Evocation in paradoxical sleep of a hippocampal conditioned cellular response acquired during waking

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This experiment was a test of the possibility that a conditioned hippocampal response acquired during wakefulness could be evoked during paradoxical sleep (PS). After one session of habituation to a tone, waking rats underwent conditioning in four sessions during which the tone was used as the conditioned stimulus preceding a footshock. Pseudoconditioned animals received unpaired tone-shock presentations. There was a 24-h intersession interval. After each session, the same tone, never awakening the animal, was presented during PS phases. Hippocampal multiunit activity (MUA) was analyzed at each tone presentation during waking and during PS. Before conditioning, tone presentation did not affect MUA. During conditioning sessions, it elicited sustained increases in hippocampal discharge. When the tone was presented during postlearning PS, it induced a short-lasting increase in MUA. On the other hand, in the pseudoconditioned animals, hippocampal activity failed to show any change in response to the tone, whether during wakefulness or during PS. These results suggest that a behaviorally significant stimulus can be recognized during PS.

The ability of the sleeping organism to process environmental stimuli remains an open question. In animal research, previous studies have concerned principally slow-wave sleep (SWS), and in most, the arousal response has been used as an index of stimulus detection during sleep. Evidence has been presented that discriminative arousal responses can be made during SWS according to stimulus relevancy (Buendia, Sierra, Goode, & Segundo, 1963; Halperin & Iorio, 1981; Rowland, 1957; Segundo, Roig, & Sommer-Smith, 1959). In the present experiment, we focused on paradoxical sleep (PS). Our purpose was to test the general assumption that behaviorally important stimuli can be detected during PS and that this can occur without arousal from sleep.

A number of studies have lent support to the idea that paradoxical sleep is a state that allows information processing. First, experimental evidence has suggested that a newly formed memory trace is reprocessed during PS phases following learning, since postlearning PS deprivation has been shown to impair performance in a variety of complex learning tasks. Moreover, an increase in the duration of PS has been observed during periods of spontaneous sleep occurring after learning (for reviews, see Fishbein & Gutwein, 1977; Hennevin & Leconte, 1977; McGrath & Cohen, 1978; Pearlman, 1979; Smith,

1985). Some data, obtained in our laboratory (see Bloch, Hennevin, & Leconte, 1979), suggest that the processes that take place during postlearning PS might be of the same kind as those acting just after the acquisition of information. For example, a promnesic treatment (a stimulation of the midbrain reticular formation) administered during the postacquisition period appeared to largely substitute for PS, since it decreased the posttraining PS elevation and abolished most of the impairment produced by PS deprivation (Bloch, Hennevin, & Leconte, 1977). When applied a few hours after training, the same treatment was only effective when delivered during PS (Hennevin, Hars, & Bloch, 1989).

Further, Maho and Bloch (1983) demonstrated that learning could occur during PS. They developed a classical conditioning paradigm in which both the conditioned and the unconditioned stimuli (CS, US) consisted of direct brain stimulation (a stimulation of the medial geniculate body was used as the CS, a stimulation of the central gray as the US). The conditioned response was an increase in multiunit activity (MUA) in the dorsal hippocampus. Although conditioning was not established during SWS, it was established during PS as easily as during wakefulness. Thus, the brain in PS appears quite able to acquire and process new information.

Yet it remains to be demonstrated that information coming from the external world can likewise be processed during PS. In animals, PS is a state of deep sleep, in that the awakening thresholds for external stimuli are higher during PS than they are during SWS (Dillon & Webb, 1965; Triglona, Ciancia, & Bloch, 1968; Van Twyver &

We are pleased to acknowledge the advice of Dominique Lépine concerning statistical analysis. Correspondence should be addressed to Elizabeth Hennevin, LPN2, C.N.R.S., 91198 Gif-sur-Yvette Cedex, France.

Garrett, 1972). It is not clear whether or not the relevance of the stimulus is an important factor in its ability to arouse. Indeed, there are some reports of lowered arousal thresholds following conditioning, relative to when the stimulus is neutral (Siegel & Langley, 1965). In contrast, other experimenters have failed to detect differential arousal responses regardless of whether the stimuli were meaningful or neutral (Halperin & Iorio, 1981; Van Twyver & Garrett, 1972).

On the other hand, there is evidence that the organism in PS is not totally shut off to the outside world and can detect nonawakening stimuli, after the stimuli have acquired significance during the waking state. For instance, Ciancia, Trigona-Leisinger, and Bloch (1980) used the electrodermal response as an index of reactivity to a tone. They found that pairing the tone with electrical shock during waking enhanced responsiveness to the tone when it was subsequently presented in PS. Moreover, we showed that when the stimulus used as a CS during avoidance conditioning was given as a reminder in postlearning PS, subsequent retention performance was improved. No effect was detected when the stimulus had not been previously associated with the learning task (Hars, Hennevin, & Pasques, 1985). Introducing the cue during SWS impaired retention (Hars & Hennevin, 1987). Most recently, we showed that 1 month after learning, presentation of the cue during PS was still effective in modifying retention performance, but that it had no effect during SWS (Hars & Hennevin, 1990).

To account for the efficiency of cuing during PS, it is necessary to admit that the cue stimulus has been detected and recognized during PS. The present experiment was conducted to identify a central index of this detection. For this purpose, we studied modification of hippocampal cellular activity induced by a conditioned stimulus presented during postlearning PS.

