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Effects of medial forebrain bundle stimulation on classical trace and delayed eyeblink conditioning

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EFFECTS OF REWARDING ELECTRICAL STIMULATION OF LATERAL HYPOTHALAMUS ON CLASSICAL CONDITIONING OF THE NICTITATING MEMBRANE RESPONSE

JUHA ARIKOSKI, TAPANI KORHONEN, MARKKU PENTTONEN, TIMO RUUSUVIRTA and JAN WIKGREN

Department of Psychology, University of Jyväskylä, Jyväskylä, Finland

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Abstract

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1. Adult New Zealand albino rabbits were prepared with chronic hypothalamic stimulating electrodes and hippocampal recording electrodes.
2. Rabbits were restrained and classically conditioned by a tone CS and an airpuff US either followed or preceded by a hypothalamic stimulation (HS). Control rabbits were conditioned without the HS.
3. It was found that HS following the CS facilitated both behavioral and hippocampal responses, while HS preceding the CS inhibited them.
4. Enhanced hippocampal learning-related unit firing to the CS may represent an early indication of conditioning before the behavioral activity produces any observable change.

Keywords: classical conditioning, dentate gyrus, lateral hypothalamus, rewarding brain stimulation

Abbreviations: conditioned response (CR), conditioned stimulus (CS), hypothalamic stimulation (HS), interstimulus interval (ISI), long-term potentiation (LTP), multi-unit activity (MUA), nictitating membrane (NM), unconditioned response (UR), unconditioned stimulus (US).

Introduction

Classical conditioning is based on the view that learning can be seen as the development of an association between two stimuli presented in a specific order (a conditioned stimulus preceding an unconditioned stimulus) and in close temporal proximity. Adding a third stimulus which is biologically similar to the US used in classical conditioning can produce either facilitation or retardation in the development of the association between the CS and the US. Such stimulation can be applied either before the CS-US pairing (US pre-exposure or pre-stimulation studies) or after the CS-US pairing (post-stimulation studies). The connection of an additional stimulus of this type to the CS-US pairing can be either temporally close (within few seconds) or distant (within minutes, hours or days) and at the same time represents a fixed (experimenter-administered, constant interstimulus interval, ISI) or variable (self-stimulation, subject-administered ISI) connection between these events.

Temporally closely tied, rewarding hypothalamic post-stimulation has been found effective in the facilitation of associative learning (Major and White 1978, Kim et al 1983, Hirano et al 1987). Kim et al. (1983) have shown that associative eye blink conditioning to a click CS and glabella tap US in cats could be rapidly produced by presenting a hypothalamic stimulation at a relatively short (10-msec to few seconds) fixed ISI after the CS-US pairing. In these studies repeated pairings of the CS and US followed by the HS produced CRs in less than 30 pairings. Repeated pairings of the click CS and glabella tap US without associated delivery of the HS usually require 500-1000 CS-US pairings for CR acquisition (Brons and Woody 1980). Furthermore, Major and White (1978) have reported that a self-stimulation HS given immediately but at variable intervals (during a few seconds) after the training trial improved retention of a learning task in rats. Even longer intervals (from tens of seconds to days) between the CS-US pairing have been found to be effective: Mondadori et al. (1976) observed that a HS presented 30 seconds after a trial facilitated learning in an avoidance task setting. It has also been shown by Laroche and Bloch (1982) that long-term electrical stimulation of the mesencephalic reticular formation during 90 seconds after training sessions facilitated learning. These studies indicate that a post-trial HS can have a facilitative effect on associative learning, which

is not dependent on the length of the ISI between the HS and the CS-US pairing.

Another possibility is to administer the additional stimulus prior to the CS-US pairing. Both Kim et al. (1983) and Hirano et al. (1987) found that reversing the order of experimenter-administered HS presentation as a pre-trial HS reduced the magnitude of the conditioned response (CR). In other words, a pre-trial HS given at a fixed ISI inhibited learning. In a typical US pre-exposure study the unpaired US or the sequence of USs are given at a variable ISI before the paired conditioning trial. Berthier and Woody (1984) have defined US pre-exposure as a stimulus that normally serves as an US producing a conspicuous motor response resembling the one to be conditioned. Both Siegel and Domjam (1971) and Mis and Moore (1973) found that a repetitive presentation of an US immediately before the learning trial retarded subsequent conditioning in rabbits. In contrast, Matsumura and Woody (1982) found, however, that the repetitive presentation of 1500 glabella-tap USs one week before conditioning facilitated subsequent eye-blink CRs.

Long-term potentiation (LTP) studies can also be interpreted as representing the pre-stimulation type of effect. Berger (1984) noted that LTP of the hippocampus before paired training increased the rate at which rabbits subsequently learned the conditioning task. In his study eight trains of 400 Hz stimulation of the perforant path of the hippocampus were given two days before pairing a tone CS and an airpuff US.

Thus, according to previous studies, both pre-trial HS and US pre-exposure have either a facilitative or inhibitive effect on learning, depending on whether the ISI between the additional stimulus and the CS-US pairing is long or short, respectively.

The growth of the hippocampal unit response has been found to represent an invariable and predictive concomitant of subsequent behavioral learning (Berger and Thompson 1978a, Berger et al 1980a, Berger and Thompson 1982). Although, according to various studies (Thompson et al 1982, Thompson 1991, Nordholm et al 1993, Krupa et al 1993), the primary memory engram for short delay classical conditioning of the nictitating membrane (NM) and of the eyelid is probably localized within the ipsilateral cerebellum, it has been shown that the hippocampus may exert some modulatory influence on the cerebellar expression of associative learning - there is a pathway

from the hippocampus to the cerebellum via the subiculum to the cingulate gyrus to the pontine nuclei (Berger et al 1980a).

Sensory evoked neural responses in the dentate gyrus have been shown to reflect the inputs of two major hippocampal afferents, the septo-hippocampal fibers and the perforant path (Deadwyler et al 1982). Segal (1973) discovered that dentate cells required fewer trials to develop conditioned unit responses than CA3 cells and that dentate cells also appeared to be responsible for both the acquisition and extinction of cells in the CA3. The role of the dentate activity in reflecting associative processes at the neural level has been pointed out in many studies (Weisz et al 1982, Segal and Olds 1973, Rose 1980, Laroche et al 1983, Berger 1984).

Few experiments have studied the effects of rewarding brain stimulation on hippocampal activity during classical conditioning. Hirano and Yamaguchi (1985) recorded the hippocampal unit activity and the movements of rats during the pairing of an auditory CS and a lateral hypothalamic stimulation US. They found that dentate and hippocampal units increased their firing rates to the CS tone (during the CS-US interval) before the behavioral change. In the present study, the effects of the HS were studied by comparing the behavioral and hippocampal responses of a standard classical NM conditioning group of rabbits to pre-trial and post-trial HS groups of rabbits.

The following questions were asked: 1) Does post-trial HS, given after a short fixed delay, have a facilitative effect on classical conditioning? 2) Does a pre-trial HS facilitate or retard classical conditioning if the HS precedes the CS-US pairing by a short, fixed interval? 3) If the effect is facilitation, should it be interpreted as nonspecific or specific activation of the nervous system? 4) What is the relation between the changes in hippocampal multi-unit activity (MUA) and the behavioral responses observed?

Methods

Animals

New Zealand albino rabbits (n=23) weighing 2.9 to 4.0 kg served as subjects. The experiment was carried out according to the regulations

of the European Union for animal health and care in laboratories. The rabbits were individually housed and maintained on a 12:12 hr light/dark cycle, and they received food and water ad lib in a temperature-controlled vivarium. The animals were cared for by University veterinarians. All behavioral procedures were carried out during the daylight portion of the cycle.

Surgery

Surgical anaesthesia was initiated and maintained with i.m. injections of ketamine-zylazine cocktail (Ketalar®, 50 mg/ml; Rompun®, 20 mg/ml). Atropine® (1 mg/ml) was given s.c. against salivation. Each rabbit was first positioned in a stereotaxic headholder with the bregma 1.5 mm above lambda.

