Hippocampal event-related potentials to tone-CS during classical conditioning of the rabbit nictitating membrane response

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Abstract

Hippocampal event-related potentials (ERPs) and nictitating membrane movement were measured during CS period of delay classical conditioning of the rabbit nictitating membrane response (NMR). A 350-ms tone served as the conditioned stimulus (CS) and a 100-ms corneal airpuff as the unconditioned stimulus (US). Comparison of NMR amplitudes before and after conditioning revealed a robust conditioned response (CR) preceding US onset by approximately 100 ms. By comparing principal component analysis (PCA) factor scores and subject average ERP amplitudes before and after conditioning with paired t-tests, changes in hippocampal ERPs due to learning were found to occur within very short time windows immediately following CS onset and CR onset. No sign of a temporal neural model of the CR, present in numerous multiple-unit activity (MUA) studies, was found. Based on previous experimental results and theories, it is concluded that the timing of the brief ERP changes reflects hippocampus' role in associating the CS with the context of conditioning and with the US, and in timing the CR adaptively.

Keywords: Delay classical conditioning; Rabbit nictitating membrane response; Hippocampus; Event-related potentials; Principal component analysis

1. Introduction

The procedure used in rabbit nictitating membrane response (NMR) conditioning was first introduced by I. Gormezano in the 1960's (Gormezano, 1966). During NMR conditioning a conditioned stimulus (CS, usually a tone) is repeatedly paired with an unconditioned stimulus (US, usually an airpuff directed towards the cornea of the rabbit's eye). The tone-CS precedes the airpuff-US and the two stimuli can either overlap and coterminate (delay paradigm) or there can be a gap between them (trace paradigm). As a consequence of the recurring paired presentations, the animal develops a conditioned response (CR) to the CS, that is, the rabbit starts to cover its eye in response to the tone alone. Rabbit NMR conditioning is a simple and discrete model of mammalian learning (Thompson, Berger, Berry, Hoehler, Kettner & Weisz, 1980), and is therefore widely used in the study of the neural basis of learning and memory. Several studies on humans using the same conditioning paradigms and stimuli have also been conducted (see for example Gabrieli, Carrillo, Cermak, McGlinchey-Berroth, Gluck & Disterhoft, 1995; Woodruff-Pak, 1993).

The neural basis of eyeblink classical conditioning is well known today: the cerebellum – both cerebellar cortex and the interpositus nucleus (IPN) – together with the brain stem circuitry connected to it are a necessary and sufficient part of the brain for learning to occur (Krupa, Thompson & Thompson, 1993; Thompson, 1991; Thompson & Kim, 1996). Hippocampus is thought to impose only a modulatory role, dependent on an intact IPN (Sears & Steinmetz, 1990).

The more complex the conditioning paradigm, the more influence on learning results hippocampus seems to have (Kim, Clark & Thompson, 1995; McLaughlin, Skaggs, Churchwell & Powell, 2002; Port, Mikhail & Patterson, 1985; Solomon, VanderSchaaf, Thompson & Weisz, 1986). More precisely, hippocampus has been found to possess a time-limited role in controlling the adaptive timing of the learned CR (Akase, Alkon & Disterhoft, 1989; James, Hardiman & Yeo, 1987; Moyer, Thompson & Disterhoft, 1996; Port, Romano, Steinmetz, Mikhail & Patterson, 1986; Solomon et al., 1986; Thompson, Moyer & Disterhoft, 1996).

The role of hippocampus has mostly been studied using delay and trace conditioning paradigms, and measuring multiple- or single-unit activity from the pyramidal cells of hippocampal fields CA1 and CA3. Delay NMR conditioning accompanied by multiple-unit activity (MUA) measuring was the method also used by Berger, Alger and Thompson (1976) who first discovered, that during conditioning of the NMR a rapid change in hippocampal pyramidal cell unit activity preceding behavioral CR performance is first present in the 250 ms time period following US presentation (US period), and as a consequence of continuated conditioning moves forward in time to the CS period forming a preceding (25-35 ms) temporal neural model of the conditioned NMR. Alternative measures of activity, like event-related potentials (ERPs) derived by averaging from electroencephalography (EEG), have been used relatively rarely (see Múnera, Gruart, Muñoz, Fernández-Mas & Delgado-García, 2001; Múnera, Gruart, Muñoz & Delgado-García, 2000; Power, Thompson, Moyer & Disterhoft, 1997), although their value as an information source has been identified (Bullock, 1997). MUA and EEG differ in terms of the electrical activity measured: MUA reflects high frequency short duration action potentials, whereas EEG is a measure of graded postsynaptic and afterpotential slow waves (Buchwald, Holstein & Weber, 1973). Compared to MUA, that is virtually impossible to measure from humans, ERP measures provide an opportunity to more straightforwardly compare animal experiment results with results of studies using humans as subjects.