Indeed, a number of studies of waking animals have reported learning-induced changes in hippocampal neuronal activity. It has been shown with a large diversity of preparations that each time a sensory stimulus acquires a predictive value, its presentation elicits consistent increase in the frequency of unit or multiunit discharges of hippocampal neurons. Since the pioneering work of Olds and his co-workers (Olds, Disterhoft, Segal, Kornblith, & Hirsh, 1972; Olds & Hirano, 1969; Olds, Mink, & Best, 1969; Segal & Olds, 1972), an increasing amount of evidence has attested to the associative nature of such changes. The hippocampal response develops gradually across successive CS-US pairings; it does not develop with the repeated presentation of the to-be-conditioned stimulus alone, or in control animals given unpaired CS-US presentations; differential responses to the CS+ and CS- have been observed. (See, for reviews, Berger, Berry, & Thompson, 1986, and Disterhoft & Buchwald, 1980. See also Berger & Thompson, 1978; Bloch & Laroche, 1981; and Edeline, Neuenschwander-El-Massioui, & Dutrieux, 1990.)

Until now, no attempt has been made to examine the possibility that such hippocampal response could be evoked during sleep. To address this question in the present study, we subjected waking rats to classical conditioning with a tone used as the CS. The tone alone was subsequently presented during PS phases. Hippocampal responses to tone presentations in PS were compared with those obtained in pseudoconditioned rats. If, as we have argued above, the PS state provides the conditions for detecting and processing significant stimuli coming from the external environment, we should expect that hippocampal associative cellular responses might be reinstated by CS presentation during PS.

METHOD

Fifteen male Wistar rats, weighing 280–300 g at the time of surgery, served as subjects. For the duration of the experiment, they were housed in the colony room in individual cages, under a 12:12-h light:dark cycle, with ad-lib access to food and water. The experiments were run during the light cycle.

Implantation and Recording Procedure

The animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), which was supplemented as necessary during implantation.

They were implanted with cortical and neck-muscle electrodes. Two extradural-cortical (ECoG) electrodes, made of small silver spheres, were placed along the interhemispheric suture with a large frontoparietal contralateral derivation. Two other silver electrodes were sewn through the dorsal neck muscles for the recording of electromyographic (EMG) activity. A small brass screw fixed into the frontal bone was used as the ground.

Two recording electrodes were stereotactically implanted in the dorsal hippocampus. The electrodes were made of 62- μ m Nichrome wires that were insulated except for 80 μ m at the tip, mechanically sharpened under microscopic control to obtain about a 5- μ m diameter at the extremity. Two electrodes were inserted into a stainless steel guide microtube (0.3 mm in external diameter) whose noninsulated sharpened extremity was used for differential recordings. These electrodes were implanted in either the CA1 or the CA3 field of the hippocampus. The stereotaxic coordinates, from the Albe-Fessard, Stutinsky, and Libouban atlas (1966), were as follows: anterior, 3.8 mm; lateral, 3.6 mm; and vertical, +7.3 mm for CA1 and +6.6 mm for CA3. The MUA was acoustically monitored and displayed on an oscilloscope during implantation to further control for electrode location. All the electrodes were connected to the pins of two miniature sockets fixed to the skull with dental acrylic cement. At least a week of recovery was allowed before the start of the experiment.

During the experiment, hippocampal MUA was recorded through field effect transistors (Siliconix U404; input 15 pA, output 3 K Ω) placed on the animal's head. Neuronal activity was fed to a preamplifier (bandwidth 500–10000 Hz). The output, displayed on an oscilloscope, was recorded on a magnetic tape during the 2-sec pretone and the 2 sec of tone, for subsequent off-line analysis. The EMG activity was fed to a preamplifier (bandpass 3–75 Hz) of a Grass polygraph. The output was recorded on a magnetic tape during the 2-sec pretone and the 2-sec tone period.

Apparatus

The experimental box (25×25×50 cm) was placed in a sound-attenuating chamber. Both had a transparent front door, which al-

lowed a visual control of the animal. The top of the experimental box was equipped with a loudspeaker (5 cm in diameter, bandpass 20–20000 Hz). The grid floor was made of stainless steel rods 0.5 cm in diameter, spaced 1.5 cm center to center. Counterbalanced cables connected to the animal were relayed at the top of the box through a multichannel rotating connector.

Procedure

Familiarization period. For 5 days, the animals were familiarized with the experimental conditions. Each animal was placed in the experimental box and connected to recording cables for 4 h each day. During this period, the intensity of the tone that was to be used subsequently was determined for each animal. This procedure was performed to take into account possible differences in reactivity to the tone. ECoG and EMG activity were polygraphically monitored so that vigilance states could be recognized as they occurred. The tone (10 kHz, 2 sec in duration) was presented during periods of SWS. The intensities were varied and delivered in a random order. There were 10 tone presentations on the average. The chosen intensity was never arousing, and it induced little or no modification in hippocampal MUA. This intensity was maintained during all subsequent experimental phases. Tone intensity varied among rats from 55 to 70 dB SPL.

Habituation and conditioning. For 5 consecutive days, at the same time of the day, each rat was placed in the experimental box and connected to recording cables. ECoG and EMG activity were permanently monitored.

