HIPPOCAMPAL THETA ACTIVITY, LEARNING AND BEHAVIORAL TENDENCIES

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Trace eyeblink conditioning is a method that models a declarative, awareness-dependent form of learning. Hippocampal theta activity, an approximately 3.5 - 8.5 Hz oscillatory pattern in rabbits, has been associated with learning-related phenomena: arousal, attentional processes and association forming. In some studies theta-contingent training, given during a brain state rich in hippocampal theta oscillation, has improved the initial learning in trace eyeblink conditioning. However, other laboratories have failed to replicate this effect. This study explored the effects of theta-contingent conditioning and extinction on learning, and the connection between the magnitude of spontaneous hippocampal theta activity and the behavioral tendencies or individual dispositions of a subject.

In this study, Open Field test (OFT) was used to measure behavioral tendencies. Spontaneous hippocampal theta ratio was recorded, and theta-contingent conditioning was performed in three groups: high (T+) and low-theta training (T-) and yoked controls (Y). Unexpectedly, spontaneous hippocampal theta activity did not correlate significantly with the OFT variables. Moreover, while the amount of conditioned responses (CRs) increased as a function of training, the training group had no effect on the learning rate. However, the T- group had the highest proportion of subjects that reached the 60-% learning criterion, and the mean CR% in the best session was significantly higher in the T- group than in the T+ group. In extinction, the T+ group learned significantly faster than the Y group. When classifying the subjects as active versus inactive based on the OFT, T- training significantly improved the learning rate of only the active subjects. The results show that high-theta training did not improve learning in trace eyeblink conditioning, and suggest that theta-contingent training may affect differently subjects with distinct behavioral tendencies.

Key words: Hippocampus, theta activity, theta-contingent conditioning, trace eyeblink conditioning, extinction, Open Field test

INTRODUCTION

Classical eyeblink conditioning (EBC) is a method that can be used to model two distinct forms of learning. In delay eyeblink conditioning, a conditioned stimulus (CS) is usually a tone, in the end of which an overlapping unconditioned stimulus (US), a mild airpuff directed to the eye, is presented. In trace conditioning there is a temporal gap between the tone-CS and the airpuff-US. In both variants, the eyeblink, an unconditioned response (UR) to the airpuff, gets conditioned to the tone-CS. Learning is thus indicated by conditioned eyeblink responses (CRs) before the onset of the impending US. Learning in delay conditioning is regarded as implicit, requiring no awareness of the connection between the two stimuli. On the contrary, learning in trace conditioning is thought to require awareness of the relationship, and thus to model declarative memory formation (Clarks, Manns, & Squire, 2002). Sometimes extinction, where the CS is no longer followed by the US, is used to measure the ability to unlearn the CS-US relationship.

After decades of search for the neural correlates of both delay and trace EBC, beginning from the work of Gormezano, Schneiderman, Deaux and Fuentes (1962), the knowledge is still evolving. However, there seems to be a general agreement about the most influential structures and mechanisms affecting learning (see Christian, & Thompson, 2003, for a review). In both delay and trace conditioning, the cerebellum has an essential role (McCormick, Clark, Lavond, & Thompson, 1982). For instance, a lesion inactivating the interpositus nucleus (IP) prevents the retrieval of the conditioned responses and obstructs future learning (Green, & Woodruff-Pak, 2000). In learning of delay conditioning, also a long-term depression of the cerebellar cortex has an important role (Woodruff-Pak, & Disterhoft, 2007; Pakaprot, Kim, & Thompson, 2009).

In the more complicated trace EBC, which requires forming an association between the two stimuli, a more extensive circuit is needed (see Woodruff-Pak, & Disterhoft, 2007, for a review). Along with the cerebellum, the septo-hippocampal system has a critical role, one that it does not have in delay conditioning (Moyer, Deyo, & Disterhoft, 1990). A functioning hippocampus is indeed necessary in the associative process needed for successful trace learning, but its role diminishes as the memory trace becomes consolidated elsewhere (Takehara, Kawahara, Takatsuki, & Kirino, 2002). Additionally, the role of the medial prefrontal cortex (mPFC) and its connections via the lateral pontine nuclei (LPN) to the cerebellum are nowadays seen as bridging the temporal gap between the CS and the US (Weible, McEhron, & Disterhoft, 2000; Siegel, Kalmbach, Chitwood, & Mauk, 2012).

After acknowledging the importance of the hippocampus in learning trace eyeblink conditioning, attention has shifted to its oscillatory properties. Hippocampal or limbic theta activity, oscillating between 3.5 - 8.5 Hz in rabbits, has been related to functions such as arousal and alertness (Green, & Arduini, 1954), voluntary movement (Vanderwolf, 1969), attention and orientation (Klemm, 1976), motivational state (Berry, & Swain, 1989), selective attention (Vinogradova, 1995, see for a review), spatial cognition and navigation (Buzsáki, 2005) and learning and memory (Berry, & Seager, 2001; Vertes, 2005). According to Winson (1972), in rabbits, theta activity is seen to take place particularly during an alert or aroused state, novel sensory stimuli presentation, paradoxical sleep and in the presence of motion. It has also been suggested that hippocampal theta activity would be related to the attentional state of the animal (Nokia, Penttonen, Korhonen, & Wikgren, 2009) and to the production of associations and generalizations (Nokia and Wikgren, 2010). Whether the natural amount of hippocampal theta activity of a rabbit is related to its behavioral tendencies or not, remains uncertain.

A high magnitude of spontaneous pre-training hippocampal theta activity has been linked to success in learning both delay and trace eyeblink conditioning (Berry, & Seager, 2001, see for a review), with a distinction that in trace conditioning it only predicts the onset of learning (Nokia, Penttonen, Korhonen, & Wikgren, 2009). Moreover, trial presentation that is contingent upon high amount of hippocampal theta activity (theta-contingent training) seems to produce faster learning in the initial stage of conditioning, but does not have an effect on the asymptotic learning (Seager, Johnson, Chabot, Asaka, & Berry, 2002; Griffin, Asaka, Darling, & Berry, 2004). A high magnitude of theta also seems to enhance neural activity in the structures critical for the acquisition of trace EBC (Darling, Takatsuki, Griffin, & Berry, 2011; see Berry and Hoffman, 2011, for a review). However, the recent results of Nokia, Penttonen and Wikgren (2010) have challenged the idea that solely high-theta training improves early learning. They showed that conditioning contingent upon sharp-wave ripples, which occur specifically during a low-theta state, produced significantly faster learning in the initial phase when compared to a yoked control group.

The effects of theta-contingent training on extinction, the ability to unlearn the conditioned CS-US relationship, remain largely uninvestigated. According to Nokia and Wikgren (2010), in a discrimination-reversal task that resembles extinction, a high pre-training theta ratio predicted high responding to both CSs and did not thus aid the process of unlearning. On the other hand, also ripple-contingent training seems to result in slower extinction in comparison to the yoked controls (Nokia, Penttonen, & Wikgren, 2010). While having such few experiments investigating theta-contingent extinction, it remains unclear what to expect. Anyhow, at least the activation in the hippocampal regions of CA1, CA3 and dentate gyrus is seen to decrease during extinction

(Schmajuk, & DiCarlo, 1992). According to Bouton (2004), the success of extinction is actually the most importantly dependent on the context of learning.

The need for this experiment was created by the controversy over the results related to theta-contingent conditioning and extinction, as well as by the small amount of research investigating the relationship between spontaneous hippocampal theta activity, behavioral tendencies and learning. The main research problems were thus, 1) how does theta-contingent training affect learning in trace eyeblink conditioning and extinction and 2) what is the relationship between spontaneously occurring hippocampal theta activity and behavioral tendencies. Our main hypotheses were the following. First of all, due to the inconsistencies in the previous research, we hypothesized that either high or low-theta training might improve initial learning, but would not necessarily affect the final performance. Secondly, we expected not to particularly detect the benefit of either high or low-theta training in extinction. Instead, we assumed to see an enhancing effect of a context change on the performance, since the theta-group of approximately half of the subjects was changed in extinction. Finally, we expected that the higher the magnitude of spontaneous hippocampal theta activity, the more anxious and less active the subjects would be in the OFT.

METHOD

Subjects

The subjects of this experiment were 36 female New Zealand White rabbits obtained from Lidköping's Kaninfarm (Lidköping, Sweden). In the beginning of the procedures, the rabbits were 2.5 months old and weighed ~ 2 - 2.5 kilograms. The experiments were executed in the premises of the animal research unit of the University of Jyväskylä (Jyväskylä, Finland), where the rabbits were housed in individual metal cages. The rabbits were offered a free access to food and water. In addition, they were provided with pieces of wood to chew and hay to eat. The room temperature and humidity were controlled. The light-dark cycle of the rabbits was set to 12 hours, with lights on at 6 a.m. All the procedures were conducted during the light period.

