SINGLE NEURONAL ACTIVITIES FROM CA_3 REGION OF HIPPOCAMPUS DURING CONDITIONING, IN MOBILE UNANAESTHETISED CONSCIOUS RABBITS

SUBHRA DATTA*

Department of Physiology,
University College of Medical Sciences,
Ring Road, New Delhi - 110 029

(Received on April 27, 1988)

Summary : Single neuronal activities of 93 units from CA_3 region of hippocampus were studied in unanaesthetised mobile rabits. Effects of repeated reinforced conditioned stimuli (CS+) were observed on these neuronal firing pattern. The conditioned stimuli (CS) consisted of a tone 600 Hz for 6 seconds which was reinforced by a subcutaneous electrical shock (0.4 V, frequency 250 sec, and pulse width 300 μsec) for one second duration (CS+). Ten such CS+ were applied at the gap of 5 min, in between. As majority of CA_3 neurons showed irregular spontaneous activities - the histograms drawn after calculation of interspike intervals showed a definite pattern of discharge which could be compared before, during and after multiple CS+. Two groups of neurons could be identified by their resting firing pattern. One group consisting of 21 neurons (22.5% approximately) showed complex spikes, with spike frequency < 2 to 8 per sec. They were complex spike cells (CSC). The other group consisting of majority of neurons (72 neurons, 77.5% approximately) showed comparatively high spike frequency > 8 to 40 per sec (θ cells). Both the group of neurons reacted tonically to CS+. All complex spike cells and 54% of θ cells showed inhibitory reaction and 46% of θ cells showed excitatory reaction to CS+. But with repeated presentation (4th to 5th) of CS+ the reaction gradually declined and finally after tenth CS+ it disappeared and resting firing pattern was observed. Thus it seems that the neurons of CA_3 region have an intrinsic habituation capability. The probable cause, mechanism and the significance for the habituation has been discussed here.

Key words : hippocampus CA_3 conditioning

INTRODUCTION

During the last forty years various workers have associated hippocampus with behaviour, conditioning, learning and memory. However, even a multidisciplinary approach (morphological. clinical, bio-chemical, behavioural and electrophysiological) failed to prove unequivocally the role of hippocampus in these complex functions (3,5,22,27,28). In 1974, Simonov described the hippocampus as "an organ of hesitation and doubt" (23). In hippocampectomised men and animals, long or short-term memory volume did not change.

Present address : Department of Physiology, University College of Medical Sciences and G. T. B. Hospital, Shahadara, Delhi - 110 032

But "the process of registration of new information" was deranged. The 'new' informations did not become 'old' and the habituation of the orienting reflex response to novelty was absent. So, new learning became difficult (28).

Thus "the process of registration of new information" could be taken at a cellular level of the hippocampus. Electrophysiologically, the neurons of hippocampus have been studied in both conscious as well as in anaesthetised animals of different species. Behavioural correlations to these electrophysiological studies by EEG (7,8,25) as well as single neuronal activities were done by various workers (9,10,17,19,24,27). Ranck *et al.*, observed two types of neurons electrophysiologically from different regions of hippocampus in mobile, conscious rats which were named as theta (θ) cells and complex spike cells (CSC) They also observed and interpreted various behavioural correlates of these neurons (21 22). But in conscious rabbits, Vinogradova *et al.*, only considered neurons which were comparable to θ cells in rats, but CSCs, if present, were branded abnormal and not considered in their studies (27,28). Also, in behavioural correlation to neuronal activities, one finds a lot of discrepancies in the observations as well as interpretations from different laboratories (7,8,11,20,22,25, 28).

In all the above mentioned experiments in conscious animals the number of single neuronal activities recorded from any particular region of hippocampus were comparatively few and the conditioning techniques were variable. The behaviour of the animals were also interpreted differently by these workers. Thus these wide range of discordance in the observations and interpretations found in the literature, could be attributed to the species difference, various techniques adopted and the bias of the workers.