The aim of this study is to further explore the functions of hippocampus in classical conditioning of the rabbit NMR by measuring and comparing NM movement and hippocampal ERPs during the 250-ms period following CS onset (CS period, Berger at al., 1976) before and after conditioning. Following delay paradigm, a 350-ms tone CS is coupled with a coterminating 100-ms airpuff US. In control group, the two stimuli are presented separately in random order. The ERPs recorded are explored using principal component analysis (PCA) and paired t-tests. PCA is expected to detect and depict the component structure of the ERP more objectively compared to traditional peak or area measures. It is assumed that the possible conditioning dependent changes in hippocampal ERPs resemble those previously observed by measuring MUA and correlating it with NMR data (Berger et al., 1976). Some unexpected and deviating results can also be anticipated, since the source of information is different from that of MUA measures (Buchwald et al., 1973).

2. Methods

2.1. Subjects

Twelve female adult New Zealand albino rabbits weighing 2.7-3.9 kg at the time of surgery were used as subjects. The rabbits were housed in individual metal cages in the research animal laboratory of University of Jyväskylä. Food and water was provided ad libitum and room temperature and humidity controlled. Subjects were maintained on a 12:12-h light:dark cycle, with lights on at 6.00 am. All operations were conducted during the light portion of the cycle. All experimental procedures were put into practice in accordance with the European Communities Council Directive (86/609/EEC) on care and use of animals for research aims.

2.2. Surgery

The rabbits were anesthetized with an i.m. injection of ketamine-xylazine cocktail (Ketaminol, 50 mg/ml, 5.6 ml; Rompun®, 20 mg/ml, 2.2 ml; physiological saline, 2.2 ml). Before surgery an injection of 1 ml/kg was given and then 1 ml every twenty minutes to keep the level of anesthesia steady. Eyedrops (Oftan®) were used to prevent the eyes from drying or getting infected. At the beginning of the surgery the rabbit was placed in a stereotaxic instrument (Kopf Instruments) with bregma 1.5 mm over lambda. A longitudinal incision was made to the scalp and four stainless steel anchoring screws attached to the skull. The screws were connected in pairs and served as reference measuring points for EEG. Next, Teflon insulated recording electrodes were implanted chronically into the right hippocampus. For six rabbits in the conditioning group four electrodes (β5.0, R4.0, H+6.0; β5.0, R5.0, H+6.0; β5.0, R6.0, H+5.5; β5.0, R7.0, H+5.5), for four rabbits both in conditioning and control groups one electrode (β5.0, R5.0, H+5.5) and for the remaining two rabbits also both in conditioning and control groups three electrodes (β-5.0, R4.0, H+5.5; β-5.0, R5.0, H+5.0; β-5.0, R6.0, H+4.5) were implanted[1]. During implantation, EEG was monitored to define the preferred depth of the electrode. Finally, the electrodes were attached to two pin connectors and the whole construction cemented in place with dental acrylic mass. A subcutaneous injection of analgesic solution (1 ml; 1 part Temgesic® and 9 parts physiological saline) was given at the end of the surgery to provide for analgesia. The rabbits were then moved to their cages and their condition monitored regularly. At least one week was reserved for recovery from the surgery before starting the experiments.

Either accompanying electrode implantation or on a separate occasion, a nylon loop was sutured into the nictitating membrane of the rabbit's right eye to measure the NM movement during training. Oftan® Obucain eyedrops were used for local anesthesia.

2.3. Behavioral training

Prior to the experiments, the rabbits were placed (approximately twenty minutes) in a Plexiglas restraining box located in a ventilated, electrically insulated and soundproof conditioning chamber to familiarize them with the situation. The stimuli used were a 1000-Hz, 85-dB, 350-ms tone CS and a 2.1-N/cm² source pressure, 100-ms corneal airpuff US delivered through a nozzle (diameter 2 mm) placed approximately 1 cm away from the eye. The unpaired sessions consisted of 140 trials: 70 tone and 70 airpuff stimuli were presented separately in a pseudorandom order with an intertrial interval (ITI) averaging at 20 seconds. During delay conditioning trials, CS and US were presented coterminating, CS onset preceding US onset by 250 ms. The conditioning sessions consisted of 80 trials: 60 conditioning, 10 CS-alone and 10 US-alone trials were presented in a pseudorandom order. The ITI varied randomly between 30 and 50 seconds, averaging at 40 seconds. A computer was used to control the stimulus presentation and the rabbit's behavior was monitored via video camera during the experiments. A robust CR on eight out of nine consecutive paired or CS-alone trials had to be present to meet conditioning criterion. Sessions were conducted one per day on consecutive days. For the six rabbits only in conditioning group, ten conditioning sessions were conducted. The remaining six rabbits both in conditioning and control groups first went through

[1] For six rabbits with four recording electrodes in hippocampus, four recording electrodes were also implanted to the lateral hypothalamus (β1.0, R2.0, H-6.0; λ0.0, R2.0, H-5.0; λ1.0, R2.0, H-4.0; λ0.0, R3.0, H-3.0). For four rabbits with one recording electrode in hippocampus, three recording electrodes were implanted to the lateral hypothalamus (β-1.0, R2.0, H-6.0; β0.0, R2.0, H-5.0; β1.0, R2.0, H-4.0), one to area H VI (λ-0.5, R5.0, H-6.0), one to the interpositus nucleus (λ0.5, R5.0, H-14.5), one to the lateral pons (λ8.0, L2.5, H-20.0), and one cooling probe to the dentate gyrus (λ0.5, R6.5, H-14.0). For the remaining two rabbits with three recording electrodes in hippocampus, two recording electrodes were also implanted to the prefrontal cortex (β4.0, L0.8, H-3.0; β5.0, L0.8, H-4.0), one to area H VI (λ-0.5, R5.0, H-6.0), one to the interpositus nucleus (λ0.5, R4.5, H-16.0), and for one of them two to lateral hypothalamus (β-1.0, R2.0, H-6.0; β0.0, R2.0, H-5.0).

unpaired training (nine sessions for four rabbits and five for two rabbits) and then were trained to criterion (ten sessions for four rabbits and five for two rabbits). The subjects thus formed a conditioning group of twelve individuals of whom six individuals also formed the control group.