After drilling a hole over the cerebral cortex, four Teflon-insulated, stainless steel stimulation electrodes (100 µm exposed tips) were lowered stereotaxically into the lateral hypothalamus (A0.0, R2.0, H-5.0; A0.0, R3.0, H-5.0; A1.0, R2.0, H-5.0; and A1.0, R3.0, H-5.0). Four Teflon-insulated, stainless steel MUA recording electrodes (100 µm exposed tips) were implanted to the hippocampal formation. One of the electrodes located in the dentate gyrus was selected to be used in the experiment. An indifferent reference electrode for monopolar recordings consisted of two symmetrically implanted skull screws on both sides of the midline.

The electrodes were connected to two 15-pin D-type connectors that were cemented onto the skull with dental acrylic together with four anchoring screws. The implantation was done by the method developed by Korhonen (1991). Also a headstage designed to hold a minitorque potentiometer, an air puff delivery nozzle and tone tubing were attached to the complex.

Finally a small loop of 4-0 nylon thread was sutured into the right NM. Analgetics (Temgesic®, 0.3 ml/kg) was given s.c. at the end of surgery and every eight hours for the following 48 hours. All the rabbits were given 1-2 weeks for recovery before behavioral training began.

Apparatus

During training the rabbits were restrained in a plexiglas box located in a ventilated, sound-attenuating and electrically shielded box. The rabbits were observed during the experiments through a video monitor. Low-noise pre-amplifiers with a gain of 10 (Analog Devices, AD524) were attached directly to the connector in the acrylic mass on the head of the animal. The neural signals were further amplified, bandpassfiltered (500-6000 Hz) and digitized at 15 kHz by separate equipment, and they were then fed to an A/D converter. For the NM movement measurements, a small wire with a tiny stainless steel hook was attached to a nylon loop sutured into the NM. The wire was connected to a stainless steel crank. The movement of the crank rotated the cylinder of a minitorque potentiometer connected to a holder on the head. A flexible shielded cable connected the rabbit to a stimulation source. Head movements were recorded with a solid-state piezoresistive accelerometer (ICSensor, 3021-002-P). A tone generator was placed outside the training box.

A microcomputer delivered trials at randomised intertrial intervals as well as the sequence of the stimuli. It also controlled isolated brain stimulation pulse trains. The identification data generated by the computer was displayed on the video-screen together with a time-graph bar display used for timing the phases of the NM and head movements. All this alpha-numeric information was superimposed on the video picture of the rabbit in the training box.

Procedure

At least for one week following surgery, each animal was observed in the chamber for the purpose of determining the behavioral threshold current levels of the HS electrodes and making certain that the electrode and current level selected for conditioning would not induce struggling while under restraint. The HS consisted of electrical stimulation of the medial forebrain bundle at a pulse width of 0.5-msec, a train duration of 250-msec, a stimulus intensity typically of 100-250 μA, and a pulse frequency of 100 Hz. Stimulation typically resulted in heighened exploratory activity (sniffing the floor and chewing movements similar to earlier findings by Bruner (1967) and Kim

et al. (1983) or sometimes attempted escape followed by freezing). In the present study, a current level inducing orienting and approach movements, without any aversive effects, was selected. If the HS did not elicit positive approach movements the rabbit was placed in the control group.

The rabbits were then placed in a standard Plexiglas restraint box in a conditioning chamber for two 45-min adaptation sessions. The subsequent training consisted of daily paired CS-US sessions. The CS was a 350-msec tone (1 kHz, 78 dB SPL, 350-msec) delivered via a plastic tube placed in front of the left ear at a distance of 1 cm. The US was a 100-msec air puff (2.1 N/cm^2, measured from the cornea) directed to the right eye. CRs were defined as 0.5 mm of NM movement that occurred after onset of the CS but before onset of the US.

The first five daily conditioning sessions consisted of 60 CS-US paired (training) trials followed (post-trial HS) or preceded (pre-trial HS) by the 250-msec train of HS (Fig 1). Each session also included 10 pseudorandomly given CS-only and and 10 US-only test trials. Control (CC) rabbits received the same set of stimuli without the HS. Conditioning was performed in delayed paradigm fashion, where the CS and US (overlapping the last 100-msec of the CS) terminated simultaneously, thus creating a 250-msec interstimulus interval. The intertrial interval was random, ranging from 30-50 sec (m=40 sec).

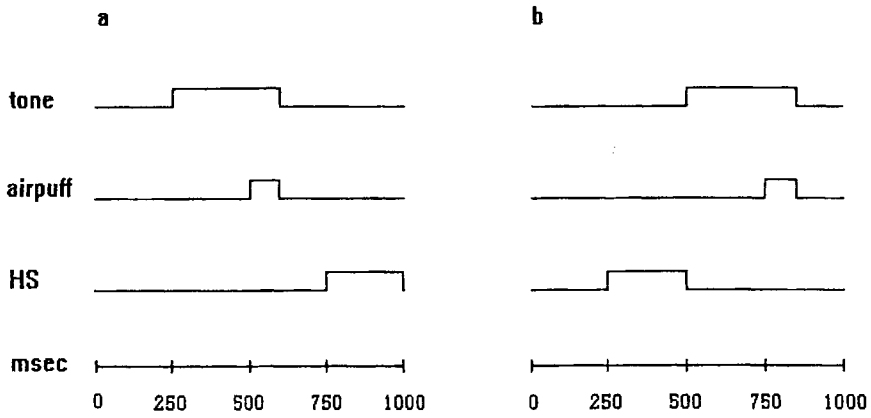

Fig 1. Schematic illustration of the training procedures. a) Post-stimulation trial. b) Pre-stimulation trial. In the control group the training procedure was the same as in the post-stimulation group, but without the HS.

After the first five conditioning sessions the order of HS presentation was reversed in 11 rabbits for five more days. The pre-trial HS group thus received the HS after the CS-US pairing (as post-trial HS) and vice versa. MUA was recorded for 13 rabbits during the first five daily sessions.

Histology

After the experiments, the animals were anesthetized with a lethal dose of sodium pentobarbital (6-10 cc i.m. and i.v.) and perfused via the ascending aorta with saline followed by 10% formalin. The HS electrode locations were marked by passing a 10 μA direct current through the electrode for 20 sec. The brain was removed and fixed in formalin-sucrose solution for at least one week. Frozen coronal 100-μm sections were taken from the sites of the electrodes. The slices were mounted on gelatinized slides and stained with cresyl violet and potassium ferrocyanide. The exact locations of the tips were compared to the coordinates of the stereotaxic atlas of Shek et al (1986).

Data Analysis

The signal analysis was based on a 1,000-msec period, which included 250-msec preCS, 250-msec CS, 250-msec US, and 250-msec postUS periods. The MUA was analyzed off-line by a customized DTVee program which highpass filtered the signal and identified and counted the spikes. The spike baseline was set to a level at which the spontaneous activity was approximately 15 spikes/sec. The baseline correction was performed by subtracting the average of the prestimulus time from the average of the last ten bins (10 ms) of the CS-period. Each trial thus obtained a numerical value, comparable to the data from other sessions and subjects. This data was pooled inside each group. SPSS 6.1 for Windows was used for all numerical processing and analysis of variance. The Greenhouse-Geisser conservative F test was applied when needed.

Results

Behavioral Responses

The behavioral data are shown in Fig 2. It was found that the pre-trial HS group showed a minimal increase in CRs, while in the control (CC) and post-trial HS groups the increase in the CR was much larger. During the CS periods of both the paired trials ($F(2, 20) = 4.33$, $p < .05$) and the CS test trials ($F(2, 20) = 3.70$, $p < .05$) the effect of the treatment (pre-trial HS, post-trial HS and CC) was statistically significant. For the CS test trials the session (day) effect was significant ($F(1.50, 40) = 22.48$, $p < .001$). Most importantly, the treatment x session (day) interaction was also significant ($F(2.99, 40) = 3.56$, $p < .05$).

