ACTA NEUROBIOL. EXP. 1978, 38: 45-61

PROACTIVE AND RETROACTIVE EFFECTS OF HIPPOCAMPAL STIMULATION ON ACTIVE AVOIDANCE LEARNING, HIPPOCAMPAL EEG AND BRAIN ACETYLCHOLINESTERASE ACTIVITY IN CATS

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Abstract. The subject of this investigation were the effects of electrical stimulation of the hippocampus on the acquisition of active avoidance response (AAR) in a shuttle-box. The stimulation (200 μA, 50/s, negative rectangular pulses of 1.0 ms duration) was applied once for 10 s before or after each training session. It was found that the application of hippocampal stimulation before each session facilitated the acquisition of AAR; discontinuation of the stimulation after training did not cause a decrease of AAR performance. Application of the stimulation after each session inhibited learning in four out of six cats. However, the level of AAR performance increased rapidly in these cats after inversion of the trials–stimulation sequence. It was also found that the intensity of the somatic and vegetative symptoms evoked by stimulation (stupor, salivation, twitching of facial muscles, pupil dilatation, crying) increased gradually in successive experimental sessions, suggesting the development of the kindling effect in cats stimulated before each session. In cats stimulated after each session the intensity of these symptoms was greatly diminished as compared to sessions where the stimulation was not preceded by the avoidance training, or they did not

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appear at all. However, normal sensitivity to stimulation returned after several applications of hippocampal stimulation before each experimental session. Electroencephalographic studies showed that hippocampal stimulation with the use of the same parameters as those used during training evoked hippocampal afterdischarges lasting 5-60 s. No changes of aceltycholinesterase activity in different brain regions were found in consequence of such stimulation.

INTRODUCTION

In spite of a great number of experiments performed with the use of ablation or lesion techniques, the hippocampal participation in memory has not been proved definitely. In these circumstances the results obtained with the use of stimulation methods become to gain more interest. It has been shown that chemical (Metrazol) or electrical stimulation of the hippocampus may cause a significant facilitation of learning in many experimental situations and in different species (5, 6, 9, 17, 24, 28, 36, 37). The results obtained in our Laboratory also confirm a positive influence of electrical hippocampal stimulation on learning; we found that such stimulation applied simultaneously with conditioned stimulus (CS) could facilitate the acquisition of active avoidance response (AAR) in a shuttle-box in cats (15) and decrease the latency of AAR in an unidirectional avoidance apparatus (14). Some observations made during these experiments allow to suppose that the facilitatory influence of hippocampal stimulation may last for a comparatively long period of time (several minutes) after stimulation. Such proactive effects were observed in the case of weak as well as strong stimulation. It might be expected then that hippocampal stimulation applied before each experimental session could facilitate the learning of AAR. In the following experiments we attempted to study the influence of a single train of electrical pulses applied to the posterior part of the hippocampus in cats on the acquisition of AAR in a shuttle-box. In most previous studies the attention of authors was focused on retroactive effects of hippocampal stimulation. Therefore we decided to compare the influence of stimulation applied before each training session with the effect of an identical stimulation applied immediately after each session. In our previous work the most distinct proactive effect was observed after strong stimulation (200 μA, 50/s, negative rectangular pulses of 1.0 ms duration). Therefore, only such stimulation was used in the present experiments. Some additional investigations were undertaken in order to find out the possible changes of EEG and acetylcholinesterase (AChE) activity that might occur in the consequence of hippocampal stimulation. Such changes were expected on the basis of the

well known similarities between the effects of ECS and hippocampal stimulation on learning in some experimental situations (19, 26, 27), suggesting that a common mechanism is being involved in both cases. ECS is known to cause a long lasting increase of AChE activity in the brain (1, 7). There is some evidence suggesting that the effects of hippocampal stimulation may also be mediated through the cholinergic system (8, 9).

METHODS

Subjects. The experiment was conducted on 45 male cats weighing 2.5–3.5 kg. Conditioning studies were performed on 18 animals randomly divided into three groups. Group I ($n = 6$) was composed of cats stimulated before each experimental session. The cats of group II ($n = 6$) were stimulated after each session. Group III ($n = 6$) served as unoperated control. EEG recordings were performed before and after the conditioning experiment on cats of group I and II. Seven additional naive animals were used for electroencephalographic studies only. AChE activity was studied in 20 animals divided into three groups. Group A ($n = 8$) was composed of unoperated cats; group B ($n = 6$) served as an implanted control and group C ($n = 6$) as an implanted stimulated group.

