even though neural processing of stimuli in the hippocampus is altered between the phases of hippocampal theta, this might or might not affect behavioral learning regarding the stimuli. In detail, in Study I learning in TEBC was retarded when the CS was timed at the hippocampal theta peak monitored from the fissure. However, timing the CS to the trough did not seem to accelerate encoding of the association to the memory compared to a random neural state of the hippocampus. Retrieval of the newly formed memories neither in the test session of Study I nor in Study II was more effective during the theta peak as the theory suggests (Hasselmo et al., 2002). Interestingly, theta-contingent delay conditioning did not accelerate learning in Study II.

Furthermore, there is a connection between some rapidly changing bodily states (e.g., the cardiac cycle and respiratory phases), the neural states of hippocampus and learning. When conditioning trials in TEBC were timed at the diastolic vs. systolic phase of the cardiac cycle of animals and humans, the neural processing of the stimulus was modulated differently. Moreover, when the CS was timed at the diastolic phase the animals learned more efficiently than in the control group where the CS was timed at a random phase of the cardiac cycle. Finally, Study IV showed that learning can be optimized by timing the CS and US in TEBC to the expiration phase. Both the diastolic phase and the expiration phase occur during epochs of decreased activation of cardiorespiratory cells in the brainstem. Hence, the results of studies III and IV address a crucial question: is the limbic system also in an altered neural state during these epochs or is there another mechanism that explains the results of these studies?

4.1 The role of hippocampal theta phases in encoding and retrieving newly formed memories

Comparing the results of studies I and II, and the computational model of Hasselmo et al. (2002) gives us a reason to discuss the role of the phases of hippocampal theta in the encoding and retrieval of memories. During the trough phase of hippocampal theta in the fissure, the LTP is strong between the CA1 (postsynaptic) and CA3 (presynaptic) neuronal activity (Huerta & Lisman, 1995; Hyman, Wyble, Goyal, Rossi, & Hasselmo, 2003; Hölscher, Anwyl, & Rowan, 1997). This should be a favorable time window for encoding a new association between, for example, temporally closely presented CS and US in classical conditioning.

It is interesting that some studies (Griffin et al., 2004) support the importance of hippocampal theta during the encoding of new association and some do not (Nokia & Wikgren, 2014). It could be that during or in contingency of SPW-states, when hippocampal theta oscillations are weak, association are most effectively encoded (Nokia, Mikkonen, Penttonen, & Wikgren, 2012; Nokia, Penttonen, & Wikgren, 2010). This view is indirectly supported with the results of Study IV. Learning was better when the CS and US were presented during the expiration phase. It has been shown that sharp wave-ripples can be observed in the hippocampus more likely during the expiration phase of respiration (Liu et al., 2017) and in Study IV learning was enhanced during expiration.

Considerations about the role of hippocampal theta in learning can be made. The rising awareness towards the external world manifests in the hippocampus as dominating theta oscillations. This is a stage, according to Buzsáki (1989), when the "weak" associations are held (in the working memory). When the awareness is turned momentarily inwards, such as during immobility, grooming and eating, the theta activity decreases and sharp wave-ripples emerge. This happens in a stage where the awareness is turned to the internal world, even in slow-wave sleep. Low theta-activity in the hippocampus enables the working of the CA3 auto-association network that re-activates the same cells as in the "weak" memory association and strengthens plasticity, that is, the synaptic connections within the hippocampal CA3 and CA1 regions (Buzsáki, 2015). Sharp wave-ripples are suggested to play a key role when the association of CS and US and the properties needed for CR are consolidated into long-term memory (Buzsáki, 1986). However, supplementary investigations need to be carried out to reveal how the direction and level of awareness is connected to the oscillations of the limbic system and what is the optimal oscillatory state in the hippocampus during the early and late stages of learning.

4.2 Body and brain interaction

As has been stated, the brain has harmonic oscillatory frequencies but there is also rhythmical harmony between bodily and brain rhythms (Klimesch, 2013). Komisaruk (1970) observed a temporal correlation between hippocampal theta and the phases of the cardiac cycle. Neural modulation of stimulus processing during the cardiac cycle has also been studied (Walker & Sandman, 1982). Later studies have shown a relationship between behavior and cardiac cycle (Gray et al., 2012; Martins et al., 2014; Park et al., 2014). The modulation of neural processing of stimuli and learning from it was investigated in Study III with rabbits and human participants using trace eyeblink conditioning as a learning task. In the experimental groups the CS was timed at either the systolic or diastolic phase of the cardiac cycle. Electroencephalography was recorded from humans and LFPs in the hippocampal CA1 of the rabbit throughout the learning process. The results of Study III suggest that neural processing of external stimuli varies during systolic and diastolic phases. The ERPSs were attenuated during the diastolic phase and amplified during the systolic phase. Curiously, in human participants the cardiac cycle had an effect on the sensory processing of the external stimuli but not on the behavioral level. The learning task was easy to learn because the attention of the participants was probably on the stimuli, hence the effect of the cardiac cycle phase could not be detected. However, learning in rabbits was enhanced when the CS was timed at the diastolic phase. The results at the neural and at the behavioral level seem to be contradictory because attenuated responses were carried over to enhance behavioral learning but they were actually in line with the results of Study IV.

The cardiac cycle and respiration pattern has rhythmic coupling (Bregher & Hubay, 1955) that is called respiratory sinus arrhythmia. The purpose of this mechanism is to optimize oxygen levels in the periphery and to save energy (Yasuma & Hayano, 2004). Cardiorespiratory regulation is driven by the neural network in the brainstem (Dergacheva et al., 2010). To study if respiration has an effective time window for learning, human participants watched a silent movie during respiratory phase-contingent TEBC. In the experimental groups the CS and US onsets were both timed at the inspiration or the expiration phase. The main result was that learning was optimal when the CS and US were presented during the expiration phase. Additionally, respiratory sinus arrhythmia was confirmed by comparing the R-peak intervals between the inspiration and expiration phases. Taken together, the results of studies III and IV suggest that the neural state of the brain and body is in an optimal state for learning when prominent stimuli are perceived in the diastolic phase during expiration. The neural plasticity of the brain could be in a favorable state during this period. One possible explanation is that the firing of the large neuronal populations in the cardiorespiratory cells of the brainstem during the systolic phase and inspiration impact the overall neural states of the hypothalamus and other regions of the limbic system.

4.3 Limitations and future directions

One of the serious issues considering studies I–III is the timing of the CS and the US during phase-contingent TEBC. CS onset was timed either at the peak or the trough of the ongoing hippocampal theta oscillation in Study I. However, the CS onset–offset period exceeded one theta phase (e.g., see Figure 2) and in addition, US timing was totally random. The CS timing was improved in Study II by shortening the tone to 40ms length to keep the CS onset-offset within one theta phase. Still, US timing was not optimal. The short tone also affected the learning rates because only 7 out of 13 animals reached the criterion of well learned state (> 60 % of learned responses during one session). In Study III the CS was timed at the systolic or diastolic phase, but again, the US timing was random. Because the temporal CS and US contingency should be associated on the neural level, this leads to a serious impact on the results for behavioral learning. The CS and the US were both timed in TEBC at the inspiration or expiration phase in Study IV. The major limitation of Study IV was that the neural activity of the nervous system was not recorded. There could have been some interesting findings in, for example, ERPs but there was no solid hypothesis to test in humans without intracranial EEG. This is why it was excluded from the experiment. Using an animal model would have been more suitable because variations in neural activity in the hippocampus have been detected during inspiration and expiration (Liu et al., 2017; Zelano et al., 2016). Varying the trace period according to the theta frequency (Studies I and II) and heart rate (Study III) to time the US onset properly could be considered an improvement to the experimental setups. Yet changing the length of the trace period between trials (Rescorla & Wagner, 1972) has an effect on the learning process (Bangasser, Waxler, Santollo, & Shors, 2006), and should be considered in the analysis.

There are some further, minor issues to be pointed out. First, how do you study memory retrieval in TEBC? Is the retrieval to be tested with CS+US trials (as in Study I) or with CS-alone trials (as in Study II)? If memory retrieval means a well-timed CR, CS-alone should suffice. CS-alone trials can also be considered extinction training, but even though 60 trials were presented in the early and late stages of training (i.e., reached the criterion of > 30% or > 60% in CRs during one session) there was only a small effect visible in the learning rates during the following session (see Study II, Figure 3B). Second, the experimental environment can have an effect on the overall state of the nervous system in humans as well as animals. In fact, synaptic plasticity in the hippocampus might be lower during stress (Kim & Diamond, 2002). The effects of the stress level in learning and neural processing of external stimulus should be investigated further and considered when designing the experimental setup and also taken into account in the analysis.

There are several logical paths for further studies from this point. One would be to study neural events during learning in regions of the hippocampus

other than CA1 and the fissure. Curiously, dentate spikes seem to play an important role in memory consolidation (Nokia, Gureviciene, Waselius, Tanila, & Penttonen, 2017). In addition, the sensory inputs via lemniscal and non-lemniscal pathways to the hippocampus are to be studied (Bickford et al., 2002) during the diastole vs. systole and inspiration vs. expiration phases in TEBC to explore the modulation of sensory information. Study IV will be replicated in animal model, and it is crucial to analyze the possibly synchrony between the hippocampus preBötzinger complex (see Study IV). At the least, SPW-Rs should be recorded from the CA1 region during TEBC in respiratory phase–contingent training (see Liu et al., 2017) to see if there is more probable occurrence of SPW-Rs during expiration. One study (Pedemonte et al., 2003) has reported phase-locking between the cardiac cycle, firing of medullary cells that drive the cardiac function and the hippocampal theta oscillation in awake, sleeping and anesthetized guinea pigs. It is very possible, that cardiac cycle and breathing are driven by medullary units that both have synchronous activity with each other and at the same time with the hippocampal oscillations. Thus, the synchrony will be studied with an animal model.

4.4 Conclusions

The findings of this thesis demonstrate that the body and brain work in harmonic rhythms. Altered neural states in the hippocampus and rapidly changing bodily states, such as the phases of the cardiac cycle and respiration, have an effect on the learning and neural processing of information about external stimuli. These findings should be examined widely in experimental psychology and neuropsychology. In the future, understanding these mechanisms could lead us to applications where we enhance learning in impaired learners as well as in healthy humans in complex tasks in which the cognitive capacity is challenged. One prominent possibility is to use a noninvasive method, such as following the rhythms of the cardiac cycle and respiration and timing the essential visual, auditory or other type of sensory information accordingly to optimize learning.

YHTEENVETO (FINNISH SUMMARY)

Nopeat muutokset kehon ja hermoston tiloissa vaikuttavat oppimiseen

Muisti on keskeinen perusta oppimiselle. Pitkäkestoiseen muistiin tallennetaan informaatiota, säilytetään informaatiota ja tarvittaessa palautetaan sitä tietoisuuteen. Deklaratiivinen muisti sisältää tietoa menneistä tapahtumista (episodinen muisti) ja faktatietoa (semanttinen muisti), jota voidaan palauttaa mieleen. Klassisen silmäniskuehdollistamisen variaatiota, trace-ehdollistamista käytetään assosiatiivisen oppimisen tutkimiseen. Siinä esitetään toistuvasti ehdollista ja ehdotonta ärsykettä ajallisesti lähekkäin, jotka toistojen kautta opitaan yhdistämään toisiinsa. On havaittu, että trace-ehdollistamisessa oppimisen kannalta tärkein aivoalue on hippokampus ja teorioiden mukaan hippokampuksen rytmiset hidasaaltovärähtelyt (theta oskillaatiot) liittyvät olennaisesti muistijälkien prosessointiin. Väitöskirjan jokaisessa tutkimuksessa käytettiin trace-ehdollistamista joko hippokampuksen hermostollisen tilan eri vaiheissa tai kehon tilan eri vaiheissa.

Väitöskirjan ensimmäisessä tutkimuksessa testattiin teoriaa jonka mukaan hippokampuksen hidasaaltovärähtelyissä on vaihe, jossa hermosto on informaation muistiin koodaamisen kannalta optimaalisessa tilassa. Samalla selvitettiin, miten ehdollisen ärsykkeen aiheuttamat vasteet muokkautuivat hidasaaltovärähtelyn eri vaiheissa. Tulokset tukivat teoriaa siltä osin, että hidasaaltovärähtelyssä on vaihe, jolloin hermostollinen tila on opittavien ärsykkeiden muistiin koodaamiseen kannalta epäedullinen. Tämä näkyi trace-ehdollistamisen käyttäytymisvasteissa, eli oppimisessa, ja ehdollisen ärsykkeen aiheuttamissa hermostollisissa vasteissa. Hippokampuksessa ajallisesti huonommin organisoituneet vasteet näkyivät käyttäytymisen tasolla oppimisen heikentymisenä. Oppimisen ei havaittu tehostuvan teorian väittämässä optimaalisessa tilassa, vaikka hippokampuksen vasteet ulkoiseen ärsykkeeseen olivat paremmin organisoituneita kuin toisessa koe-ryhmässä ja kontrolliryhmässä. Toisessa tutkimuksessa samaa teoriaa testattiin muistiin palauttamisen osalta. Trace-ehdollistamisessa opittujen assosiaatioiden muistiin palatutusta testattiin oppimisen alku- ja loppuvaiheessa, sekä poisoppimisen aikana. Tulokset eivät tukeneet teoriaa oppimisen osalta, vaikka hermoston vasteiden muokkautuminen oli yhdenmukainen teorian ja edellisen kokeen kanssa.

Kolmannessa tutkimuksessa siirryttiin tutkimaan kehon nopeita tilavaihteluja suhteessa oppimiseen. Sydämen sykkeen on havaittu olevan vaihesynkroniassa hippokampuksen hidasaaltovärähtelyjen kanssa. Lisäksi on havaittu, että aivoissa informaation prosessointi muokkautuu eri tavoin riippuen sydämen toimintavaiheesta, joko systolisesta tai diastolisesta vaiheesta. Trace-ehdollistamista opetettiin joko sykkeen systolisessa tai diastolisessa vaiheessa. Havaitsimme, että hermostolliset vasteet hippokampuksessa ja aivokuorella muokkautuivat eri tavoin riippuen ehdollisten ärsykkeiden ajoituksesta suhteessa sykkeeseen vaikka vaihesynkroniaa ei hidasaaltovärähtelyn ja sydämen toimintavaiheiden välillä havaittukaan. Tämä näkyi myös kanien oppimistuloksissa. Sydämen

toiminnan diastoliseen vaiheeseen ajoitettu ehdollistaminen opittiin tehokkaammin kuin kontrolliryhmässä, jonka ajoitus sydämen toimintavaiheeseen oli satunnainen.

On olemassa näyttöä siitä, että hippokampuksen hermostollinen tila vaihtelee hengityksen eri vaiheissa. Tämän lisäksi, sydämen toimintavaiheiden ja hengityksen vaiheilla on synkroniaa. Mekanismi mahdollistaa kehon optimaalisen kaasutasapainon (happi/hiilidioksidi) ylläpitämisen ja energian säästämiseen. Neljännessä tutkimuksessa selvitettiin, onko hengityksen vaiheella merkitystä oppimisessa. Trace-ehdollistamisessa ehdollinen ja ehdoton ärsyke ajoitettiin joko sisäänhengitykseen tai uloshengitykseen samalla kun tutkittavat katsoivat mykkäfilmiä. Uloshengitys osoittautui optimaaliseksi vaiheeksi oppimisen kannalta. Osoitimme, että sydämen toimintavaiheet ja hengitysrytmi toimivat synkronisesti kokeen aikana. Tämän väitöskirjan tutkimukset osoittavat, että tietyt hermoston ja kehon nopeat tilavaihtelut voivat vaikuttaa oppimiseen ja että hermoston tila vaihtelee merkittävästi erilaisten kehon tilamuutosten kanssa synkronisesti. Näitä tuloksia voitaisiin helposti hyödyntää oppimisen tehostamisessa. Esimerkiksi haastavissa oppimistilanteissa voitaisiin kehon ulkopuolelta mitata sykettä ja hengitystä, ja ajoittaa opittavan informaation esiintyminen oppimisen kannalta suotuisan hermostollisen tilan aikana.