The ethical permission for this research was provided by National Animal Experiment Board of Finland. All the procedures were executed in accordance with the European Communities Council Directive (86 / 609 / EEC) on the care and use of animals for research purposes.

Surgery

30 minutes before the beginning of a surgery, subcutaneous injections of an analgesic solution [0.1 ml of 0.3 mg/ml buprenorphine (Temgesic, Schering-Plough Europe, Brussels, Belgium) diluted in 0.9 ml of 0.9% NaCl, dose: 2 ml] and an anti-inflammatory drug [carprofen (Rimadyl vet, Pfizer Inc. Animal Health, Espoo, Finland), dose: 0.1 ml / kg] were given. The anesthesia was produced with an intramuscular injection of ketamine-xylazine cocktail [7.8 ml of 50 mg / ml Ketaminol vet (Intervet International B.V., Boxmeer, Netherlands) mixed with a 2.2 ml dose of 20 mg / ml Narcoxyl vet (Intervet International, B.V.)]. During the surgery, a 0.8 ml booster of ketamine and a 0.8 ml booster of the cocktail were administered subcutaneously to maintain anesthesia.

To perform the surgery, a rabbit was placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA) with the bregma 1.5 mm higher than the lambda. Eyedrops were given in order to protect the rabbit's eyes from drying. In the surgery, a longitudinal incision was made to the scalp and four stainless steel anchoring screws (5 mm anterior and 5 mm lateral to the bregma; 12 mm posterior and 5 mm lateral to the bregma) were implanted on the surface of the scull. As connected together, the screws formed a reference measuring point for the electrophysiological recordings.

Next, four monopolar recording electrodes made of Teflon-insulated stainless steel wire (bare diameter 125 μm), enclosed inside a 27-gauge hypodermic stainless steel tubing, were implanted into the left hippocampus (5 mm posterior and 4 / 5 / 6 / 7 mm lateral to the bregma; depths -6.5 - 7.2 mm). In addition, two electrodes were inserted into the left retrosplenial cortex (6 mm posterior and 2/3 mm lateral to the bregma; depths 3.5 / 4.5 mm). Monitoring the local-field potentials (LFPs) during the implantation assisted in indicating the preferred depths of the electrodes. After the implantation, all the electrodes were attached to a pin connector, and dental acrylic was used to form a solid foundation for the measuring instruments. On average, the surgery lasted 1 hour.

After the surgery, the rabbits were injected subcutaneously with metoclopramide [dose 0.1 ml / kg, concentration 5 mg / ml; Primepran (Sanofi Winthrop Industrie, Quétigny, France)] to facilitate normal feeding and drinking. Analgesic (buprenorphine, see above for details) was administered every 8 hours for the next 24 - 28 hours depending on the recovery rate. Post-surgical recovery time was one week at minimum.

Open Field Activity test (OFT)

The Open Field Activity test (OFT) is a method to measure exploratory behavior, general activity and anxiety, mostly with rats (Prut, & Belzung, 2002). In this study, the behavioral measures of the OFT were investigated in relation to the learning rate and spontaneously occurring hippocampal theta activity.

A square-shaped open arena of 200 x 200 cm with 50 cm high plywood walls was used. The field was divided in 16 50 x 50-cm squares, which allowed the records of three dependent variables expected to reflect the exploratory behavior, anxiety and activity levels of the subjects: I) motility (line-crossings), II) time spent in the central zone (the four central squares) and III) number of enters to the central zone. A fourth possible variable, the amount of droppings, often used with rats, was excluded since only one rabbit produced results in this aspect.

In the procedure, the rabbits were released consistently in the middle of the same edge of the arena and left alone in the room for 10 minutes. The activity was recorded by a web camera attached to the ceiling. The arena was fully and evenly lit. Both the line-crossings and the number of enters in the central zone were calculated with a tally counter from the recordings afterwards. A line-crossing was defined to have taken place when the rabbit's imaginary center of gravity crossed a line. Time spent in the central zone was measured with a stopwatch (accuracy 0.01 s). For each of the variables, values of mean units / minute (for example mean number of line-crossings / minute) for the 5- and 10-minute periods were calculated. In addition, the number of line-crossings was also calculated only for the first minute.

Conditioning procedure

The conditioning procedure began with a habituation of approximately 20 minutes to the experimental situation. A subject was placed into a Plexiglas restraining box, which was then positioned into a ventilated, electrically insulated and sound-attenuated conditioning chamber. During all the procedures, a steady background noise of approximately 65 dB was produced by a fan located in the back of the chamber for appropriate ventilation. In habituation, the functioning of the implanted electrodes was verified simultaneously as baseline hippocampal activity with no stimuli was recorded. For each individual, from the four electrodes implanted into the hippocampus,

the one producing the clearest signal and showing theta activity was chosen as the trigger channel, a channel from which the theta-ratio was calculated later during conditioning.

On the following day, baseline responses to the conditioning stimuli (CS) were recorded in an unpaired session where the CS and the US were randomly presented with an intertrial interval (ITI) of 15 - 25 seconds. The stimuli were 60 200-ms tones (CS; 4 kHz, 85 dB) and 60 100-ms corneal airpuffs (US; 0.35 bar source pressure, 64-dB sound pressure level). The airpuff, delivered through a plastic tube, was delivered through a nozzle with a 2-mm inner diameter and a distance of approximately 1 cm from the right eye. The rabbit was placed in the chamber so that the distance between the wall-mounted speaker and the rabbit's head was approximately 10 cm.

Trace eyeblink conditioning was begun on the third day of measurements. The rabbits were assigned in four groups so that, based roughly on the OFT results, each group would have rabbits with a similar range of activity. From the experimental groups, the T+ group (n = 11) was conditioned during high theta activity (theta ratio > 90 %) and the T- group (n = 10) during low theta activity (theta ratio < 30 %). Additionally, the yoked control rabbits (n(T+) = 8, n(T-) = 7) received the stimuli simultaneously with their pairs, irrespective of the oscillatory state of their hippocampus. The theta-ratio was calculated using a LabView program (National Instruments Corporation, Austin, TX). First, a Fast Fourier Transform (FFT) was calculated, and consequently the theta-ratio was calculated from the produced power spectrum ([theta / (theta + delta)] = (3.5 - 8.5 Hz) / (0.5 - 8.5 Hz). Both the FFT and the theta ratio were calculated from 1-second sweeps.

All groups received a minimum of 10 and maximum of 16 sessions of trace eyeblink conditioning, each session including 60 paired trials with a minimum ITI of 15 s. As said, the conditioned stimulus (CS) was a 200-ms tone and the unconditioned stimulus (US) was a 100-ms corneal airpuff. The CS onset occurred 700 ms before the US, leaving thus a stimulus-free trace period of 500 ms. The order in which the subjects were given the daily sessions was randomized, to avoid any effects due to the time of the day.

In order to be assigned to the extinction phase, a rabbit had to exceed a 60% success rate in the CRs in 2 / 3 consecutive conditioning sessions. The widely used criteria of 5th CR for initial learning and 8 of 9 consecutive CR's for asymptotic learning were regarded as insufficient for our purposes, which is why a stricter criterion was chosen to be followed. Indeed, only 19 rabbits were included in the extinction based on the criterion.

In the extinction sessions, the tone alone was presented contingent upon theta or lack of it 60 times with a minimum ITI of 15 s. 10 extinction sessions were executed for each rabbit. Extinction was thought to be successful when the CR-percentage was again under 20 – 30 %. To further measure the effects of T+ and T- training and the effect of a context, the group was changed for 2

T+ and 4 T- rabbits. The aim of the change was to test if a procedure-specific inner state itself has an explaining effect on learning, instead of either T+ or T- particularly.

Recordings and data analysis

The implanted electrodes recording the neural data were attached to an electrode coupler, which was anchored with dental acrylic to the rabbit's head. A low-noise preamplifier connected to the electrode coupler sent signals through an insulated cable to the amplifier (Axon Cyberamp 380, Molecular Devices Corporation, Union City, CA), to which also the bipolar electromyogram (EMG) was connected. The EMG signal was measured using smooth stainless steel wire hooks that were positioned around the upper and lower eyelids of the rabbit during each session. The signals from both the electrodes and the EMG were digitized (Digidata 1322A) and recorded by the AxoScope software (Molecular Devices Corporation) with a 2.02-kHz sampling rate. When entering the AxoScope, the EMG-signal was band-pass filtered between 30 and 400 Hz. The neural data was band-pass filtered between 0.1 and 400 Hz.