2.4. Recordings and data reduction

To acquire EEG measures, low-noise pre-amplifiers were attached directly to the electrode coupler anchored with dental acrylic mass to the rabbit's head. A flexible, insulated cable was used to connect the animal to the amplifiers. In addition to amplifying, the neural signals were band-pass filtered (0.1–200 Hz) and digitized at a rate of 500 samples/s.

In order to measure NM movement during the experiments, the nylon loop attached to the rabbit's NM was linked to the swivel arm of a minitorque potentiometer by a rigid stainless steel hook. Movements of the NM were converted to voltage by the potentiometer: 1 mm equaled 1 V. Appearance of the CR was monitored by measuring maximum nictitating membrane movement during CS period. All NM movements exceeding 0.5 mm were counted as responses.

BRACE software was used to record the data. For four rabbits in the control group during the nine sessions of unpaired treatment only 20 to 28 trials per session were recorded. Otherwise all trials were recorded and stored.

2.5. Histology

In order to perform histology the rabbits were first anesthetized with an i.m. injection of ketamine-xylazine cocktail and then overdosed with an i.v. injection of pentobarbital. After the rabbit's death, circulation was perfused by letting in saline and then 10% formalin through the ascending aorta. Next, the brain was removed and stored in 10% formalin solution for approximately two weeks. The brains were then frozen and coronally sectioned with a microtome into 100 μm thick slices. The slices were attached to gelatinized slides and stained with cresyl violet. At last, the electrode tip locations were determined from the slides with the help of a microscope and a stereotaxic atlas.

2.6. Statistical analyses

All analyses were performed with SPSS 11.5 for Windows. An alpha-level of .05 was used in all statistical testing primarily, but test results were also considered relevant when p-value fell below .1. SigmaPlot 8.0 was used to visualize the data and PCA factor structure. EEG and NMR movement measured every two milliseconds during CS period were treated as dependent variables (125). The selection of the time period under analysis was based on previous existing data concerning the timing of hippocampal activity changes (see for example Berger et al., 1976; Múnera et al., 2001). Bad trials were eliminated using |0.5| mm NMR movement during pre-stimulus period (100 ms) as criterion. 3.8% of all trials in conditioning group and 3.3% in control group had to be eliminated. Only paired and CS-alone trials were selected for further statistical analyses.

Behavioral data analysis: Paired t-tests. Tests were administered to conditioning and control group NMR data separately. Maximum values of NMR average amplitudes defined for each subject on first and last conditioning or unpaired sessions (session one versus five, and in the case of four rabbits in control group sessions one and two versus eight and nine) were compared with paired t-test. NMR amplitudes averaged by subjects and sessions at the measured 125 time points (representing 2 ms in real time) following CS onset on first and last sessions were also compared to determine the latency of the possible differences in amplitude. The CR appearance (NM movement exceeds 0.5 mm) was defined from the grand average NMR amplitude on conditioning session five.

ERP data analysis: PCA and paired t-tests. Baseline correction was conducted by averaging EEG from the 100 ms prior to CS onset and subtracting the obtained mean value from all following time points. All analyses were performed separately for control and conditioning groups, and also for hippocampal field CA1 and dentate fascia data due to the fact that not all subjects had recording electrodes from both hippocampal areas. Also, the ERP values obtained from the two hippocampal areas were opposite in polarity which would have affected the resulting factors of the PCA. Thus, four separate PCAs were conducted, using ERP data from single CS-alone and paired trials (CS period, 250 ms) measured on first and last unpaired or conditioning sessions as cases, and the measured 125 time points as variables. Covariance matrix was used to extract factors, and the number of factors determined with the help of scree test and preceding factor extraction from correlation matrix (eigenvalues over 1). Varimax-rotation was conducted to minimize the temporal overlap of the obtained factors (van Boxtel, 1998). Factor scores were restored and used to explore the differences in factor loadings due to unpaired treatment or conditioning: Scores on first and last unpaired or conditioning sessions were averaged by subjects and sessions, and compared using paired t-test.

Paired t-test was also performed on subject average ERP amplitudes at 125 time points on first and last unpaired or conditioning sessions. This was done in order to create more comparable results in relation to the previously published MUA studies, and also in order to compare the results of the study at hand obtained with PCA and traditional means.

3. Results

3.1. Histology

It was confirmed that the recording electrodes were located in hippocampal field CA1 and/or in dentate fascia (df) in all rabbits. In the case of two rabbits, that were kept alive for further experimental purposes, the electrode locations were deduced from the histological and ERP data of the other rabbits: Data displaying a negative first peak in response to the tone-CS was inferred to reflect hippocampal field CA1 activity, and data displaying a positive first peak hippocampal dentate fascia (df) activity. Four rabbits had electrodes in both areas, two had electrodes only in dentate fascia, and six had electrodes only in CA1.