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ORIGINAL PAPERS

I

PHASE MATTERS: RESPONDING TO AND LEARNING ABOUT PERIPHERAL STIMULI DEPENDS ON HIPPOCAMPAL θ PHASE AT STIMULUS ONSET

by

Miriam Nokia, Tomi Waselius, Jarno Mikkonen, Jan Wikgren
& Markku Penttonen, 2015

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Research

Phase matters: responding to and learning about peripheral stimuli depends on hippocampal θ phase at stimulus onset

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Hippocampal θ (3–12 Hz) oscillations are implicated in learning and memory, but their functional role remains unclear. We studied the effect of the phase of local θ oscillation on hippocampal responses to a neutral conditioned stimulus (CS) and subsequent learning of classical trace eyeblink conditioning in adult rabbits. High-amplitude, regular hippocampal θ-band responses (that predict good learning) were elicited by the CS when it was timed to commence at the fissure θ trough (Trough group). Regardless, learning in this group was not enhanced compared with a yoked control group, possibly due to a ceiling effect. However, when the CS was consistently presented to the peak of θ (Peak group), hippocampal θ-band responding was less organized and learning was retarded. In well-trained animals, the hippocampal θ phase at CS onset no longer affected performance of the learned response, suggesting a time-limited role for hippocampal processing in learning. To our knowledge, this is the first study to demonstrate that timing a peripheral stimulus to a specific phase of the hippocampal θ cycle produces robust effects on the synchronization of neural responses and affects learning at the behavioral level. Our results support the notion that the phase of spontaneous hippocampal θ oscillation is a means of regulating the processing of information in the brain to a behaviorally relevant degree.

θ oscillation (3–12 Hz) (for review, see Buzsáki and Moser 2013) characterizes hippocampal local-field potentials in awake animals. Its amplitude is strongest near the hippocampal fissure, and its phase reverses between the hippocampal fissure/dentate gyrus and the CA1 pyramidal layer (Buzsáki 2002). θ is thought to reflect a state in which information about the surroundings is actively acquired (Buzsáki 1989). Animals exhibiting dominant hippocampal θ during spontaneous recordings prior to training tend to learn better (Berry and Thompson 1978; Nokia et al. 2009, 2012b), and high-amplitude, well phase-locked θ-band hippocampal responses to the conditioned stimulus early in eyeblink conditioning predict good learning (Nokia et al. 2009, 2010, 2012a; Nokia and Wikgren 2014). Training contingent on transient episodes of hippocampal θ or its explicit absence has produced significant but somewhat inconsistent effects on learning (Griffin et al. 2004; Nokia and Wikgren 2014). In summary, both spontaneous and evoked hippocampal θ oscillations are connected to simple associative learning. Yet the mechanism behind these effects remains largely unknown.

According to a computational model by Hasselmo and colleagues (Hasselmo et al. 2002; Hasselmo and Stern 2014), the phase of hippocampal θ oscillation determines specific and separate time windows for the efficient encoding and retrieval of memories. During the trough of the fissure θ cycle, the hippocampus preferentially processes input arriving from multimodal cortical areas through the entorhinal cortex, leading to long-term potentiation (LTP) at the CA3–CA1 synapses (Huerta and Lisman 1995; Holscher et al. 1997; Hyman et al. 2003), while CA1 output back to the neocortex is suppressed (Kamondi et al. 1998). This, according to the model, supports the encoding of new information. Conversely, stimulation at the peak of fissure θ leads to depotentiation at the CA3 to CA1 synapses (Huerta and Lisman 1995; Holscher et al. 1997; Hyman et al. 2003) and to the firing of CA1 pyramidal cells (Skaggs et al. 1996; Kamondi et al. 1998), thereby relaying information processed in the hippocampus back to the entorhinal cortex. According to the model, the retrieval of already encoded information is favored at the peak of fissure θ. A recent study suggested that electrical stimulation of the hippocampus at the θ trough and peak enhances the encoding and retrieval, respectively, of a spatial memory (Siegle and Wilson 2014). Based on the above discussion, the timing of intracranial stimulation relative to the local θ phase, at the very least, affects its consequences for hippocampal synaptic plasticity and output at the cellular and possibly also at the behavioral level. Whether θ phase also relates to learning about and responding to peripheral stimuli remains elusive.

Here, we studied whether the ongoing θ phase affects the hippocampal processing of peripheral stimuli and, if so, whether such effects carry over into learning. An overview of the experiment is presented in Figure 1A. First, we aimed to determine whether hippocampal responses to a neutral peripheral stimulus are modulated by θ phase. Female rabbits were implanted with recording electrodes in the hippocampus (see Fig. 1B), and, during a single session, presented with a 200-msec tone either during periods of spontaneously occurring hippocampal θ activity or irrespective of neural state. We expected to see better phase-locked hippocampal θ-band responses when the tone onset overlapped with the fissure θ trough compared with when it started at θ peak. Next, we

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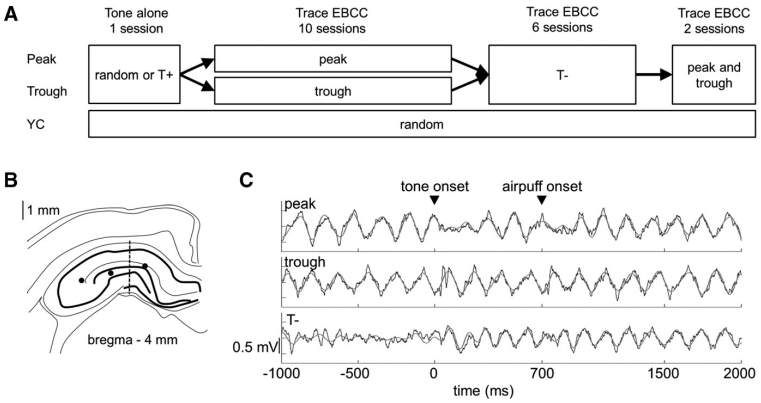

Figure 1. The effects of the phase (peak versus trough) of hippocampal fissure θ oscillation on hippocampal responding and trace eyeblink classical conditioning (EBCC) were studied (*A*). Local-field potentials (LFPs) recorded from near the hippocampal fissure (*B*) were used to trigger trials during trace eyeblink conditioning (*C*). (*A*) Adult female rabbits were divided into three groups. For animals in the Peak group ($n = 6$), the tone alone was presented either contingent on θ (T+) or irrespective of neural state. This was followed by trace eyeblink conditioning contingent on the peak of the θ oscillation (peak) and then contingent on the absence of θ (T−). Finally, the effect of θ phase on memory retrieval in this group was tested by presenting conditioning trials both to the peak and trough of the θ oscillation (peak and trough). For animals in the Trough group ($n = 5$), treatment was identical to that in the Peak group, except during the first 10 conditioning sessions, trials were presented contingent on the trough of the θ oscillation (trough). The yoked control group (YC, $n = 6$) was trained irrespective of their neural state (random) at all times. (*B*) Representative example of the approximate placement of a linear probe (dashed line) used in recording hippocampal LFPs. Exact locations of single electrodes used for detecting θ in three animals are marked with filled circles. (*C*) Examples of hippocampal fissure LFPs prior to and during the presentation of the tone (conditioned stimulus) and the air puff (unconditioned stimulus). The *top* and *middle* panels depict single-trial LFPs in representative animals in the Peak (*top*) and Trough (*middle*) groups recorded during the first session of conditioning (beginning of "Trace EBCC 10 sessions" shown in 1*A*). The *bottom* panel in *C* represents LFP from the same animal as in the *top* panel (peak) from session 11 (beginning of "Trace EBCC 6 sessions" in *A*). Note that now the conditioned stimulus was triggered by the absence of θ (T−). In *C*, the raw LFPs are printed in black and the θ-band (4–8 Hz) filtered signal in gray. Arrowheads indicate the onset of the 200-msec tone used as a conditioned stimulus and the onset of the 100-msec air puff toward the eye used as an unconditioned stimulus.

studied whether the phase of the θ oscillation at stimulus onset might have effects on learning about that stimulus. To this end, the rabbits were trained for 10 sessions in trace eyeblink classical conditioning, with the conditioned stimulus (CS, the same tone as before) timed to start at either the peak (Peak group) or trough (Trough group) of the fissure θ cycle (see Fig. 1C). Yoked control (YC) animals were trained simultaneously and received trials irrespective of their neural state. We expected higher phase-locking of hippocampal θ-band responses to the CS in the Trough compared with the Peak group, and better learning in the Trough group compared with both the Peak and the YC groups. We then conditioned all the rabbits for another six sessions to maximize the number of animals that learned. Now, animals previously trained contingent on θ phase were trained in the explicit absence of θ (T−) because in our previous study (Nokia and Wikgren 2014) it led to a greater proportion of animals learning compared with random presentation of training trials. Animals in the YC group continued to be trained irrespective of their neural state. We expected animals in the experimental groups (Peak and Trough) to learn the task during the T− training, if they had not already done so. Last, to study the effects of θ phase on memory retrieval, we presented conditioning trials to well-learned animals: A total of two sessions were conducted, each with 30 trials to the peak and 30 to the trough of θ, in random order. Better memory retrieval, that is, a higher number of learned responses, was expected when the CS was presented to the peak of the fissure θ cycle compared with when it was presented to the trough of the θ cycle.

Results

Hippocampal θ activity was recorded from near the fissure

In all animals, recording electrodes (32-channel linear probes or single wire electrodes) were inserted into the dorsal hippocampus (Fig. 1B). The data from animals implanted with single electrodes were combined with the data from the animals implanted with linear probes, as, to time the presentation of stimuli, the oscillatory state in the hippocampal fissure was monitored in each animal by the signal from only one electrode or probe recording site. The source of the signal was determined online based on the known phase reversal of hippocampal θ between the CA1 and the dentate gyrus, the variation in θ amplitude along the hippocampal cell layers, and the occurrence of ripples (Chrobak and Buzsáki 1996) in the CA1 region and in the CA3 region. The recording site/electrode with maximal θ amplitude in a given animal was selected to trigger trials based on the phase of θ oscillation (see Fig. 1C). The signal from the same single recording site/electrode per animal was also used in analyzing hippocampal responses to the CS.

The results of off-line analyses of the θ amplitude and phase variation from a representative animal implanted with a 32-channel linear probe are depicted in Figure 2, panels C,D. As in rats [see for example (Bragin et al. 1995)], θ is highest in amplitude near the hippocampal fissure and its phase reverses between the CA1 pyramidal layer and the fissure/dentate gyrus. Curiously, in rabbits, the phase of θ oscillation remains constant across the fissure, the dentate gyrus and the hilus. Note that in rats, comparison of the dentate gyrus and the hilus also shows a phase-shift in θ (Buzsáki et al. 1983).

Hippocampal θ-band responses to a neutral tone were modulated by ongoing, spontaneous θ oscillations

During a single session conducted prior to any conditioning, all the rabbits were presented with a 200-msec, 80-dB, 4-kHz tone. For the animals later assigned to the experimental groups, the tone was presented 300 times either during periods of spontaneously occurring hippocampal θ activity (T+, $n = 5$) or irrespective of neural state (random, $n = 6$). For the animals assigned to the YC group ($n = 6$) for reasons of technical difficulties in obtaining neural recordings, stimuli were presented irrespective of neural state. This session was carried out to find out if θ phase, and the neural state at large (T+ versus random), affects θ-band hippocampal responses to a neutral peripheral stimulus. By θ-band responses we refer to responses occurring at the frequency band of 4–8 Hz. Phase-locking and relative amplitude, quantified as the θ ratio (%) for these responses, is analyzed separately. For details on these measures, please see Materials and Methods.

First we analyzed the effects of the preceding neural state (T+ versus random) on the relative amplitude and phase-locking of the hippocampal fissure θ-band responses to the neutral tone

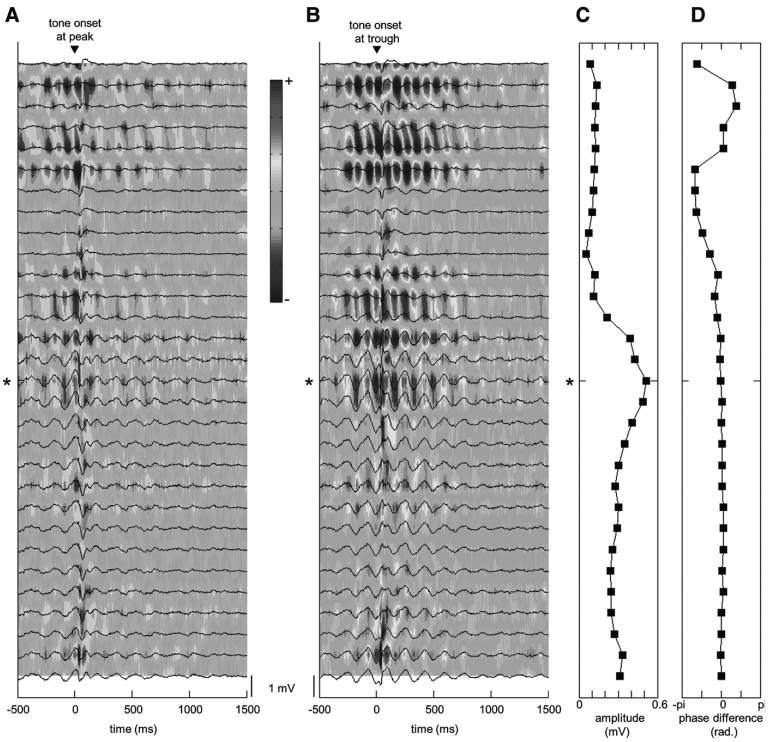

Figure 2. θ Phase modulates hippocampal responses to peripheral stimulation. In naïve animals, the conditioned stimulus (200-msec tone) alone elicited bigger and better phase-locked hippocampal θ-band responses when presented to the trough (*B*) of the hippocampal fissure θ cycle than when presented to the peak (*A*) of the cycle. The amplitude (*C*) of the θ oscillation was maximal near the hippocampal fissure. The phase (*D*) of θ oscillation reversed between the CA1 and the fissure and remained robust within the entire dentate gyrus. All data depicted are from a single representative animal. In *A* and *B*, raw (band-pass filter: 1–500 Hz) local-field potentials averaged over 60 trials are drawn in black superimposed on the corresponding current source density plot. The asterisk marks the recording point used for triggering trials, i.e., the θ channel. Red indicates current source and blue indicates current sink. In *C*, the amplitude of the θ cycle immediately preceding tone onset is plotted across the recording points. In *D*, the difference in phase of the θ cycle at tone onset compared with the phase of the θ cycle at the bottom-most recording point (in the lower blade of the dentate gyrus/hilus) is plotted across the recording points.

alone. The hippocampal θ-band responses were equally well phase-locked to the tone onset whether it was presented during θ (phase-locking value, mean ± standard error of mean: 0.20 ± 0.04) or to a random brain state (0.25 ± 0.05; one-way ANOVA: $F_{(1,9)} = 0.43$, $P = 0.531$, group data not shown in figures). The amplitude of the θ-band response to the tone was higher when it was delivered during θ (θ ratio: 86% ±2 percentage units) compared with when it was presented irrespective of neural state (73% ±4 percentage units; $F_{(1,9)} = 8.41$, $P = 0.018$). That is, θ-contingent presentation elicited stronger θ-band hippocampal responses to the tone.