In view of the existing controversial data available, it was interesting to study a large number of neurons from the CA_3 region of hippocampus, in order to solve the mysteries in "the process of registration of new information" and to add a new dimension in the understanding of conditioning, in conscious rabits, over the vast background knowledge already available.

CA_3 region (regio inferior) of hippocampus has been selected because this region receives "processed informations" from the various cortical association areas indirectly through the mossy fibres of the dentate granule cells (5,15,19,26,27). Biochemically also the neurons of CA_3 (pyramical cells) are known to increase their rate of protein synthesis after conditioning (13,14).

MATERIAL AND METHODS

Unanaesthetised adult rabbits (weighing between 1.5 to 2 *kg*) of either sexes were used for the present study. After injecting 3 *ml* of local anaesthetic (2.5% procaine hydrochloride) in the scalp, the skull was cleaned and a burr hole of 3 mm diameter was made 5 *mm* lateral and 3.5 *mm* posterior to bregma by a hand driven drill gently. A small nut was fixed by

dental cement, guiding the hole where the microdrive to hold the microelectrode could be placed later. Dura was removed carefully and sealed by one drop of agar agar 4% in gel form. One small screw was fixed in the occipital region to connect the indifferent ground electrode. One inverted screw was also placed near the frontal region to hold the operational amplifier from the input of the microelectrode. The nut and the two screws were kept in position by dental cement. Two fine needle stimulating electrodes were placed in the thigh subcutaneously. The animals were kept in a cage mobile and fairly comfortable.

A glass capillary microelectrode with external diameter 1.2 *mm* and tip diameter 1 μ, filled with 3 M NaCl solution was placed in the microdrive which was secured over the nut guiding the hole in the skull of the rabbit. The electrode tip was guided in the CA_3 region of dorsal hippocampus at the depth of 6 *mm* by manoeuvring the microdrive.

Single neuronal activities were picked up and amplified by a small operational amplifier and then displayed on the screen of cathode ray oscilloscope (CRO) through a Nihon Kohden amplifier. Audio monitoring of the neuronal activities was done throughout the experiment and simultaneous recording was done in a FM tape recorder.

The conditioning stimuli (CS) consisted of auditory stimuli for 6 seconds. which was given by an improvised audio source, an 'INCO' research stimulator with output connected to an 'Ahuja' audio-amplifier. Frequency of the sound was kept at 600 Hz per sec. At the end of 5 sec the CS was reinforced (CS+) by a subcutaneous mild electric shock of 0.4v, frequency 250/sec with each pulse width 300 μ sec for one second by an electronic stimulator (Nihon Kohden, SEN 3201). Each animal received ten such CS+, at the gap of minimum 5 min in between. The whole set up is shown diagrammatically in Fig. (1).