On the 1st day, each rat was put through a 30-min habituation session. Ten tones (10 kHz, 2 sec) were presented to the waking animal with variable intertrial intervals (range, 2–5 min). After this session, two groups were formed, equalized in terms of responsiveness of hippocampal MUA to tone presentations during both wakefulness and PS.

For the next 4 days, the animals in the conditioned group ($n = 8$) were submitted to a daily session of classical conditioning during waking. On each trial, the tone (CS, 2 sec in duration) was paired with a scrambled electrical shock delivered through the grid floor. The footshock (0.5 sec in duration) was given at CS offset; the intensity was adjusted for each individual (range, 0.2–0.3 mA). There were 10 trials per session, with a mean intertrial interval of 3 min (range, 2–5 min).

For animals in the pseudoconditioned group ($n = 7$), the four sessions consisted of 10 presentations of the same tone and footshock but explicitly unpaired, with the same intertone intervals as for the first group. Mean tone intensity was similar to that delivered in the conditioned group.

Test trials in PS. After the habituation session and after each session of conditioning or pseudoconditioning, each animal was kept in the experimental box and monitored for sleep phases. Eight test tones were given in PS. They had exactly the same characteristics (in terms of both duration and intensity) as those used during wakefulness. After the onset of a PS phase, 15–20 sec were allowed to elapse before tone presentation. Within a PS phase, intertone interval varied from 20 to 30 sec. A single PS phase was too short for eight tone presentations, so test trials were distributed among several phases, from two to six according to their duration. Therefore, intertone interval could be up to 2 h. For each group, mean duration of postsession recordings was 4.5 h.

Data Analysis

For off-line analysis, the hippocampal MUA was passed through a voltage window discriminator, adjusted independently for each animal and each MUA channel. The adjustment of the triggering level provided a spontaneous mean rate of 20 to 30 spikes per second. For each channel, the voltage window was set the same in the different phases of the study. The output pulses of the trigger were stored on each trial by a microcomputer in successive 50-msec

bins during the 1-sec pretone and the 2 sec of tone. Standard computer programs allowed construction of peristimulus time histograms for each electrode placement, as well as group histograms by averaging across MUA channels. EMG activity was treated in a similar manner, except that triggers were adjusted to provide a spontaneous mean activity of 25 to 40 counts per second.

A few trials (approximately 1%) were discarded from analysis: (1) trials on which a movement was polygraphically detected in the waking animal during the 1-sec pretone period, and (2) trials on which the sleeping animal woke up during pretone or tone periods. In order to eliminate possible electrical artifacts, bin counts exceeding 12 for hippocampal MUA and 33 for EMG activity were discarded during the construction of individual histograms.

At each tone presentation, whether during waking or during PS, mean activity during the 1-sec pretone period (20 bins) and during the first 600 msec (12 bins) of tone was analyzed. For the tone period, three successive subperiods of 200 msec each were considered (reasons for this choice are explained below).

Statistical comparisons were carried out with a contrast analysis of variance (Rouanet, Bernard, & Leroux, 1990), performed on the averaged values obtained for the trials of each recording session. The data were subjected to analyses of variance with each recorded electrode placement as a subject factor and orthogonal repeated measures factors of sessions (5 levels: 1 habituation and 4 conditioning or pseudoconditioning) and time periods (2 levels: pretone and a given subperiod of tone). First, in order to detect possible shifts in spontaneous firing rate across days, the pretone firing rate was subjected to an analysis of variance across sessions. Second, in order to study the tone effect on MUA activity, within-session analyses were done by comparing, for each session, the number of spikes per bin during the pretone period with the number of spikes per bin of the tone period. Third, between-group analyses were conducted to compare differential changes in responsiveness due to conditioning and pseudoconditioning.

Similar analyses were done for EMG data, except that between-group comparisons were conducted on normalized scores (Z scores). For this, scores were computed at each trial by subtracting, for each animal, mean activity of the pretone period from mean activity of a given subperiod of tone. Then the difference was divided by the standard deviation of the pretone activity recorded for the entire session. These normalized data were used to take into account differences in pretone EMG activity observed across days in the two groups.

Histology

At the end of the experiment, the animals were perfused (intracardiac perfusion) under deep pentobarbital anesthesia with 0.9% saline followed by 10% formalin. The brains were kept for 2 weeks in 10% formalin and then frozen, sliced at 60 μ m, and stained with Cresyl violet for Nissl preparation.

RESULTS

Conditioned Group

Behavioral data. In the habituation session, no movement was observed during tone presentation. Pairing the tone with footshock rapidly induced a variety of motor responses to the tone (such as head movement, flinching, freezing, etc.). Many such responses, too subtle to be seen by mere observation, were detected by monitoring neck muscle EMG activity, which showed increases during tone presentations. These motor responses were present as early as the first conditioning session. Statistical analysis of EMG data supported these observations.

Figure 1. Position of hippocampal recording electrodes used in the present study. Filled circles depict placements for the conditioned group. Empty circles depict placements for the pseudoconditioned group.

First, a global analysis of variance across sessions, performed on the 20 bins of the pretone period, revealed a significant modification of spontaneous EMG activity across the 5 days [$F(4,28) = 3.845, p < .025$]. Pretone activity did not change across the four conditioning sessions [$F(3,21) < 1$], but it was higher during conditioning than during habituation [$F(1,7) = 26.070, p < .005$].