3.2. Behavioral data

Comparing the subject maximum NMR amplitudes during CS period on first and last unpaired sessions with paired t-test did not reveal a statistically significant difference, $t(5) = 2.20$, $p > .05$ (Fig. 1). The same procedure administered to conditioning group data revealed a clear effect of learning, $t(11) = 4.12$, $p = .01$ (Fig. 1). Paired t-tests administered to conditioning group subject average NMR amplitudes at 125 originally measured time points representing 2 ms in time resulted in a statistically significant difference between first and last session at 102 to 108 ms, $t(11) = 2.28–2.97.$, $p < .05$ and from 110 ms onward, $t(11) = 3.20–4.03$, $p < .01$. Taken together, no effect of unpaired treatment was evident, but conditioning resulted in a robust conditioned NMR beginning about 100 ms after CS onset and reaching CR limit of 0.5 mm movement at approximately 150 ms after CS onset, thus preceding US onset by approximately 100 ms (Fig. 1).

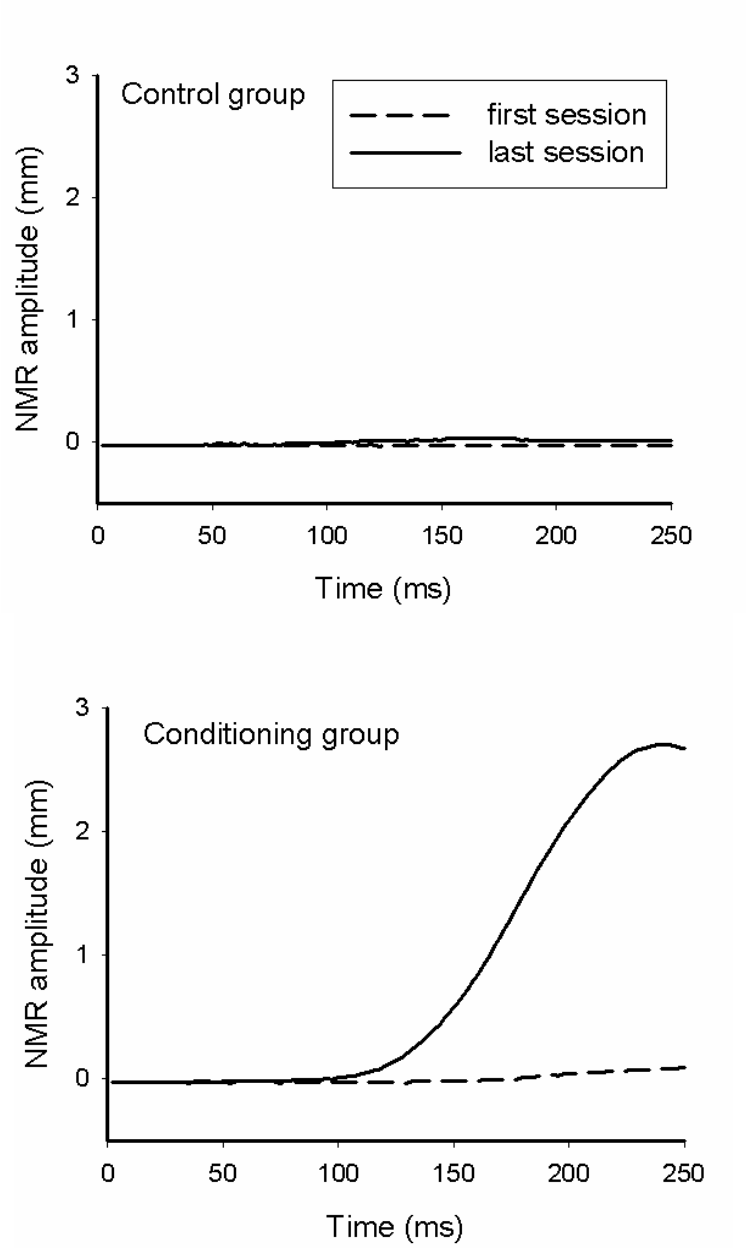

Fig. 1. Nictitating membrane response (NMR) amplitudes during 250 ms CS period. As a consequence of conditioning (lower graph, N = 12), but not unpaired treatment (upper graph, N = 6), a robust CR preceding US onset by approximately 100 ms was developed.

3.3. ERP data

Four subgroups were created based on treatment type and measuring area: control group CA1 (five rabbits), conditioning group CA1 (ten rabbits), control group df (three rabbits), and conditioning group df (six rabbits). All analyses were performed separately for the four subgroups.

Principal component analysis. PCA was conducted using single trials as cases and 125 time points representing 2 ms in time as variables. Factor loadings of greater than .8 in specific time points were used to identify the latency area of variability best explained by that factor. For example, Factor 1 of PCA conducted for conditioning group CA1 accounting for 22.3% of total variance rose above .8 at 106 ms, reached it's maximum value at 120 ms, and declined at 136 ms. Paired t-test was used to explore the differences in factor scores between first and last session.