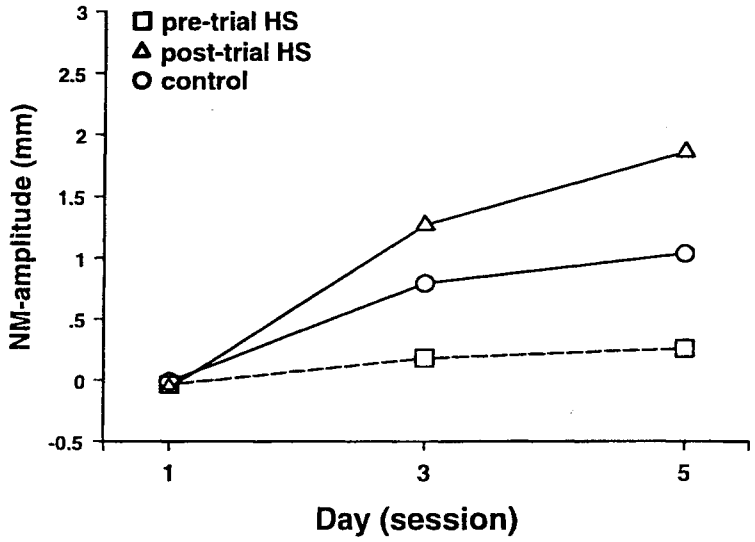

Fig 2. The post-trial HS group showed faster CR acquisition than the control group whereas the CR performance of the pre-trial HS group remained close to zero during the sessions.

The effects of the reversal of the pre-trial and post-trial HS treatments during conditioning were also tested (n=11). After the first five days of conditioning only a small CR in the pre-trial HS

group was observed. Instead, during the last five conditioning sessions, when HS was given after the CS-US pairing (as post-trial HS), the CR increased. In contrast, a conspicuous CR developed in the post-trial HS group during the five days of conditioning, but when the order of HS presentation was changed the CR decreased. The treatment x session (day) interaction was significant ($F(2, 18) = 4.72$, $p < .05$) (Fig 3). The results of examining the histological material are shown in Fig 4.

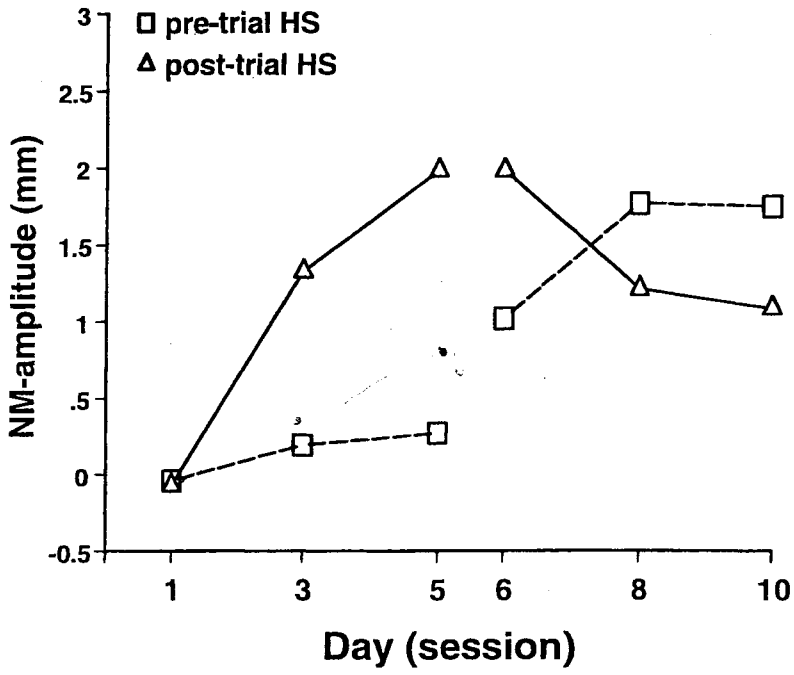

Fig 3. While no CRs developed during the initial pre-trial HS training, CR amplitudes increased when the animals received post-trial HS treatment. Rapidly developing and large CRs in the post-trial HS group decreased during subsequent pre-trial training.

In addition, it was noted that for the US test trials the session (day) effect was significant ($F(1.99, 40) = 44.93$, $p < .001$), while no significant difference was found in the group x session interaction ($F(3.97, 40) = .06$, $p = .993$).

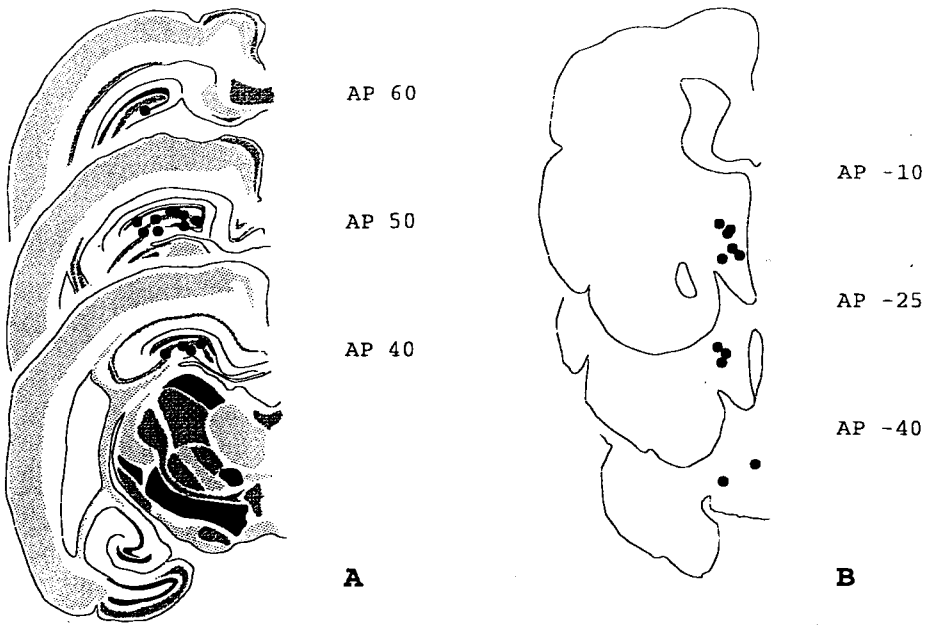

Fig 4. (A) Locations of electrode tips used to register the MUA of the dentate gyrus. (B) Locations of stimulation electrodes used to deliver the HS. Effective loci were close to the lateral hypothalamus.

Multi-Unit Activity

Multi-unit activity (MUA) was registered from the dentate gyrus for the first five daily sessions. The group X session (day) interaction was significant ($F(3.39, 44) = 3.53$, $p < .05$), indicating that the post-trial HS group showed the fastest increase in MUA during learning when compared to the other groups.

The significant difference in MUA was already observable after the first day of conditioning (F (2, 11) = 73.59, $p < .001$), when the MUA had increased in the post-trial HS group but remained at zero in the control and pre-trial HS groups. Further from the day one, the MUA in the post-trial HS group was higher than in the other groups during each session. Moreover, like the behavioral data, the activity in the pre-trial HS group remained close to zero on each day similarly to the behavioral data (Fig 5). The mean conditioned responses (CRs) to the

CS tone by each group and the group-effect by MANOVA for each day are shown in Table 1.

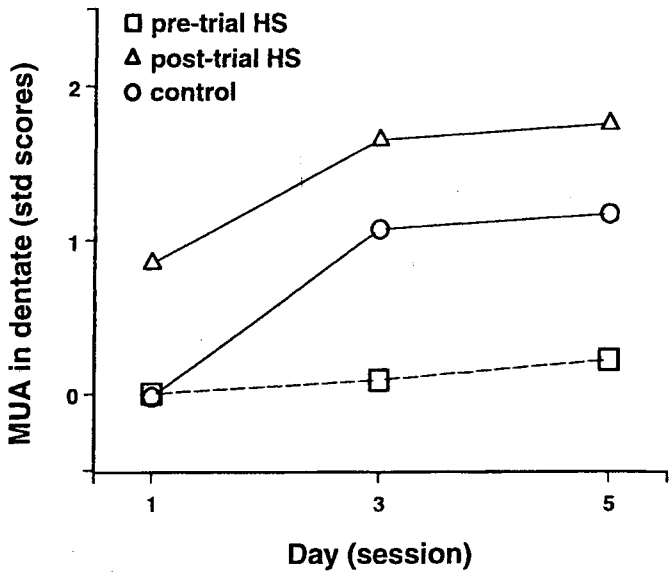

Fig 5. MUA grew most rapidly in the post-trial HS group.