Next, the effects of θ phase on hippocampal responses to the tone were examined in the five animals in which the tone was presented contingent on θ (T+). We expected to see better phase-locked and possibly also bigger hippocampal θ-band responses when tone onset overlapped with the fissure θ trough compared with when it started at θ peak. Equally strong θ-band responses were elicited during the trough (87% ±2 percentage units) and the peak of the cycle (85% ±2 percentage units; paired samples *t*-test: $t_{(4)} = 1.74$, $P = 0.156$). That is, the relative amplitude of the θ-band responses did not differ according to the phase of the ongoing θ oscillation at tone onset. However, as hypothesized, better phase-locked responses were elicited when the tone onset fell at the trough of the θ cycle (0.53 ± 0.05) compared with the peak of the θ cycle (0.29 ± 0.05). This effect was evident in four out of the five animals. Figure 2 depicts examples of event-related averages and current source density plots (please see Materials and Methods) of local-field potentials (LFPs) recorded from the hippocampus with a linear probe in response to the tone alone when it commenced at the peak (A) or the trough (B) of the fissure θ cycle in one representative animal. Visual comparison of A and B suggests better phase-locking of θ-band responses to the tone when it was presented to θ trough compared with θ peak. Although the effect was not quite statistically significant at a group level [$t_{(4)} = 2.62$, $P = 0.059$], we carried on with the experiment as planned.

Baseline responses to the tone, spontaneous θ ratios and intertrial intervals did not differ between groups

The 11 animals with functional recording electrodes were assigned to two experimental groups (Peak, $n = 6$ versus Trough, $n = 5$) for subsequent trace eyeblink conditioning. Half the animals in the Peak and Trough groups had, prior to conditioning, received tone-alone presentations to a random brain state and half during θ. There was no statistically significant difference between the Peak and Trough groups in phase-locking (0.19 ± 0.04 versus 0.28 ± 0.05) or relative amplitude (75% ±4 percentage units versus 83% ±3 percentage units, respectively) of the hippocampal fissure θ-band responses to the tone presented alone prior to conditioning ($F_{(1,9)} = 2.16$, $P = 0.175$ and $F_{(1,9)} = 2.30$, $P = 0.163$, respectively). That is, animals in both groups responded similarly to the tone before it was used as a conditioned stimulus in trace eyeblink conditioning.

Previous studies indicate that the spontaneous level of hippocampal θ activity predicts subsequent learning (Berry and Thompson 1978; Nokia et al. 2009). Thus, to ensure there was no underlying difference in baseline θ between the Peak and the Trough groups, data from a 5-min stimulus-free recording conducted immediately before the first conditioning session were analyzed. Hippocampal θ ratios representing the relative power of θ in the Peak and Trough groups were comparable (74% ±4 percentage units versus 69% ±3 percentage units, respectively; one-way ANOVA: $F_{(1,9)} = 1.03$, $P = 0.337$).

During subsequent conditioning, there were no differences between the groups in intertrial intervals (ITIs, repeated-measures [rm] ANOVA, main effect of group: $F_{(2,14)} = 1.45$, $P = 0.267$; interaction of group (3) and conditioning block (5): $F_{(8,56)} = 0.67$, $P = 0.718$). However, the ITI increased across the first five blocks (i.e., 10 sessions) of conditioning in all groups ($F_{(4,56)} = 6.36$, $P < 0.001$). Mean ITI was 50.4 ± 0.9 sec during the first conditioning

session and 57.6 ± 1.7 sec during the tenth conditioning session. This change was not part of the experimental design but it was expected in light of our previous observations indicating non-θ (T−) periods occur more often as conditioning proceeds (Nokia and Wikgren 2014). In other words, periods of prominent spontaneous θ activity become less frequent across conditioning as the animal presumably becomes less attentive toward the, now familiar, situation. Further analyses indicated that no changes across training blocks or differences between groups in ITIs occurred during the six sessions (three blocks) of T− conditioning (rm ANOVA: main effect of block: $F_{(2,26)} = 2.11$, $P = 0.142$, interaction of block and group: $F_{(4,26)} = 0.16$, $P = 0.955$ and main effect of group: $F_{(2,13)} = 0.52$, $P = 0.607$). The average ITI during this latter training phase was 53.2 ± 2.6 sec.

All in all, these analyses indicate no differences in baseline responding to the tone before it was used as the CS and no differences in either the level of spontaneous hippocampal θ activity or ITIs between the groups. This implies that any differences or changes across conditioning in hippocampal responding to the tone-CS or learning can be interpreted to be a result of our experimental manipulations. Note that as the tone elicited larger θ-band responses in animals in which it was presented contingent on θ (T+ versus random), this tone-alone treatment type was included as a covariate in the repeated-measures ANOVAs when examining differences between the experimental groups and changes across conditioning.

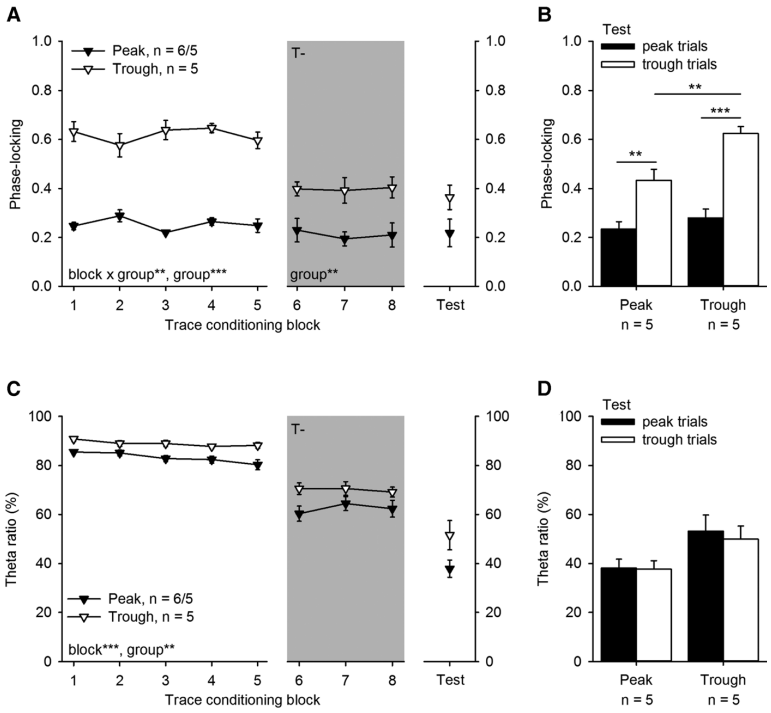

Figure 3. Trace eyeblink conditioning contingent on the phase of the hippocampal fissure θ cycle affects both the phase-locking (*A,B*) and relative amplitude (θ ratio %, *C,D*) of hippocampal fissure θ-band (4–8 Hz) responses to the conditioned stimulus (CS). (*A*) Hippocampal θ-band responses to the CS were highly phase-locked if the CS was presented to the trough of the fissure θ cycle. In contrast, phase-locking remained low throughout training if the CS was presented to the peak of the θ cycle during the first 10 sessions of conditioning (i.e., the first five blocks). Note that during T− training (gray background) animals previously trained contingent on θ trough or peak were all trained contingent on the absence of θ. Also note that in the Peak group ($n = 6$), one animal was dropped after the initial five blocks of θ phase contingent conditioning due to breaking of the linear probe. Hence the number of animals in this group that proceeded to T− conditioning and the test phase is 5. Asterisks refer to the results of repeated-measures (rm) ANOVA indicating an interaction of block and group (**) $P < 0.01$ and a main effect of group (***) $P < 0.001$. (*B*) Presenting the CS contingent on the θ cycle trough to well-trained animals (Test) resulted in higher phase-locking of fissure θ-band responses. However, phase-locking remained higher in the group initially trained contingent on the θ trough (Trough group). Asterisks refer to results of paired samples *t*-tests (peak versus trough trials) and one-way ANOVA (Peak versus Trough groups). (*C*) The relative amplitude of the hippocampal fissure θ-band responses (θ ratio %) to the CS was higher in the Trough than Peak group and consistently decreased across conditioning. Again, asterisks refer to repeated-measures ANOVA indicating significant main effects of block and group. (*D*) Presenting the CS to the trough or the peak of the θ cycle in well-trained animals had no effect on the relative amplitude of the hippocampal θ-band response. In all subplots vertical lines depict standard error of mean.

Fissure θ trough-contingent conditioning enhanced hippocampal θ-band responses to the CS while peak-contingent training impaired learning

Our main aim in this experiment was to study whether the phase of θ oscillation at stimulus onset might have effects on learning about that stimulus. To this end, rabbits were trained for 10 sessions in trace eyeblink classical conditioning (see Materials and Methods), with the tone-CS timed to start either at the peak (Peak group) or the trough (Trough group) of the fissure θ cycle. The YC animals were trained simultaneously and received trials irrespective of their neural state. The phase-locking and relative amplitude of the hippocampal fissure θ-band responses to the CS as a function of the trace eyeblink conditioning are presented in Figure 3A–D.

Our hypothesis was that the hippocampal responses would be better phase-locked to the onset of the CS and possibly also higher in amplitude in the Trough group than in the Peak group. Both expectations were realized. First, phase-locking of hippocampal θ-band responses to the CS was greater in the Trough group compared with the Peak group (rm ANOVA, main effect of block: $F_{(4,32)} = 0.93$, $P = 0.457$; interaction of block and tone-alone treatment type: $F_{(4,32)} = 0.93$, $P = 0.460$; interaction of block and group: $F_{(4,32)} = 4.31$, $P = 0.007$, $\eta_p^2 = 0.35$; main effect of tone-alone treatment type: $F_{(1,8)} = 0.42$, $P = 0.536$; main effect of group: $F_{(1,8)} = 110.35$, $P < 0.001$, $\eta_p^2 = 0.93$) (see Fig. 3A, left-most panel). Phase-locking remained stable across conditioning in both groups (Peak: $F_{(4,20)} = 2.33$, $P = 0.091$; Trough: $F_{(4,16)} = 2.84$, $P = 0.059$). Second, the relative amplitude of the hippocampal θ-band responses to the CS was greater in the Trough group compared with the Peak group (main effect of group: $F_{(1,8)} = 11.46$, $P = 0.010$, $\eta_p^2 = 0.59$) (see Fig. 3C, left-most panel). In addition, the relative amplitude of the responses decreased as a

function of conditioning in both groups (main effect of block: $F_{(4,32)} = 6.85$, $P < 0.001$, $\eta_p^2 = 0.46$; interaction of block and group: $F_{(4,32)} = 1.96$, $P = 0.124$; interaction of tone-alone treatment type and block: $F_{(4,32)} = 1.32$, $P = 0.285$; main effect of tone-alone treatment type: $F_{(1,8)} = 0.03$, $P = 0.873$).

Intrigued by the high level of phase-locking in the Trough group we also ran some analyses comparing our current data to results obtained in our previously published studies: In fact, the hippocampal responses elicited in the Trough group were more regular than those we have previously observed (Nokia and Wikgren 2014), either when the CS was presented contingent on hippocampal θ [T+, independent samples *t*-test on phase-locking of θ-band responses to the CS elicited during the first conditioning block: $t_{(14)} = 9.93$, $P < 0.001$], contingent on the absence of θ [T−, $t_{(13)} = 5.21$, $P < 0.001$] or irrespective of ongoing neural state [random, $t_{(11)} = 7.85$, $P < 0.001$]. In contrast, the responses to the tone-CS presented at the θ peak were markedly less phase-locked and smaller in amplitude, and did not differ from those elicited in any of the groups in our earlier study (see [Nokia and Wikgren 2014], comparison to T+: $t_{(15)} = 1.30$, $P = 0.212$; comparison to T−: $t_{(14)} = 0.25$, $P = 0.804$; comparison to random: $t_{(12)} = 0.58$, $P = 0.576$).

In sum, as when presented alone, the tone presented as a CS during trace eyeblink conditioning elicited better phase-locked hippocampal θ-band responses when it was timed to start at the trough of the spontaneously occurring θ oscillation than when it was timed to start at the θ peak (or irrespective of ongoing neural state, in the absence of θ or randomly during θ, [Nokia and Wikgren 2014]). This effect was consistent across conditioning. In addition, the CS also elicited larger amplitude hippocampal θ-band responses when it was presented to the trough compared with when it was presented at the peak. The amplitude of the θ-band responses decreased across conditioning. This attenuation took place irrespective of the timing of the CS in relation to θ phase.

These results above suggest that θ phase-contingent conditioning might also affect learning at the behavioral level. Our hypothesis was that animals trained at the trough of hippocampal fissure θ oscillation would acquire trace eyeblink conditioning at the behavioral level faster/better than animals trained at the peak of oscillation or irrespective of their neural state. The behavioral results of the experiment are depicted in Figure 4A-C. Learned responding increased across the first five blocks of conditioning in all groups (rm ANOVA, main effect of block: $F_{(4,52)} = 19.00$, $P < 0.001$, $\eta_p^2 = 0.59$; interactions of block and tone-alone treatment type and block and group: $F_{(4,52)} = 2.20$, $P = 0.132$ and $F_{(8,52)} = 0.98$, $P = 0.436$, respectively; main effect of tone-alone treatment type: $F_{(1,13)} = 1.68$, $P = 0.218$) (see Fig. 4A, left-most panel). However, the animals in the Peak group elicited significantly fewer conditioned responses (CRs) across the conditioning sessions compared with those in the YC and Trough groups (main effect of group: $F_{(2,13)} = 5.33$, $P = 0.020$, $\eta_p^2 = 0.45$; pairwise Bonferroni-corrected comparisons: Peak versus Trough: $P = 0.053$, Peak versus YC: $P = 0.063$, Trough versus YC: $P = 1.000$). Analysis of the cumulative percentage of CRs at the end of the first 10 sessions of conditioning confirmed this observation (one-way ANOVA: $F_{(2,14)} = 6.08$, $P = 0.013$; Bonferroni-corrected pairwise comparisons: Peak versus Trough: $P = 0.096$, Peak versus YC: $P = 0.014$, Trough versus YC: $P = 1.000$) (Fig. 4B). The animals in the YC group made a conditioned response (CR) in 31% (±0.03 percentage units) of the trials compared with 27% (±0.02) in the Trough and only 16% (±0.04) in the Peak group. Of the YC animals five out of six (83%) reached the learning criterion of 60% CRs within the first 10 sessions of θ-contingent conditioning. In contrast, in the Peak group only 1 out of 6 (17%) and in the Trough group only two out of five (40%) animals

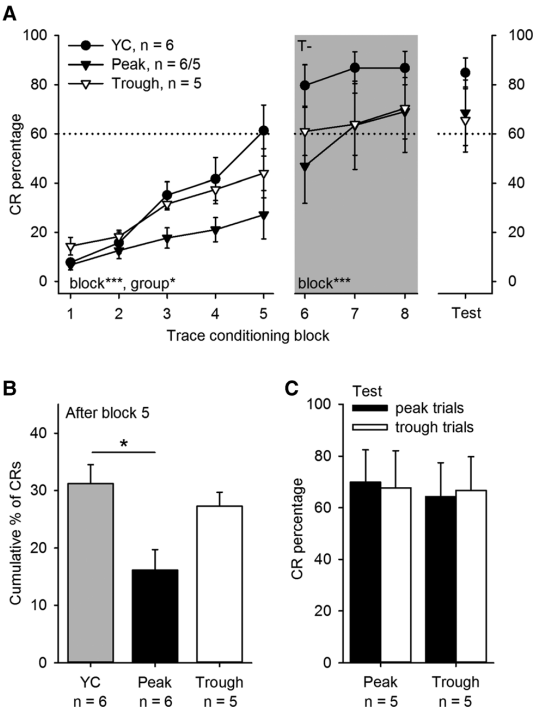

Figure 4. Trace eyeblink conditioning was retarded if the conditioning trials were consistently presented to the peak of hippocampal fissure θ (*A,B*). In well-trained animals, presenting the conditioning trials contingent on hippocampal θ phase had no effect on conditioned responding (*C*). (*A*) Conditioned response (CR) percentage across the different phases of the experiment. Gray background indicates conditioning contingent on the absence of θ (T−). Note that during T− training animals previously trained contingent on θ trough or peak were all trained contingent on the absence of θ. For animals in the yoked control group, training was always random in relation to their neural state. Also note that in the Peak group ($n = 6$), one animal was dropped after the initial five blocks of θ phase contingent conditioning due to breaking of the linear probe. Hence the number of animals in this group that proceeded to T− conditioning and the test phase is 5. Asterisks refer to the results of repeated-measures ANOVA indicating that conditioned responding increased in all groups across the conditioning blocks (***) $P < 0.001$ and that there was a difference between groups during the first five conditioning blocks $P < 0.05$. (*B*) The cumulative percentage of CRs in the Peak group was smaller than that in the YC group after the first five conditioning blocks. (*C*) In well-trained animals, conditioned responding was comparable whether the CS onset was timed to the trough or the peak of fissure θ. Vertical lines depict standard error of mean.

reached criterion within the first 10 conditioning sessions. According to the binomial test, learning was significantly retarded in both the Peak and the Trough groups compared with YC: Peak: $P = 0.001$; Trough: $P = 0.035$. The YC animals performed the fifth CR after an average of 72 ± 9 paired trials whereas those in the Peak group needed 107 ± 31 trials and those in the Trough group 48 ± 19 trials to accomplish the same ($F_{(2,14)} = 1.83$, $P = 0.197$).