In control group CA1 PCA, five factors accounted for 88.2% of the total variance in the ERPs. Paired t-test administered on Factor 1 (21.8% of total variance explained, loading over .8 at 48 to 86 ms after CS onset) scores revealed an effect of unpaired treatment, $t(4) = 4.01$, $p < .05$ (Fig. 2). This was thought to represent pseudoconditioning to the recurring presentations of the aversive airpuff US. In conditioning group CA1 PCA, five factors accounted for 92.5% of the total variance. When Factor 4 (16.8% of total variance explained, strongest at 0 to 36 ms after CS onset) factor scores were compared with paired t-test, a statistically significant effect of conditioning on hippocampal ERPs was found, $t(9) = 3.03$, $p < .05$ (Fig. 2).

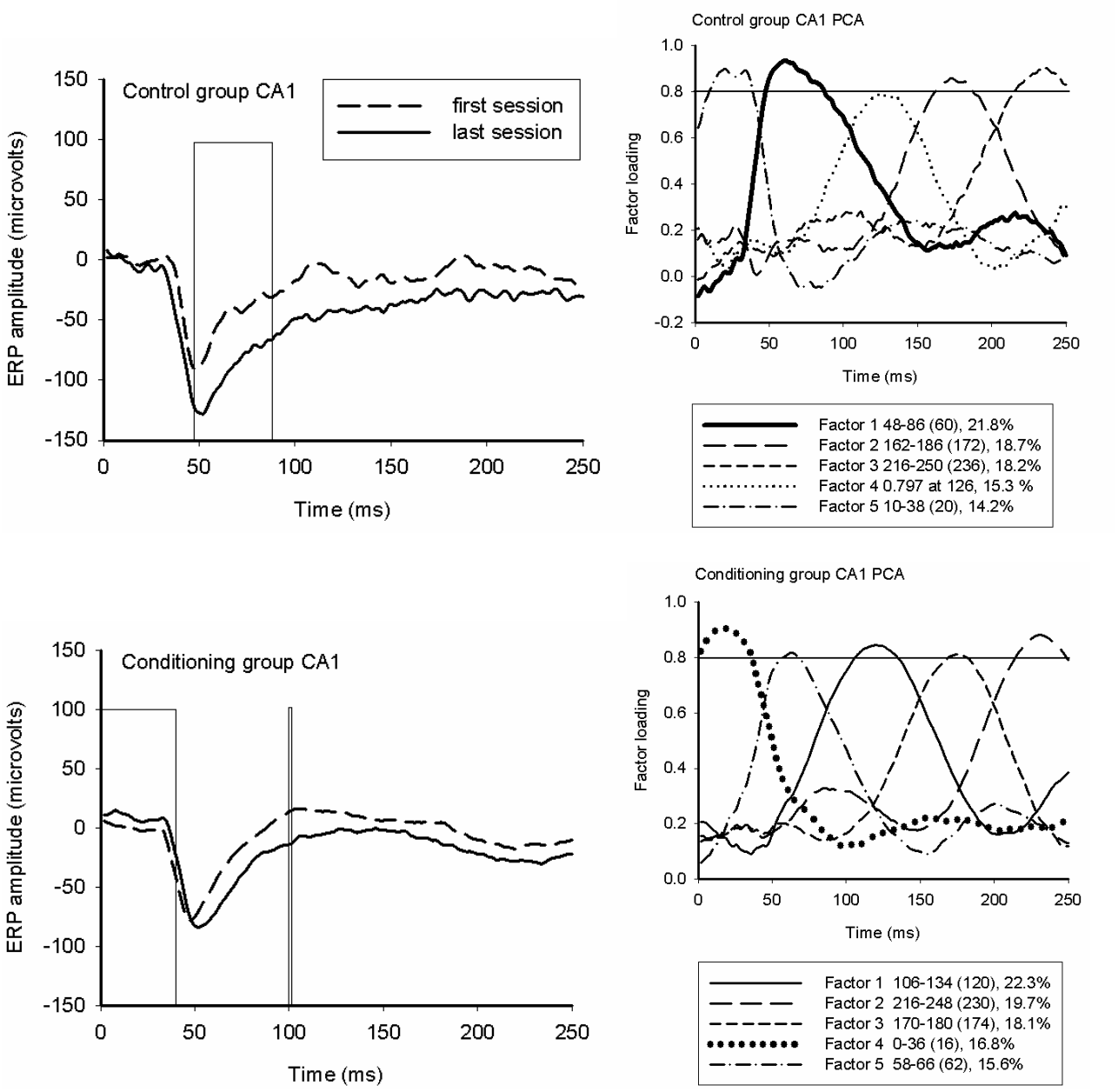

Fig. 2. Hippocampal field CA1 ERP amplitudes and factor (principal component) loadings during 250 ms CS period. Boxes indicate the latency range of the differences (PCA/t-test) in hippocampal field CA1 ERP amplitude due to unpaired treatment (upper graphs, 48 to 86 ms, $p < .05/50$ to 64 ms, $p < .1$, N = 5) and conditioning (lower graphs, 0 to 36 ms, $p < .05/36$ to 40 ms, $p < .1$ and at 102 ms, $p = .1$, N = 10). Factors indicating change in ERPs are highlighted in the PCA graphs.