Table 1

Mean CRs Represented as Average Amount of Spikes in Dentate MUA

group	day 1	day 2	day 3	day 4	day 5
control	-0.02	0.03	1.07	0.71	1.17
pre-trial HS	0.00	0.03	0.09	0.05	0.22
post-trial HS	0.85	1.04	1.65	1.51	1.75
F-value	73.59	13.14	5.14	11.0	9.47
Sign. of F	.000	.001	.026	.002	.004

Discussion

The results of this study indicated that post-trial HS facilitated classically conditioned NM responses. In contrast, associative learning was retarded when the HS preceded the CS-US pairing.

Behavioral Effects

The facilitative effect of the post-trial HS on learning can be explained in different ways. Both the pre- and post-trial HS results could represent either a specific or nonspecific effect on learning. On the one hand, it is possible that reinforcing brain stimulation might improve learning by inducing a non-specific or general activation of the central nervous system during a critical period for memory consolidation. On the other hand, the temporal proximity or close contingency between the CS-US pairing and the HS might be a critical factor in facilitating learning. This hypothesis is supported by the results of several studies (Major and White 1978, Kim et al 1983, Hirano et al 1987, Aldavert-Vera et al 1996), which showed that post-trial HS had facilitative effect on CR performance when the ISI between the CS-US pairing and the HS was short.

In contrast, studies using a pre-trial presentation of the HS and US pre-exposure have shown retardation of the CR when a relatively short ISI is used between the preceding additional stimulus and the CS-US stimulus pair (Siegel and Domjam 1971, Mis and Moore 1973, Terry 1976, Kim et al 1983). Ewing et al (1985) investigated the associative consequences of backward conditioning using rat subjects and a conditioned emotional response procedure. In this CS pre-exposure study comparisons were arranged so that CSs preceded the CS-US pairing by either a relatively short ISI (60-s) or by a relatively long ISI (600-s). Ewing et al (1985) found that shorter ISIs produced less CR acquisition and more extinction than the longer ones. Exceptionally, longer intervals between the unpaired US presentation and subsequent CS-US pairing have been reported to yield a facilitative effect on paired learning (Matsumura and Woody 1982).

Studies of LTP (Berger 1984, Brown et al 1990) have indicated that electrical brain stimulation given before the training session increases the rate at which a conditioning task is learned. However,

in the present study HS given before the CS-US pairing decreased the rate of CR performance. This result can be considered as a pairing-specific effect between the preceding HS and subsequent CS-US pairing comparable to observations where an unsignaled US has been interpreted as resulting in a reduction in the validity of the CS as a predictor of US occurrence (Rescorla 1967). The present study supports such an interpretation of the behavioral effects when the pre-trial and post-trial HS treatments were reversed.

An attempt to interpret the results of the present study as a LTP type of change meets with some difficulties. In general, associative LTP has been suggested to result from strong stimulation (US) preceding weak input (CS) (Wigström and Gustafsson 1986). However, as Rescorla (1988a) has stated, such a paradigm models backward conditioning, which has been found to be ineffective in producing paired associative learning at the behavioral level. In addition, Brown et al. (1990) have noted that associative long-term stimulation can produce enhancement in transmission in the case of both weak and strong inputs. This observation suggests that classical conditioning might enhance the behavioral response to both the CS and US. However, as it is well known, Pavlov (1928) reported that in classical conditioning there was a change in response to the CS, but no change in response to the US. More generally, Diamond and Rose (1994) have stated that associative LTP does not model contemporary views of classical conditioning.

In the present study the facilitative effect of the post-trial HS and the inhibitive effect of the pre-trial HS on learning can be interpreted as specific activation of the nervous system. The location of the stimulation electrodes used in the present study bear out the hypothesis of Major and White (1978), who have proposed that activation of the medial forebrain bundle of the lateral hypothalamus, which is part of the specific arousal system, produces a facilitatory effect on learning.

Multi-Unit Activity

The results showed that in the pre-trial HS group the dentate activity as in the case of the behavioral data, remained close to zero each day. However, in the post-trial HS group an increase in the

multi-unit responses appeared already during the CS period after the first day of conditioning, in contrast to their relative absense in the control and pre-trial HS groups. This difference was not detected in the behavioral data. Enhanced multi-unit firing as a response to the CS was also observed in the dentate gyrus before the behavioral activity produced any observable change. An example of the MUA and behavioral data is shown in Fig 6.

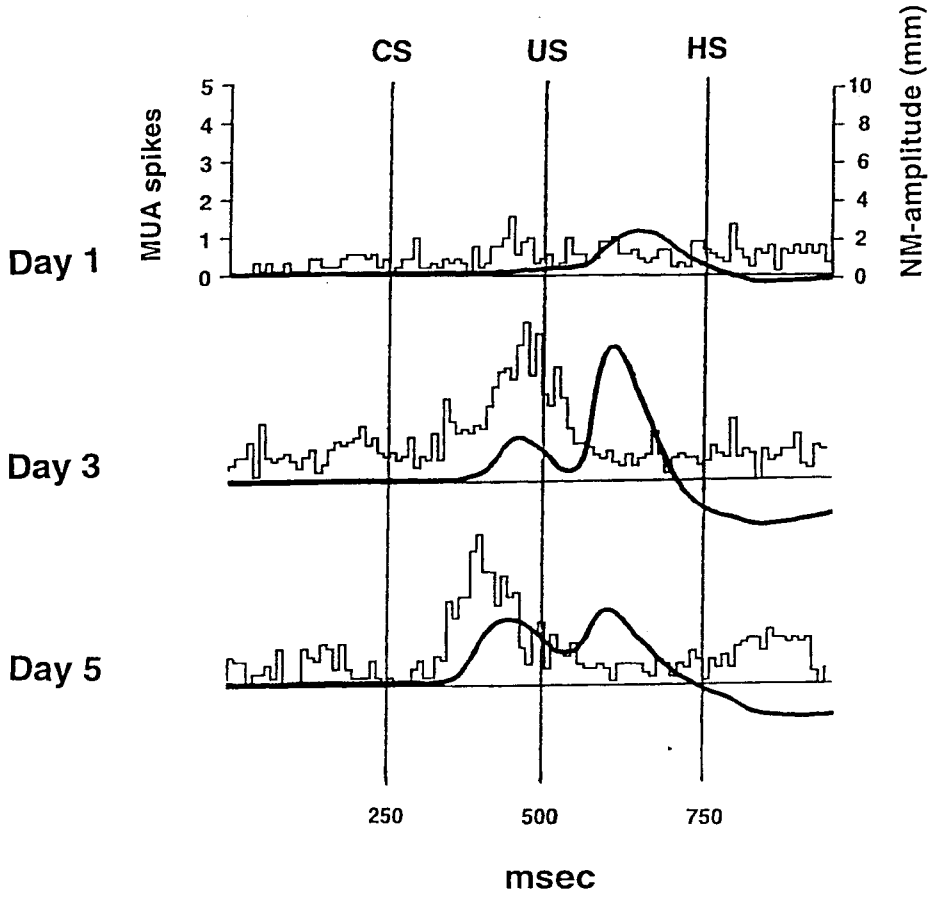

Fig 6. An example of the MUA (spikes) and behavioral (NM) data from one animal from sessions (days) 1, 3 and 5 (post-trial HS phase).

The above-mentioned results are comparable to the effects reported earlier by the group of Thompson (Berger et al 1980b, Thompson et al 1980). In these studies it was found that in conditioning of the rabbit NM the activity of hippocampal neurons increased in the early phase of training before behavioral conditioning was established. Hirano (1984) also found enhanced hippocampal unit activity to the CS before the behavioral activity produced any observable difference, when measuring hippocampal unit responses to the tone CS^{+} after HS-reinforced differential conditioning in rats.

The studies by the group of Thompson (Berger et al 1980b, Thompson et al 1980) showed that delayed multi-unit responses emerged during the US period of CS-alone trials. A similar onset-delay in the emergence of multi-unit responses was also found in the present study.