To ensure theta-contingent conditioning, signal from the chosen channel was sent straight from the amplifier to a custom-made application in the LabVIEW software, which continuously calculated the theta-ratio (details above). The training trials were presented when a custom-made program created with EPrime1 software (Psychology Software Tools Inc., Pittsburgh, PA) received the trigger pulse sent by LabVIEW.

The EMG was analyzed with MATLAB (The MathWorks Inc., Natick, MA). In the beginning, the signal was band-pass filtered to 100 - 400 Hz. After that, Hilbert transform was used to determine the absolute value of the signal. For each subject's each trial, the peak value during the pre-CS time (250 ms before the tone) was determined. Next, the mean of the peak pre-CS values (MAXpre) was calculated for each individual session. Similarly, the standard deviation of the pre-CS EMG was first calculated for each trial, after which the mean SD (SDpre) for the pre-CS EMG of each session was derived. This way a baseline EMG activity of each rabbit was obtained.

Using these standards, an eyeblink was determined as a signal that was bigger than the threshold (MAXpre + (4 * SDpre)). Moreover, only the trials fulfilling the following criteria were classified as conditioned responses: I) the eyeblink must occur during the last half of the trace period (250 ms before the US), II) the signal must exceed the threshold and III) the signal must stay above the threshold value over 10 ms.

The OFT results were analyzed in regard to the three variables mentioned above (line-crossings, time in the central zone, enters into the central zone). From each variable, a measure of units per minute during the first 5 minutes and during the 10-minute time was formed. Additionally, 1-minute line-crossings were calculated.

Statistical analyses

The statistical analyses were done with IBM Statistics SPSS 20 (SPSS Inc., Chicago, IL). The correlation tests of the OFT variables and spontaneous hippocampal theta activity were calculated using Spearman's rho, since all the variables were not normally distributed according to the Shapiro-Wilk test of normality. The value of spontaneous hippocampal theta activity of only 29 subjects was included in the analyses, because the other subjects had had misplaced electrodes.

In addition to the CR% of the sessions, some additional learning variables (the trial for showing the 5th and the 10th CR in CC; the number of trials needed to reach the learning criterion in CC; the best CR% in CC and EXT sessions) were calculated. Moreover, because the subjects had their individual number of conditioning sessions (less for the fast learners, more for the slow ones), the learning rate in conditioning was measured with the mean CR% of the first, the centermost and the last session of each subject. If the number of sessions for a rabbit was even, a mean of the two centermost sessions was calculated. In the extinction, when having the same number of sessions for each subject, five blocks of learning were created by calculating the mean CR%s of each two consecutive sessions [for example, block1 = (session1 + session2) / 2, block2 = (session3 + session4) / 2].

When examining the learning rates, a repeated measures multivariate analysis of variance (MANOVA) was utilized, having the three sessions (in conditioning) and five blocks (in extinction) as within-subjects factors, and the group (T+, T- and Y in conditioning; T+, T- and Y and same vs. changed condition in the extinction) as between-subjects factors. Also the learning in the last conditioning session and the first extinction session was compared, having the original theta-group and the change of context as between-subjects factors. Univariate analysis of variance (ANOVA) was used to compare the mean CR%s of the best sessions of different groups.

In addition, the subjects were classified in regard to whether they had reached the learning criterion or not (Mean CR% of the session > 60 in conditioning, < 20 in extinction). They were also classified as active vs. inactive using each of the 5-minute OFT values. Because the values were not

normally distributed, the median was chosen for the criterion of classification. When the effects of theta-group, context group or active versus inactive group on learning (reached the learning criterion vs. did not reach) were investigated, a χ^2-test was used.
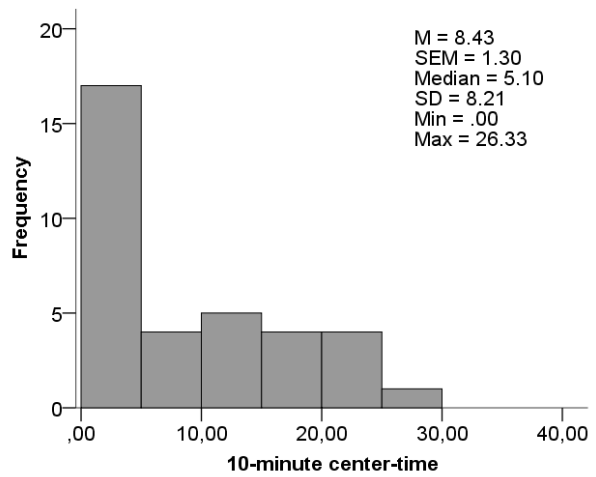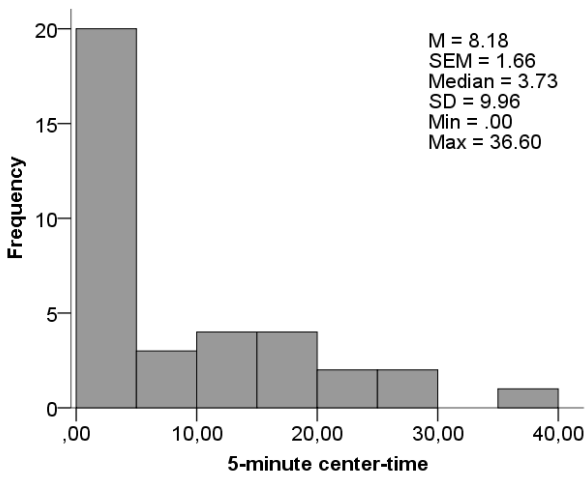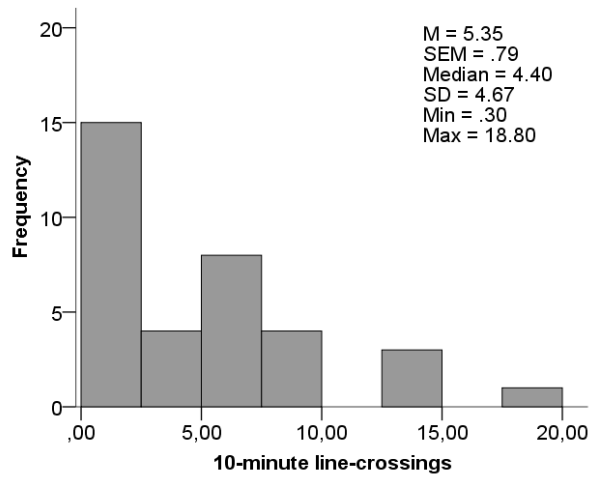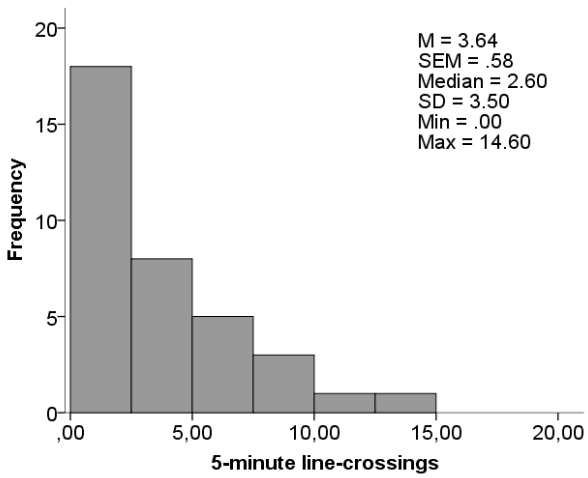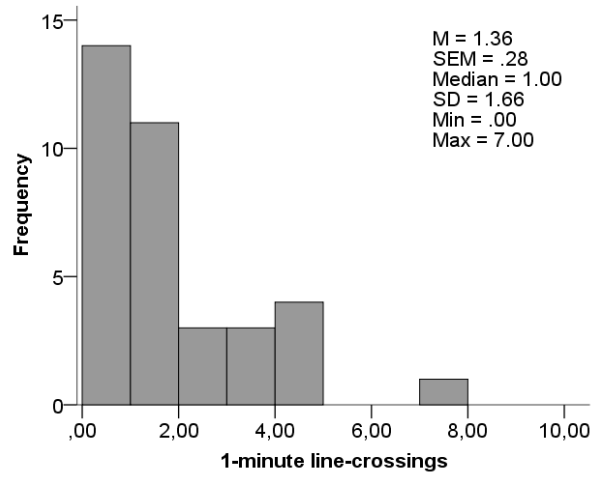
Histology

After finishing the experiments, the rabbits were anesthetized with an intramuscular injection of ketamine-xylazine cocktail, after which an overdose of pentobarbital was injected intravenously. The brains were perfused with physiological saline and 10% formalin through the ascending aorta. To mark the locations of the electrode tips, a DC current of 200 µA and 20 seconds was passed through the electrodes. After removing the brains, they were stored in a solution with 10% formalin and 30% sucrose for a week. The brains were then frozen and subsequently sectioned coronally with a microtome into slices with 100-µm thickness. Finally, after attaching the slices to gelatinized slides and staining them with Prussian blue and cresyl violet, the electrode-tip locations were defined using a light microscope and a stereotaxic atlas (Bures *et al.*, 1967).