To conclude, the acquisition of a behavioral learned response was retarded by θ-contingent conditioning, when the CS was timed to start at the peak of the θ cycle. Despite enhancing hippocampal θ-band responding to the CS, presenting conditioning trials to the trough of fissure θ had little effect on the acquisition of a behavioral CR.

Continued conditioning in the absence of θ (T−) led to attenuated and less phase-locked hippocampal θ-band responses while learned responding was further increased

To maximize the number of animals that learned, we trained all the rabbits for another six sessions. Now, the animals previously trained contingent on θ phase (Peak and Trough groups) were trained in the explicit absence of θ (T−) to maximize the number of animals that learned (Nokia and Wikgren 2014). The animals in the YC group continued to be trained irrespective of their neural state. In one animal initially assigned to the Peak group, the recording electrode broke during the night between sessions 13 and 14 and hence the animal was excluded from further training and analyses.

Our hypothesis was that T− conditioning would not change the hippocampal θ-band responses to the CS considerably. If anything, we expected to see a decrease in phase-locking in the animals previously trained at the trough of θ. First, possible changes in the θ-band response amplitudes and phase-locking as a result of switching from θ phase-contingent to T− conditioning was studied in both groups (Peak and Trough) separately. Note that in Figure 3, data are presented compressed into blocks of two sessions; for this analysis, however, a paired-samples *t*-test was conducted on measures obtained during the last θ phase-contingent session (session 10) and the first T− session (session 11). In the Peak group, the phase-locking of the hippocampal fissure θ-band responses to the CS remained the same, even when it was timed to start in the absence of θ [$t_{(5)} = 0.14$, $P = 0.897$], and the relative amplitude of the response decreased significantly [$t_{(5)} = 7.01$, $P = 0.001$] (see Figs. 3A,C, respectively). In the Trough group, both the phase-locking as well as amplitude of the hippocampal θ-band responses decreased significantly when the conditioning protocol was switched from θ trough-contingent to T− [$t_{(4)} = 7.36$, $P = 0.002$ and $t_{(4)} = 13.51$, $P < 0.001$, respectively]. To summarize, switching from θ phase-contingent to T− conditioning attenuated hippocampal θ-band responses to the CS. In addition, only in animals previously conditioned at θ trough, also the phase-locking of the responses to the CS decreased when training was switched to T−.

Next, we studied whether hippocampal θ-band responses changed across the T− conditioning (three blocks of two sessions) and whether the differences between groups in hippocampal responding (Peak versus Trough) were still evident even though conditioning was now carried out in an identical way, that is, in the explicit absence of θ. The CS continued to elicit better phase-locked responses in the animals previously trained at θ trough compared with those previously trained at θ peak also during the T− conditioning (rm ANOVA, main effect of group: $F_{(1,7)} = 15.35$, $P = 0.006$, $\eta_p^2 = 0.69$; main effect of block: $F_{(2,14)} = 0.12$, $P = 0.884$; interaction of block and tone-alone treatment type: $F_{(2,14)} = 1.67$, $P = 0.223$; interaction of block and group: $F_{(2,14)} = 0.74$, $P = 0.494$; main effect of tone-alone treatment: $F_{(1,7)} = 2.64$, $P = 0.148$) (see Fig. 3A middle panel, gray background). However, there were no significant differences between groups or changes across the T− conditioning in the relative amplitude of the θ-band responses to the CS (rm ANOVA, main effect of group: $F_{(1,7)} = 4.30$, $P = 0.077$, main effect of block: $F_{(2,14)} = 1.69$, $P = 0.220$; interaction of block and tone-alone treatment type: $F_{(2,14)} = 0.22$, $P = 0.805$; interaction of block and group: $F_{(2,14)} = 2.59$, $P = 0.110$; main effect of tone-alone treatment: $F_{(1,7)} = 0.57$, $P = 0.474$) (see Fig. 3C middle panel, gray background). To summarize, during T− conditioning, phase-locking of hippocampal θ-band responses to the CS diminished yet continued to be higher in animals previously trained at θ trough compared with those previously trained at θ peak. Hippocampal θ-band response amplitude was diminished overall compared with θ phase-contingent conditioning and a statistically significant difference between the previous Peak and Trough groups was no longer evident.

In terms of learning trace eyeblink conditioning, we expected animals in the experimental groups (Peak and Trough) to learn the task during the T− training, if they had not already done so during the first 10 conditioning sessions conducted contingent on θ. We used T− training because in our previous study (Nokia and Wikgren 2014) it led to a greater proportion of animals learning compared with random presentation of training trials (note that T+ conditioning had no such effect). Conditioned responding continued to increase in all groups during the second phase of training (rm ANOVA: main effect of block (3): $F_{(2,24)} = 12.71$, $P = 0.001$, $\eta_p^2 = 0.51$; interaction of group (3) and block: $F_{(2,24)} = 3.09$, $P = 0.062$; interaction of tone-alone treatment type and block: $F_{(2,24)} = 0.49$, $P = 0.544$; main effect of group: $F_{(2,12)} = 0.44$, $P = 0.655$; main effect of tone-alone treatment type: $F_{(1,12)} = 1.85$, $P = 0.199$) (see Fig. 4A middle panel, gray background). At the end of the T− conditioning, the cumulative percentage of CRs was 50% in the YC group compared with 32% in the Peak and 40% in the Trough group ($F_{(2,13)} = 2.72$, $P = 0.103$). By the end of 16 d of conditioning, all (100%) of the YC animals, three out of five (60%) animals previously trained at θ peak and 4 out of 5 (80%) animals previously trained at θ trough reached the learning criterion of 60% CRs during at least one session. The binomial test could not be run because all the animals in the YC group learned. Of the animals that eventually learned, those in the YC group reached the traditional learning criterion of eight CRs in nine consecutive trials after an average of 401 ± 43 trials whereas those first trained at θ peak and then in the absence of θ required 547 ± 54 trials and those first trained at θ trough and then in the absence of θ 456 ± 71 trials to perform at the same level ($F_{(2,10)} = 1.62$, $P = 0.246$). To conclude, after T− conditioning the learning outcome in the Peak and Trough groups was similar to that of the YC group.

High-amplitude, well phase-locked hippocampal fissure θ-band responses to the conditioned stimulus early in training predicted better learning of trace eyeblink conditioning

Previous studies indicate a correlation between hippocampal function and learning only, or at least especially, early in training (Nokia et al. 2009). To study the link between hippocampal θ-band responses and learning, we calculated correlations between the relative amplitude/phase-locking of hippocampal θ-band responses to the CS during the first conditioning session and the percentage of CRs elicited during conditioning (blocks 1–8). For this, animals from both the Peak and the Trough groups were pooled. A high θ ratio in response to the CS during the first conditioning session predicted a higher CR percentage during conditioning blocks 3–6 ($r = 0.656-0.694$, $P = 0.018-0.028$, $n = 11$). The correlations between the θ ratio and conditioned responding during blocks 1–2 and 7–8 were not statistically significant ($r = 0.332-0.495$, $P = 0.146-0.318$, $n = 11/10$). In addition, highly phase-locked responding during the very first conditioning session predicted a higher percentage of CRs during conditioning blocks 3 and 4 ($r = 0.760/0.693$, $P = 0.007/0.018$, $n = 11$, respectively). There was also a close to significant correlation with conditioned responding during block 5 ($r = 0.553$, $P = 0.078$, $n = 11$). Correlations with CR percentage during blocks 1, 2, and 6–8 were not significant ($r = 0.137-0.466$, $P = 0.149-0.706$, $n = 11$). To summarize, high-amplitude, temporally uniform hippocampal θ-band responses to the CS at the start of conditioning anticipated good learning. Hippocampal responding early in training was

correlated to conditioned responding specifically in the middle of training, when the conditioned response is being acquired and its performance rate starts to rise rapidly (Prokasy 1984).

θ phase at CS onset did not affect conditioned responding in well-trained animals

After 16 sessions of conditioning, two more sessions were carried out, now reverting back to the procedure of presenting the trials contingent on hippocampal θ phase for animals in the Peak and Trough groups. However, now half the trials were presented to the trough and half to the peak of the θ cycle recorded from the hippocampal fissure, in a random order. Animals in the YC group remained to be conditioned irrespective of their neural state. Our hypothesis was that a conditioned response would be more likely to occur in response to a CS presented at θ peak compared with when it was presented at θ trough. This would have indicated superior memory retrieval at θ peak (Hasselmo et al. 2002).

The CS continued to elicit better phase locked θ-band responses from the hippocampal fissure if it was timed to start at the trough of the θ cycle compared with the peak of the θ cycle (rm ANOVA with group [Peak versus Trough] as a between-subjects factor: main effect of trial type [peak versus trough]: $F_{(1,8)} = 109.83$, $P = 0.001$, $\eta_p^2 = 0.93$). The analysis also revealed a significant interaction of trial type and group ($F_{(1,8)} = 7.83$, $P = 0.023$, $\eta_p^2 = 0.50$) and a main effect of group ($F_{(1,8)} = 7.43$, $P = 0.026$, $\eta_p^2 = 0.48$) (see Fig. 3B). Due to the interaction, groups were further analyzed separately using paired samples *t*-test. This confirmed that the CS elicited better phase-locked θ-band responses in both groups when presented to the trough of θ during the test sessions (Peak: $t_{(4)} = 4.93$, $P = 0.008$; Trough: $t_{(4)} = 10.58$, $P < 0.001$; see Fig. 3B). However, the CS also still elicited better phase-locked responses in animals initially trained at θ trough compared with animals initially trained at θ peak, when it was presented to the trough of the θ cycle (one-way ANOVA: $F_{(1,8)} = 12.98$, $P = 0.007$). The phase-locking of responses to the CS was similar in both groups when it was timed to commence at the peak of the fissure θ cycle ($F_{(1,8)} = 0.93$, $P = 0.363$). Also, there were no statistically significant differences between groups (Peak versus Trough group, trials presented at the peak/trough: $F_{(1,8)} = 3.94/3.75$, $P = 0.083/0.089$) or effects of θ phase (trials presented to peak versus trough, Peak group: $t_{(4)} = 0.06$, $P = 0.578$; trials presented to peak versus trough, Trough group: $t_{(4)} = 1.95$, $P = 0.123$) on the relative amplitude of the hippocampal fissure θ-band responses (see Fig. 3D). However, the relative amplitude of the θ-band responses elicited in response to the CS in the hippocampal fissure attenuated in animals initially trained at the peak of θ when training was switched back from T− to θ phase-contingent (last T− session versus first test session, paired samples *t*-test: $t_{(4)} = 5.44$, $P = 0.006$). The same was not true for the Trough group ($t_{(4)} = 1.96$, $P = 0.122$). Overall, conditioned responding during the two test sessions remained at the same level compared with the last T− conditioning block (paired samples *t*-tests: YC: $t_{(5)} = 0.81$, $P = 0.457$; Peak: $t_{(4)} = 0.01$, $P = 0.990$; Trough: $t_{(4)} = 2.09$, $P = 0.105$; see Fig. 4A, right-most panel). Last but most important, behavioral responding did not differ between the peak and trough trials (paired samples *t*-tests: Peak group: $t_{(4)} = 0.48$, $P = 0.654$; Trough group: $t_{(4)} = 0.69$, $P = 0.526$; see Fig. 4C).

To summarize, θ phase continued to modulate hippocampal responses to the CS even after extensive training. In addition, animals initially trained at θ trough continued to exhibit better phase-locked hippocampal θ-band responses to the CS compared with animals initially trained at θ peak. This effect was limited to trials during which the CS commenced at θ trough. Most important, despite these clear effects on hippocampal responding, θ phase had no effect on memory retrieval at the behavioral level.

Discussion

Hasselmo and colleagues (Hasselmo et al. 2002; Hasselmo and Stern 2014) have proposed that the function of hippocampal θ oscillations in learning and memory is to enable separate time windows for the encoding and retrieval of memories by regulating information flow within the hippocampo-entorhinal loop. Inspired by this idea, we studied whether the phase of hippocampal θ oscillation at stimulus onset has an effect on hippocampal responding to that stimulus and whether this affects learning about that stimulus in the context of classical eyeblink conditioning in rabbits. Our results indicate that the most regular hippocampal responses occurred to stimuli presented at the trough of fissure θ, whereas the responses to stimuli presented at the peak of fissure θ were less organized. Overall, high-amplitude, temporally regular hippocampal θ-band responses early in training predicted better learning. Presenting the CS at θ peak retarded learning whereas the acquisition of a learned response was little affected in the Trough group, despite the enhanced hippocampal θ-band responses to the CS. Across continued conditioning and in well-trained animals, the association between hippocampal responses to the CS and behavioral learned responses vanished, suggesting that the role for hippocampal function in learning is time-limited. In sum, θ phase predetermines both hippocampal responding to peripheral stimuli and learning about those stimuli indicating that it is involved in regulating processing of information in the brain up to a behaviorally relevant degree.

According to the computational model by Hasselmo and colleagues (Hasselmo et al. 2002; Hasselmo and Stern 2014), the phase of hippocampal θ oscillation determines whether the hippocampus preferentially processes input from the entorhinal cortex, leading to memory encoding, or from the CA3 autoassociative network, leading to memory retrieval. To a certain degree, our results comply with the model: a brief auditory stimulus evoked large and temporally well-organized responses at the θ-band within the hippocampus when timed to start specifically at the trough of a θ cycle recorded from the hippocampal fissure. In fact, the hippocampal responses elicited were more regular than those we have previously observed (Nokia and Wikgren 2014), either when the CS was presented contingent on hippocampal θ or irrespective of ongoing neural state. In contrast, the responses to the tone-CS presented at the θ peak were markedly less phase-locked and smaller in amplitude, and did not differ from those elicited in any of the groups in our earlier study (Nokia and Wikgren 2014). The high phase-locking of θ-band responses in the Trough group resembles the θ reset observed in the dentate gyri of rats performing a working memory task involving both visual and auditory peripheral stimuli (Givens 1996). It has been demonstrated in rats that LTP is preferentially induced if stimulation is timed to occur during the peak of the CA1 θ (trough of fissure θ) not only when the oscillation occurs spontaneously (Holscher et al. 1997) but also when it is evoked and reset by a peripheral stimulus (McCartney et al. 2004). Thus, phase-locking or resetting of hippocampal θ oscillation to the stimulus onset creates windows of opportunity for enhanced processing of subsequent relevant stimuli. Together with these earlier findings our current results imply that hippocampal processing of related peripheral stimuli occurring close in time (within 500 msec to 1 sec of each other) could be brought to or near the maximum by presenting the stimuli consistently at the hippocampal fissure θ trough. In further studies, conditioning should be conducted in a manner where both the CS and the unconditioned stimulus are presented consistently to the trough/peak of the hippocampal θ oscillation. One would expect superior learning if both stimuli occur at fissure θ trough.