Second, we compared, for each session, mean pretone activity to mean activity during the first 200 msec follow-

ing tone onset. No difference was detected for any session [for all comparisons, $F(1,7) < 1$]. When the subsequent 400 msec of tone were considered, a marked increase in EMG activity was observed in response to tone presentation during conditioning sessions [$F(1,7) = 11.750, p < .025$], whereas no modification was noted during the habituation session [$F(1,7) = 2.284, n.s.$]. The increase of EMG activity was present as early as the first conditioning session and was significant for both the second and the third periods of 200 msec following tone onset [respectively, $F(1,7) = 8.226$ and $F(1,7) = 8.149; p < .025$].

Thus, pairing the tone with footshock induced myographic responses to the tone, which appeared with a latency of 200 msec after tone onset. This result led us to analyze hippocampal MUA data in successive periods of 200 msec each.

Hippocampal responses to the tone during conditioning. Of the 16 channels of MUA, 2 placements in CA1 were discarded for technical reasons. Since no difference was detected between cellular data recorded from the CA1 and CA3 fields (5 and 9 electrodes, respectively; see Figure 1), data were pooled for subsequent statistical analyses.

Samples of hippocampal MUA recorded during waking are shown in Figure 2 (left).

On the habituation day, no change was noted during the first 200 msec of tone [$F(1,13) < 1$], and a significant decrease in neuronal activity was observed during the subsequent 400 msec [$F(1,13) = 8.012, p < .025$].

Figure 2. Samples of single trial records of hippocampal multiunit activity (MUA) collected in 1 animal during waking and subsequent paradoxical sleep (PS) at two different stages of the experiment (habituation session and second session of conditioning). Triggered activity is represented above the MUA recording; the arrows indicate tone onset. Note the increased discharge to tone presentation on both the conditioning trial and the test trial given during postconditioning PS.

In contrast, during conditioning, tone presentation elicited a large increase in hippocampal activity that was significant for each of the three successive subperiods of 200 msec [respectively, $F(1,13) = 37.947$, $F(1,13) = 32.906$, and $F(1,13) = 26.432$; in all cases, $p < .001$]. This increase was present as early as the first conditioning session [respectively, for the three periods analyzed: $F(1,13) = 14.947$, $p < .005$; $F(1,13) = 15.600$, $p < .005$; $F(1,13) = 8.741$, $p < .025$]. It was maintained on the three subsequent sessions (for each session and each period of 200 msec, $p < .001$). More detailed analysis revealed that the hippocampal conditioned response began to appear during either the first or the second bin of 50 msec following tone onset. Thus, when mean activity during the first 100 msec of tone was compared with that of the pretone period, a significant increase was observed at each session of conditioning [respectively, $F(1,13) = 15.334$, 10.619 , 43.144 , and 31.030 ; in all cases, $p < .01$ or less]. (See Figure 3.)

The increased hippocampal responsiveness to the tone was not a consequence of modification of the spontaneous firing rate, since mean activity during the pretone period did not change across the 5 recording days [$F(4,52) < 1$].

Thus, pairing tone with footshock resulted in enhanced hippocampal responsiveness to the tone. As can be seen in Figure 3, the hippocampal cellular response occurred long before the myographic response.

Hippocampal responses to the tone during paradoxical sleep. Statistical analyses are restricted to data recorded during the first 200 msec following tone onset, since no significant modification was detected during the subsequent 400 msec.

A sample of hippocampal response to tone presentation in PS is shown in Figure 2 (right).

Before conditioning, tone presentation did not alter the hippocampal discharge rate [$F(1,13) = 2.796$, n.s.]. On the other hand, after conditioning, it induced a significant increase of MUA [$F(1,13) = 16.845$, $p < .005$]. This in-

Figure 3. Averaged group histograms of hippocampal multiunit activity (MUA; top panel) and of electromyographic (EMG) activity (bottom panel) recorded during the habituation session (0) and the four sessions of conditioning (1-4). For hippocampal MUA, each histogram represents the number of spikes per bin of 100 msec during the 1-sec pretone period and the 1st second of tone. For EMG activity, each histogram represents the number of counts per 100-msec bin over the same period. Note the lack of effect of tone presentation at the habituation session, contrasting with the marked increases in both hippocampal MUA and EMG activity during conditioning. Note that the increase of hippocampal activity begins largely before that of EMG activity.

PARADOXICAL SLEEP

HIPPOCAMPAL MUA

Figure 4. Averaged group histograms of hippocampal multiunit activity recorded in the conditioned (top panel) and pseudoconditioned (bottom panel) groups during paradoxical sleep phases following the habituation session (0) and the four sessions of conditioning or pseudoconditioning (1–4). Each histogram represents the number of spikes per bin of 100 msec during the 1-sec pretone period and the 1st second of tone. Note the short-lasting hippocampal response to tone presentation in the conditioned group after each of the four sessions of conditioning. Note the complete absence of response in the pseudoconditioned group.

crease was observed from the 1st to the 4th conditioning days [respectively, $F(1,13) = 11.883, p < .005$; $F(1,13) = 5.646, p < .05$; $F(1,13) = 10.695, p < .01$; $F(1,13) = 7.885, p < .025$]. As can be seen in Figure 4 (top), the hippocampal response to the tone occurred mostly during the first 100 msec of tone [respectively, for Conditioning Sessions 1, 2, and 3: $F(1,13) = 11.313, F(1,13) = 11.083$, and $F(1,13) = 13.691$, all $ps < .01$]. At Session 4, the increase appeared with a longer latency and was not significant for the first 100 msec [$F(1,13) < 1$].