Surgery and histology. A detailed description of the surgical technique can be found in previous works (15, 16). Two monopolar, stainless steel, teflon coated electrodes 120 μm in diam. and 0.5 mm bare tip length were implanted bilaterally into the posterior part of the hippocampus of cats of group I, II, B and C. The coordinates were as follows: A 2.0, L 10.0, H +4.0 according to Jasper and Ajmone-Marsan's atlas (20). The seven cats used only for EEG recordings had additional electrodes located in the dorsal and ventral part of the hippocampus, and stainless steel screws implanted epidurally above the temporoparietal cortex for recording the cortical EEG.

On completion of the experiment the operated animals (with the exception of animals used for the AChE estimations) were sacrificed, their brains removed from bones and fixed in 10% neutral formaline. Every fifth section from the place of electrode penetration was stained with cresyl violet (Nissl's method) for the purpose of histological verification of electrode placements.

Apparatus. A shuttle-box, the same as previously described (17) was used in the conditioning experiments. A 60 W light bulb located under the grid-floor of each part of the apparatus served as a source of CS. The grid-floor was connected to the source of AC electric current through an autotransformer and additionally, an isolating 1 : 1 transformer. Bila-

teral stimulation of the brain was carried out by means of a square wave stimulator and an isolation unit. Both stimulating electrodes were connected with the isolation unit output through 250 kΩ potentiometers allowing to set the current intensity passing through each electrode. The parameters of stimulation were checked with the aid of a dual beam oscilloscope.

EEG recordings were performed in a plywood box 120 ×60 × 60 cm placed in a shielded chamber. The records were made with the use of a 16-channel electroencephalograph (EEG-U-16, BIOFIZPRIBOR) with the time constant set on 0.3 s and the frequency cut-off on 50 Hz.

Conditioning procedure. All cats' were trained to avoid the illuminated part of the shuttle-box by crossing the bar during 10 s of CS (steady light) presentation. If the cat had not crossed the bar during 10 s, US (short bursts of AC) was applied. The conjoint action of US and CS was terminated immediately after the escape to the "safe" (dark) part of the shuttle-box occurred. The intensity of US was 0.5 mA higher than threshold intensity established individually for each cat before the begining of training. The value of current which evoked the first visible behavioral response to the shock (paw lifting, anxiety) was accepted as "threshold". Usually it was 0.5-0.8 mA. The daily experimental session was composed of 10 trials with 20–40 s intertrial interval. The percentage of CR, the latency of crossing and the number of intertrial responses (ITR) were measured. All animals were trained 6 days in a week.

The cats of group I were trained with the use of hippocampal stimulation applied before each experimental session. The electrical stimulation of the brain (ESB) consisted of a single 10 s train of negative rectangular pulses of 1.0 ms duration with frequency 50/s and intensity 200 μA passing through each electrode. ESB was applied immediately after placing the cat in the shuttle-box. After the disappearance of the behavioral symptoms evoked by ESB (usually it lasted 2–3 min) the training was started.

In group II the training began 2 min after the cat's entry into the shuttle-box. In this group ESB was applied at the end of each experimental session after the last trial had been performed. Immediately after stimulation the cat was removed from the training apparatus.

In group III (unoperated controls) the training was started 2 min after the entry, similarly to group II. After the last trial the animals were removed from the shuttle-box.

In group I and II the basic period of training lasted 15 days. Then a checking period followed in which the experimental procedure was changed. If the level of AAR performance during the last five sessions

of training was at least 90%, the procedure was as follows. Every experimental session was composed of two sets of 10 trials each. The first set of trials was followed by ESB and after 2 or 3 min the next set of trials was started. US was not used in these sessions. This procedure was continued during five consecutive experimental days. It enabled us to compare more closely the AAR performance in changed and unchanged stimulation conditions. In the cats which did not attain the required level of AAR performance during training the trials–stimulation sequence was reversed and five additional sessions of training were performed.