Consistent with the theory by Hasselmo et al. (2002), compared with a yoked control group, learning was severely retarded

when the conditioned stimulus was presented at the peak of fissure θ. Learning was also not improved in the group trained at the fissure θ trough. That is, even though the conditioned stimulus evoked unusually regular responding in the hippocampus, and even though high regularity, once again (Nokia and Wikgren 2014), predicted good learning in general, it was not enough to improve the acquisition of the behavioral learned response. This suggests the existence of a ceiling effect where hippocampal responding seems to support learning to a limited degree only, no matter how regular or efficient it is. In fact, in healthy young animals learning seems to be most efficient when the to-be learned associations are presented randomly or in the absence of hippocampal θ and with a long enough ITI (Nokia and Wikgren 2014). Our current and earlier results are at odds with some previous findings on enhanced learning and neural responses following θ-contingent trace eyeblink conditioning in young healthy animals (Griffin et al. 2004; Darling et al. 2011). These discrepancies in results might be explained by differences in, for example, the algorithm for detecting θ or the duration of the CS which here was 200 msec in comparison to 100 msec used in the studies cited above. It might be that the longer duration of the CS used here made its presentation less specific to a certain phase of the θ oscillation as the duration of a single full cycle of a 6 Hz oscillation is <200 msec.

In addition to the effects of θ phase on the encoding of incoming information we also studied its effects on the retrieval of already established memories. According to the theory of Hasselmo and colleagues (2002, 2014), during the peak of the θ cycle recorded near the hippocampal fissure, entorhinal input is suppressed and the CA3 autoassociative network activated, which supports the retrieval of already encoded information. However, in the present study, there was no effect of θ phase on memory retrieval in well-learned animals. Although hippocampal responses to the CS were, once again, temporally well-organized when presented to the trough of the θ cycle and less organized when presented to the peak, conditioned responding at the behavioral level did not differ according to the timing of the CS relative to the fissure θ phase. Thus, efficient hippocampal processing seems to be crucial only in the very early stage of learning, when the CS–US association is first realized and CRs start to emerge at an increasing rate (Prokasy 1984). It would seem that the hippocampus has only a modulatory, if any, role in the late stages of CR acquisition or in the retention of the CR, leaving other brain structures such as the prefrontal cortex (Weiss and Disterhoft 2011) and/or the cerebellum (Gould and Steinmetz 1996) in charge. To better pinpoint the timing of hippocampal dependence of learning, the effects of θ phase-contingent stimulus presentation on memory retrieval should be tested earlier in the conditioning process, ideally at several different intervals from the onset of conditioning, in subjects otherwise trained irrespective of their neural state. It is also noteworthy that the theory by Hasselmo and colleagues (2002, 2014) was developed to explain mechanisms of memory retrieval and encoding during episodic memory modeled by spatial navigation tasks in rodents. Although also a form of episodic learning, eyeblink classical conditioning in rabbits might be governed by slightly different rules, which might explain the contradictions between our current results and the theory by Hasselmo and colleagues (2002); Hasselmo and Stern (2014).

In conclusion, to our knowledge, this is the first study to demonstrate that the timing of a peripheral stimulus to a certain phase of hippocampal θ oscillation produces robust effects on the synchronization of neural responses and affects learning at the behavioral level. That is, the phase of the hippocampal θ oscillation seems to be a means of regulating the processing of information in the brain up to a behaviorally relevant degree. Our results also support the notion of a time- and otherwise limited role for hippocampal processing in associative learning: high-amplitude, phase-locked hippocampal responses to the CS seem crucial only for the initial stage of classical conditioning, i.e., associating the CS with the unconditioned stimulus, and enhancing these responses does not automatically lead to better learning.

Materials and Methods

Subjects

The subjects were 17 adult female New Zealand White rabbits (Lidköpings kaninfarm, Sweden) weighing ∼3.3 kg at the time of surgery. The rabbits were housed in individual cages on the premises of the animal research unit 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 a.m. All procedures were conducted during the light portion of the cycle. All the experimental procedures were implemented in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the care and use of animals for research purposes.

Surgery

Subcutaneous injections of an analgesic solution (0.1 mL of 0.3 mg/mL buprenorphine [Temgesic, Schering-Plough Europe] diluted in 0.9 mL of 0.9% NaCl, dose: 2 mL) and of an anti-inflammatory drug (50 mg/mL carprofen [Rimadyl vet, Pfizer Inc. Animal Health], dose: 0.1 mL/kg) were administered >30 min prior to surgery. The rabbits were anesthetized with an i.m. injection of ketamine–xylazine cocktail (7.8 mL of 50 mg/mL Ketaminol vet [Intervet International B.V.] mixed with 2.8 mL of 20 mg/mL Narcoxyl vet [Intervet International B.V.]). A dose of 0.8 mL/kg of the cocktail was injected i.m. before surgery. During surgery, additional doses of either the cocktail or ketamine alone were injected subcutaneously approximately every 20–30 min or as needed. At the beginning of surgery, the rabbit was placed in a stereotaxic instrument (Kopf Instruments) with the bregma positioned 1.5 mm higher than the lambda. Eye gel was administered to prevent the animal's eyes from drying. A longitudinal incision was made to the scalp and four stainless-steel anchoring screws (5 mm anterior and 5 mm lateral to the bregma; 13 mm posterior and 5 mm lateral to the bregma) were attached to the skull. The screws were connected together and served as a reference for the electrophysiological recordings. Next, a piece of skull was removed above the left hippocampus and the dura cut to expose the surface of the brain. A 32-channel linear probe (A1×32–10 mm-100–177, H32 package, NeuroNexus) attached to a microdrive (nDrive xL, NeuroNexus) was chronically implanted into the dorsal hippocampus 5 mm posterior, 5.5 mm lateral, and ∼4 mm below the surface of the brain, aiming the tip of the probe at the dentate gyrus. The opening in the skull was sealed using Kwik-Sil (World Precision Instruments) silicone. Last, the probe and microdrive were cemented in place with dental acrylic. For three animals, instead of the linear probe, six monopolar recording electrodes made of Teflon-insulated stainless steel wire (bare diameter 125 μm) mounted inside 27-gauge hypodermic stainless steel tubing were chronically implanted into the left dorsal hippocampus 5–6 mm posterior, 4–7 mm lateral, and 6.5–7.4 mm below the bregma, aiming at the hippocampal fissure and dentate gyrus. The data from these animals were combined with the data from the animals implanted with linear probes. Metoclopramide (dose 0.1 mL/kg, concentration 5 mg/mL; Primperan [Sanofi Winthrop Industrie]) was injected subcutaneously immediately after surgery to facilitate normal feeding and drinking. Analgesic (buprenorphine, see above for details) was administered every 8 h for the next 24–48 h depending on the recovery rate of the animal.

Conditioning procedure

During the recordings, the rabbits were placed in a Plexiglas restraining box allowing free movement of the head and located

in a ventilated, electrically insulated, and sound-attenuated conditioning chamber. A fan located inside the conditioning chamber behind the rabbit created a steady background noise of ~65 dB. At least 1 wk was allowed for post-surgical recovery before commencing the experiment. During the recovery period, the animals were habituated to the recording chamber and neural signals recorded briefly (10–20 min) to adjust the position of the linear probe, using the microdrive. The probe was positioned so that CA1 ripples, θ and CA3 ripples were visible.

LabView (National Instruments) and E-Prime software (Psychology Software Tools Inc.) were used to control the presentation of the stimuli. Briefly, the signal from one electrode site near the hippocampal fissure was monitored online in 1-s sweeps refreshed every 50 msec. For each sweep, the Fast Fourier Transform was calculated with a resolution of 1 Hz. The θ ratio (%) was calculated as the ratio between the power of the signal at 5–8 Hz divided by the power of the signal at 1–12 Hz.

Tone–alone session: testing response modulation according to θ phase

To study response modulation as a function of hippocampal θ phase, before conditioning, an 80-dB, 4-kHz, 200-msec tone later to be used as the CS was presented 300 times (minimum intertrial interval (ITI) 10 s). For five animals later assigned to the experimental groups, the tone was presented during periods of dominant θ (T+) recorded from the fissure. Stimuli were delivered when the θ ratio exceeded 80%. For six animals later assigned to the experimental groups, tones were presented irrespective of their brain state, using the ITIs from the first five animals (to which the tone was presented contingent on a high θ ratio). For the animals assigned to the yoked control group, stimuli were presented irrespective of their brain state. The CS-alone treatment type (T+ versus random) was included as a covariate in the repeated-measures ANOVAs when examining differences between the experimental groups and changes across training.

θ phase–contingent trace eyeblink conditioning (Peak/Trough)

During conditioning, the CS, a 80-dB, 4-kHz, 200-msec tone, was paired with the unconditioned stimulus (US), a 100-msec air puff toward the eye. A 500-msec trace period was used, creating a 700-msec onset-to-onset interval. Sixty trials per session were conducted with a minimum ITI of 35 s. For one group of animals, the CS was timed to commence at the trough of hippocampal fissure θ (Trough, $n = 5$) and for the other group it was timed to start at the θ peak (Peak, $n = 6$). Examples of the timing of the conditioning stimuli relative to hippocampal θ are presented in Figure 1B. Grouping was random. Using LabView, the CS was timed to commence at a certain phase of the θ cycle by using a combination of the θ ratio (>80%) and simple threshold triggering: the signal had to pass through two threshold values either on its way up (rising) or on its way down (falling). Although the algorithm was very simple, and by no means perfect, the setup worked well. The instantaneous phase of the θ cycle at CS onset was −0.03 radians (SEM 0.05) in the Peak group and 3.01 (SEM 0.05) radians in the Trough group during the first conditioning session, indicating very good accuracy in timing. (Note that for the trough group, negative phase values were corrected ($x + 2\pi$) so that the distribution peak was set at around π.) A group of yoked control (YC, $n = 6$) animals were trained together with animals in the Peak ($n = 3$) and Trough ($n = 3$) groups, that is, the YC group received conditioning trials irrespective of their neural state. Animals were assigned to the YC group on the basis of broken electrodes (no signal). Ten conditioning sessions were carried out on consecutive days.

Trace eyeblink conditioning contingent on the absence of θ (T−)

After the initial training period, conditioning was continued for another six daily sessions, but now trials were only triggered when the animal in the experimental group (Peak/Trough) exhibited low θ power (T−, θ ratio <35%).

Testing the effect of θ phase on memory and learned performance

Finally, the effects of fissure θ phase on retrieval of the memory trace/performance of the conditioned response (CR) (Hasselmo et al. 2002; Hasselmo and Stern 2014) were tested. Animals were presented with 60 paired trials, of which 30 were timed to commence at the peak and 30 at the trough of fissure θ, in a random order. Another test session was performed the next day to accumulate 60 trials delivered to the peak and 60 trials delivered to the trough of θ.

Recordings and data analysis

Bipolar electromyogram (EMG) was recorded using stainless steel wire hooks placed around the right upper and lower eyelids for the duration of the training session. To acquire neural measures, a low-noise preamplifier (MPA8I or MPA32, MultiChannel Systems (MCS)) was directly attached to the electrode coupler anchored with dental acrylic to the rabbit's head. A flexible, insulated cable was used to connect the animal to the filter amplifier (FA64I, filter: 1–5000 Hz, MCS). All signals were digitized (USB-ME-64 System, MCS) and recorded with Mc_Rack software (MCS), using a 20-kHz sampling rate. Finally, all signals were digitally band-pass filtered between 1 and 500 Hz (high-pass: RC, low-pass: fourth-order Bessel) and stored using a 2-kHz sampling rate. MATLAB (The MathWorks Inc.), and SPSS (SPSS Inc.) were used for offline data analysis.

Eyeblinks

The EMG signal was high-pass filtered off-line (>100 Hz) and Hilbert-transformed. An envelope curve following the peaks of the signal was calculated. Baseline EMG activity was defined for each animal and session as the mean of the peak EMG amplitude during a 500-msec pre-CS period (MEANpre). Also determined was the mean of the standard deviation of the EMG activity during the 500-msec pre-CS period (SDpre). Eyeblinks were defined as EMG activity exceeding a threshold of [MEANpre + 4 × SDpre] for at least 10 msec. Trials with eyeblinks during the 100-msec period immediately preceding CS onset were rejected. Eyeblinks during the latter half (250-msec period) of the 500-msec trace period were counted as conditioned responses. As in our previous studies (Nokia and Wikgren 2014) on trace eyeblink conditioning, the learning criterion were considered to be met when the subject performed ≥60% CRs during at least one conditioning session. Learning rate was quantified as the cumulative percentage of CRs, the number of trials to the fifth CR and the number of trials to meet the criterion of eight CRs on nine consecutive trials, which has been used to indicate asymptotic learning (see, for example, Berry and Thompson 1978).

Relative magnitude of hippocampal θ–band (4–8 Hz) oscillations: θ ratio

θ ratio (Nokia et al. 2009) was obtained from a 5-min baseline recording conducted immediately prior to the first conditioning session. To this end, the hippocampal fissure signal was analyzed in 1500-msec sweeps using the FFT. From the FFT result, the ratio (%) of the power of the signal at the θ band (4–8 Hz) compared with that at the θ and delta bands (1–8 Hz) was calculated. To study the relative amplitude of the θ-band responses to the conditioning stimuli, the hippocampal θ ratio was calculated from 700-msec period immediately following CS onset. For statistical analyses, the θ ratio during the CS and subsequent trace period (700 msec) was averaged over sessions (correlation with learning) and then over blocks of two sessions (changes across training and differences between groups).

Phase–locking of hippocampal θ–band (4–8 Hz) responses

To assess the temporal accuracy of the θ-band responses to the conditioning stimuli, a phase-locking value (PLV) (Palva et al.

2005) was calculated. The PLV is based on amplitude-normalized phase information and is thus resistant to changes or differences in signal amplitude. This allows comparable measures to be obtained from data recorded over time in multiple subjects. The hippocampal LFP data were first band-pass filtered between 4 and 8 Hz. Then, a Hilbert transform was run on the signal to obtain the phase information, and the amplitude of the signal normalized to 1 by dividing each data point by its absolute value. Finally, the PLV was obtained by averaging over 60 trials (one session) and taking the absolute value of the mean. The PLV varies between 0 and 1, 0 indicating no phase locking and 1 indicating perfect phase locking. For statistical analyses, the mean of the PLV during the CS and subsequent trace-period (700 msec) was derived and averaged over blocks of two sessions. Note that for the analysis of the CS-alone data in the animals that received the CS contingent on θ (T+, impact of peak versus trough), 60 trials during which the CS onset was closest to the peak and 60 trials during which it was closest to the trough of the cycle were selected and PLVs calculated for these. This ensured comparability of the analyses in relation to those conducted on the data obtained during conditioning. For the comparisons between the CS-alone treatment types (T+ versus random) and those between the experimental groups-to-be (Peak versus Trough), all 300 CS-alone trials were used for calculating the PLV.

Current source density

To visualize the sources and sinks of LFPs in different hippocampal cell layers, current source density analysis (for the theoretical basis see Nicholson and Freeman [1975] and Mitzdorf [1985]) was performed on data recorded during the tone-alone session in selected animals. The result for one representative animal was visualized with MATLAB (Fig. 2) and corresponds to the unscaled second derivative of the potential as a function of recording depth (see also Bragin et al. 1995).