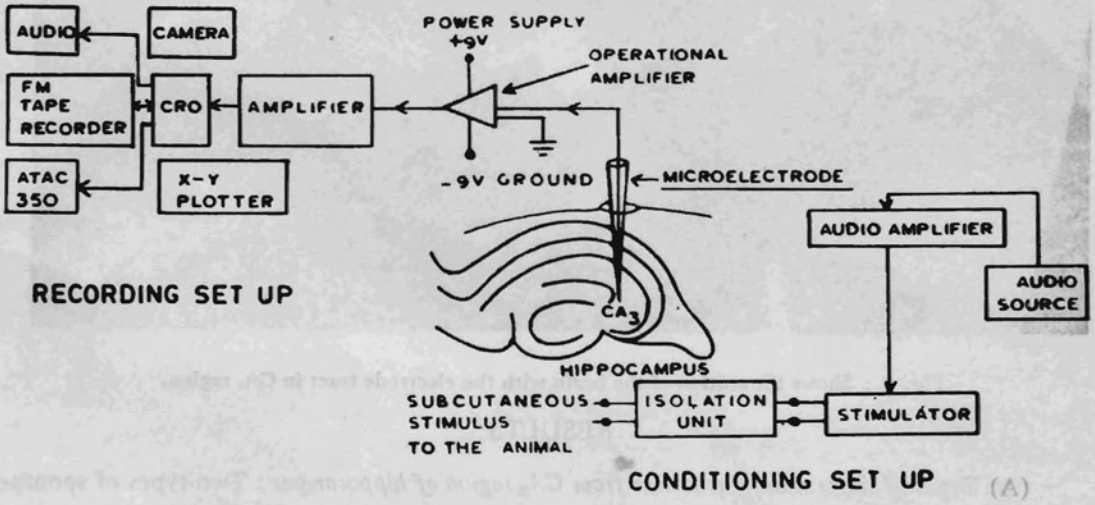

Fig. 1 : Diagrammatic representation of the EXPERIMENTAL SET UP.

The unit activities of the CA_3 region of hippocampus were recorded before, during and after each CS+. The spikes were then fed to a data processor, ATAC-350 Inter-spike intervals were calculated and histograms drawn to know the real pattern of discharge. A 'chi' square test was done to compare the result statistically in every series.

In some cases where the inter-spike intervals did not change much, frequency change were also calculated. In these series statistical analysis was done by S. D. and "Student's t" test was performed to know any significant change during and after conditioning. The behaviour of the animal was observed visually throughout the experiment. At the end of the experiments the electrode positions were confirmed by histology (Fig. 2).

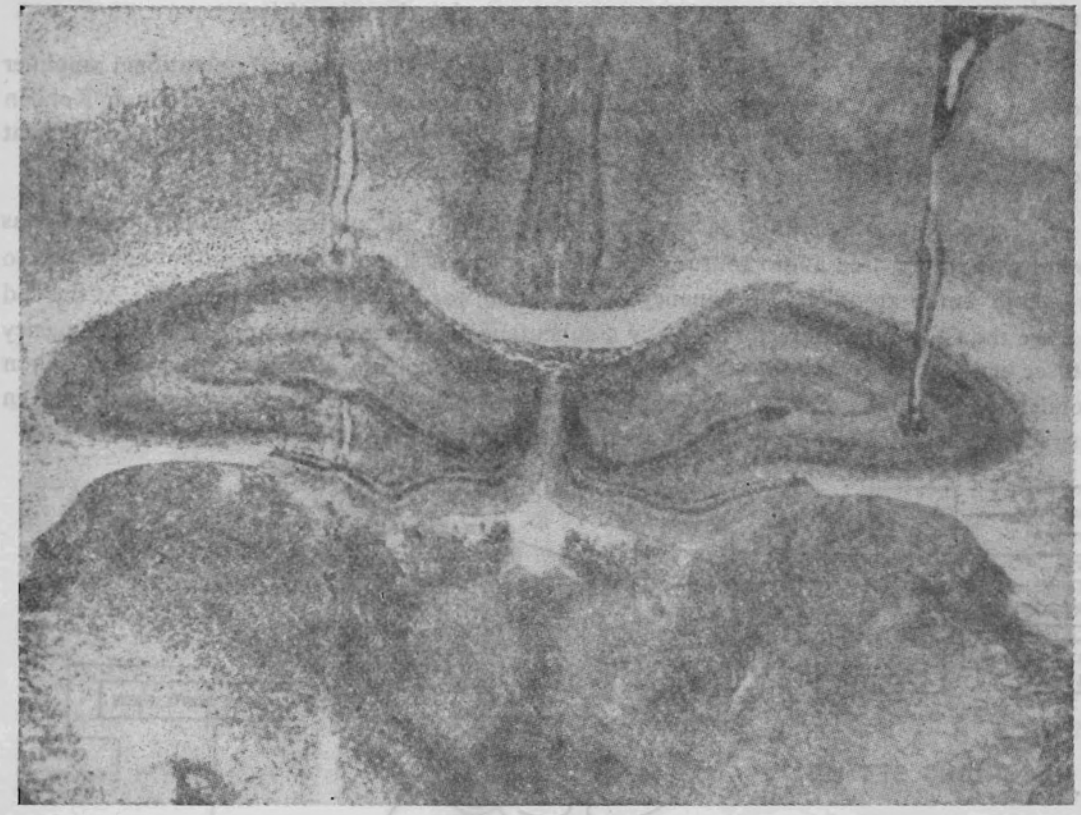

Fig. 2 : Shows the section of the brain with the electrode tract in CA_3 region.