RESULTS

Open-Field test (OFT)

The statistics of the variables used in the Open-field test are presented in the table below. All the OFT variables showed increased exploration and decreased anxiety of the subjects, when comparing the means of the 5- and 10-minute measures. The variation within the variables was great: there were both subjects who stayed relatively still and subjects who were remarkably active.

1-minute line-crossings

Frequency

M = 1.36
SEM = .28
Median = 1.00
SD = 1.66
Min = .00
Max = 7.00

5-minute line-crossings

Frequency

M = 3.64
SEM = .58
Median = 2.60
SD = 3.50
Min = .00
Max = 14.60

10-minute line-crossings

Frequency

M = 5.35
SEM = .79
Median = 4.40
SD = 4.67
Min = .30
Max = 18.80

5-minute center-time

Frequency

M = 8.18
SEM = 1.66
Median = 3.73
SD = 9.96
Min = .00
Max = 36.60

10-minute center-time

Frequency

M = 8.43
SEM = 1.30
Median = 5.10
SD = 8.21
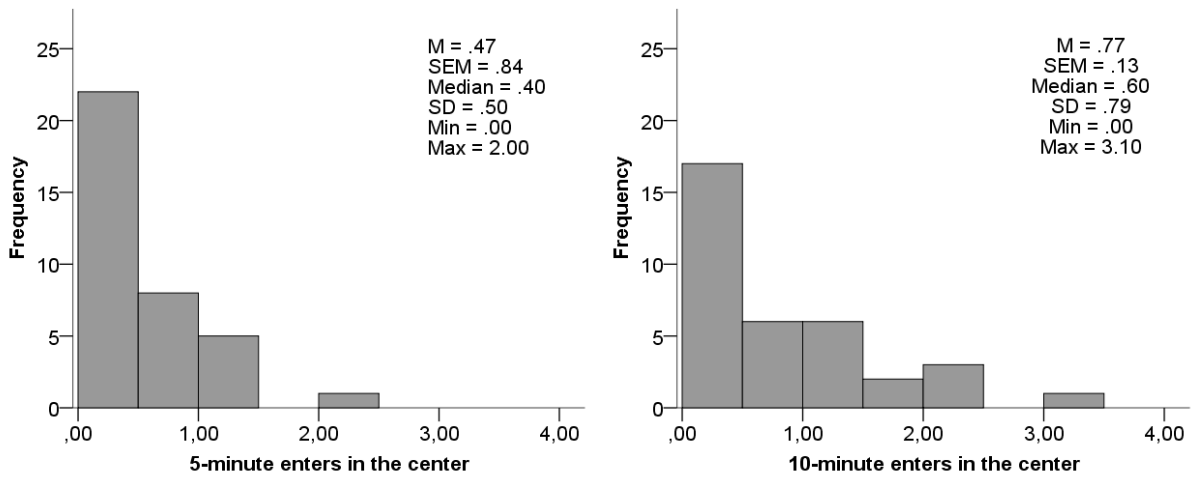Min = .00
Max = 26.33

10

FIGURE 1. The statistics of the OFT variables.

Note: all the variables are values calculated for unites / minute.

The 5- and 10-minute line-crossings (LC), center-time (CT) and enters in the center zone (EC) correlated significantly with each other. The 1-minute line-crossings correlated significantly only with the other line-crossing variables.

	5-min LC	10-min LC	5-min CT	10-min CT	5-min EC	10-min EC
1-min LC	.606***	.540***	.125	.091	.253	.313
5-min LC		.925***	.511***	.494***	.774***	.811***
10-min LC			.624***	.619***	.786***	.857***
5-min CT				.906***	.838***	.830***
10-min CT					.790***	.836***
5-min EC						.943***

Note: Spearman's rho (r_s) was used in the analyses.

***$P < 0.001$

TABLE 1. The correlations (r_s) between the OFT variables ($n = 35$)

OFT and spontaneous theta activity

Spontaneous hippocampal theta activity of the subjects ($n = 29$) had a mean of 67.84 % and varied from 58.53 % to 82.66 %. ($SEM = 0.96$, $SD = 5.17$). Spontaneous theta ratio measured before training did not correlate significantly or systematically with any of the OFT variables.

Spontaneous theta activity and learning in conditioning and extinction

When analyzing all the subjects either together or in the theta-groups (T+, T- and Y), spontaneous hippocampal theta activity did not correlate significantly with any of the variables measuring learning (CR% in the first, the centermost, the last and the best session; the trial during which the fifth and tenth CRs occurred, the trial during which the rabbit performed a CR on 8 of 9 consecutive trials) in conditioning. Similarly, in case of extinction, spontaneous theta ratio did not correlate significantly with the CR% of the first, the centermost or the last extinction session no matter if the subjects were analyzed together or in theta-groups.

Learning in theta-contingent conditioning

When including the first, the centermost and the last session of trace eyeblink conditioning in the analysis, training had a significant main effect indicating increase of CRs as a function of session [$F(2,320) = 46.14$, $p < .001$]. Neither the main effect of group [$F(2,330) = 1.76$, $p = .194$], nor the session x group interaction [$F(4,660) = 1.95$, $p = .113$] reached significance.
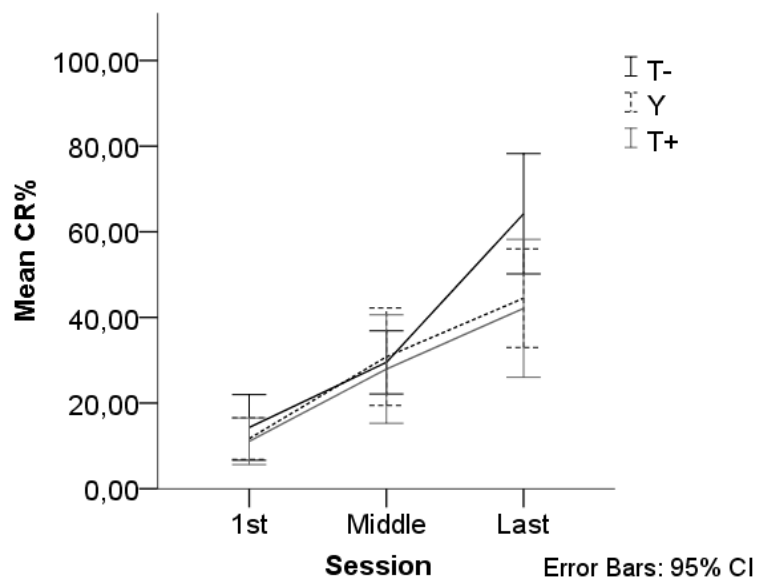

FIGURE 2. The mean CR% in the first, centermost and last session of conditioning

Altogether, 19 of the 36 subjects exceeded the 60-% criterion and were classified as learners. From the T+ group, 4 / 11 rabbits (36.0. %), from the T- group, 9 / 10 rabbits (90.0 %), and from the Y group 6 / 15 rabbits (40.0 %) reached the criterion. The proportion of learners was the highest in the T- group ($\chi^2 = 7.731$, $p < .05$). For the subjects who learned, the mean number of sessions needed was 12.95 (SEM = .19).