In control group df PCA, five factors accounted for 89.3% of the total variance. (Fig. 3) Changes in factor scores due to unpaired treatment were evident at 0 to 28 ms, $t(2) = 4.19$, $p < .1$, and at 104 to 136 ms after CS onset, $t(2) = 3.34$, $p < .1$, probably reflecting sensitization as a consequence of repeated presentations of the aversive airpuff US (Fig. 3). In conditioning group df PCA, five factors accounted for 93% of the total variance. Paired t-test revealed no significant differences due to learning (Fig. 3).

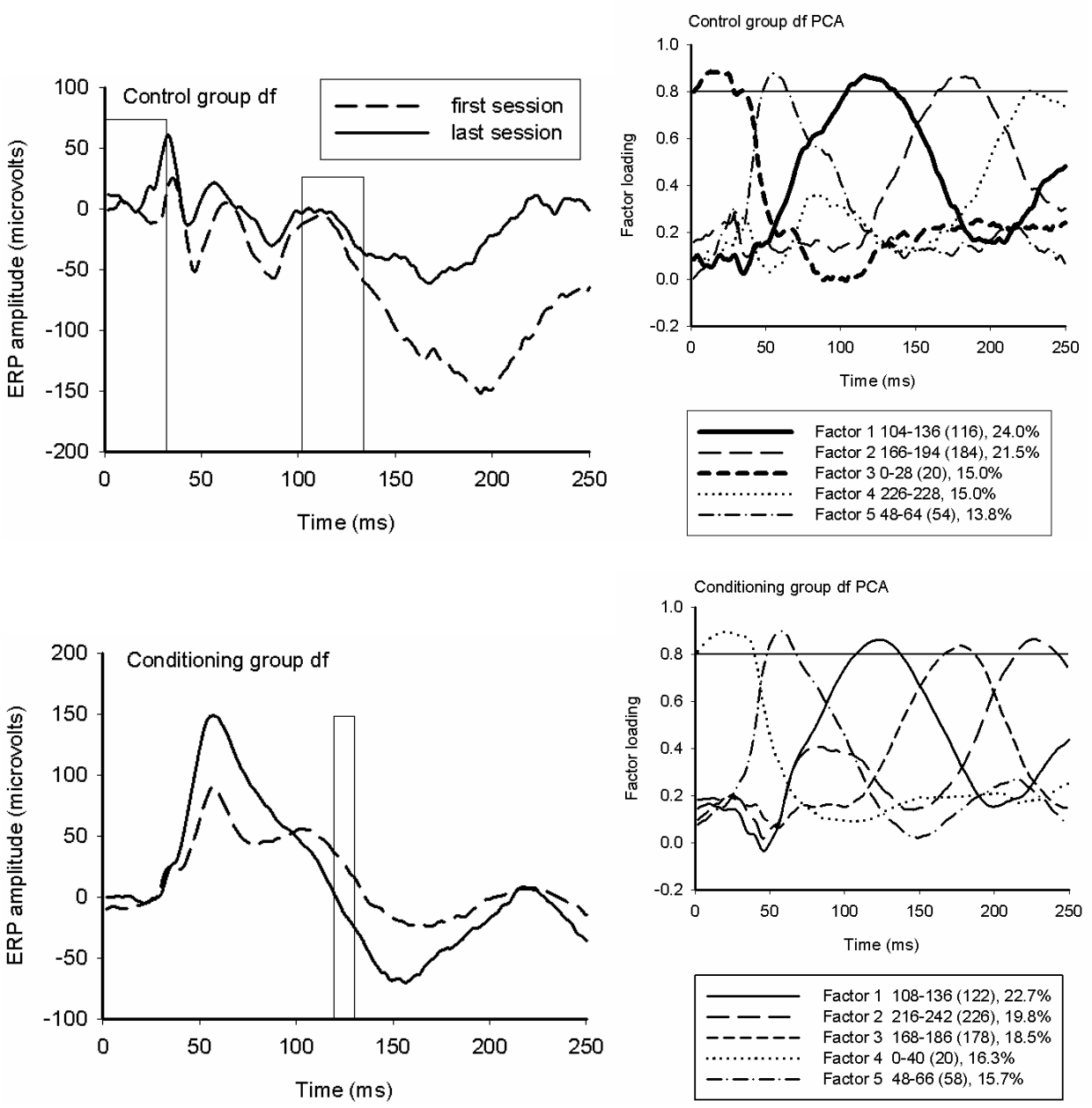

Fig. 3. Hippocampal dentate fascia ERP amplitudes and factor (principal component) loadings during 250 ms CS period. Boxes indicate an effect of unpaired treatment (upper graphs, 0 to 28 ms, $p < .05/.1$ and 104 to 136 ms, $p < .1$, N = 3), and of conditioning (lower graphs, 122 to 130 ms, $p < .1$, N = 6) on dentate fascia ERP amplitude. Factors indicating change in ERPs are highlighted in the PCA graph.