RESULTS

(A) *Types of single neuron activities from CA_3 region of hippocampus :* Two types of spontaneous neuronal activities were observed from the 93 neurons of the CA_3 region of hippocampus.

(a) 72 (77.5%) neurons showed irregular spontaneous spike discharge with frequencies varying randomly between > 8 to 40 spikes per second. These cells were θ cells (Fig. 3 E).

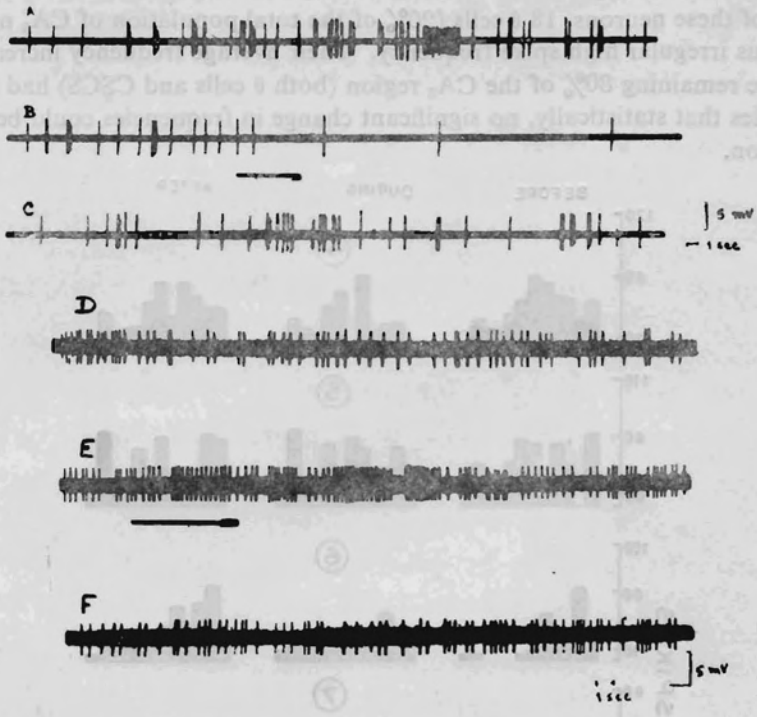

Fig. 3 : (A) Spontaneous spike discharge of a complex spike cell before CS+, (B) spike discharge during and after 3rd CS+, (C) spike discharge from the same neurons after 10th CS+, (D), (E) and (F) respectively show the θ cell discharge before, during and after 3rd CS+ and after 3rd CS+ and after 10th CS+, Two black horizontal lines marked under (B) and (E) show the points of CS+ applications (5 sec of tone, followed and accompanied by electric shock, at 6th sec.).

(b) 21 neurons 22.5%) occasional showed 2-4 complex spikes with 2-7 msec inter-spike intervals. These complex spikes firing was not a constant feature of these neurons. Each of these complex spike bursts, when present, were considered as a single action potential. These neurons were named complex spikes cells (CSCS). These neurons had irregular comparatively show firing rate < 2 to 8 spikes per sec (Fig. 3A).

(B) *Effects of reinforced conditioned stimuli (CS+) on the neurons of the CA_3 region of hippocampus :* All the 93 neurons studied from CA_3 region reacted to the initial 3-5 CS+ application, tonically, but their reaction varied :

(a) 39 neurons 54% of the θ cells) showed apparent decrease in the spike frequency. These neurons are 'inhibitory' in future.