There was no change in the spontaneous firing rate across the 5 recording days [$F(4,52) < 1$].

Further analyses confirmed that hippocampal responsiveness to the tone was different before and after conditioning. Indeed, a significant interaction was present between periods (pretone vs. tone) and sessions (posthabituation vs. postconditioning) [$F(1,13) = 7.798, p < .025$]. No interaction was found when the four postconditioning records were considered [$F(3,39) < 1$].

Thus, after the tone had been paired with footshock during wakefulness, its presentation during subsequent PS increased hippocampal neuronal activity. It is important to emphasize that the hippocampal responses observed during PS are independent of motor activity; tone presen-

tation never induced any change of EMG activity that remained at the zero level through the entire PS phase.

Pseudoconditioned Group

Behavioral data. As in the conditioned group, the level of spontaneous EMG activity was higher during the pseudoconditioning sessions than during the habituation session [$F(1,6) = 6.748, p < .05$]. No change was detected across the four pseudoconditioning sessions [$F(3,18) = 1.715, n.s.$].

But, contrary to what was observed in conditioned animals, those submitted to pseudoconditioning never exhibited myographic responses associated with tone presentations (see Figure 5, bottom). Comparisons between EMG activity during pretone and tone periods failed to reveal any significant difference at any session, whether the first 200 or the subsequent 400 msec of tone were considered; during the habituation session, as well as during pseudoconditioning, $F(1,6) < 1$, whatever the subperiod of tone examined.

Hippocampal responses to the tone during pseudoconditioning. Of the 14 electrodes implanted, 2 were discarded for technical reasons. Cellular data derived from the CA1 and CA3 fields (respectively, 4 and 8 electrodes)

WAKING

PSEUDOCONDITIONED GROUP

Figure 5. Averaged group histograms of hippocampal multiunit activity (MUA; top panel) and of electromyographic (EMG) activity (bottom panel) recorded during the habituation session (0) and the four sessions of pseudoconditioning (1–4). For hippocampal MUA, each histogram represents the number of spikes per bin of 100 msec during the 1-sec pretone period and the 1st second of tone. For EMG activity, each histogram represents the number of counts per 100-msec bin over the same time period. Note the lack of effect of tone presentation on both hippocampal and EMG activity throughout the five sessions.

were pooled for statistical comparisons. We concentrate our analysis on the first 200 msec following tone onset, since results obtained in the conditioned group, during wakefulness as well as during PS, have shown that this period was the most relevant.

The pretone firing rate presented no significant shift across days [$F(4,44) < 1$].

As can be seen in Figure 5 (top), MUA remained unaffected by tone presentation during the habituation session [$F(1,11) = 1.802$, n.s.] and during the four subsequent sessions [$F(1,11) < 1$ for Sessions 1, 2, and 4; $F(1,11) = 1.252$, n.s., for Session 3].

Hippocampal responses to the tone during paradoxical sleep. No significant modification of background activity was seen across the 5 recording days [$F(4,44) < 1$].

Contrary to what was observed in PS following conditioning sessions, no increase of MUA was detected after pseudoconditioning (see Figure 4, bottom). Tone presentation had no effect at all either after the habituation session or after Pseudoconditioning Sessions 1, 3, and 4 [$F(1,11) < 1$ in the four cases]. After Session 2, a sig-

nificant decrease of MUA was noted [$F(1,11) = 11.935$, $p < .01$].

Thus, when tone was not paired with footshock, it never elicited an increase of hippocampal activity, whether it was presented during wakefulness or during subsequent PS.

Between-Group Comparisons

First, we compared EMG data obtained from waking animals during the five sessions. The two groups did not significantly differ with regard to spontaneous pretone activity across days [$F(1,13) = 3.50$, n.s.]. Similarly, during the first 200 msec following tone onset, no difference in EMG reactivity was detected: comparisons of normalized scores obtained for this period failed to reveal any difference at any time [$F(1,13) < 1$ for each of the five sessions]. In contrast, when the subsequent 400 msec of tone were examined, a global analysis conducted on the five sessions revealed a statistically significant difference between the two groups [$F(1,13) = 20.991$, $p < .001$]. Scores obtained at the habituation session were not different [$F(1,13) < 1$]; those obtained during the sub-

Figure 6. Mean hippocampal multiunit activity recorded during wakefulness and subsequent paradoxical sleep in the conditioned group (C) and in the pseudoconditioned group (PC). White columns represent mean number of spikes ($\pm$ SEM) per 100 msec during the 1-sec pretone period. Dark columns represent the number of spikes ($\pm$ SEM) during the first 100 msec of tone. Top: Mean firing rate during the habituation session (left) and during the four training sessions pooled together (right). Bottom: Mean firing rate during posthabituation paradoxical sleep (PS; left) and during posttraining PS (right). Note that spontaneous firing rate does not differ between the waking and PS states. Note that tone presentation evokes increased discharges in the hippocampus during conditioning and subsequent PS. Note that neither habituation nor sensitization training produces this effect.

sequent sessions were greater in the conditioned group [$F(1,13) = 16.790$, $p < .005$]. This between-group difference was found for each of the four sessions [$F(1,13) = 5.341$, $p < .05$, for Session 1; $F_s(1,13) = 14.544$, 12.290 , 9.681 , $p < .01$, for Sessions 2, 3, and 4].