In group III the training consisted of 20 sessions without any change of experimental procedure.

EEG recordings. In the seven naive cats the EEG recordings were made 10 times in a period of 2 wk. One experimental session was composed of 3 to 5 min of continuous recording of spontaneous EEG preceding stimulation of the posterior part of the hippocampus. After stimulation the recording was performed until all electroencephalographic and behavioral changes had disappeared. Usually the hippocampus was stimulated only once during one session with the use of the same parameters as in the trained animals. In a some instances, however, the stimulation was repeated three or four times in order to check the changes of hippocampal sensitivity that might occur after the first stimulation. In cats of group I and II (trained animals) EEG recordings were made in the same way at the begining and at the and of the AAR training.

Investigations of the influence of ESB on acetylcholinesterase activity. After convalescence the animals used in this part of the experiment were placed in the shuttle-box. To the cats of group A immediately after the entry ESB was applied with the use of the same parameters as in the AAR training. Two minutes after ESB the animals were removed from the apparatus and decapitated with the use of a specially constructed guillotine. In the cats of B and C groups the procedure was the same, but ESB was not applied. Immediately after the decapitation the brains were removed from bones and sectioned on ice into six parts. Each part was carefully minced and 0.5 g samples were homogenized in a glass tissue homogenizer with the addition of cold (4°C) phosphate buffer (pH = 7.2). The homogenates were dilluted with the buffer so as to obtain concentration of 5 mg of brain tissue per milliliter. Samples of 1.0 ml in volume were incubated with 4 μM of acetylcholine chloride during 20 min at 37°C. The reaction was stopped by the addition of three-chloroacetic acid. The residual acetylcholine (ACh) was determined with the use of the hydroxylamine-$FeCl_3$ method of Hestrin (18). The

mean from three independent estimations of the same homogenate was accepted as the final result of each determination. The activity of AChE was expressed in μM of ACh hydrolyzed during 20 min by 5 mg of brain tissue.

RESULTS

Anatomical findings

The results of histological analysis performed on cats of group I and II (trained cats) are presented in Fig. 1. In all cases both electrodes have been found in the posterior part of the hippocampus though their placements were not always symmetrical. The differences in learning between cats found in group II cannot, however, be accounted for by the differences in the electrode placements. In cats of group B and C electrode placements were evaluated on the basis of visual scrutiny of isolated hippocampi.

Behavioral effects of hippocampal stimulation

In most instances an arrest reaction followed by stupor was observed after the begining of the stimulation. The symptoms outlasted the stimulation by several or even tens of seconds. They ended with a series of loud meows followed by the cat shaking itself, scratching the regions of postoperative scars and grooming activity. This behavior was frequently interrupted by an intensive exploration of the experimental situation. In all cats of group I and one animal of group II (cat 247) the intensity of the symptoms increased and their duration became longer in successive experimental sessions. At the end of training salivation, twitching of facial muscles and gnawing movements occurred in these cats. Finally in two of them full motor seizures were evoked by ESB. In all cats of group II except one the effects of ESB were much smaller, usually being limited to the arrest reaction. No increase of susceptibility to ESB was observed during the basic period of training. However, in four cats which have not attained the AAR criterion level and had the ESB applied before each experimental session, the symptoms gradually attained the same intensity as in the cats of group I.

AAR performance during basic period of training

The results of training are presented in Fig. 2. The statistically significant heterogeneity of group II ($P < 0.001$) allowed to exclude two animals (cats 180 and 247), from further calculations since the level of AAR attained by these cats was much higher than in the rest of this