(b) All the CSCS also showed inhibitory reaction (Fig. 3 B).

(c) 33 neurons (46% of the θ cells) showed increased rate of firing ('excitatory' neurons), out of these neurons, 18 θ cells (20% of the total population of CA_3 neurons studied) had spontaneous irregular high spike frequency. Their average frequency increased upto 100% (Fig. 3F). The remaining 80% of the CA_3 region (both θ cells and CSCS) had such irregular spike frequencies that statistically, no significant change in frequencies could be observed after CS+ application.

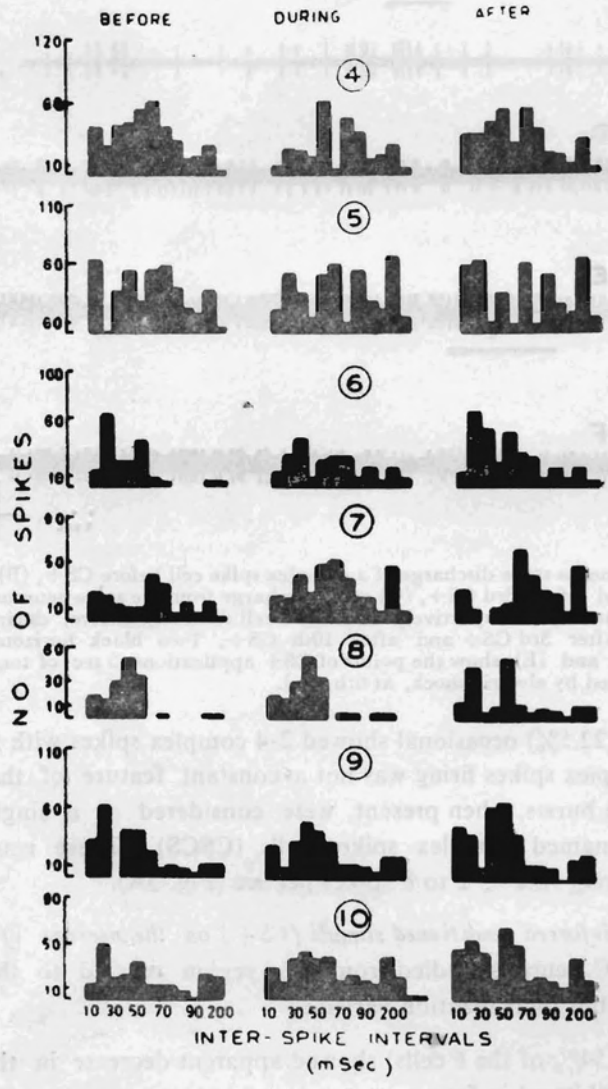

Fig. 4-A

(C) *Plotting the histograms with inter-spike intervals :* Histograms were plotted after analysing the spikes by the data processor, Atac-350. The abscissa showed the inter-spike intervals (in msec) and the ordinate indicated the number of spikes in msec. These number of spikes did not represent the frequencies of discharge of the neurons.