Statistical analyses

Repeated-measures analysis of variance (ANOVA), with training blocks of two sessions as a within-subjects factor and group as a between-subject factor, was used to analyze changes across training. Type of CS-alone treatment (random versus T+) was included as a two-level covariate (0/1). Whenever a significant interaction of block and group emerged, separate repeated-measures ANOVAs were conducted for each group, using blocks as a within-subjects factor. Greenhouse–Geisser corrected *P*-values are reported when the sphericity assumption was violated according to Mauchly's test. One-way ANOVA was used in group comparisons when single variables were tested. Bonferroni corrected *P*-values were used for post hoc comparisons. Binomial test was used to compare the proportions (%) of animals that learned in each group. Pearson's correlation coefficient was used to analyze the connection between hippocampal θ-band responses to the CS and learning. Finally, paired samples *t*-tests were used to analyze differences within subjects.

Histology

Rabbits were anesthetized with an i.m. injection of ketamine–xylazine cocktail and then overdosed with an i.v. injection of pentobarbital (Mebunat vet, Orion-Yhtymä Oyj). Next, the brain was perfused with physiological saline followed by 9% formalin solution through the ascending aorta. The locations of the electrode tips were marked by passing a DC current (200 μA, 5 sec) through them. The brain was then removed and stored in formalin for several days. The brain was coronally sectioned with a vibratome into 60-μm-thick slices. The slices were attached to gelatinized slides, dried, and stained with Prussian blue and cresyl violet. The electrode locations were determined with the help of a microscope.

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HIPPOCAMPAL THETA PHASE–CONTINGENT MEMORY RETRIEVAL IN DELAY AND TRACE EYEBLINK CONDITIONING

by

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Hippocampal theta phase–contingent memory retrieval in delay and trace eyeblink conditioning

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ABSTRACT

Hippocampal theta oscillations (3–12 Hz) play a prominent role in learning. It has been suggested that encoding and retrieval of memories are supported by different phases of the theta cycle. Our previous study on trace eyeblink conditioning in rabbits suggests that the timing of the conditioned stimulus (CS) in relation to theta phase affects encoding but not retrieval of the memory trace. Here, we directly tested the effects of hippocampal theta phase on memory retrieval in two experiments conducted on adult female New Zealand White rabbits. In Experiment 1, animals were trained in trace eyeblink conditioning followed by extinction, and memory retrieval was tested by presenting the CS at troughs and peaks of the theta cycle during different stages of learning. In Experiment 2, animals were trained in delay conditioning either contingent on a high level of theta or at a random neural state. Conditioning was then followed by extinction conducted either at a random state, contingent on theta trough or contingent on theta peak. Our current results indicate that the phase of theta at CS onset has no effect on the performance of the behavioral learned response at any stage of classical eyeblink conditioning or extinction. In addition, theta-contingent trial presentation does not improve learning during delay eyeblink conditioning. The results are consistent with our earlier findings and suggest that the theta phase alone is not sufficient to affect learning at the behavioral level. It seems that the retrieval of recently acquired memories and consequently performing a learned response is moderated by neural mechanisms other than hippocampal theta.

1. Introduction

The hippocampus is a crucial brain structure in learning [1]. According to Buzsáki's [2] two-stage model of learning and memory, the hippocampus exhibits two states related to the encoding and strengthening of memory traces. When exploring its environment and focusing attention on external stimuli, hippocampal electrophysiological activity is dominated by rhythmic slow wave activity, the theta oscillations. Theta oscillations are most prominent in the hippocampal input region, the dentate gyrus (DG) and near the hippocampal fissure [3]; for a review, see Ref. [4] and are driven by GABAergic neurons of the medial septum [5]. When the level of attention lowers and hippocampal theta activity ceases, the output region of the hippocampus, the CA1, shows activation in the form of large amplitude sharp-wave ripples. Ripples are generated when input from the entorhinal cortex (EC) via the perforant path and the DG reaches the CA3. Within the CA3 autoassociative network, recently activated neuronal assemblies then reactivate [6], and further activate the CA1 pyramidal cells. Pyramidal cells of the CA1 project the signal back to the neocortex and complete the network loop. Thus, in Buzsáki's [2] model, the role of theta oscillations is suggested to be most crucial during the encoding of new information. However, others also suggest a role for theta in the retrieval of already encoded memories [7].

Lesion studies [8,9] and pharmacological manipulations [10] indicate that the hippocampus is needed at least in the early stages of learning in eyeblink conditioning [11]. Trace eyeblink conditioning is a hippocampus-dependent task [12,13] whereas delay eyeblink conditioning can be acquired even in the absence of the whole forebrain [14]. Yet studies suggest that hippocampal electrophysiological state, namely the level of theta, correlates with learning even during delay eyeblink conditioning [15–17]. According to Berry et al., when training is carried out during theta, animals learn delay eyeblink conditioning faster than do those trained in the absence of or during low levels of hippocampal theta activity [18]. This is a finding so far not reported elsewhere.

A computational model by Hasselmo et al. [7] proposes that not only the presence or absence of hippocampal theta might be important but that the phase of the theta cycle determines the optimal time

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windows for hippocampal encoding and retrieval of memories. First, the model suggests that encoding associations in memory is most effective when synaptic output from the EC to the hippocampus is strong. Second, the model postulates that retrieval of the learned associations is most effective when the EC input is weak. The fluctuation in the input from the EC to the hippocampus is congruent with the hippocampal fissure theta phase, the peak of theta corresponding to low EC input. Presenting the CS at the peak or trough also affected learning rate, since acquisition of conditioned response (CR) was slower when the CS onset coincided with the theta peak. However, in contradiction to the hypothesis, our results suggested that in well-learned animals the behavioral performance of the CR is not dependent on the theta phase regardless of a clear effect on neural responses [19]. However, a possibility remains that an effect might have been seen if memory retrieval had been probed at different stages of learning. Lesion studies suggest that the hippocampal contribution to eyeblink conditioning is strongest early in learning [11].

The studies reported here were conducted to further test if hippocampal theta phase has an effect on the retrieval of a recently acquired memory trace and the consequent performance of a learned response in various stages of the acquisition process as well as during extinction training. It might well be that although no behavioral effect was detected in our previous study on well-trained animals [19], there could be an effect when the memory trace is still in the labile state early in training [11]. This notion was addressed in Experiment 1. In line with the model of Hasselmo et al. [7], our hypothesis was that retrieval would be more efficient if the CS was presented at fissure theta peak. In addition to simple acquisition of a learned response, in Experiment 1 we also studied if the phase of theta moderates the conditioned responses during different stages of extinction training. Again, more efficient retrieval of the previously acquired memory trace manifested as a conditioned response was expected if the CS was presented at fissure theta peak. In Experiment 2, we first sought to confirm the results of the study by [18] on theta-contingent delay conditioning and then addressed the question of whether extinction of the learned response is also affected by theta phase. To do so, we first trained rabbits in delay eyeblink conditioning either during theta or regardless of neural state. We expected animals trained during theta to learn faster and/or better than those trained at random [18]. Then, in well-trained animals we conducted extinction either by presenting the CS alone randomly or always at the theta trough or at the theta peak. Compared to animals trained at a random state, we expected to see impeded extinction learning in animals trained contingent on fissure theta peak [19].

2. Materials and methods

2.1. Subjects

The subjects were 29 (Experiment 1, 13 rabbits; Experiment 2, 16 rabbits) adult female New Zealand White rabbits (Lidköpings kaninfarm, Sweden) weighing approximately 2.8 kg at the time of surgery. The rabbits were housed in individual cages at 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 a.m. All experiments were carried out during the light part of the cycle. All the experimental procedures, care and handling were executed in accordance with Directive 2010/63/EU of the European Parliament and of Council on the protection of animals used for scientific purposes. Animal handling was performed only by trained personnel and the rabbits were introduced to human contact and handling for a sufficient amount of time before the surgery.

2.2. Surgery

Before the surgery, rabbits were treated with subcutaneous injections (s.c.) of an anti-inflammatory drug (50 mg/mL carprofen [Rimadyl vet, Pfizer Inc. Animal Health], dose: 0.1 ml/kg) and with 2 ml of an analgesic drug (0.3 mg/ml buprenorphine [Temgesic, Schering-Plough Europe] diluted with 0.9 ml of 0.9% NaCl) to moderate acute pain after surgery. The rabbits were anesthetized with an intramuscular injection (i.m.) of ketamine–xylazine cocktail (7.8 ml of 50 mg/ml Ketaminol vet [Intervet International B.V.] mixed with 2.8 ml of 20 mg/ml Narcoxyl vet [Intervet International B.V.]). A dose of 0.8 ml/kg of the cocktail was injected i.m. before surgery. During surgery, additional doses of either the cocktail or ketamine alone were injected subcutaneously approximately every 20–30 min or as needed. Before the surgery, the rabbit's fur was shaved from the top of the head. Then, the rabbit was positioned in a stereotaxic instrument (Kopf Instruments) with the bregma 1.5 mm higher than the lambda. Eye gel was inserted into the rabbit's eyes. At this point, 2.0 ml of lidocaine (10 mg/ml Lidocain [Orion pharma]) was injected s.c. in the area of surgery before making the opening incision.

A longitudinal incision was made to the scalp and a local anesthetic (2 g of lidocaine-hydrochloride Xylocain [AstraZeneca]) was administered to the wound. Eight holes for electrodes were drilled into the skull along with 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 posterior and anterior screws were connected together and served as a reference and the ground, respectively, in the electrophysiological recordings. Eight monopolar recording electrodes (Teflon-insulated stainless steel wire; 0.125 mm uninsulated diameter [A-M Systems]) were chronically implanted into the left dorsal hippocampus, aiming four electrodes to 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). Wires, skull screws, 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 s.c. and the rabbit was returned to her 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.

2.3. Experimental procedure

The experimental procedures are illustrated in Fig. 1. After one week of recovery from surgery, the animals were accustomed to a plexiglas restraining box without restraining and overall behavior was monitored. On the second day, restrained animals were habituated to the recording chamber and 30 min of spontaneous hippocampal local-field potentials (LFPs) were recorded. LFPs and electromyography (EMG, please see Section 2.4 below) from the right eye were recorded 5 min prior to, during and 1 min after each session. The inter-trial interval always varied randomly between 30 and 60 s. LabVIEW (National Instruments) was used to monitor neural activity and blinking online, to execute the experimental procedures and to present stimuli. The percentage of learned responses performed by each animal was analyzed after every session using MATLAB (The MathWorks Inc.). Please see Section 2.4 for further information.

2.3.1. Experiment 1: trace eyeblink conditioning, memory retrieval testing and extinction

During the first training session, 60 tone-alone (40-ms, 5-kHz, 75-dB tone) trials were presented regardless of neural state. 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

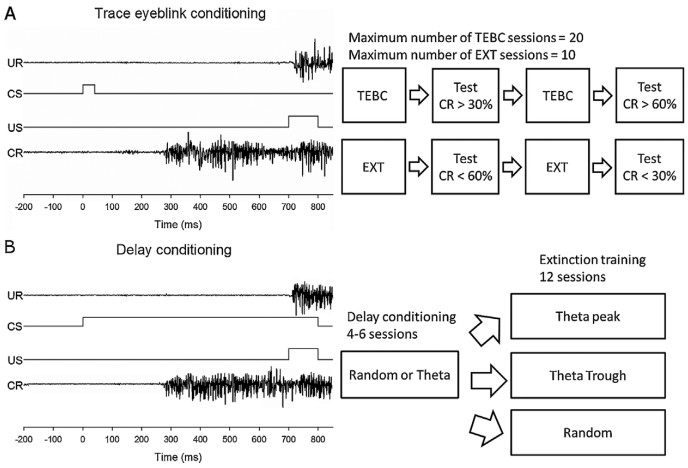

Fig. 1. Outline of the experimental procedures for Experiment 1 (A) and Experiment 2 (B).
A) Animals (n = 13) in Experiment 1 were trained in trace eyeblink conditioning (TEBC) with a 40-ms, 5-kHz, 75-dB tone as a conditioned stimulus (CS) and a 100-ms air puff as an unconditioned stimulus (US). The trace period was 660 ms. Examples of an unconditioned response (UR) and a conditioned response (CR) during one conditioning trial are illustrated. After conditioning, rabbits that had learned (n = 7) were subjected to extinction training. Memory retention depending on the phase of the hippocampal fissure theta oscillation was tested at four different stages of learning. B) Animals (n = 16) in Experiment 2 were trained in delay eyeblink conditioning with an 800-ms tone as a conditioned stimulus (CS). The CS and the US overlapped during the last 100 ms of the tone and co-terminated. Half of the animals were trained in a random neural state (Random, n = 8) and half were trained contingent on hippocampal theta (Theta, n = 8). After conditioning, animals were divided into three groups for extinction training: Random (n = 5), Theta peak (n = 6) and Theta trough (n = 5).

above as the CS and a 100-ms air puff (0.35 bar source pressure) to the right eye as an unconditioned stimulus (US). A trace period of 660 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.

Memory retrieval was tested at two stages of acquisition, first when the animal performed a learned response at a rate of 30% and second when learned responding took place during 60% of the trials. Test sessions were conducted within the same day after reaching the set criterion. During each test session, the CS was presented alone to limit the test to memory retrieval. For CS presentation to take place, robust theta oscillations had to be present for at least one second (theta ratio > 80%). The CS onset was timed either to the peak (20 trials) or the trough (20 trials) of the hippocampal fissure theta oscillation, in a random fashion and with an ITI of at least 30 s. On the next day, trace eyeblink conditioning continued as usual.

Animals were trained with trace eyeblink classical conditioning (CC) until they made at least 60% learned responses during two consecutive sessions or up to 20 sessions. Animals that reached the two-session criterion or made more than 60% learned responses during at least one session were considered to have learned the task. In these animals, extinction training was then started. Sixty CS-alone trials per session were presented irrespective of the neural state of the hippocampus. These sessions were carried out until learned responses took place during less than 30% of the trials or up to 10 sessions. The effect of theta phase on memory retrieval was tested as before on two occasions during extinction training. First when learned responding dropped below 60% and last when (or if) it dropped below 30%.

2.3.2. Experiment 2: delay conditioning and extinction

Delay eyeblink conditioning was carried out with a tone-CS (800 ms, 5 kHz, 75 dB) and a 100-ms air puff (0.35 bar source pressure) to the right eye as a US. The US was presented starting at 700 ms from CS onset and thus overlapping and co-terminating with the CS. Sixty trials per session were presented. For eight animals, conditioning was conducted in a random neural state and for the other half when theta was dominant in the hippocampus (the theta ratio had to be over 80% during a 1-s pre-trial period, please see Section 2.4). Training trials were only presented when the animal was not already blinking. Four of the sixteen animals were used to pilot the experiment. They were trained for six sessions regardless of learning rate. Two of the rabbits were trained in a random neural state and two when theta was prominent in the hippocampus. Delay conditioning was re-modelled due to poor extinction rates (see Results) for the remaining 12 animals. Now, delay conditioning was terminated as soon as an animal performed > 80% learned responses during two consecutive conditioning sessions.

After delay conditioning, 12 sessions of extinction training with 60 trials per session were conducted. The CS was either presented to a random neural state (n = 5), or the onset of the CS was timed to the peak (n = 6) or the trough (n = 5) of the hippocampal fissure theta oscillation.

2.4. Recordings and data analysis

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 and test sessions. The raw EMG signal was conveyed to a filter-amplifier (A-M Systems Model 2100), amplified 1000 x and band-pass filtered from 100 to 500 Hz. For neural recordings, a ten-fold amplification was executed with a preamplifier (MPA8I, MultiChannel Systems [MCS]) attached to the electrode connector in the rabbit's head. Then the signal was band-pass filtered (1–5000 Hz) with a 64-channel filter amplifier (MCS). Last, the signal was digitized at a rate of 20 kHz, low-pass filtered (500 Hz) and downsampled at a rate of 2 kHz with a MCS USB-ME64 System (MC_Rack software). SPSS (IBM) and MATLAB (MathWorks) were used for offline data analysis.