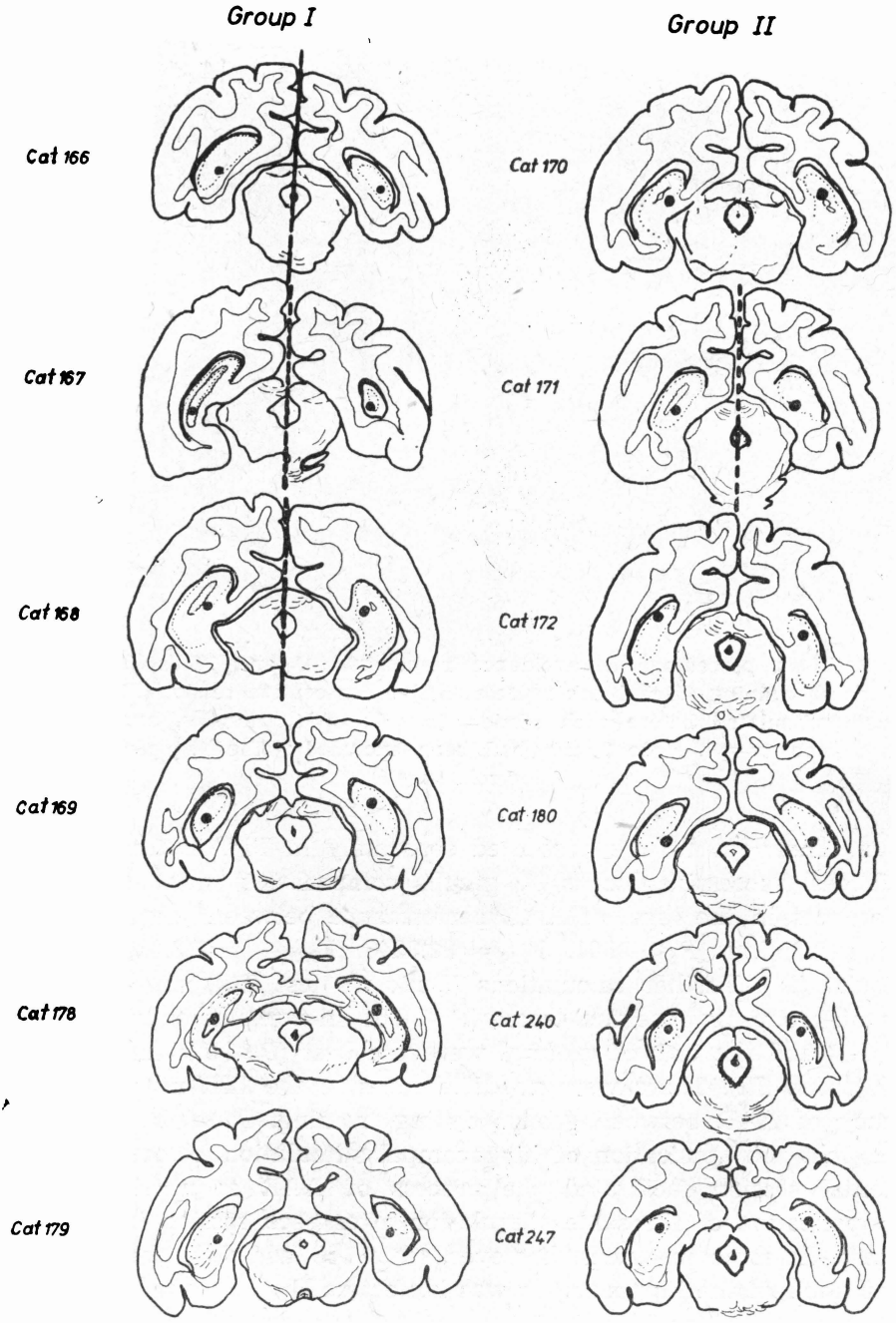

Fig. 1. Schematic drawings of frontal brain sections showing the location of uninsulated tips of stimulating electrodes (black dots) within the hippocampus in cats of group I and II.