The mean CR% in the best session for the T- group was 72.24 % ($SEM = 4.75$, $SD = 15.03$, min = 64.29, max = 92.98), for the T+ group 49.50 % ($SEM = 6.11$, $SD = 20.27$, min = 64.41, max = 86.21) and for the Y group 55.23 % ($SEM = 5.46$, $SD = 21.15$, min = 60.34, max = 89.29). Even though the group did not have a significant effect on the previously presented variables, it had a significant effect on the mean CR% in the best session of the subjects [$F(2,330) = 3.91$, $p < .05$]. The mean CR% was significantly higher in the T- group than in the T+ group ($p < .05$).
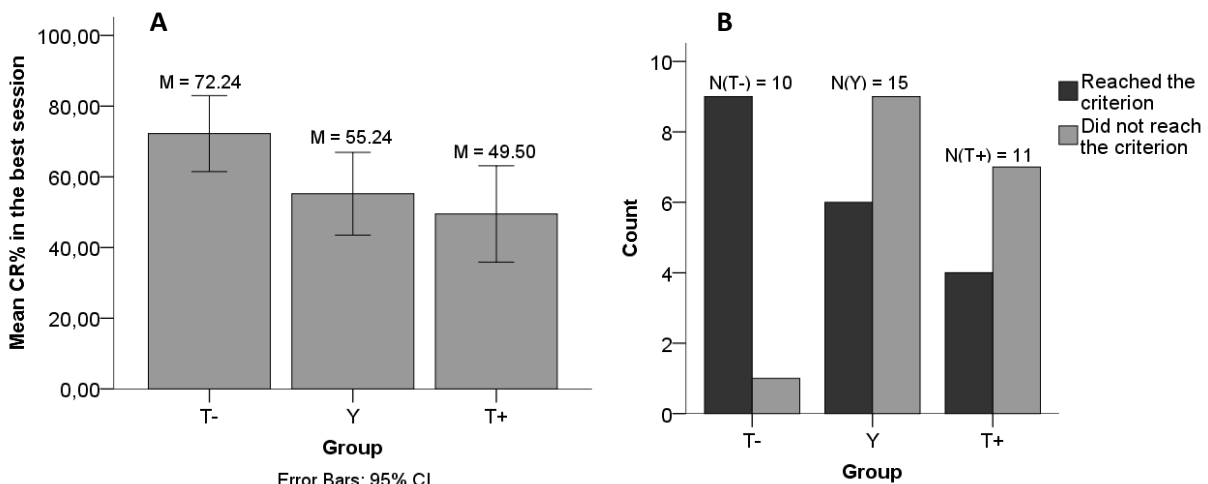

FIGURE 3.

A. The mean CR% in the best session of conditioning

B. Reaching the 60-% learning criterion in conditioning

Learning in extinction

Using the 5 training blocks, each consisting of two consecutive sessions, both the session [$F(4,130) = 8.55$, $p = .001$] and the group [$F(2,160) = 4.06$, $p < .05$] had a significant main effect on learning. In each group the amount of CRs was thus decreased by extinction training, but the T+ ($n = 6$) group learned significantly faster than the Y group ($p = .048$; $n = 6$), however not when compared to the T- group ($n = 7$).
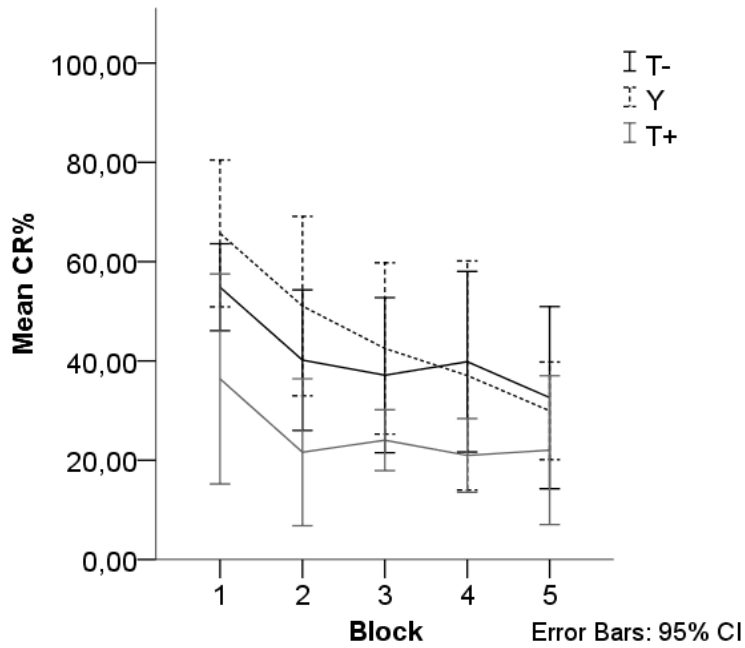
FIGURE 4. The CR% in the five extinction blocks by theta group

When examining learning with respect to the context group [same (S), $n = 7$; different (D), $n = 6$; and yoked control (Y), $n = 6$], the main effect of training was again significant [$F_{(4,130)} = 9.12$, $p = .001$]. Learning was thus improved only by the amount of practice, not by the context group.
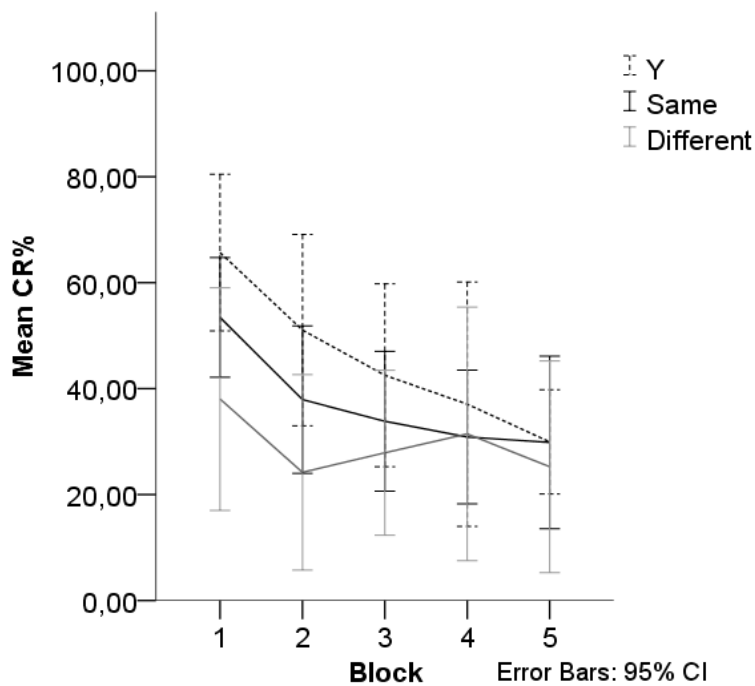

FIGURE 5. The mean CR% in the five extinction blocks by context group

Altogether, 15 of the 19 rabbits who were qualified to participate in extinction reached the 20-% learning criterion. From the T- group, 4 / 7 rabbits (57.1 %), from the Y group 5 / 6 rabbits (83.3 %) and from the T+ group all the 6 rabbits (100 %) learned the extinction to the criterion. The proportion of learners was the highest in the T+ group and the lowest in the T- group, but the difference was not statistically significant ($\chi^2 = 3.672$, $p = .159$). The mean CR% in the best session was 22.87 (*SEM* = 5.09, *SD* = 13.47, min = 1.85, max = 42.11) in the T- group, 14.98 (*SEM* = 5.10, *SD* = 12.39, min = 5.26, max = 39.29) in the Y group and 9.82 (*SEM* = 2.14, *SD* = 5.28, min = 1.75, max = 16.07) in the T+ group. The T+ group learned the best, but the differences were not significant [$F(2,160) = 2.25$, $p = 0.138$].