Conclusion

The major finding of the present study was that a weak electrical stimulation of the lateral hypothalamus given immediately after the paired trials (post-trial HS) enhanced CR performance on the behavioral level and facilitated learning related changes in neural activity in classical NM conditioning. It was also found that pre-trial HS decreased CR performance and inhibited learning.

This conclusion was supported on a behavioral level when post-trial HS was substituted to pre-trial HS; retardation was changed to facilitation or vice versa when the order of the HS presentation was reversed.

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Inquiries and reprint requests should be addressed to:

Juha Arikoski
Department of Psychology
University of Jyväskylä
P.O. Box 35
FIN-40351 Jyväskylä
FINLAND

Running head: RESPONSES DURING TRACE AND DELAYED CONDITIONING

Behavioral Responses During Classical Trace and Delayed Conditioning When Using Unilateral Medial Forebrain Bundle Activation in Rabbits

Juha Arikoski, Korhonen, T., Ruusuvirta, T. and Wikgren, J.

Department of Psychology, University of Jyvaskyla, Jyvaskyla, Finland

Abstract

Adult New Zealand albino rabbits (n=11) underwent acquisition of the classically conditioned nictitating membrane response in a trace paradigm, in which conditioned stimulus (CS) offset preceded unconditioned stimulus (US) onset, by a period when no stimuli intervened between the CS and the US. Rabbits were prepared with chronic hypothalamic stimulating (HS) electrodes and they were restrained and classically conditioned by a tone CS and an airpuff US. In the present study we examined, whether behavioral nictitating membrane response (NMR) acquisition to a trace CS was facilitated or inhibited by insertion of a third stimulus (HS) either after or prior to the CS-US pairing. It was found that HS following the CS-US pairing facilitated conditioned response acquisition, whereas HS preceding the CS inhibited conditioned response acquisition. These results are similar to the results of an earlier study, in which a delay conditioning paradigm (US onset occurs prior to the CS offset) was used (Arikoski et al., 1995; 1997).

Behavioral Responses During Classical Trace and Delayed Conditioning When Using Unilateral Medial Forebrain Bundle Activation in Rabbits

Rabbit nictitating membrane/eyeblink conditioning has served as the most widely used behavioral model system for studying associative learning, with two variants, trace- and delayed conditioning. In the present experiment, the influence of hypothalamic stimulation (HS) on classical trace conditioning of nictitating membrane responses is evaluated. The question was whether a trace paradigm could produce association between the CS-US pairing in a situation where an extra stimulus, rewarding HS, was given either before or after the pairing. The results of an earlier delayed conditioning study (Arikoski et al., 1995; 1997) showed that the HS following the CS-US pairing facilitated the behavioral responses, while HS preceding the pairing inhibited them.

The temporal proximity (i.e. contiguity) or close contingency between the CS and US and the following HS have been found essential in the facilitation of associative learning (Major and White, 1978; Kim et al., 1983; Hirano et al., 1987; Aldavert-Vera et al., 1996; Arikoski et al., 1995; 1997). In contrast, Laroche and Bloch (1982) and Mondadori et al. (1976) have shown that brain stimulation that is presented 30-90 seconds after CS-US pairing can facilitate learning, indicating that a close contingency between the CS-US pairing and the HS is not critical.

On the contrary, pre-trial HS (Kim et al., 1983; Hirano et al., 1987) or US pre-exposure (Siegel and Domjam, 1971; Mis and Moore, 1973) (a stimulus that normally serves as an US producing a conspicuous motor response resembling the one to be conditioned)

given immediately before the CS-US pairing have been found to inhibit learning. On the other hand, Matsumura and Woody (1982) found that the repetitive presentation of USs alone one week before conditioning facilitated subsequent eye-blink CRs. Thus, according to these studies, both pre-trial HS and US pre-exposure can have either facilitative or inhibitive effects on learning, depending on whether the ISI between the additional stimulus and the CS-US pairing is long or short, respectively.

In a delayed conditioning procedure the CS is presented until the start of the US, without a gap between the two stimuli. Hence, there is a period during which the CS and US overlap. Generally, it has been found that delay conditioning takes place faster than trace conditioning (e.g. Akins & Domjan, 1996; Solomon & Groccia-Ellison, 1996; Thompson & Kim, 1996). In a delay conditioning paradigm it is assumed that only the onset process will gain associative strength and hence, only unimodal CRs will occur .

In a trace conditioning procedure the CS offset precedes the US onset, and there is a gap between the end of the CS and the start of the US. Therefore, a trace CS has two stimulus changes, onset and offset, which could each serve as a source of internal stimuli that jointly govern CR generation (Desmond & Moore, 1988). Hence, both CS-onset and CS-offset-evoked stimulus trace processes can acquire associative strength by acting in synchrony in generating unimodal CR waveforms during classical conditioning (e.g. Desmond, 1991; Moore et al., 1989).

It is now an interesting question to see whether the post-trial HS has still facilitative and pre-trial HS has inhibitive effects on NMRs when using the trace conditioning, in which the learning procedure is more complex. Therefore, in the present

study the behavioral effects of the trace conditioning paradigm and the behavioral effects of previously published delayed conditioning paradigm are compared to each other.

Method

Animals

New Zealand albino rabbits (n=11) weighing 2.9 to 3.8 kg served as subjects. The experiment was carried out according to the regulations of the European Union for animal health and care in laboratories. The rabbits were individually housed and maintained on a 12:12 hr light/dark cycle, and they received food and water ad lib in a temperature-controlled vivarium, where they had the possibility to play with aspen sticks. Twice a week the rabbits were also given straw. The animals were cared for by University veterinarians. All behavioral procedures were carried out during the daylight portion of the cycle.

Surgery

Surgical anaesthesia was initiated and maintained with i.m. injections of ketamine-zylazine cocktail (Ketalar®, 50 mg/ml; Rompun®, 20 mg/ml). Each rabbit was first positioned in a stereotaxic headholder with bregma positioned 1.5 mm above lambda.

After drilling a hole over the cerebral cortex, three Teflon-insulated, stainless steel stimulation electrodes (100 µm exposed tips) were lowered stereotaxically into the lateral hypothalamus (P1.0, R2.0, H-6.0; A0.0, R2.0, H-5.0 and A1.0, R2.0, H-4.0).

The electrodes were connected to two 15-pin D-type connectors that were cemented onto the skull with dental acrylic together with four anchoring screws. The implantation of the electrodes was

done by the pressure fitting method developed by Korhonen (1991). Also a headstage designed to hold a minitorque potentiometer, an air puff delivery nozzle and tone tubing were attached to the complex.

Finally a small loop of 4-0 nylon thread was sutured into the right NM. Analgetic (Temgesic®, 0.3 ml/kg) was given s.c. at the end of surgery and every eight hours for the following 48 hours. All the rabbits were given 1-2 weeks for recovery before behavioral training began.

Apparatus

During training the rabbits were restrained in a plexiglas box located in a ventilated, sound-attenuating and electrically shielded box. The rabbits were observed during the experiments with a video monitor. Low-noise pre-amplifiers with a gain of 10 (Analog Devices, AD524) were attached directly to the connector in the acrylic mass on the head of the animal. The neural signals were further amplified, bandpass filtered (500-6000 Hz) and digitized at 15 kHz by separate equipment (Axon amplifier), and they were then fed to an A/D converter. For the NM movement measurements, a small wire with a tiny stainless steel hook was attached to the nylon loop sutured into the NM. The wire was connected to a stainless steel crank. The movement of the crank rotated the cylinder of a minitorque potentiometer connected to a holder on the head. A flexible, shielded cable connected the rabbit to a stimulation source. Head movements were recorded with a solid-state piezoresistive accelerometer (ICSensor, 3021-002-P). A tone generator was placed outside the training box and the tone directed through plastic tubing to the rabbit.

A microcomputer delivered trials at randomised intertrial intervals and delivered the sequence of training stimuli. The microcomputer also controlled delivery of the isolated brain stimulation pulse trains. Identification data generated by the computer was displayed on the video-screen together with a time-graph bar display used for timing the phases of the NM and head movements. All this alpha-numeric information was also superimposed on the video picture of the rabbit in the training box and recorded on video tape.