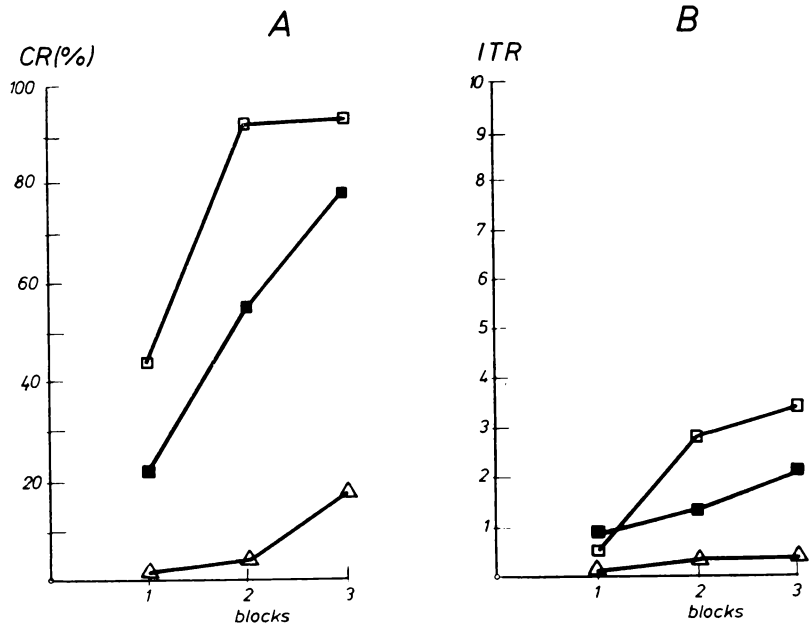

Fig. 2. Mean percentage of avoidance responses (*A*) and ITR's (*B*) during 15 sessions of training. Each block represents five consecutive sessions, Open squares, animals stimulated before each session ($n = 6$); open triangles, stimulated after each session ($n = 4$, since 2 cats had been excluded); filled squares, unoperated controls ($n = 6$).

group. Their results are presented separately in Fig. 3. An analysis of variances (successive blocks $\times$ groups comparisons) showed significant differences in the level of AAR performance between the three groups during training ($P < 0.001$, $F_{2,11} = 32.3248$, $F_{2,11,0.001} = 13.81$, Type I of Linquist (25). Further calculations (Duncan's test (33)) showed that the cats of group II were the worst ($P < 0.01$ in comparison with group I and III) and the cats of group I were the best ($P < 0.05$ in comparison with group III). There were no differences in the ITR number and the latency of AAR between groups during training. It might be assumed then, that the application of hippocampal stimulation before each experimental session facilitated the process of AAR acquisition, whereas the application of the same stimulation after each session inhibited this process. In some instances, however, (cats 180 and 247) the postsession stimulation of the hippocampus was inefective.

AAR performance after the change of stimulation procedure

A factorial analysis of variances was performed in order to evaluate the data obtained in this part of the experiment. The effects of the following factors were evaluated: A, the change of stimulation procedure

(five sessions preceding (A_1) and five sessions following the change (A_2) were taken into account)): B, days in succession; C, groups. (Note: in group I only the first sets of 10 trials from the sessions performed after the change of stimulation procedure were analyzed here). A significant interaction of A and C factors was found with respect to the level of AAR performance ($P < 0.001$, $F_{2,14} = 32.137$, $F_{2,14,0.001} = 11.78$). The same was found with respect to the ITR number ($P < 0.001$, $F_{2,14} = 20.935$).

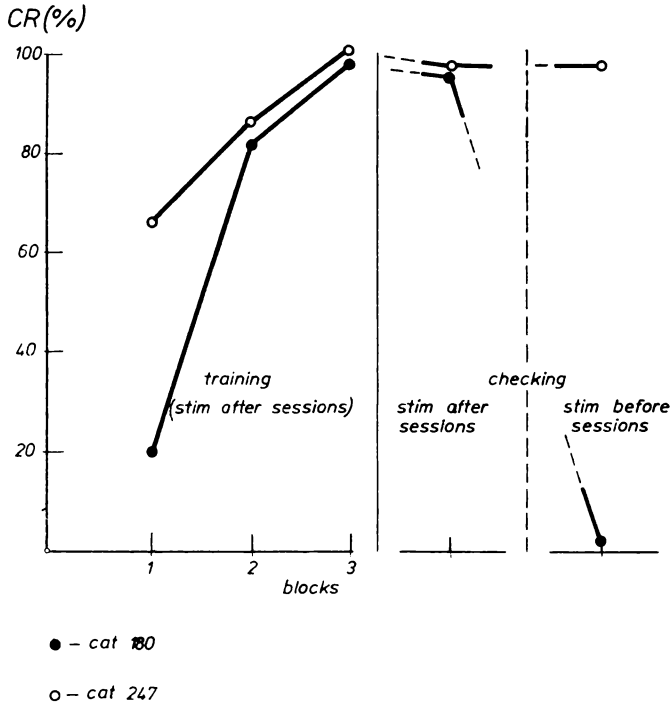

Fig. 3. The level of AAR during training and during the checking period in cats 180 and 247.