Second, we compared hippocampal responses to the tone presented during wakefulness. A global analysis across days performed on mean pretone activity showed that spontaneous firing rate was similar in the two groups throughout the experiment [$F(1,24) < 1$].

Difference in responsiveness to the tone was studied by comparing the two groups' mean activity during the first 200 msec of tone with mean pretone activity. A

global analysis performed on the five sessions revealed a strong interaction of groups $\times$ periods [$F(1,24) = 20.723$, $p < .001$]. Responsiveness to the tone was similar at the habituation session [$F(1,24) < 1$], but it was higher during conditioning than during pseudoconditioning [$F(1,24) = 26.172$, $p < .001$]. This difference was observed at each of the four sessions [respectively, $F_s(1,24) = 11.140$, 17.048 , 18.595 , and 24.478 ; in all cases, $p < .005$ or less].

Third, we compared hippocampal responses to the tone given during PS. Both groups presented similar spontaneous discharge rates during the pretone period [$F(1,24) < 1$].

A significant interaction between group and period factors was found on the 5 recording days [$F(1,24) = 16.413$, $p < .001$], indicating a higher responsiveness to the tone in the conditioned group. Spontaneous responsiveness was similar, since the interaction was not significant at the habituation session [$F(1,24) = 1.759$, n.s.]. In contrast, relative to the pretone level, hippocampal responses to the tone were higher after conditioning than after pseudoconditioning [$F(1,24) = 16.781$, $p < .001$]. The interaction of group $\times$ period was significant for Sessions 1, 2, and 3 [respectively, $F(1,24) = 6.033$, $p < .025$; $F(1,24) = 15.728$, $p < .001$; and $F(1,24) = 6.024$, $p < .025$]; it did not reach significance for the fourth session [$F(1,24) = 3.640$, n.s.].

The results obtained during both waking and PS in the two groups are summarized in Figure 6.

DISCUSSION

The main findings from the present experiment can be summarized as follows. Pairing a previously habituated tone with a footshock induced enhanced responsiveness of hippocampal MUA to the tone presented during conditioning and subsequent PS phases. No such changes occurred when unpaired tone-shock presentations were given.

A number of authors, on the basis of electrophysiological and behavioral correlates, have classified hippocampal cells into two major categories—complex spike cells and theta cells. They have shown that the firing rate changes differentially with behavioral state in these two classes of hippocampal cell types (Delacour, 1980; Ranck, 1973; Suzuki & Smith, 1985). Complex spike cells fire slowly most of the time; they are most active during SWS and least active during waking and PS. Theta cells generally fire much faster than complex spike cells and increase their discharge rate during waking and PS. In the present study, from analysis of the spontaneous firing rate of the multiunits recorded, it appears that the cellular pools present high activity during waking and PS (25–30 spikes/sec) and a lower activity during SWS (15–20 spikes/sec). This suggests that in the pool of hippocampal cells recorded in the MUA, most behave as theta cells. Previous researchers have emphasized that this hippo-

campal cell type exhibits associative changes in response to the conditioned stimulus in tone-discrimination learning (Christian & Deadwyler, 1986; Delacour, 1982).

The increased hippocampal firing in response to the CS during conditioning is comparable to that previously observed when the same conditioning procedure was used (Bloch & Laroche, 1981; Edeline, Dutrieux, & Neuenchwander-El-Massioui, 1988). It develops as a result of learning because it is dependent on the temporal contiguity of the CS and the US. It did not occur in response to the repeated presentation of the to-be-conditioned stimulus, either alone or in control animals given unpaired CS-US presentations. The hippocampal response emerged after very few trials (within four to five) and persisted throughout the four training sessions. It appeared with a mean latency of 50–60 msec after tone onset and was sustained through the whole tone duration.

On the behavioral level, it has commonly been observed that presentation of a stimulus that has acquired a predictive value in aversive conditioning elicits a variety of emotional and motor responses. This raises the traditional problem of whether or not the conditioning-related cellular change is dependent on these behavioral conditioned responses. Such a consideration is especially appropriate for hippocampal neuronal changes, because certain types of neurons of dorsal hippocampal formation are known to increase their firing with movement (Ranck, 1973; Rose, 1983; Sinclair, Seto, & Bland, 1982; Suzuki & Smith, 1985).

Whether or not learned increases in hippocampal activity are dependent on the expression of specific behavioral responses elicited by the CS has received much attention in the literature and has been addressed through the use of several strategies. For instance, in a conditioning paradigm that required the animal to remain motionless during the CS in order to obtain the appetitive US, Olds and Hirano (1969) found increased firing of hippocampal neurons accompanying the conditioned behavioral inhibition. In a temporal single alternation paradigm, Hoehler and Thompson (1979) observed greater magnitude of hippocampal responses to the CS on reinforced trials relative to nonreinforced trials, while nictitating membrane responses did not differ. Lastly, Laroche, Neuenchwander-El-Massioui, Edeline, and Dutrieux (1987), using a transfer-of-control technique, showed that the hippocampal responses established during classical conditioning were maintained while two opposite behavioral responses to the CS occurred (suppression vs. enhancement of leverpressing).