Procedure

After at least one week of recovery from surgery, each animal was observed in the chamber for the purpose of determining the behavioral threshold current levels of the HS electrodes and making certain that the electrode and current level selected for conditioning would not induce struggling while under restraint. The HS consisted of electrical stimulation of the medial forebrain bundle at a pulse width of 0.5-msec, a train duration of 250-msec, a stimulus intensity typically of 100-250 μA, and a pulse frequency of 100 Hz. Stimulation typically resulted in heightened exploratory activity (sniffing the floor and chewing movements similar to earlier findings by Bruner (1967) and Kim et al. (1983) or sometimes attempted escape followed by freezing). In the present study, a current level inducing orienting and approach movements, without any aversive effects, was selected. Before training the rabbits were placed in a standard Plexiglas restraint box in a conditioning chamber for two 45-min adaptation sessions.

The first three daily sessions consisted of 60 explicitly unpaired CS-only, US-only and HS-only presentations. The CS was a 50-msec tone (1 kHz, 78 dB SPL) delivered via a plastic tube

placed in front of the left ear at a distance of 1 cm. The US was a 100-msec air puff (2.1 N/cm^2, measured from the cornea) directed to the right eye. CRs were defined as at least 0.5 mm of NM movement that occurred after onset of the CS but before onset of the US.

After the three unpaired sessions the trace conditioning began. Each of five consecutive trace conditioning daily sessions consisted of 60 CS-US paired (training) trials followed (post-trial HS) or preceded (pre-trial HS) by a 250-msec train of HS (Fig. 1). Each session also included pseudorandomly given 10 CS-only, 10 US-only and 10 HS-only test trials. Conditioning was performed by using a trace paradigm, where CS offset preceded US onset (150 m-sec stimulus-free period, trace interval), thus creating a 200-msec interstimulus interval. The intertrial interval was random, ranging from 30-50 sec (x=40 sec).

Insert Fig 1. about here.

After the first five conditioning sessions the order of HS presentation was reversed for five more days. Thus, the former pre-trial HS group received the HS after the CS-US pairing (as post-trial HS) and vice versa. The last three days consisted of unpaired sessions similar to the first three ones.

Histology

After the experiments, the animals were anesthetized with a lethal dose of sodium pentobarbital (10-15 cc i.m. and i.v.) and perfused via the ascending aorta with saline followed by 10% formalin. The HS electrode locations were marked by passing 10 μA of direct current through the electrode for 20 sec. The brain was

removed and fixed in formalin-sucrose solution for at least one week. Frozen coronal 100-μm sections were taken from the sites of the electrodes. The slices were mounted on gelatinized slides and stained with cresyl violet for cell bodies and potassium ferrocyanide for the electrode marking lesions. The exact locations of the tips were compared to the coordinates of the stereotaxic atlas of Shek et al (1986).

Data Analysis

The signal analysis was based on a 1,500-msec period, which included 250-msec preHS, 250-msec HS, 250-msec preCS, 250-msec CS, 250-msec US, and 250-msec postHS periods. SPSS 7.0 for Windows was used for all numerical processing and analysis of variance. The Greenhouse-Geisser conservative F test was applied in every analysis.

Results

During the first five paired sessions the post-trial HS group showed significant gradual increases in the amplitudes of conditioned trace NMRs ($F(1.38, 24) = 5.47$, $p < .05$). In contrast, significant increases in the amplitudes of the NMRs were not detected in the pre-trial HS group during the first five paired sessions.

The effects of the reversal of the pre-trial HS and post-trial HS treatments were tested during the last five paired conditioning sessions. During these last five days, when the HS was given after the CS-US pairing (as post-trial HS) the CR amplitude increased gradually in the former pre-trial HS group. In contrast, when the order of HS presentation in the post-trial HS group was changed as a pre-trial HS, the CR amplitude decreased gradually (Fig. 2). These changes were not, however, significant

due to the small amount of animals especially in the pre-trial HS group (n=3).

Insert Fig. 2. About here.

At the end of the study, during the last three unpaired sessions (days), the amplitudes of the conditioned NMRs measured during the 250 -msec CS periods of the CS test trials were found to be larger than those measured during the first three unpaired sessions ($F(1, 8) = 14.72$, $p < .01$). This was detected in both groups (pre-trial HS and post-trial HS). Therefore, despite some extinction that was detected during the last three sessions, there still could be found NM responses.

Reversal of the pre-trial HS and post-trial HS treatments in either group did not change the NM responses during the 250 -msec HS periods of the HS-test trials (i.e. the HS periods of the HS-alone trials). However, during the 250 -msec CS periods of the HS-test trials (i.e. the CS periods in the HS-alone trials) the treatment x session interaction effect was significant ($F(2.78, 36) = 6.76$, $p < .01$) in both the pre- and post-trial HS groups during the last five days of conditioning, in spite of the fact that the treatment was reversed (pre-trial HS treatment was changed to post-trial HS treatment and vice versa). This reversal of these treatments proves that an association was established not only between the CS-tone and US-airpuff but also between the brain stimulation (HS) and the CS-tone.

Finally, the CRs of the CS-test trials of the trace paradigm data was compared to the CRs of the CS-test trials of the previously presented (Arikoski et al., 1997) delayed paradigm

data. The results of the present trace conditioning study were found to be generally consistent with those of the previous delayed conditioning study. However, the amplitudes of the CRs were, as expected, weaker when using the trace paradigm (Fig. 3).

Insert Fig. 3. About here.

Significant differences in CR acquisition emerged between the delayed and trace groups during the first five daily conditioning sessions. During the CS periods of the CS-test trials the effect of the treatment (post-trial HS delayed versus HS trace treatment) was statistically significant ($F(1, 11) = 6.75$, $p < .05$). For the CS-test trials the session (day) effect was significant ($F(1.45, 22) = 19.27$, $p < .001$). Most importantly, the treatment x session (day) interaction was also significant ($F(1.45, 22) = 7.58$, $p < .01$). During the last five days of paired conditioning (after reversal of the site of the HS) the effect of the treatment was also statistically significant ($F(1, 11) = 7.10$, $p < .05$). Also the session effect (day) for the CS-test trials was significant ($F(1.62, 22) = 5.75$, $p < .05$). However, the treatment x session (day) interaction was only close to significant ($F(1.62, 22) = 3.16$, $p = .076$).

During the first five days of paired conditioning the effect of the treatment between pre-trial HS delayed versus trace groups was, as expected, not statistically significant. Similarly, there could not be found either a session effect or a treatment x session interaction. However, during the last five days of paired conditioning the effect of the treatment between pre-trial HS and

delayed versus trace groups was statistically significant ($F(1, 6) = 16.83$, $p < .01$).

Discussion

At a general level basic associative learning describes the way in which organisms learn about causal relationships in the world (Rescorla, 1988). More accurately, associative learning is defined as a relatively permanent change in behavior that results from the temporal conjunction of two events (e.g. Gormezano, 1984). However, it could be argued whether a pairing specific change in an existing response during classical conditioning represents sufficient evidence for associative learning. It is possible that not every two events that occur together will become associated. In this view of classical conditioning, the conditioned stimulus (CS) is said to signal the unconditioned stimulus (US). If an organism shows an augmented response to the CS as a result of being exposed to the relationship between the CS and US, then an association is said to have been formed between the two events (e.g. Rescorla, 1988).

However, an occurrence of a response to the CS may not only have specific associative properties but may also result from nonassociative processes. For instance the baseline level of activity may influence responding. Second factor is the elicitation by the CS of unconditioned responding in the target or other responses systems. The third nonassociative contributor is the sensitizing effect that US presentation may have on CS-elicited responses. To assess the contribution of nonassociative processes to responding control procedures incorporating CS and US as well as HS alone (probe) presentations were adopted in the present study.

The present findings showed that the post-trial HS facilitated trace conditioned NM response acquisition, while NM response acquisition was retarded when the HS was given prior to the CS-US pairing. These results are consistent with the results of our previous study (Arikoski et al., 1995; 1997), in which delayed conditioning procedure was used. Control group (CS-US pairing without HS) was not used in the present trace contditioning study. However, conntrol animals were used in the previous delayed conditioning study. During the fist five days of conditioning the control animals showed faster NMR acquisition than the pre-trial HS animals, but slower NMR acquisition than the post-trial HS animals.