Paired t-tests: Average ERP amplitudes. In control group CA1, an alpha-level .1 difference due to unpaired treatment was evident at 50 to 64 ms after CS onset, $t(4) = 2.16–2.42$, $p < .1$ (Fig. 2). In conditioning group CA1, differences were found at 36 to 40 ms after CS onset, $t(9) = 1.95–2.06$, $p <$

.1 and at 102 ms after CS onset, t(9) = 1.83, p = .1 (Fig. 2). In control group df, an effect of unpaired treatment was evident at 0 to 6 ms after CS onset, t(2) = 2.92–6.09, p < .05/.1 (Fig. 3). In conditioning group df, a difference statistically significant at alpha-level .1 was found between 122 to 130 ms after CS onset, t(5) = 2.05–2.45, p < .1 (Fig. 3).

The results of the subject average ERP t-tests were in accordance with PCA factor score t-test results. Changes in control group ERPs most likely reflect sensitization due to the recurring presentations of the aversive airpuff US. Changes in conditioning group ERPs were timed almost analogous with the CS onset and CR onset.

4. Discussion

The results obtained with the help of PCA and paired t-tests indicate, that delay classical conditioning of the rabbit NMR affected NM movement and hippocampal field CA1 ERP amplitudes at different time windows: No sign of a temporal neural model of the CR (Berger et al., 1976) was observed in hippocampal ERPs. The average conditioned NMR started at 102 ms after CS onset reaching .5 mm amplitude at 150 ms after CS onset and continuated to grow in amplitude towards the end of the CS period, whereas changes due to conditioning in hippocampal ERPs were present between 0 to 36 ms (CA1) after CS onset. When also less robust differences (alpha-level .1) were examined, changes in hippocampal field CA1 and dentate fascia ERPs were found at approximately the same time period as the CR onset, at around 102 ms (CA1) and at 122 to 130 ms (dentate fascia) after CS onset. Also an effect of unpaired treatment was evident in hippocampal ERPs, most likely reflecting sensitization due to the repeated presentation of the aversive airpuff US. However, in control group, the NMR amplitude showed no change and remained below 0.1 mm at all times, indicating that no noticeable behavioral reactions to the tone were present due to unpaired treatment.

4.1. Hippocampus' role in delay conditioning

It was expected that due to delay classical conditioning of the rabbit NMR the hippocampal ERPs would demonstrate changes in amplitude similar to the ones reported by Berger et al. in 1976 using MUA as a measure of activity. However, compared to changes in hippocampal pyramidal cell MUA (Berger et al., 1976; Berger, Clark & Thompson, 1980; Berger, Rinaldi, Weisz & Thompson, 1983; Hoehler & Thompson, 1980), the changes in average hippocampal field CA1 ERPs related to delay conditioning now evident were short-lived, and limited to the beginning of the CS and CR, implicating that they are more closely related to the CS and CR onset, than to the actual performance of the CR. On the basis of several studies (see for example Orr & Berger, 1985; Weikart & Berger, 1986), Berger, Berry and Thompson (1986) suggested that hippocampal activity might be involved in modulating the amplitude and/or latency of the CR. The hippocampus' modulatory role in timing the CR has been turn out to be important especially when the temporal relations of the CS and US are complex, as in trace conditioning (James et al., 1987; Port et al., 1986; Sears & Steinmetz, 1990; Solomon et al., 1986), but changes in CR latency and acquisition have also been detected when the stimuli are presented continuously and coterminating, as in delay conditioning (Akase et al., 1989; Port et al., 1985). This makes it reasonable to assume that the ERP changes following delay conditioning now observed are also related to the timing of the CR by making it possible to infer the adaptive timing of the CR from the CS onset.

The timing of the hippocampal ERP changes now evident suggests that they have something to do with what Hoehler and Thompson (1980) call "a temporal map" of biologically important events (in this case the CS and the US) presumably generated in the hippocampus. Further suggestions about hippocampus' role in association formation is provided by Múnera et al. (2001), who – using

cats as subjects – discovered that hippocampal pyramidal cell firing is most probable after CS presentation, irrespective of the sensory modality of the stimulus, complexity of the conditioning paradigm, and of the timing and topography of the CR. Múnera et al. (2001) even managed to show that the CS-related activation was connected to the predictive value of the CS, that is to the CS-US association; not to the CS (or US for that matter) as a single stimulus. Using trace paradigm, McEchron and Disterhoft (1997) came to the same conclusions about hippocampus as coding the properties and temporal relations of the CS and the US, instead of modulating the performance of the CR. The investigations by Romano (1999) and Katz, Rogers and Steinmetz (2002) considering latent inhibition add to these assumptions by suggesting that hippocampal multiple-unit activity provides a reliable index of CS associability, and that hippocampus' role in associating the CS and the US (and the context of conditioning) is related to the novelty of the stimuli. The results and conclusions reviewed above lend support to the assumption, that the now observed changes in hippocampal ERPs are a sign of hippocampus' contribution in associating the CS with the US.