2.4.1. Eyeblinks

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). In addition, the mean of the standard deviation of the EMG activity during the 500-ms pre-CS period (SDpre) was determined. Eyeblinks were defined as EMG activity exceeding a threshold of [MEANpre + 7 × SDpre] for at least 10 ms. Trials with eyeblinks during the 100-ms period immediately preceding CS onset were rejected. Eyeblinks during the last 250 ms of the trace period were counted as conditioned responses.

2.4.2. Phase-locking of hippocampal theta-band (4–8 Hz) responses

To assess the temporal accuracy of the theta-band responses to the conditioning stimuli, a phase-locking value (PLV) [20] was calculated. PLV is a measure of temporal consistency of a signal from trial to trial, a higher value meaning a higher probability for the signal to be in the same phase at a given time point after the stimulus onset. In terms of neuronal activity, high phase-locking to an event means that the responding of groups of neurons evoked by the external

stimulus–reflected in LFP– is temporally regular and predictable. Predictable firing of groups of neurons within a brain structure can be viewed as a precursor of forming neuronal assemblies across brain structures. Synchrony of neuronal firing can enhance communication between anatomically connected brain regions [21].

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

2.5. Statistical analyses

Repeated-measures analysis of variance (ANOVA), with training sessions or blocks of two sessions as a within-subjects factor and group as a between-subject factor, was used to analyze changes across training. For post-hoc comparisons, Bonferroni correction p values are reported. Paired t tests were used to analyze differences within subjects regarding the memory retrieval and phase-locking in either the peak or the trough of the theta. All of the t tests passed normality tests (Shapiro-Wilk).

2.6. Histology

Rabbits were anesthetized with an i.m. injection of a ketamine-xylazine cocktail. The rabbit's left ear was shaved and it was then overdosed with pentobarbital (Mebunat vet, Orion-Yhtyma Oyj) by injecting intravenously (i.v.) a large vein in the ear. Then, the brain was perfused with physiological saline followed by 9% formalin solution through the ascending aorta. The locations of the eight electrode tips were marked by passing a DC current (0.2 mA, for 10 s) through them. The brain was then removed and stored in formalin for several days. The brain was coronally sectioned with a vibratome into 60-μm-thick slices. The slices were attached to gelatinized slides, dried, and stained with Prussian blue and cresyl violet. The electrode locations were determined with the help of a microscope.

3. Results

Histological examinations confirmed that the recording electrodes for theta were in or near the hippocampal fissure, as intended, in 27 animals (see Fig. 2A). Two rabbits with broken electrodes were used in the Random group in Experiment 2.

3.1. Experiment 1: phase contingent trial onset affects neural but not behavioral responses in trace eyeblink conditioning

All training sessions included, 11 out of 13 animals learned to blink in response to the CS in over 30% of training trials per session. However, only seven out of thirteen rabbits reached the criterion of more than 60% learned responses. These seven animals went through extinction training and 5 of them met the extinction criterion, that is, they made less than 30% learned responses at the end of extinction training. These learning results meant that memory retrieval tests to study the effects of theta phase on neural and behavioral responses to the CS were conducted first on 11 animals (early acquisition, > 30% learned responses), then on seven animals (successful acquisition, > 60%), then on seven animals (early extinction, < 60%) and finally on five animals (successful extinction < 30%).

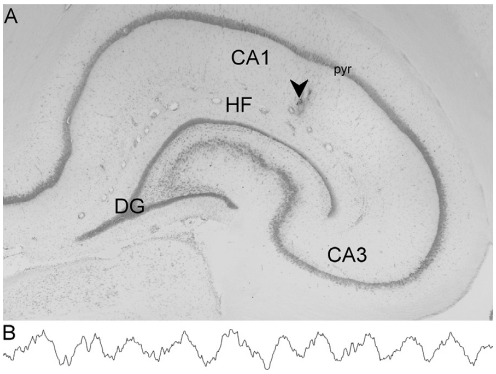

Fig. 2. In both experiments, hippocampal local-field potentials were recorded from the fissure (A) and displayed characteristic theta oscillations (B).
A) A 60-μm cresyl violet–stained slice of a rabbit hippocampus with the locations of CA1, CA3, dentate gyrus (DG), hippocampal fissure (HF) and pyramidal cell layer (pyr) indicated. The recording electrode tip location is marked with an arrow. B) Example of theta recorded from the fissure.

Hippocampal responses and conditioned responses to the CS onset taking place at the peak vs. the trough of the theta oscillation in hippocampal fissure was tested with paired samples t tests. The results are summarized in Fig. 3. Phase-locked hippocampal theta-band responses to the CS (see Ref. [19] were moderated by the phase of the theta early in acquisition when animals performed > 30% learned responses, $t(10) = 4.98, p < 0.001, d = 0.25$. Namely, phase-locking was higher when the CS onset was timed to the fissure theta trough compared to when it was timed to the theta peak. This was the case also early in extinction training, < 60% learned responses, $t(6) = 3.82$, $p = 0.009$, $d = 0.17$, and after successful extinction, < 30% learned responses, $t(4) = 4.91$, $p = 0.008$, $d = 0.32$. After successful acquisition of the learned response (> 60% learned responses), phase-locking of hippocampal theta-band responses to the CS was similar regardless of whether the CS onset was at the peak or the trough of the theta cycle, $t(6) = 1.47$, $p = 0.192$. The performance of the conditioned response was not moderated by the phase of the fissure theta cycle at any stage of acquisition or extinction of the learned response; paired samples t tests: > 30% learned responses: $t(10) = 1.10$, $p = 0.296$; > 60% learned responses: $t(6) = 1.45, p = 0.199$; < 60% learned responses: $t(6) = 0.97$, $p = 0.370$; < 30% learned responses: $t(4) = 0.17$, $p = 0.876$.

3.2. Experiment 2: the presence of theta did not affect learning in delay conditioning and theta phase did not affect extinction

As seen in Fig. 4c, the degree of phase-locking of hippocampal theta-band responses to the CS did not differ between the Theta ($n = 8$) and Random group ($n = 6$) and also did not change during the delay conditioning. See Fig. 4a; interaction of group and session: $F(3, 36) = 0.64$, $p = 0.592$; main effect of session: $F(3, 36) = 0.91$, $p = 0.446$; main effect of group $F(1, 12) = 1.48$, $p = 0.247$. In addition, the proportion of learned responses increased across the first four sessions of delay eyeblink conditioning equally in both groups, rabbits trained irrespective of neural state (Random, $n = 8$) and those trained in the presence of theta (Theta, $n = 8$). See Fig. 4c; repeated measures ANOVA, interaction of group and session: $F(3, 42) = 0.82$, $p = 0.493$; main effect of session: $F(3, 42) = 67.86$, $p < 0.001$; main effect of group $F(1, 14) = 0.07$, $p = 0.800$. That is, there was no difference between groups in hippocampal responses to the CS, and hence no effect of theta on delay eyeblink conditioning.

After delay conditioning, all rabbits were trained in extinction. For

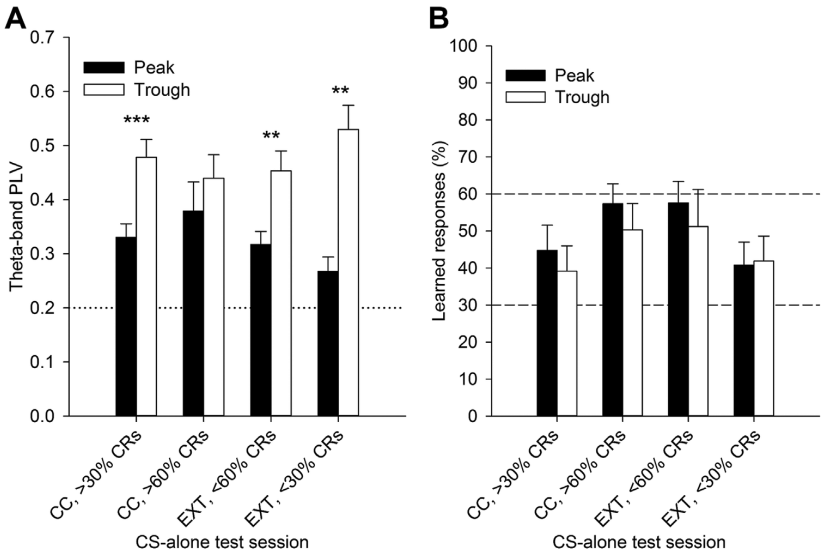

Fig. 3. In Experiment 1 we found no effect of theta phase on memory retrieval at the behavioral level. The effects of theta phase on memory retrieval were tested at four different stages of learning: when animals performed more than 30% conditioned responses (CRs) during trace eyeblink classical conditioning (CC), when animals performed more than 60% CRs during CC, or when the percentage of CRs dropped below 60% and below 30% during extinction training (EXT). During test sessions, the conditioned stimulus (CS) was presented alone 40 times: 20 times to the trough of theta (Trough) and 20 times to the peak of theta (Peak). The order of the trials was random. A) Overall, hippocampal theta-band (4–8 Hz) responses to the CS were more temporally uniform from trial to trial (indicated by a higher phase-locking value, PLV) if the CS was presented to the hippocampal fissure theta trough compared to theta peak. Asterisks refer to statistically significant differences between Peak and Trough conditions (paired samples *t*-test): *** $p < 0.001$, ** $p < 01$. B) Despite the difference in hippocampal responding, there was no difference in the rate of behavioral learned responding (%) between trials presented at theta trough and theta peak.

five animals, the CS alone was always presented at theta trough (Trough) and for six animals it was always presented at theta peak (Peak). For the remaining five animals, the CS was presented irrespective of the neural state (Random). Phase-locked hippocampal theta-band responses to the CS were different between the trough, the peak, and the random group during extinction training (repeated measures ANOVA, interaction of group and session: $F(10, 55) = 1.26$, $p = 0.28$; main effect of session: $F(5, 55) = 0.86$, $p = 0.517$; main effect of group $F(2, 11) = 9.32$, $p < 0.004$; see Fig. 4b). Specifically, the phase-locking value (PLV) was higher, when the CS onset was timed to the fissure theta trough ($n = 5$) compared to when it was timed to the theta peak ($n = 6$) or when the CS was presented in a random neural state ($n = 3$). In the beginning of extinction (Block 1, first two sessions) phase-locked hippocampal theta-band responses to the CS observed in the Trough group were statistically different from those observed in the Peak group, $F(1, 9) = 5.63$, $p = 0.04$, but did not differ from those observed in the Random group, $F(1, 6) = 4.26$, $p = 0.09$. During subsequent extinction training (Block 2 and onwards), hippocampal theta-band responses in the Trough group were statistically significantly better phase-locked to the CS compared to responses in the Peak group, $F(1, 9) = 5.87$–15.70, $p < 0.05$–0.005, and in the Random group, $F(1, 6) = 6.13$–15.31, $p < 0.05$. Despite the difference in neural responding, behavioral learned responses decreased equally in all of the groups during extinction training; repeated measures ANOVA, interaction of group and session: $F(10, 65) = 1.08$, $p = 0.392$; main effect of session: $F(5, 65) = 5.79$, $p = 0.004$; main effect of group $F(1, 13) = 3.52$, $p = 0.060$). Note that none of the animals accomplished full extinction during the 12 sessions that were conducted (see Fig. 4d).

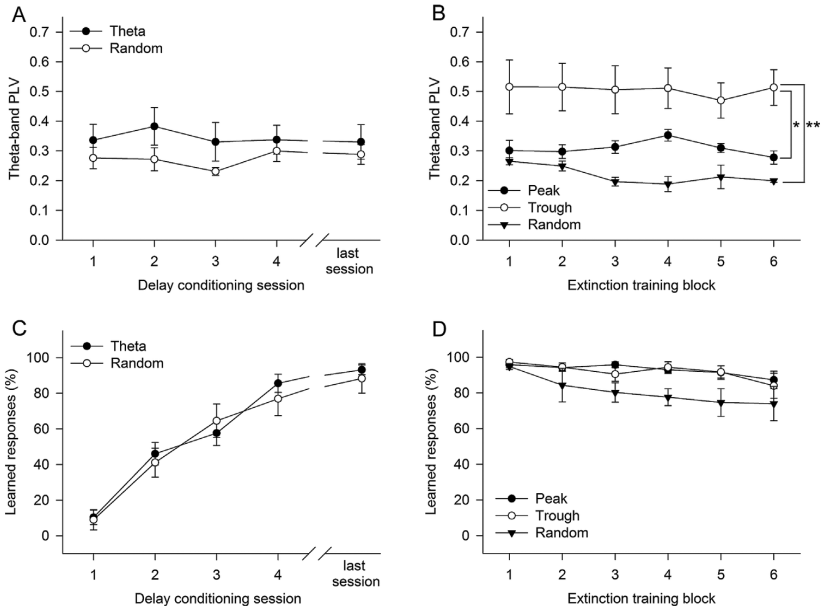

Fig. 4. In Experiment 2 we found no effect of theta-contingent training on learning delay eyeblink conditioning. We also did not find an effect of theta phase–contingent training on extinction learning at the behavioral level, despite more uniform hippocampal responses elicited in the group receiving the conditioned stimulus always at theta trough. A) Hippocampal theta-band (4–8 Hz) responses to the CS were equally temporally uniform from trial to trial (quantified as the phase-locking value, PLV) in the Theta ($n = 6$) and Random ($n = 8$) groups during delay eyeblink conditioning. B) During extinction training theta-band phase-locking was higher in the Trough group ($n = 5$) compared to the Peak ($n = 6$) and the Random ($n = 3$) groups. Asterisks refer to statistically significant differences between groups (Bonferroni-corrected post-hoc comparisons): ** $p < 0.01$, * $p < 0.05$. C) Theta-contingent training had no effect of learning delay eyeblink conditioning at the behavioral level. D) Theta phase–contingent training had no effect on learning extinction at the behavioral level.

4. Discussion

Our main aim in the two experiments reported here was to find out if the behavioral memory trace of a recently formed association would be more readily retrieved if the conditioned stimulus was presented at the peak of the hippocampal fissure theta oscillation. The peak of fissure theta represents a moment in time during which input from the EC to the hippocampus is suppressed, and during which the hippocampus is assumed to be in an optimal state for memory retrieval [7]. We found that the behavioral performance of a learned response was not moderated by hippocampal theta phase either early or late in learning trace eyeblink conditioning (Experiment 1) or during extinction learning (both experiments). However, hippocampal responses to the conditioned stimulus were overall temporally more uniformly organized, that is, better synchronized, when the CS was presented to the hippocampal fissure theta trough compared to when it was presented to the theta peak (both experiments). Thus, it seems that the two phases, peak and trough, of the theta oscillation represent differential microstates of the hippocampus, leading to differential responses at the level of the hippocampus, but this is not sufficient to exert an effect on retrieving the conditioned response acquired during eyeblink conditioning.

Our results indicating no effect of theta phase on memory retrieval across conditioning and extinction are consistent with our earlier findings in well-learned animals [19] and suggest that retrieval of recently acquired memories and consequently performing a learned response is moderated by neural mechanisms other than hippocampal theta. Earlier attempts to link the model by Hasselmo et al. [7] to behavioral data have produced opposing results: Hasselmo [22] explains the results of M'Harzi et al. [23] with his model of memory retrieval. In a study by Hasselmo et al. [23], rats were trained in a T-maze task before and after fornix lesions. The assumption in Hasselmo [22] was that the lesion could damage the properties of the theta rhythm and cause long-term potentiation in the CA3 auto-associative network and in the synapses between the CA3 and CA1 pyramidal cells. Indeed, at the behavioral level, after the fornix lesion, rats had problems learning a new reward location in the maze. That is, as would be guessed based on the computational model [7], encoding to memory was impaired and retrieving old memories was strengthened when input from the EC to the hippocampus was eliminated [23]. Thus, evidence exists both for and against the computational model of Hasselmo et al. [7].