The temporal proximity or close contingency between the CS-US pairing and the following HS seems to be a critical factor in facilitating learning. Several studies (Major & White, 1978; Kim et al., 1983; Hirano et al., 1987; Aldavert-Vera et al., 1996) have indicated that post-trial HS has facilitative effect on CR performance when the ISI between the CS-US pairing and the HS is short.

In contrast, studies using a pre-trial presentation of the HS or US pre-exposure have shown retardation of the CR when a relatively short ISI is used between the preceding additional stimulus and the CS-US stimulus pair (Siegel and Domjam, 1971; Mis and Moore, 1973; Terry, 1976; Kim et al., 1983). Ewing et al. (1985) investigated the associative consequences of backward conditioning using rat subjects in a conditioned emotional response procedure. In this CS pre-exposure study comparisons were arranged so that CSs preceded the CS-US pairing by either a relatively short ISI (60-sec) or by a relatively long ISI (600-

sec). Ewing et al. (1985) found that shorter ISIs produced less CR acquisition and more extinction than the longer ones. In contrast, longer intervals between the unpaired US presentation and subsequent CS-US pairing have been reported to yield a facilitative effect on paired learning (Matsumura and Woody, 1982).

Even though it is possible that reinforcing post-trial HS might improve learning by inducing a non-specific or general activation of the central nervous system during a critical period for memory consolidation, it is more likely that the facilitative effect of the post-trial HS as well as the inhibitive effect of the pre-trial HS on learning can be interpreted as specific activation of the nervous system. The location of the stimulation electrodes in the present study bear out the hypothesis of Major and White (1978), who have proposed that activation of the medial forebrain bundle of the lateral hypothalamus produces a facilitatory effect on learning.

Trace vs. delayed conditioning paradigm.

In the trace procedure, where a period of no stimuli intervened between the CS and US, the post-trial HS group showed an increase in conditioned NMRs during CS-tests, while in the pre-trial HS group the increase in CRs was minimal. Although conditioning developed in the post-trial HS groups in both trace and delayed procedures, the topography of the CR was different. There is a general tendency to acquire delayed conditioning faster than trace conditioning (e.g. Akins & Domjan, 1996; Solomon & Groccia-Ellison, 1996; Thompson & Kim, 1996). The increase in CRs of the trace conditioned post-trial HS group in the present study was minor when compared to the increase in CRs of the delay

conditioned post-trial HS group. This might be due to the fact that longer CSs of the delay paradigm study set up hypothetical "eligibility traces" that are better able to span the trace interval (Blazis & Moore, 1991). In the present study the duration of the CS was only 50 -msec. Despite this short duration, there still could be found CRs. In addition, response acquisition to a trace CS can be facilitated substantially by inserting a second stimulus at the end of the trace interval just before the US (e.g. Kehoe et al., 1979; Rescorla, 1982). These observations indicate that bridging the gap between the CS and US increases perception of temporal contiquity and allows conditioning to occur.

Hence, it seems that the stimulus-free period, a trace interval, is a crucial factor between delay and trace conditioning. Pavlov (1927) originally stated that to perform trace conditioning the animal must maintain an image or "trace" of the CS long enough to be associated with the US. Similarly Clark and Squire (1998) more recently have stated that in trace conditioning the CS must leave some trace in the nervous system for a CS-US association to be established. These formulations suggests that a within-trial memory component is necessary for CS-US association in trace conditioning (Solomon & Groccia-Ellison, 1996).

The trace paradigm differs from the delayed paradigm in terms of not only the trace interval but also the CS-US interval (ISI). Sasse et al. (1991) have noted differences in delay conditioning as a function of ISI. They have found that longer ISIs constitute more difficult tasks for animals.

Taken together, the difference in CR acquisition between our trace and delayed paradigm groups seems related to the within-

trial interval. This seems to be consistent with the finding of Solomon and Groccia-Ellison (1996) who have noted that the crucial difference between the trace and delayed paradigm groups in terms of age-related differences in acquisition is related to the trace interval. In addition, Akins and Domjam (1996) have shown that the trace interval may govern the topography of the conditioned response. More generally, it can be stated that the CR generation is sensitive to the characteristics of the continuing CS. However, the CS onset gives rise to internal mechanisms that by themselves are powerful determinants of responding. Clark and Squire (1998) have found in human subjects that trace conditioning differs from delay conditioning by requiring knowledge of the CS-US relationship to build up and be remembered across many trials.

Taken all together, in classical trace conditioning the acquisition of a CR is possible even though the trace interval intervenes between the CS and US. This implies that some neural representation of the CS is able to support the association between the two temporally separated stimuli events. In addition, trace conditioning may require declarative knowledge (awareness of the CS-US association) because the trace interval between the CS and the US makes it difficult to process the CS-US relationship in an automatic, reflexive way, as in delay conditioning (Clark and Squire, 1998).

Effect of the HS

Earlier in this study we reported that reversal of the pre-trial HS and post-trial HS treatments did not have any effect on HS-alone responding. However, NMR amplitudes to HS of both the pre- and post-trial HS groups increased significantly during the last five conditioning days apart from the fact that the

procedures were different. This could be interpreted as a nonassociative sensitizing effect of the HS. Hence, the reinforcing post-trial HS might have improved CR acquisition by inducing a non-specific or general activation of the central nervous system.

However, unlike the HS given prior to the CS-US pairing, the HS given after the CS-US pairing did have a positive, facilitative effect on CR acquisition. How could the effect of the post-trial HS be explained? It seems that the CS-US-HS trace or delayed conditioning depends on the associative strength of HS rather than its presence (see Gibbs et al., 1991). Hence, the results of the present study may be construed as indicating that responding to CS relies on a CS-HS linkage that is connected to the response system either directly or indirectly through a linkage with a representation of the US.

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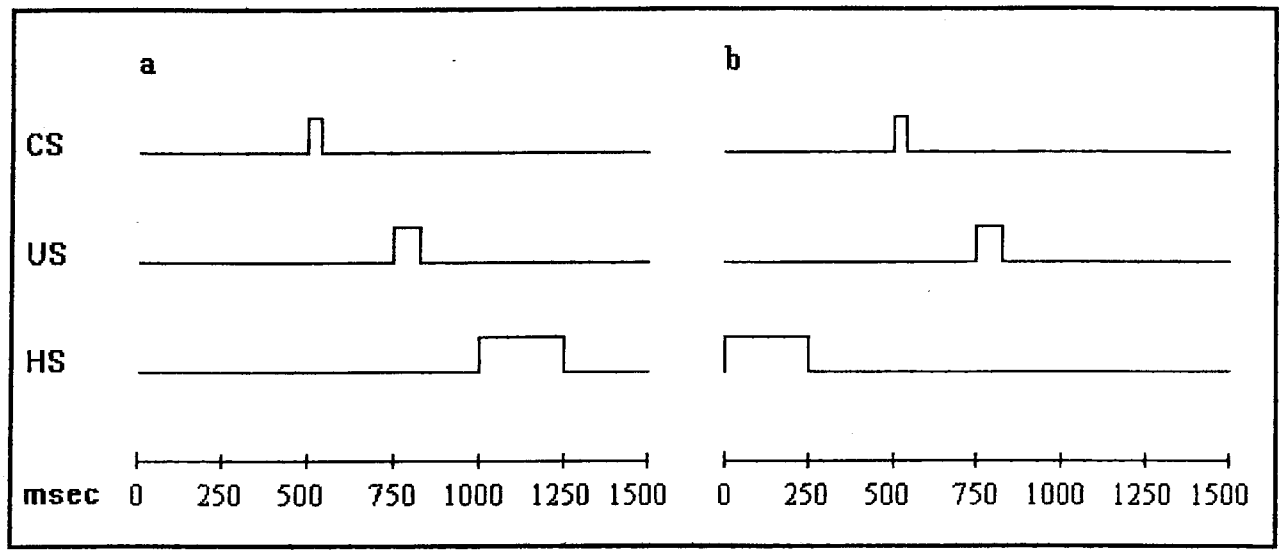
Figure Caption

Figure 1. Schematic illustration of the training procedures. a) Post-stimulation trial. b) Pre-stimulation trial.