When splitting the data by context groups, it can be seen that 5 / 6 Y-rabbits (83.3 %), 5 / 7 S-rabbits (71.4 %) and 5 / 6 D-rabbits (83.3 %) reached the criterion in extinction. None of the groups reached it significantly better than the others ($\chi^2 = .377$, $p = .828$). In addition, the mean CR% in the best session was 14.98 (*SEM* = 5.10, *SD* = 12.49, min = 5.26, max = 39.29) for the Y group, 18.29 (*SEM* = 4.10, *SD* = 10.86, min = 1.85, max = 37.04) for the S group and 15.16 (*SEM* = 5.89, *SD* = 14.42, min = 1.75, max = 42.11) for the D group. The differences between the groups were again not significant [$F(2,160) = 0.15$, $p = 0.865$].
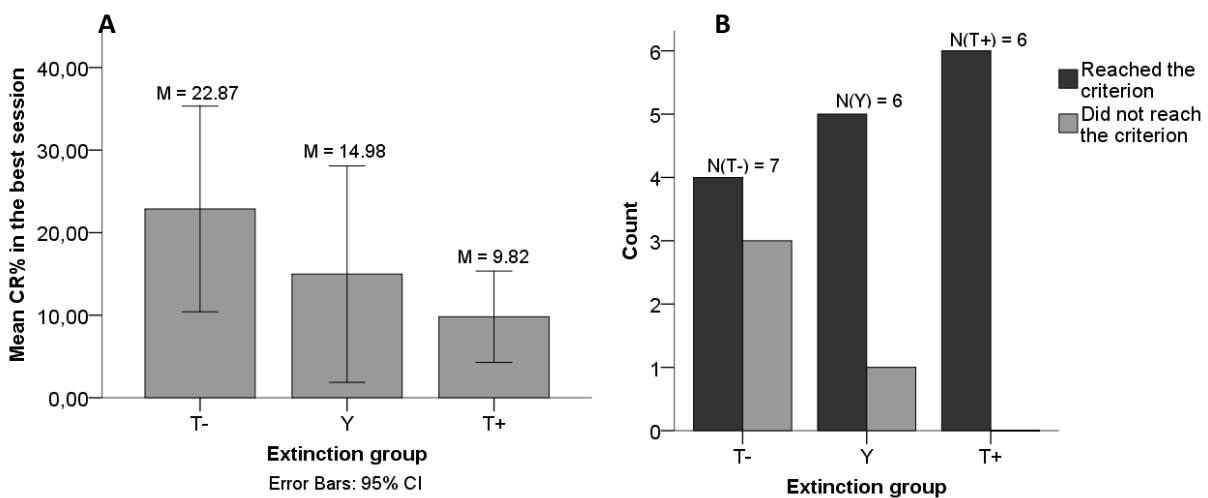

FIGURE 6.

A. The mean CR% in the best session in extinction by group

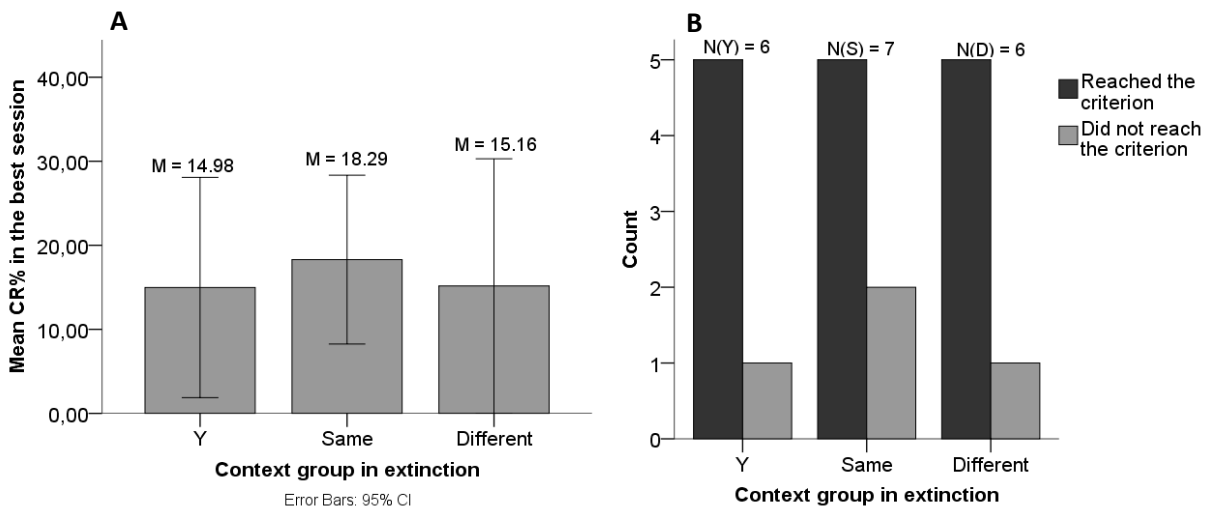B. Reaching the 20-% learning criterion in extinction by group

FIGURE 7.

A. The mean CR% in the best session in extinction by the context group

B. Reaching the 20-% learning criterion in extinction by the context group

The last session of conditioning vs. the first session of extinction

When comparing the mean CR% in the last conditioning session and the first extinction session, only training had a significant main effect, indicating decrease of CRs as a function of session [with the theta-groups $F(1,160) = 5.15$, $p < .05$; with the context-groups $F(1,160) = 7.47$, $p < .05$)]. From the theta-groups the T- group learned the best and the Y-group the worst; from the context-groups the D-group the best.
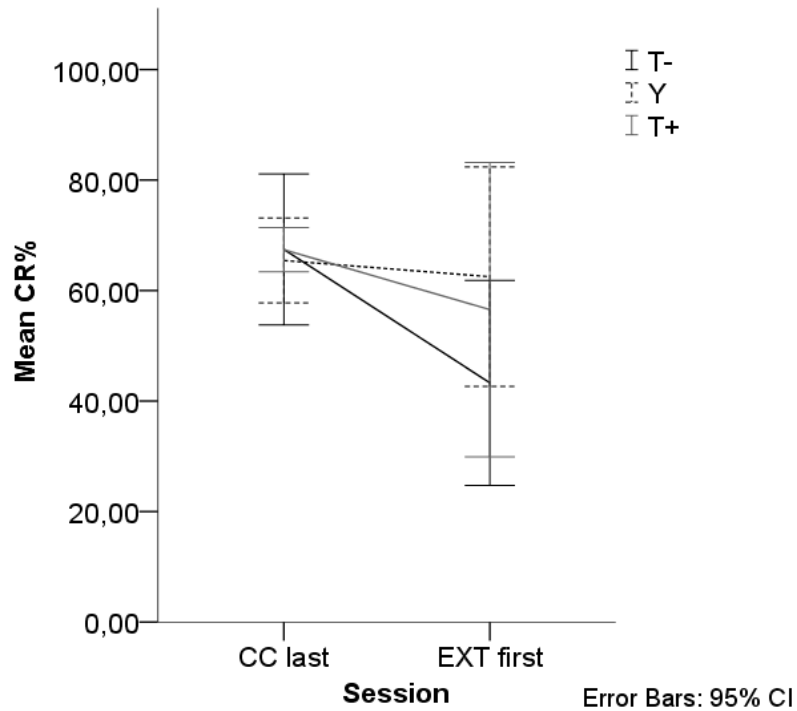
FIGURE 8. The mean CR% in the last session of conditioning and the first session of extinction by the original theta group
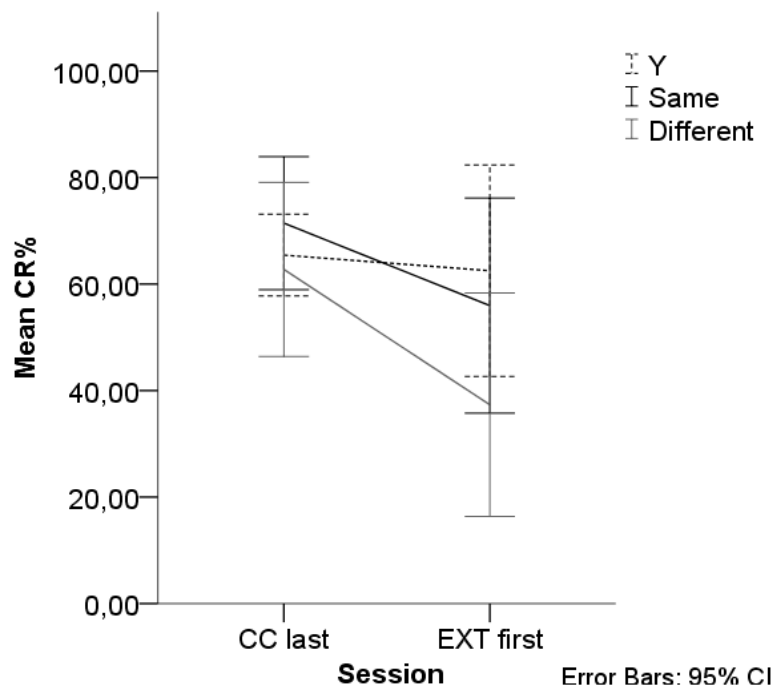

FIGURE 9. The mean CR% in the last session of conditioning and the first session of extinction by the context group

Learning of the active and inactive subjects

The subjects were classified as active versus inactive with respect to each of the 5-minute OFT results. The three active-inactive variables correlated significantly with each other (for EC and CT $r_s = .671$, $p < .001$; for EC and LC $r_s = .783$, $p < .001$; and for CT and LC $r_s = .444$, $p < .01$). In the inactive group, theta-contingent conditioning (T- and T+ groups) did not significantly affect reaching the 60-% learning criterion (for line-crossings, $\chi^2 = 1.667$, $p = .197$; for center-time $\chi^2 = .020$, $p = .887$; for enters in the center $\chi^2 = 1.111$, $p = .292$). However, in the active group the T-subjects reached the criterion drastically better than the T+ subjects (for line-crossings $\chi^2 = 7.693$, $p < .01$; for center-time $\chi^2 = 10.000$, $p < .01$; for enters in the center $\chi^2 = 7.543$, $p < .01$).