Contrary to the prediction based on the model by Hasselmo et al. [2] we could also have hypothesized that more CRs would have been performed when the CS was presented to the hippocampal theta trough. This is because, in our previous study, higher phase-locking of hippocampal theta-band responses and better learning was acquired if the CS was presented to theta trough compared to when it was presented to the theta peak [19]. It is also known that high relative hippocampal theta amplitude indicates phase synchrony of theta oscillations between the hippocampus and the cerebellum [24], the structure that is critical in CR acquisition and performance in both delay and trace conditioning [25]. Based on these findings and the assumption that attention is crucial for learning, we have tentatively interpreted the phase-locked theta-band activity in the hippocampus to be a measure of attention targeted towards the CS. However, in our present experiments, even though the CS seemed to elicit more attention when presented at the trough of the theta cycle than when it was presented at the peak, there was no difference in how often the animals produced conditioned responses. A possible explanation for this is the time-limited role of the hippocampus in learning. It might be that at the time we started to test for retrieval of the CR, the animals had already passed the stage of learning where hippocampal contribution is thought to be critical, that is, the phase of 'contingency detection' [26]. That is, even though phase-locked hippocampal theta might serve as an index of attention, hippocampal processing may not be relevant in memory retrieval after the association between the CS and the US has been established. In support of this, lesion studies suggest that hippocampal contribution to learning eyeblink conditioning is most important early in training, whereas later in memory consolidation the role of the neocortex becomes more crucial [11].

In Experiment 2 we took on another unresolved question, namely whether or not spontaneously occurring hippocampal theta oscillations promote learning. We trained rabbits in delay eyeblink conditioning either during the dominant theta state or during a random neural state. Interestingly, we did not find any effect of theta-contingent training on learning. This is in contrast to the results in Seager et al. [18], who trained rabbits in delay eyeblink conditioning contingent on theta and in the absence of theta and found faster learning in the group trained contingent on theta. The discrepancy in results could in part be explained by differences in methods between the two studies: Seager et al. [18] trained rabbits with a freely varying ITI and used a 350-ms tone-CS co-terminating with a 100-ms air puff. In our study, we used a 30–60 s ITI and an 800-ms tone-CS. Regardless of these differences in ITI and CS duration, we would have expected to see an effect of theta contingent training on learning if theta is beneficial for learning overall. Note that in Experiment 2 we chose not to expose the animals to a CS-alone session (as we usually do). This choice was made in order to minimize methodological differences between our study and that of Seager et al. [18]. Last, in the present experiment, we used female rabbits whereas the sex of the animals was not reported in the Seager et al. [18] paper. If Seager et al. [18] used males, it could possibly explain the difference in learning as differences in hippocampal theta have been reported between male and female rats (for example, see Ref. [27]. Although the differences of hippocampal theta properties between sexes have not been studied in rabbits, the correlation between overall baseline theta power and learning is present in both male [28] and female rabbits [16]. Thus, it is possible but perhaps unlikely that sex differences would account for discrepancies between the results of our current experiment and those reported earlier Seager et al. [18].

As discussed above, our current results indicate no improvement in learning if eyeblink conditioning is conducted contingent on hippocampal theta. Quite the contrary this is consistent with previous findings from our lab: rabbits trained in a random neural state [19] or in the explicit absence of theta [29] learn better compared to those trained contingent on theta. As already mentioned, hippocampal theta has traditionally been thought to indicate attention or a heightened state of vigilance [2]. However, it is not possible to say, for certain, to what is the subject paying attention when theta is observed in the hippocampus. Thus, the spontaneously occurring theta oscillations might actually indicate episodes during which attention is targeted towards aspects of the training situation (for example background noise, lighting) irrelevant for learning the task at hand. This might explain why theta-contingent training does not enhance learning or memory retrieval. It might also be that in healthy young rabbits enhancing learning is difficult because the animals already perform as well as they can (ceiling effect). A different outcome could be possible when using i.e. elderly rabbits or rabbits with impaired ability to learn.

Last, in both experiments reported here we also conducted extinction training after eyeblink conditioning. We anticipated that rabbits would successfully develop extinction from both trace (Experiment 1) and delay (Experiment 2) trained association. For the delay-conditioned animals this did not happen even after 12 sessions of CS-alone training (720 trials). Correspondingly, Kehoe [30] succeeded in extinction of well-learned delay-conditioned rabbits by presenting only approximately 60 CS-alone trials. The reason for the poor extinction in our studies is unknown.

Although our results show that at the neuronal level the phase of the hippocampal theta rhythm modulates the neuronal activation to the CS during eyeblink conditioning, further experiments have to be conducted to demonstrate this effect at the behavioral level.

Acknowledgements

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III

LEARNING BY HEART: CARDIAC CYCLE REVEALS AN EFFECTIVE TIME WINDOW FOR LEARNING

by

Tomi Waselius, Jan Wikgren, Hanna Halkola, Markku Penttonen
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RESEARCH ARTICLE | *Higher Neural Functions and Behavior*

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

Tomi Waselius,[1]* Jan Wikgren,[1,2]* Hanna Halkola,[1] Markku Penttonen,[1] and Miriam S. Nokia[1]
[1]*Department of Psychology, University of Jyväskylä, Jyväskylä, Finland; and* [2]*Centre for Interdisciplinary Brain Research, University of Jyväskylä, Jyväskylä, Finland*

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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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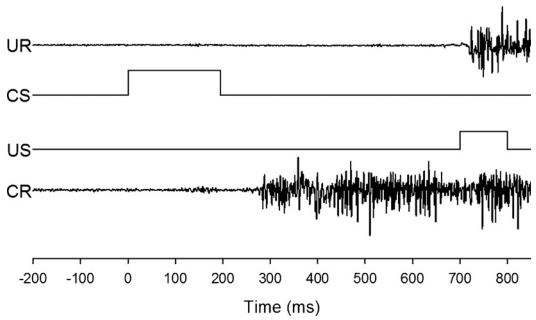

Fig. 1. Trace eyeblink conditioning. Rabbits (n = 25) and humans (n = 29) were trained in trace eyeblink conditioning with tone as a conditioned stimulus (CS) and an air puff aimed toward the eye as an unconditioned stimulus (US). The trace period was 500 ms for the rabbits and 600 ms for the human participants.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

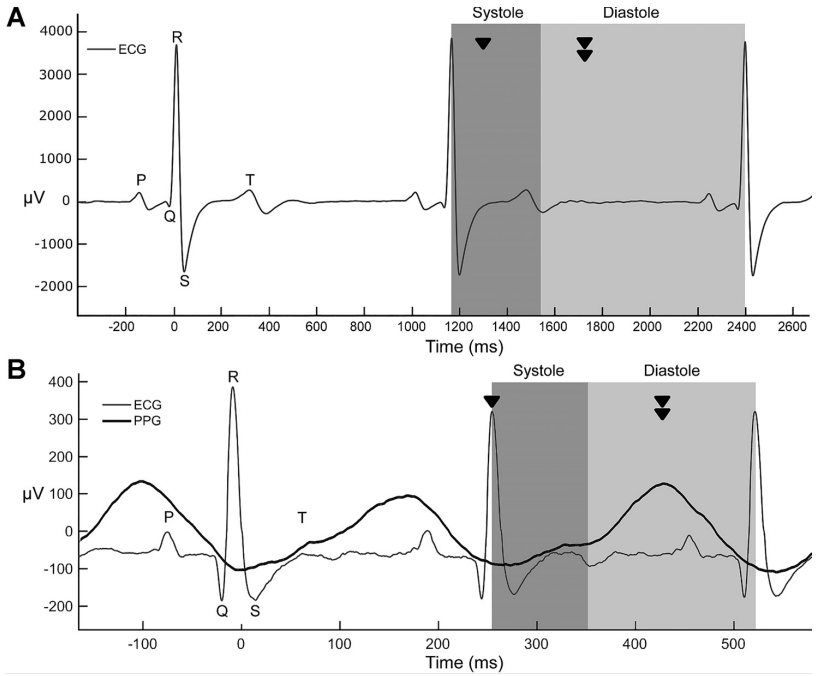

Fig. 2. In both human participants (*A*; *experiment 1*) and rabbits (*B*; *experiment 2*), the conditioned stimulus (CS) was timed to either the diastole or the systole. The cardiac cycle can be divided into 2 phases, systole and diastole. During systole, the heart contracts and pumps blood to arteries.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

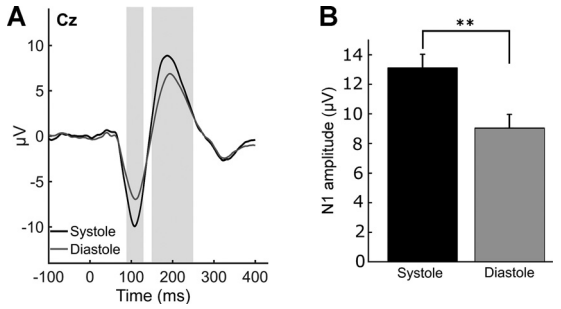

Fig. 3. Topographies of the event-related potentials to the tone-conditioned stimulus (CS; *A*) and maximum amplitudes of N1 and P2 responses measured at the Cz electrode (*B*) in human participants in *experiment 1*. The N1 response was significantly larger when the tone onset was contingent with the 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).

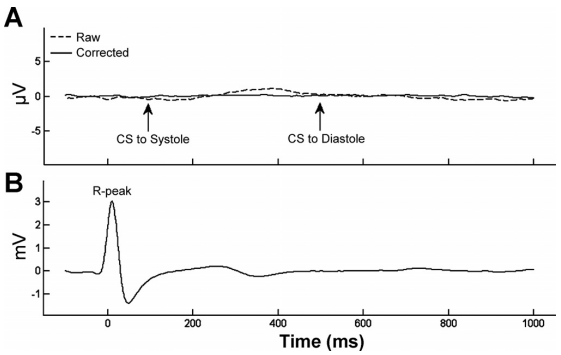

Fig. 4. *A*: effect of independent component analysis (ICA)-based artifact correction on EEG recorded at the Cz electrode. The EEG traces are grand-average responses to the heartbeat. Arrows mark the onsets of conditioned stimulus (CS) in the systole and diastole groups. There are minor deflections related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

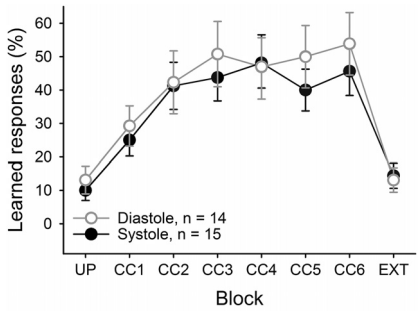

Fig. 5. Human participants in *experiment 1* learned trace eyeblink conditioning at the same rate regardless of group (systole vs. diastole). Error bars are SE. UP, unpaired; CC, conditioning; EXT, extinction.

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.

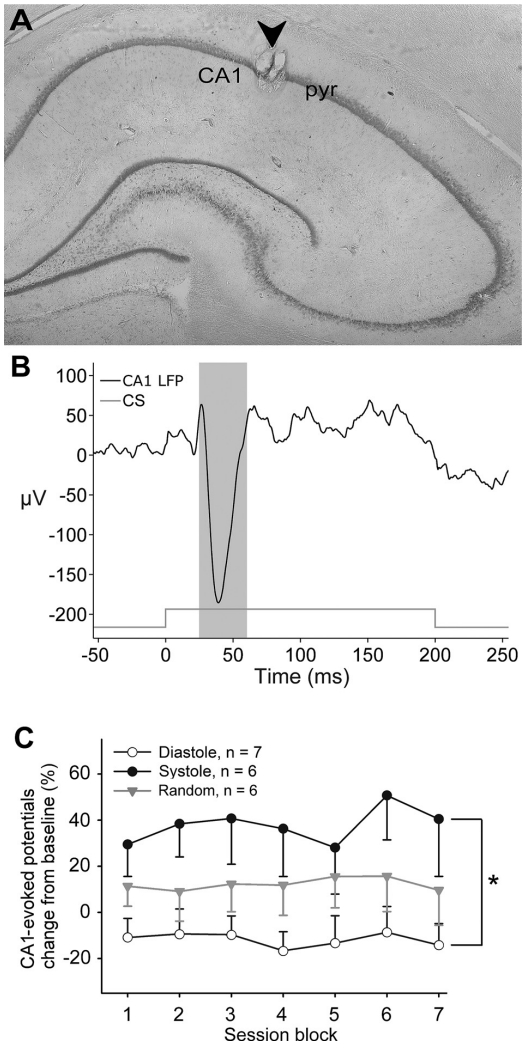

Fig. 6. In *experiment 2*, relative amplitudes of event-related potentials (ERPs) to the conditioned stimulus (CS) recorded from the rabbit hippocampal CA1 were larger in the systole group compared with the diastole group. *A*: a 60-μm cresyl violet-stained slice of a rabbit left dorsal hippocampus with 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

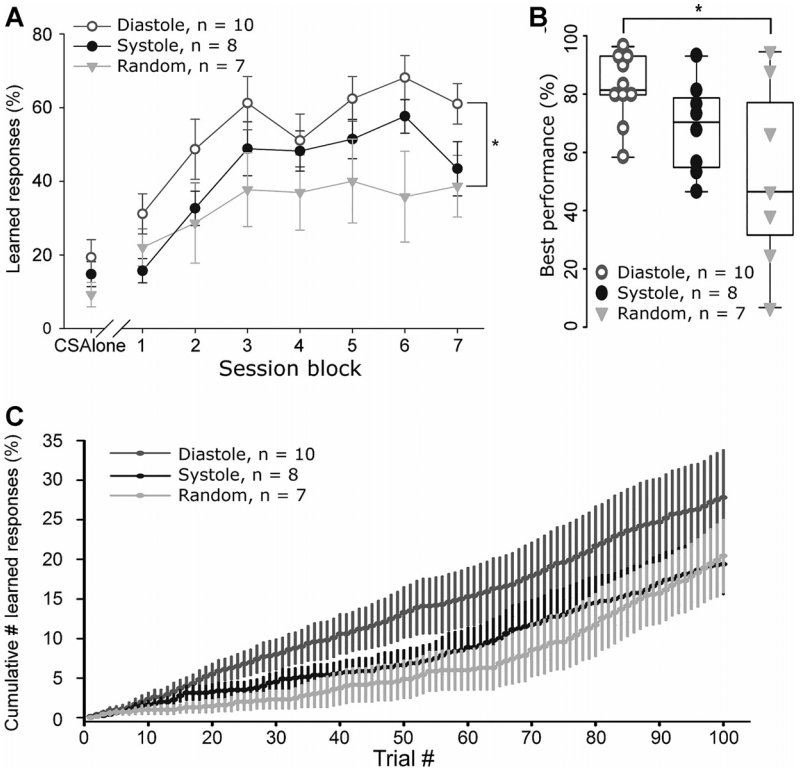

Fig. 7. In *experiment 2*, rabbits trained at diastole learned better than those trained irrespective of cardiac cycle phase. *A*: learning was faster and conditioned responding remained at a higher level in the diastole group throughout trace eyeblink conditioning. $*P < 0.05$, repeated-measures ANOVA 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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GRANTS

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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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IV

BREATHE OUT AND LEARN: EXPIRATION -CONTINGENT STIMULUS PRESENTATION FACILITATES ASSOCIATIVE LEARNING

by

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