Thus, all these experiments demonstrate that the increase in hippocampal responsiveness to the CS may be dissociated from the specific behavioral response that the CS elicits. However, none of them preclude the possibility that cellular responses might be linked to nonspecific behavioral responses related to conditioning.

In an attempt to address this question in the present experiment, we recorded dorsal neck muscle EMG activity. It is obvious that discrete movements or postural shifts

cannot be accurately detected by observation of overt behavior of the animal. Recording of the neck muscle EMG activity revealed substantial changes in muscle tension, which were present, as are hippocampal cellular responses, as early as the first few trials of conditioning. This myographic response was associative, since animals given unpaired tone-shock presentations showed no signs of such response. However, an examination of the temporal relationships between the electrical activity recorded in the hippocampus and the dorsal neck muscle EMG activity revealed that the increase in cell firing began well ahead of the increase in EMG activity. The latency from tone onset to hippocampal cellular discharge was approximately 150 msec shorter than the latency to onset of the conditioned EMG response.

Thus, the late component of hippocampal response—that is, the portion of response that occurred 200 msec after tone onset—is possibly linked to changes in posture and tonic muscular tension related to CS presentation. More generally, such a possibility might account for late, sustained hippocampal cellular increases reported in most studies of conditioned hippocampal changes. None of them included control of EMG activity at the time of recording. Our results show the necessity of such control in the assessment of whether recorded cellular changes are actually independent of motor activity. On the other hand, the early component of hippocampal responses observed in the present experiment cannot be considered a consequence of motor activity. It remains an open question whether these early changes are correlates of some preparatory motor processes or internal autonomic responses related to shock, or whether they are correlates of associative mnemonic representation evoked by the CS. As will be discussed below, results obtained during PS seem to argue in favor of the latter interpretation.

The possibility of evoking learned hippocampal responses during paradoxical sleep had not as yet been investigated. Our results show that a tone that has acquired significance during wakefulness elicits a reliable increase in hippocampal activity when presented during subsequent PS phases. This response is associative, since it did not occur when the tone had not been paired with the US—that is, before conditioning and after the pseudoconditioning procedure. It cannot be attributed to motor activity. The postural atony, characteristic of the PS state, was maintained throughout the PS phases in which tones were delivered. Moreover, no muscle twitches elicited by tone presentations were detected by polygraphic recording.

It could be argued that cellular changes observed in response to the tone during PS were caused by arousing stimuli presented to the sleeping animal; these changes would result from enhanced ability of the conditioned tone to arouse the animal from sleep relative to when the tone was neutral. It is well known that modifications of arousal influence cellular activity in most structures—especially in the hippocampus, whose relationships with ascending arousal systems are important (Lindsley & Wilson, 1975;

Winson & Abzug, 1977). Indeed, it has been demonstrated that awakening from sleep is a potent variable for the production of changes in dorsal hippocampal unit activity (Best & Best, 1976; Delacour, 1980, 1982; Mays & Best, 1975; Ranck, 1973; Suzuki & Smith, 1985). However, even if some of the hippocampal neurons were found to enhance their firing discharge upon awakening, most neurons exhibit marked reduction in firing when the animal is aroused from sleep. Moreover, arousal-dependent changes in unit responses are long in duration; they typically outlast the stimulus and can last several seconds (Delacour, 1980; Mays & Best, 1975). This contrasts with the short-lasting hippocampal responses observed in PS, which were restricted to the first 200 and even mostly the first 100 msec of tone. Finally, it is important to recall that, in the present experiment, ECoG and EMG activities were continually monitored, and that the few trials on which awakening was suspected were discarded from analysis. Taken together, these arguments affirm that the increase in stimulus-evoked responsiveness observed during PS is not a result of arousal from sleep.

When hippocampal responses obtained in PS are compared with those observed in waking, one striking feature of the changes in PS is their short duration; the increased firing during sleep appeared not to extend beyond the first 200 msec of tone, whereas it lasted throughout tone presentation during the waking state. It is relatively easy to account for this discrepancy if one accepts that the late, sustained portion of increase in hippocampal activity observed during wakefulness is a consequence of a motor response. So, reduced duration of hippocampal responses might be explained by absence of motor feedback during PS. It is tempting to think that the earliest increase, the only one present in PS, might reflect the cognitive component of hippocampal cell response—that is, the acquired CS-US association representation.

Another feature of hippocampal modifications in PS that deserves further mention is the low magnitude compared to that observed in the waking state. Several possibilities might account for these comparatively smaller responses.

First, the tone was never reinforced, which might have led to partial extinction of its predictive value. If so, one should expect a progressive decrease in the amplitude of hippocampal responses on successive test trials. But comparisons performed between the first and the second block of four trials of each session failed to reveal a significant difference. Thus, there is no evidence for an extinction process developing during PS.

Second, according to the conventional concept of state-dependent learning (Overton, 1964), information may be retrievable only under the same physiological state as that during acquisition. The changes in state that occur between sleep and waking may be expected to produce state-dependent deficits in the evocation of learned information. This type of hypothesis has already been proposed to account for the well-known difficulty in recalling dreams (Goodenough, 1978). It is also possible that the response

of hippocampal cells to the tone was more difficult to evoke during PS because of a state-dependent retrieval failure.