	T+	T-
Active	0 / 6	4 / 4
Inactive	4 / 5	5 / 6

TABLE 2. The proportion of the subjects who reached the 60-% learning criterion

Note: The active-inactive classification is based on the results of 5-minute center-time

When executing a repeated-measures MANOVA for conditioning, training had a significant, learning improving main effect in both the active [F(2,140) = 19.23, $p < .001$] and the inactive group [F(2,140) = 31.56, $p < .001$] of 5-minute center-time. In the active group, also the theta-group improved learning significantly [F(2,150) = 5.50, $p < .05$]. The active T- subjects learned significantly better than the active T+ subjects ($p < .05$). With the other 5-minute OFT variables, the results were parallel but not significant.

When taking a look at the early phase of learning (the mean CR% of the centermost session), it seems, but is not statistically significant, that theta-contingent conditioning affected learning differently in the active and inactive groups. For the active subjects, T- training was the most beneficial, whereas for the inactive subjects, T+ training provided better learning. However, in the late phase of learning (the CR% of the last session) the effect of the theta group nearly vanished for the inactive group, setting all the T- subjects and the inactive T+ subjects approximately on the same level. Only the CR% of the active T+ subjects remained visibly, but not significantly, below the level of the others.
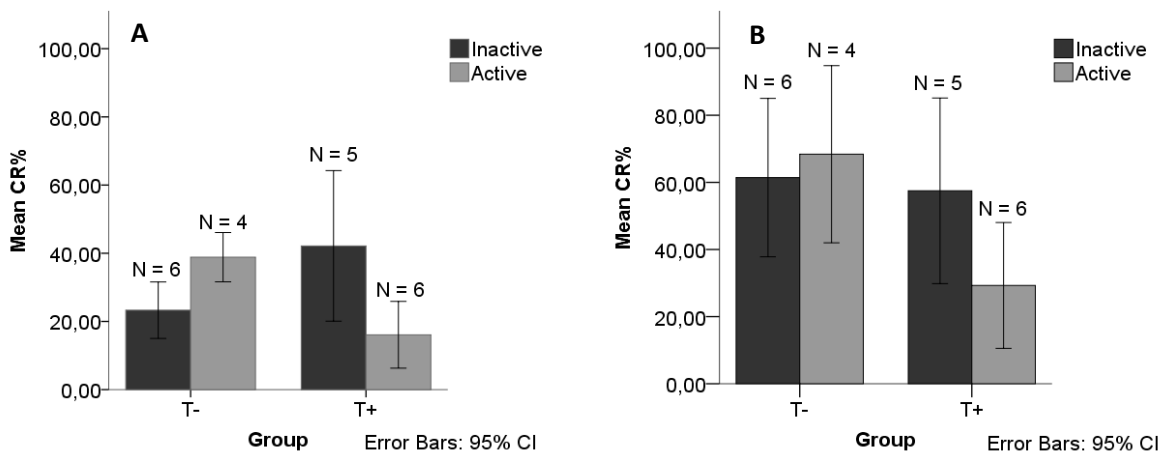
FIGURE 12.

A. Mean CR% in the centermost session of conditioning for the active and inactive groups

B. Mean CR% in the last session of conditioning for the active and inactive groups

DISCUSSION

This experiment focused on investigating the effects of theta-contingent conditioning on learning in trace eyeblink conditioning and extinction, and exploring the relationship between spontaneously occurring hippocampal theta activity and behavioral tendencies of the subjects. It was assumed that either the high or low-theta training would improve the initial learning in conditioning, but not necessarily affect the final performance or learning in extinction. In extinction we expected to see that the change of the theta-group, and thus the training context, would improve learning. It was also supposed that high magnitude of spontaneous hippocampal theta activity would be related to higher anxiety and lower motility in the Open Field test.

In contrast with our hypotheses, the amount of conditioned responses in conditioning increased only as a function of training. However, the theta-group (T- > Y > T+) had a significant effect on reaching the 60-% learning criterion and on the CR% in the best session. In extinction, the amount of CRs decreased both as a function of training and due to the training group (T+ < T- < Y). Finally, none of the OFT measures correlated significantly with spontaneous hippocampal theta activity. However, using an OFT-based active-inactive classification in the case of conditioning, the active subjects seemed to profit significantly better from T- training than from T+ training.

OFT and spontaneous hippocampal theta activity

Even though the OFT values correlated significantly with each other and seemed to provide us with a consistent measure of anxiety and exploration (the behavioral tendency of a subject), none of them correlated significantly with spontaneous hippocampal theta activity. It is possible that the behavioral tendencies do not correlate with the natural magnitude of spontaneous hippocampal theta activity as we supposed. It is also possible that we did not measure the relationship correctly. The first question is, does the empty, square-shaped OFT arena really motivate the subjects to behave as they would in a more natural situation? Prut and Belzung (2002) defend the validity of the OFT by explaining that the element of anxiety is created when the animal is separated from its social group and natural environment, and placed on a large arena. Staying in the center reflects lower anxiety, and increased locomotion explorativity. Another type of analysis is proposed by Meijsser, Kersten, Wiepkema and Metz (1999). They name three factors in the behavior of sub-adult rabbits: boldness (hopping using large part of the field; rearing up), prudence (walking; moving on the forelegs) and fear (standing stretched; low locomotion). Perhaps some other type of classification or "personality test" could have produced different results with respect to spontaneous hippocampal theta activity.

It is also possible, that the situation in which spontaneous hippocampal theta activity was recorded affected the mental state of the rabbits very differently from the OFT. During the recordings, the rabbits were placed in Plexiglas restraining boxes in a dim conditioning chamber. As rabbits live normally in small tunnels (Prut, & Belzung, 2002), the recording situation might have been less intimidating and therefore have produced a different mental state than the anxiety-provoking OFT. A more stressful situation can indeed produce more hippocampal theta activity, as Berry and Swain (1989) have shown using water deprivation. However, the main question is, whether the individual differences are manifested equally in a relatively neutral and in a more stressful situation.

Another problem in measuring spontaneous hippocampal theta activity and trying to associate it with the behavioral tendencies in the OFT might have been that even though the 20-minute recording situation was new for the rabbits, it remained unchanged and absent of stimuli. The hippocampal theta activity related to attention and arousal is normally habituated with time (Green, & Arduini, 1954; Bland, & Oddie, 2001). Furthermore, according to Vinogradova (1995, pp. 571), "theta appears when new and potentially significant information is singled out from the continuous and relatively stable background noise during the orienting response -." The lack of stimuli could at least diminish the individual differences in the magnitude of spontaneous hippocampal theta. It may

be that the anxiety-provoking OFT produces a different mental state reflected by theta activity and thus more differences between the subjects, than a calm and stable recording situation. This could explain why we found no connection between spontaneous hippocampal theta activity and behavioral tendencies of the subjects. Perhaps the spontaneous theta activity should have been recorded in the presence of some sort of stimuli, or even during the OFT.

Theta-contingent conditioning

The results of theta-contingent conditioning were surprising, since the theta group had no significant effect on the course of learning. This is against the findings of Griffin, Asaka, Darling and Berry (2004), where the T+ group learned significantly the best in both the early and the late phase of learning. Later it has been suggested that high-theta training enhances learning only in the early phase (Darling, Takatsuki, Griffin, & Berry, 2011). We found neither of these effects. Quite the contrary, the group that was the best in both reaching the 60-% learning-criterion and having the highest CR% of the best session, was the T- group whereas the T+ group was the worst one. This provides support for the findings of Nokia, Penttonen and Wikgren (2010) who found that ripple-contingent training, occurring during a low-theta state, improved learning significantly when compared to a yoked control group. They speculated that during this period the hippocampus is in a more sensitive state, without subcortical inhibition. Following the logic, training during this "vulnerable" brain state could produce better results. It seems now that the high magnitude of theta is not the single key for better learning, as assumed before.