Figure 2. Both the pre-trial HS and post-trial HS groups showed CR acquisition during their post-trial HS treatment phases.

Figure 3. CRs of the CS-test trials of the trace paradigm data was compared to the CRs of the CS-test trials of the previous delayed paradigm data.


a
b
CS
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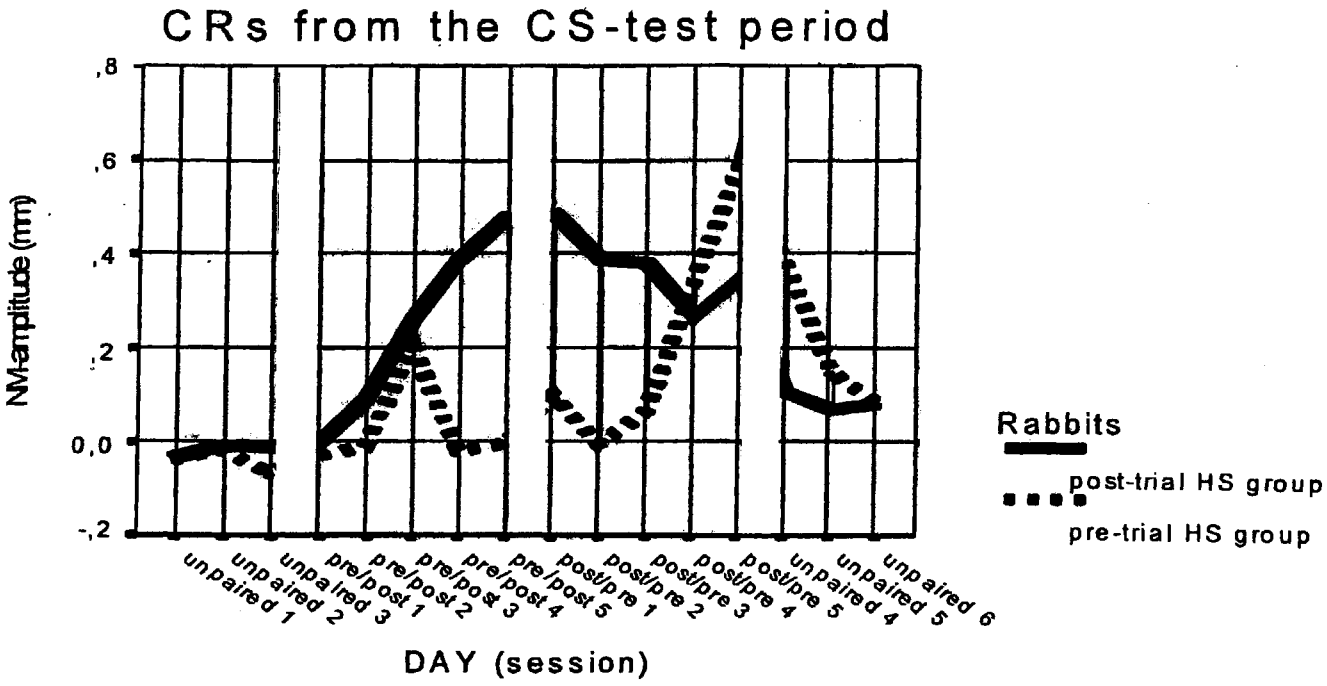
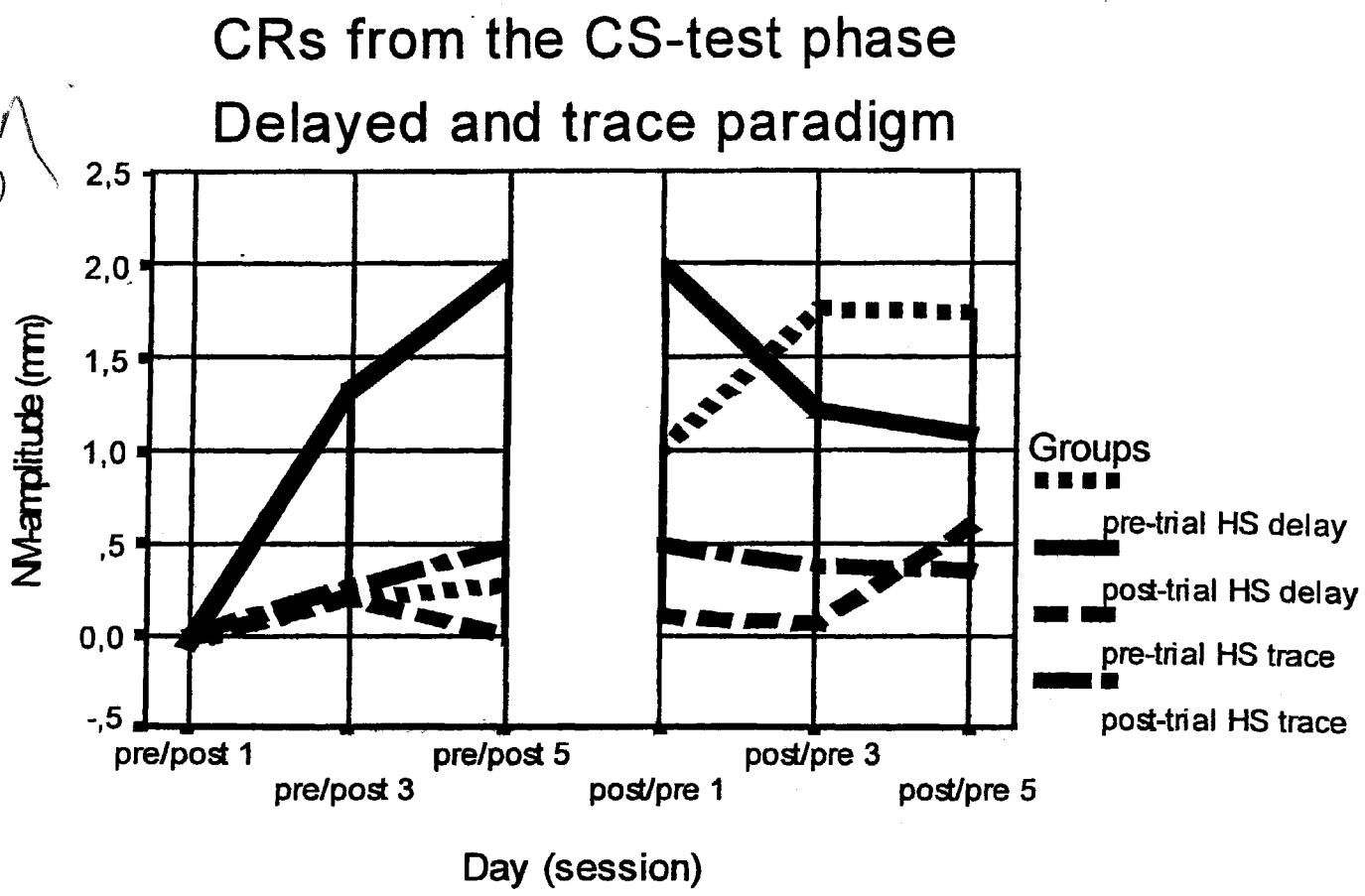

CRs from the CS-test period
,8
,6
,4
,2
0,0
-,2
Mamplitude (mm)
unpaired 1
unpaired 2
unpaired 3
pre/post 1
pre/post 2
pre/post 3
pre/post 4
pre/post 5
post/pre 1
post/pre 2
post/pre 3
post/pre 4
post/pre 5
unpaired 4
unpaired 5
unpaired 6
DAY (session)
Rabbits
post-trial HS group
pre-trial HS group


CRs from the CS-test phase
Delayed and trace paradigm
NM-amplitude (mm)
2,5
2,0
1,5
1,0
,5
0,0
-,5
pre/post 1
pre/post 3
pre/post 5
post/pre 1
post/pre 3
post/pre 5
Day (session)
Groups
pre-trial HS delay
post-trial HS delay
pre-trial HS trace
post-trial HS trace