Third, reduction of hippocampal responses in PS could be attributed to alterations in incoming sensory inputs reaching the hippocampus. Intuitively, it seems obvious that the way in which the central nervous system handles sensory information must change between sleeping and waking. In fact, little is known about information processing capacities in the auditory system during sleep. Most investigators have addressed this question by studying the effects of sleep on potentials evoked by acoustic stimuli at various sites in the auditory pathway. Concerning PS, results are not clear-cut, and they are rather contradictory (Hall & Borbely, 1970; Herz, 1965; Molnar, Karmos, & Csepe, 1986; Wickelgren, 1968; Winters, Mori, Spooner, & Kado, 1967). Nevertheless, there is evidence that a high level of activity in the middle ear muscles exists during PS, with strongly enhanced contractions occurring during the phasic episodes (Dewson, Dement, & Simmons, 1965). Contractions of this muscle group cause reductions in microphonic potentials, neural cochlear responses, and central responses to clicks (Baust, Berlucchi, & Moruzzi, 1964; Berlucchi, Munson, & Rizzolatti, 1967). Thus, it is likely that auditory signals are attenuated during PS. In the present experiment, the tone was delivered at the same intensity during wakefulness and PS. If the stimulus value of tone is reduced during PS, this could explain why the tone is less effective in evoking a cellular response in PS than in wakefulness.

A last possibility is that the responsiveness *per se* of hippocampal neurons might be depressed during PS. There is no convincing evidence to support or to rule out this proposal. We have noted, in the present experiment, that spontaneous hippocampal firing rate did not differ between the waking and the PS states. But this observation does not rule out differences in neuronal excitability. Some data suggest that, contrary to SWS, the PS state provides conditions that allow for hippocampal functioning as in waking. For example, there are indications that the capacity for synaptic plasticity, as measured by induction of long-term potentiation in the perforant path-dentate gyrus system, is comparable during PS and the still-alert state, whereas it is depressed during SWS (Bramham & Srebro, 1989). Moreover, by analyzing field potentials evoked by stimulation of the perforant path, Winson and his co-workers (for review, see Winson, 1986) demonstrated that neuronal transmission through the hippocampal trisynaptic circuit is restricted during PS as it is during waking, whereas it is totally unrestricted during SWS. On the other hand, Hobson and Schmajuk (1988) suggested that the hippocampus could make different use of input signals in PS and wakefulness because it is aminergically deafferented during PS. The fundamental assumption of these authors is that the hippocampus is involved in processing stimulus relevancy and that

noradrenergic input to the hippocampus improves this processing. Learning about the relevancy of stimuli would depend on the level of locus coeruleus (LC) activation. In agreement with this proposal, Segal and Bloom (1976) found that experimental activation of LC in waking animals augments excitatory response in hippocampal unit activity to a behaviorally significant tone. It could be speculated that natural deactivation of LC that occurs during PS (Aston-Jones & Bloom, 1981; Jacobs, 1986) has opposite effects and is in part responsible for the attenuation of hippocampal responses to the tone observed during PS.

However that may be, the most important finding of the work reported here is that presentation of a stimulus that has acquired a predictive value during conditioning does induce a response from hippocampal cells during PS. The hippocampal response in PS was observed after each of the four conditioning sessions, which attests to its reliability. This result is consistent with our previous data showing that retention of a new memory can be enhanced by presenting, during postlearning PS, the stimulus used as the CS during conditioning (Hars et al., 1985). In both cases, when the stimulus given in PS was not behaviorally relevant, it had no effect either on hippocampal activity recorded during PS or on retention performance subsequently tested in waking.

Taken together, these results demonstrate first that an external stimulus can "penetrate" during PS, at least when it has acquired meaningfulness. But, if the brain in PS is able to discriminate a relevant stimulus from the whole set of inputs, this implies that external stimuli can be processed during PS even though the organism is behaviorally unresponsive. This idea is strongly supported by our recent demonstration (Maho, 1990) that learning can be achieved during PS even when an external stimulus is used as the CS. Pairing a tone with a stimulation of the central gray during PS was shown to induce a clear conditioned increase in hippocampal MUA response to the tone. Thus, an external stimulus can be detected and processed during PS.

Our results suggest further that access to a newly acquired memory is possible during postlearning PS. According to the widely accepted view that memories may be in either an active or an inactive state (Lewis, 1979), elsewhere we have developed arguments to support the assumption that PS constitutes a preferential time for a newly formed memory trace to enter into an active state (Hennevin & Hars, 1985; Hennevin et al., 1989). For example, in order to account for the improvement in retention induced by presentation of a cue stimulus (the CS) during postlearning PS, we reconsidered the hypothesis generally advanced to account for the facilitative effect of cuing treatment when it is given to awake subjects—that cuing enhances retrieval by reactivating memory. This reactivation could give rise to some reprocessing of the previously stored information that aids in maintenance of memory (for empirical support and thorough discussion,

see Spear, 1978). So we proposed that presentation of the cue stimulus during postlearning PS might have reactivated the new memory, thus enhancing its later retrieval. Within this framework, we speculate that the hippocampal cellular response observed in PS might be viewed as an index of this reactivation elicited by CS presentation.

In conclusion, the fact that learned hippocampal cellular response can be evoked by CS presentation during postlearning PS demonstrates that the significant value of the stimulus has been recognized during PS. We suggest that this response might reflect reactivation of the newly acquired information.

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(Manuscript received January 17, 1991;
revision accepted for publication May 10, 1991.)