Further calculations in which the AAR performance before and after the change of stimulation procedure was compared in consecutive groups, revealed that in group II the level of AAR performance and ITR number after the change of stimulation procedure was significantly higher in comparison with the last five sessions of basic training ($P < 0.001$ and $P < 0.05$ respectively). In group I the level of AAR performance and ITR number in the first set of trials (before stimulation) as well as in the second set (after stimulation) did not differ from that observed at the end of the basic period of training (Table I). After the change of stimulation procedure the performance of group I and II did not differ from that of controls. In the two cats of group II, excluded from

TABLE I

The comparison of the AAR performance before and after the change of stimulation procedure

		AAR during the final phase of training (the last five sessions)			AAR after the change of stimulation-trials sequence (16th–20th session)		
		Stimulation procedure			Stimulation procedure		
		Stimulation before each session	Stimulation after each session	Without stimulation	Stimulation before each session	Stimulation after each session	Without stimulation
Group I	CR%	94.0 (80–100)			89.5 (60–100)	73.3 (30–100)	
$n = 6$	ITR	3.5 (1–8)			5.3 (0–36)	2.3 (0–8)	
Group II	CR%		11.7 (0–40)		76.0 (50–100)	—[a]	
$n = 4$	ITR		0.1 (0–3)		5.0 (0–18)	—[a]	
Group III	CR%			79.7 (20–100)			88.0 (60–100)
$n = 6$	ITR			(2,4) (0–6)			3.4 (0–9)

a, The animals did not attain 90% level of AAR performance during the basic period of training. Therefore. he conditioned response before stimulation was not checked (see text).

statistical evaluations due to their high level of AAR performance attained during the basic period of training, application of stimulation in the middle of each 20-trial session caused a complete inhibition of AAR in one (cat 180) and no change in the other (cat 247). Differences of AAR latency have not been found, probably due to a wide differentiation of this measure in each animal.

EEG recordings

An example of EEG record is shown in Fig. 4. In all cases studied (including the cats of group I and II) ESB evoked the characteristic hippocampal afterdischarges in the dorsal, posterior and, in most cases, in the ventral part of hippocampus. Fast, low amplitude activity was recorded from cortical leads during hippocampal afterdischarges. No abnormal changes were observed in the cortical EEG unless motor seizures were evoked. The hippocampal afterdischarges ended abruptly after 5–60 s (being shorter at the begining of the experiment and longer at the end) and were followed by a period of depressed activity. After a time (several tens of second) short bursts of theta-like waves appeared. Gradually,

they become more regular and after 1.5–5 min the record was practically undistinguishable from that before stimulation. The recovery time was dependent on the duration of afterdischarges; the longer the afterdischarges, the longer was the recovery time. The ventral part of the hippocampus showed the shortest time of recovery, the longest was observed in the posterior (stimulated) part. Next stimulation applied after full recovery of spontaneous EEG once again evoked afterdischarges of usu-

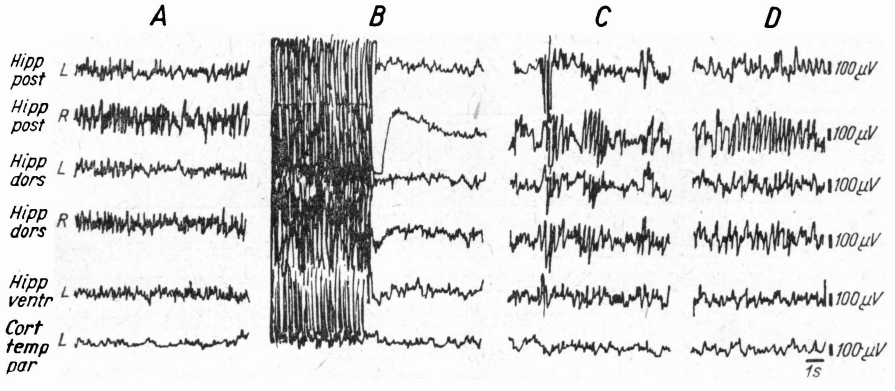

Fig. 4. A representative EEG record. *A*, before stimulation of the hippocampus; *B*, the end of hippocampal afterdischarges; *C*, 1.5 min after the end of afterdischarges; and *D*, 3 min after the and of afterdischarges.