Congruent with Eichenbaum's (2001, 2003) notions about the hippocampus as specialized in linking together discrete experiences and using the associations in problem solving when faced with new situations, Green and Woodruff-Pak (2000) suggested that the hippocampal activity related to CR performance and topography (Berger at al., 1976) reflects formation of an association between the CS and the context of conditioning. This association is only crucial in complex conditioning paradigms like trace conditioning: During delay conditioning, CR models formed in the interpositus nucleus (Clark, Zhang & Lavond, 1992; McCormick, Clark, Lavond & Thompson, 1982), and in the associated circuitry (red nucleus: Clark & Lavond, 1993; entorhinal cortex: Berger et al., 1980) are sufficient for the adaptive performance of the CR, and supersede the impact of the hippocampal model (Green & Woodruff-Pak, 2000). According to Green and Woodruff-Pak (2000) this would explain, why consistent changes in hippocampal activity – like the ones observed in the current study – are found following basic delay conditioning, although learning of a simple stimulus-response association is not dependent on hippocampus, but relies solely on an intact interpositus nucleus of the cerebellum as evidenced by lesion studies (Krupa et al., 1993; Sears & Steinmetz, 1990).

4.2. Methodological considerations

On account of studies reviewed in the previous chapter, it is unlikely that nothing happens in the hippocampus during delay conditioning, and therefore it is suspected that something in the experimental arrangements or in the application of statistical procedures caused the statistical weakness of the differences in hippocampal ERP amplitudes. First of all, the data used in the present study merits some evaluation. Most of the data had been acquired during 1990's for use in other studies observing NMR conditioning from variable viewpoints. It is possible that the results of this study are somehow affected by the diversity of locations of measuring electrodes implanted then, but not considered now, or by the somewhat incompatible training procedures administered to the subjects. For the most, the fact that not all subjects had went through unpaired training before conditioning, that not all subjects had the same amount of training either in unpaired or in conditioning phase, and that not all subjects had the same amount of measuring electrodes in the same locations made implementation of statistical methods rather limited and complex. It also makes the interpretation of results quite hesitative. However, since even with this combination of data, statistically significant changes were observed, more robust effects of conditioning on hippocampal ERPs can be expected to be detected if the data is more homogenous.

As it can be seen from the results of paired t-tests performed on PCA factor scores and on ERP amplitude subject averages, the methods yielded compatible results. Since most of the differences in ERPs were significant only at the alpha-level of .1, not much can be said about the mutual superiority of either one of the methods used. It might be that the paired t-tests conducted on subject

average ERPs and on PCA factor scores failed to find more statistically ($p < .05$) significant differences because the number of subjects, determining degrees of freedom in the t-tests, was so small. There are some considerations related to the statistical abilities of PCA, also, that must be taken into account when the results are interpreted. First, PCA is incapable of extracting factors with temporal overlap, even though it can handle component overlap better than peak or area measures traditionally used in ERP studies (van Boxtel, 1998). Second, PCA cannot take into account the temporal variability of ERP measures in extracting factors. This means, that differences in ERP component latencies between rabbits and between single trials are ignored, which may confound the resulting PCA factor structure (van Boxtel, 1998). Despite these weaknesses that might have had part in the lack of statistical differences in factor scores, a factor structure explaining approximately 90% of variance was extracted in all cases in the present study.

The somewhat deviating results of this study compared to studies measuring MUA are probably also related to the differences between MUA and ERP as measures: MUA is a measure of the net activity of a population of neurons reflecting action potential discharge. EEG, and thus ERP, measures the potential for activity reflecting postsynaptic and afterpotential slow waves in neurons located near the extra cellular recording electrode (Buchwald et al., 1973). Buchwald et al. (1973) state very clearly that the difference in the causal mechanisms underlying MUA and EEG may cause a lack of parallelism in the results obtained using the two measures. It is also suggested (Buchwald et al., 1973) that MUA seems to be much more sensitive to altered neural responsiveness compared to EEG. This might explain the brief duration of the changes in ERPs now evident compared to MUA: It could be thought that the ERP changes now obtained reflect only the most prominent – and possibly the functionally most important – changes in the neural activity of the hippocampus. Although ERPs might be less efficient in detecting subtle changes in neural activity, they are to be considered valuable, since they provide a means of relating data from animal studies with data from studies using humans as subjects.

4.3. Conclusions

The fact that no evidence of the hippocampal temporal neural CR model present in numerous MUA studies was found when ERPs were used as a measure of activity, is most surprising, and points to a differential role of hippocampus in delay classical conditioning than that suggested by Berger et al. in 1976: The findings and theories reviewed above support the interpretation, that the short-duration CS period changes in hippocampal ERPs due to delay conditioning now observed are related to the establishing of CS–context and CS–US associations, and to the modulation of the CR performance by timing it adaptively, but not to the actual performance of the CR. In the future, a study conducted using more homogenous data would be informative in trying to replicate and confirm the results now obtained. Using a more complex conditioning paradigm, and exploring the relation of hippocampal ERPs and NM movement within and between single trials, and within and between all conditioning sessions would give information about the changes in the relationship of ERP and NMR as they change as a consequence of proceeding conditioning dependent on hippocampus. Comparing MUA and ERP measures obtained from the same subjects during classical conditioning would shed light on the relationship of the two activity measures: It would clarify, whether the two activity measures correlate or not, and what kind is the temporal relationship of the changes in activity indicated by the two measures. In addition, measuring ERPs during classical conditioning of the NMR from the rabbit's scalp, and comparing the data with human ERPs obtained during aversive eyeblink conditioning would reveal possible differences between species.

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