When further discussing the results, Prokasy's (1984) two-phase model of conditioned learning offers us an important framework. According to this model, contingency detection and response selection occur in the first phase of learning, whereas response acquisition and fine response shaping take place in the relatively independent second phase. The general agreement is that hippocampus (Clark, Manns, & Squire, 2002) and its theta activity (Asaka, Mauldin, Griffin, Seager, Shurell, & Berry, 2005; Nokia, & Wikgren, 2010) are related to the associative process of trace but not delay conditioning, thus the first phase of learning. We also know that the hippocampo-cerebellar synchrony increases in relation to the power of hippocampal theta oscillation, which would be especially important for the first phase (Wikgren, Nokia, & Penttonen, 2009; Hoffman, & Berry, 2009). Consequently, it seems very logical why either high or low-theta

training could improve learning in the early phase more than in the late phase. Why the difference was not significant for either of the groups remains thus a very important question.

Our results of the late-phase learning are also confusing. Based on Prokasy's model (1984), in the second phase there occur response acquisition and response shaping – processes that resemble delay-conditioning and do not necessarily require hippocampus (Cheng, Disterhoft, Power, Ellis, & Desmond, 2008; see Christian, & Thompson, 2003, for a review). We also know that as the time after learning increases in trace eyeblink conditioning, the hippocampus has less and less effect on performance (Takehara, Kawahara, & Kirino, 2003). Thus, if after the first phase of learning the significance of hippocampus on learning diminishes, the theta-state should theoretically not have such a great effect on learning. However, the differences between the learning of the groups, although not significant, continued to increase during this late phase of learning.

Theta-contingent extinction

In extinction, both the theta-group and the amount of training affected learning significantly. The T+ group learned in total significantly better than the Y group, but hardly differed from the T- group. The result of the benefit of theta-contingent training in extinction is interesting. In fact, Schmajuk and DiCarlo (1992, see for a review) noticed that activation of the hippocampal regions of CA1, CA3 and dentate gyrus normally decreases during extinction. Nokia and Wikgren (2010) detected in their discrimination-reversal study that theta activity was related to contingency detection in conditioning but not in reversal conditioning, in which the task was also to unlearn the original CS-US relationship. However, the experiment of Nokia, Penttonen and Wikgren (2010) did produce rather parallel results with our study. They found that ripple-contingent training (during a low-theta state) retarded extinction when compared to a yoked control group. According to the researchers, one explanation is that a low-theta state free of subcortical control might rather favor excitatory than inhibitory processes related to learning, and therefore hinder extinction. As the significance of our results suffers from the fact that the theta-group of altogether 6 of our subjects was changed for the extinction, the research of optimal brain state for extinction has to be continued.

When exploring the effect of the theta-group change (T+ → T-; T- → T+) on learning in extinction, we found no significant differences between the same versus different context groups. Because extinction is thought to be a relatively context-specific phenomenon, even more than

conditioning (Bouton, 2004), we had a vague hypothesis that the subjects with a changed theta-context could learn better than the others. However, this effect was not significant. Perhaps only the change of the oscillatory context is not sufficient for the effect to occur. Furthermore, the effects of neither the context group nor the theta group were significant when taking into account only the last session of conditioning and the first session of extinction. None of the groups (T+, T-, Y, S or D) learned thus better than the others in the very beginning of the extinction. However, as we had such small groups in the end, abandoning any hypothesis related to the context-dependent learning, or the effect of theta-contingent conditioning on learning in the extinction, is not clever.

Learning in the active and inactive subjects

Some rather interesting results were obtained when examining the conditioned learning of the subjects divided in two groups (active vs. inactive) based on their 5-minute activity in the OFT. First of all, the theta-groups of the inactive subjects did not remarkably differ from each other in reaching the 60-% learning criterion. However, within the active subjects, the T- group reached the criterion significantly better than the T+ group: in the T- group nearly all of the subjects learned, in the T+ group nearly none. The same effect was reproduced when analyzing the whole course of learning: the active T- subjects learned significantly better than the active T+ subjects. This is again against the observations of various experiments that emphasize the power of either high pre-training level of hippocampal theta (Berry, & Seager, 2001) or T+ training at least in the early phase of learning (Griffin, Asaka, Darling, & Berry, 2004).

Purely based on the graphs, it seems that in the early phase of learning, high-theta training was the most beneficial for the inactive subjects and low-theta training for the active ones. In the late phase of learning this inverse effect seems to disappear, perhaps related to the diminishing role of the hippocampus (Clark, Manns, & Squire, 2002). Why would there be a differential effect of theta-contingent training for the active and inactive subjects? After observing that tailshocks improved conditioning, Beylin and Shors (1998) proposed that exposure to stressors improves learning via an enhanced attention to the discrete cues in the environment. Also a motivational state created by water deprivation can increase pre-training theta activity and learning (Berry, & Swain, 1989). Oppositely, also a low-theta state rich in sharp-wave ripples has been proposed as a beneficial state of learning as a consequence of hippocampal sensitivity (Nokia, Penttonen, & Wikgren, 2010). The question is, if both of the theories are true, is the function of hippocampal theta activity indeed

mediated by the behavioral tendencies, such as anxiety or alertness of the subjects? Perhaps a high-theta state is more beneficial for the learning of the inactive, more anxious subjects and vice versa. Learning of the individuals with different behavioral tendencies is a fascinating area of research, in which also the role of sensory inhibition and activation of hippocampal theta activity needs to be reconsidered (Sainsbury, 1998).

Strengths and limitations

This experiment, examining the hippocampal theta activity, learning and behavioral tendencies, is special with its multidimensionality. As already discussed, our results differ greatly from some previous research, but are at the same time consistent with some other, recent results. The extensiveness of this study is clearly a strength in the field of theta-contingent conditioning research. In this study, learning was not only analyzed for the initial phase but investigated until reaching the learning criteria (CR% > 60 in conditioning, < 20 in extinction), whenever the subjects learned. The number of subjects was also relatively high. Instead of only high and low theta groups, or only a high theta and a control group, altogether three groups were included in the experiment, analyses and report: high and low-theta groups and their yoked controls. The planning was done carefully.

However, the multidimensionality of the experimental design causes also some limitations for the interpretations. First of all, even though the number of subjects in each group was sufficiently high in conditioning, the analyses of the extinction might suffer from the lack of subjects in each theta and context group. Therefore the results of extinction are preliminary and should be interpreted with caution. Secondly, one weakness is the somewhat arbitrary classification of the subjects as active versus inactive in the OFT. The division was based on the median in order to have enough subjects in each group, which could have been done differently as well.

It is not yet clear why the outcomes of this study differed so much from the previous, largely unquestionable work. One explaining factor might be the learning criteria that were used – they were now stricter than what has been used before. However, any differences in the learning criteria do not explain the results of the initial learning, which now differed from the previous work the most. A lack of subjects in the previous experiments (for example Seager, Johnson, Chabot, Asaka, & Berry, 2001), compared to our study, might affect the results. Also, many articles (for example Griffin, Asaka, Darling, & Berry, 2004) have only included the results of the early learning, leaving

out the final level of performance in different groups. Now that all these points have finally been taken into account, further research related to hippocampal theta activity, personality and learning is highly recommended.

As a consequence of a well planned multidimensional experimental design, we managed to provide firm evidence against the power of high-theta training in conditioning. At the same time we introduced some new viewpoints and questions to be considered in the future. While acknowledging the limitations of this experiment, our results clearly point out some tempting subjects of research. With this experiment we still could not figure out what spontaneous hippocampal theta activity reflects, but we now know that the effect of theta-contingent conditioning on learning is definitely not as simple as thought before. The differential effect of theta-contingent training for active and inactive subjects is also something to be considered in further studies. Finally, the potential of the research related to hippocampal theta activity and learning is huge. With this study we thus wish to contribute to the developing knowledge of learning and memory, hoping that persistent research would lead to creating tools for human learning as well.

Conclusion

Our results in theta-contingent trace eyeblink conditioning and extinction differ remarkably from previous research. We did not find the positive effect of high-theta training on learning rate even in the early phase in conditioning, but in contrast low-theta training had positive effects on the final performance. In extinction, high-theta training enhanced learning significantly, but theta context change did not have a significant effect on the performance of the subjects. We were not able to show that spontaneous hippocampal theta activity and behavioral tendencies of the subjects would be related to each other. However, our classification of the subjects to active versus inactive showed that theta-contingent training might affect differently the subjects with distinct behavioral tendencies. Even though the results are preliminary, it is possible that the controversial results of the previous research could partly be explained by the distinct needs of the individuals with different behavioral tendencies. Indeed, it is now time to reconsider the role of hippocampal theta activity in sensory inhibition and activation. It is a tempting possibility that the inconsistent results related to theta-contingent conditioning could be explained by differences in the personalities of the subjects.

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