ally shorter (but it was not a rule) duration than that observed after the first stimulation. Behavioral changes were identical as those observed during the conditioning experiments. During afterdischarges the animal stood motionless with eyes wide opened and pupils dilated. Immediately after the end of afterdischarges a series of loud meows (outcries) appeared. This symptom was observed each time when the afterdischarges were evoked. Therefore it could be regarded as a good index of their appearance. The period of grooming and exploratory activity was partially correlated with the gradual recovery of EEG. Some behavioral changes (heightened motor activity, enhanced pleasure reactions) however, could persist longer than the EEG changes. In all instances, when the stimulation was repeated, the duration of afterdischarges was prolonged. At the end of experiments full motor seizures were evoked in three cats. In these cases, after the end of afterdischarges the hippocampal EEG was completely flat for a period as long as 5 min. Full recovery was not seen even after one hour.

AChE activity after hippocampal stimulation

The results obtained are presented in Table II. No significant differences were found between the three groups of cats. There were stati-

TABLE II

AChE activity in different regions of cat brain expressed in μM of ACh hydrolyzed by 5 mg of brain tissue during 20 min

	Brain stem	Cerebellum	Diencephalon	Temporal cortex	Hippocampus	Remainings
Unoperated controls $n = 8$	1.244 (±0.06)	2.371 (±0.06)	1.144 (±0.10)	0.657 (±0.09)	0.805 (±0.04)	0.824 (±0.07)
Operated unstimulated $n = 6$	1.196 (±0.05)	2.580 (±0.07)	0.990 (±0.08)	0.758 (±0.08)	0.736 (±0.37)	1.210 (±0.13)
Operated stimulated $n = 6$	1.293 (±0.06)	2.410 (±0.06)	1.200 (±0.08)	0.738 (±0.07)	0.716 (±0.05)	0.963 (±0.04)

stically significant differences of AChE activity between different areas of the brain ($P < 0.001$, $F_{5,85} = 162.5006$; $F_{5,120,0.001} = 4.42$, Linquist. Type I design). The highest activity was found in the cerebellum and the lowest in the hippocampus and the temporal cortex.

DISCUSSION

The experiment has shown that a single application of a strong hippocampal stimulation before each experimental session may facilitate the AAR acquisition in a shuttle-box. An identical stimulation applied immediately after each session may inhibit this process. The facilitatory influence of hippocampal stimulation seems to be analogous to similar effects obtained in some experiments with the use of hippocampal stimulation applied simultaneously with CS (15), during intertrial intervals (9) or after training (37). The inhibition of AAR acquisition by stimulation applied after each session resembles probably the "amnesic" effects observed by some authors (4, 21–23, 26, 34, 40). It should be emphasized that both facilitatory and amnesic effects were obtained with the use of seizure as well subseizure hippocampal stimulation (22, 35). The behavioral symptoms observed in our experiment as well as the electroencephalographic investigations left no doubt that hippocampal stimulation applied before sessions evoked seizure discharges. Moreover, the gradual increase of the intensity of symptoms suggested that the kindling effect (11, 12) developed. Among the cats stimulated after each session, this effect appeared only in one animal. The scantiness of behavioral changes evoked by stimulation in the remaining cats of this group suggested that the afterdischarges lasted for a very short time or did not appear at all. This result seems to be analogous with the attenuation of effects evoked

by ECS (13, 31, 32) or electrical stimulation of some limbic structures (2) by application of antecendent footshock or an aversive brain stimulation. Thus, it appears that the multiple application of US in the cats of group II was the cause of decreased sensitivity of the hippocampus to electrical stimulation.

Apart from the facilitation of AAR acquisition it was expected that the application of hippocampal stimulation before each session would lead to state dependent learning similar to that produced by some pharmacological agents (30), and also by strong hippocampal stimulation applied simultaneously with CS (15). As a result of this, a decrease of AAR performance should appear in sessions not preceded by hippocampal stimulation. This effect was not observed. It is possible to assume that the lack of the state dependency was caused by a gradual decline of the changes evoked by stimulation during the time when the training trials were performed. Therefore only a part of them was performed during the changed state. In consequence it might protect a correct performance in sessions not preceded by stimulation. However, an alternative explanation may be proposed. It is very likely that some permanent functional changes produced by stimulation (suggested by the presence of kindling effect) were of the same character as those appearing immediately after stimulation. Their development could protect the high level of AAR performance in trials not preceded by stimulation. The fact that hippocampal stimulation applied repetitively during 10 daily sessions preceding the begining of training may also lead to a significant facilitation of learning in a shuttle-box (15) strongly supports the above supposition. The assumption that the development of kindling may be the cause of the attenuation of state dependency is also confirmed by the fact that among the cats of group II only the one animal in which the kindling effect was observed responded to the CS on the same level in both sets of trials (preceding and following the ESB) during the checking period.

The results obtained on the remaining animals of group II, however, might suggest the existence of state dependency. The rapid increase of AAR performance in the four cats which showed retardation of AAR acquisition during training suggested a facilitation of retrieval rather than learning. This result might be explained on the basis of some observations obtained with the use of ECS. It was found that the memory of a learned habit is undisturbed shortly after the treatment but is absent 24 h later (10, 41). Similar observations were obtained when hippocampal stimulation was used (4, 23). It has been hypothesized that the consolidation process is not disturbed by the treatment but the "memory trace" is formed on the background of a changed functional state of the brain.

Therefore, reinstitution of this state become necessary for retrieval (38, 39). Some experimental data supported this supposition (38, 39). It might be assumed then, that in the four cats from group II which did not learn during training the consolidation process and the training took place on the background of different states of the brain. Therefore, the information acquired could not be utilized until the changes accompanying the consolidation process were reinstituted by application of stimulation before training sessions. An alternative explanation of the retardation of AAR acquisition by posttraining hippocampal stimulation could be the supposition proposed by Berman and Kesner (4) suggesting that posttrial hippocampal stimulation disturbs long-term but not short-term memory. However, the results obtained on cat 180 contradict both the above explanations. In this animal the acquisition of AAR during training was not disturbed and ESB applied before trials during the checking period caused a complete inhibition of AAR.

The EEG data obtained in this experiment suggest that in most cases ESB caused hippocampal afterdischages. It might be presumed therefore, that the observed changes of AAR acquisition were somehow related with some functional changes of the hippocampus (and related structures) appearing in consequence of afterdischarges. For example, the facilitated acquisition of AAR in group I as well as the rapid increase of AAR performance in group II after the change of stimulation–training sequence, could be regarded as result of "desinhibition" due to temporary deactivation of the hippocampus. Facilitatory effects of hippocampal lesions on AAR acquisition and performance in rats are well known in literature (29). However, there are several reasons which do not allow to accept the above explanation. First, the time between ESB and the begining of training seemed to be sufficiently long for the recovery of hippocampal EEG at least in first sessions of training. Second, it has been found that hippocampal lesions do not facilitate AAR acquisition in cats (3, 15). And third, in our recent experiments (in preparation) we have found that an identical stimulation applied before experimental sessions can facilitate the AAR performance at the early phase of training (when the normal level of AAR is low) and inhibit it after the stabilization of this response. Moreover, both effects can be observed in the same animal. This results strongly opposes the "desinhibition" explanation and suggests that the changes which are crucial for the obtained results occur on elements directly involved in the formation of conditioned connections.

Our preliminary investigations failed to discover any changes of AChE activity in the brain regions studied which could be comparable to those obtained after ECS application (1). It is very likely that the changes

evoked by a single hippocampal stimulation were too small to be detectable or the time elapsing between ESB and the decapitation was too short. It is worth mentioning that in the studies of Adams et al. (1) performed with the use of ECS the AChE activity was determined after application of four ECS during 20 min. It is also possible that the evoked changes were masked by individual differences of AChE levels. Further investigations should explain this problem.

This investigation was supported by Project 10.4.1.01 of the Polish Academy of Sciences. We wish to thank Dr P. Jastreboff for the statistical evaluation of the results.

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Accepted 1 September 1977

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