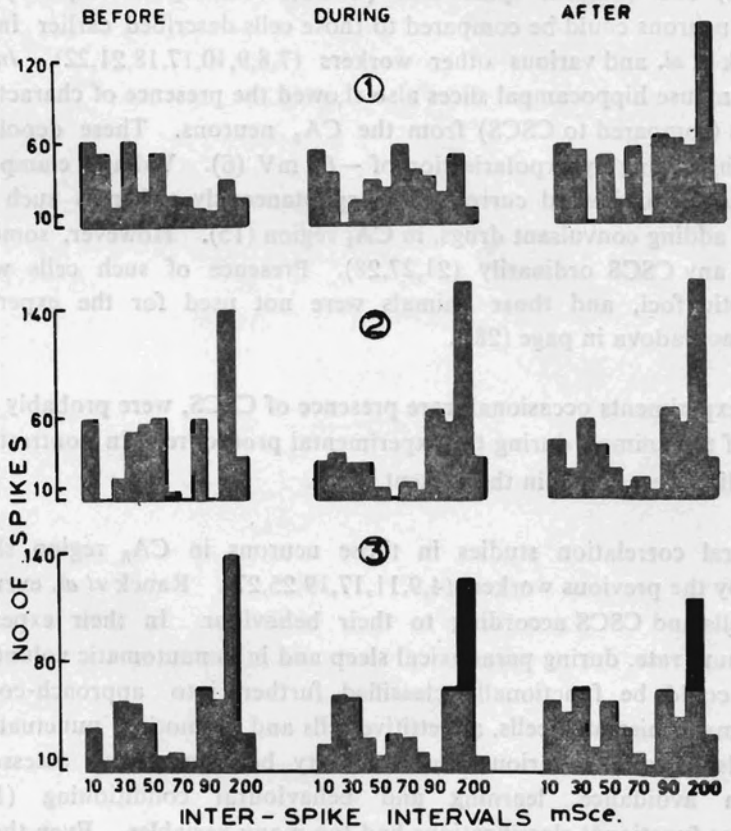

Fig. 4-B : Histograms show the number of spikes in the Y-axis and inter-spike intervals (in msec.) in the X-axis, before during, and after CS+. Figures in the circles above the histograms show the number of CS+ application. (Number of spikes do not represent the frequency).

Histograms plotted in this manner could be compared before, during and after CS+ applications (Figs. 4 A and 4 B). These histograms clearly showed the change of pattern of these inter-spike intervals, during and after initial three CS+ applications. Beyond that further exposure to CS+ application did not change the pattern and at the end of 10th CS+, it was quite similar to the pre-conditioning pattern. Statistical analysis by Chi square test (X^2) also confirmed the findings. The whole result is summarised in Table 1, Fig. 3 confirms the electrode position of this study.

DISCUSSION

In these experiments two types of neurons could be identified in the CA_3 region of hippocampus electrophysiologically in conscious, mobile rabbits. These two types of neurons are theta cells (θ) and complex spike cells (CSCS). The spike frequency and discharge pattern of these neurons could be compared to those cells described earlier in unanaesthetised rats by Ranck *et al.* and various other workers (7,8,9,10,17,18,21,22). *In vitro*, intracellular studies of mouse hippocampal slices also showed the presence of characteristic burst of action potentials (compared to CSCS) from the CA_3 neurons. These depolarisations were followed by a long lasting hyperpolarisation of −85 mV (6). Voltage clamp techniques in these neurons showed an inward current flow spontaneously, whereas such activities were only found after adding convulsant drugs, in CA_1 region (15). However, some workers, did not come across any CSCS ordinarily (21,27,28). Presence of such cells were considered abnormal epileptic foci, and those animals were not used for the experiments [during discussion of Vinogradova in page (28)].

In those experiments occasional, rare presence of CSCS, were probably due to restricted movements of the animals during the experimental procedure. In contrast, 22.5% of all the neurons studied were CSCS in the present series.

Behavioural correlation studies in those neurons in CA_3 region showed various interpretations by the previous workers (4,9,11,17,19,25,27). Ranck *et al.* even described the functions of θ cells and CSCS according to their behaviour. In their experiments θ cells fired at a maximum rate, during paradoxical sleep and in nonautomatic voluntary behaviour, whereas CSCS could be functionally classified further into approach-consumate cells, approach-consumate-mismatch cells, appettitive cells and a motion punctuate cells (21,22). Other workers also correlated various consummatory behaviours and assessed the role of hippocampus in avoidance, learning and behavioural conditioning (17,19,25,27,28). However, all these functional classifications had too many variables. Even the interpretation of a particular behaviour by different workers was different, which made the functional classification even more incomprehensible.

Precisely for these reasons, to study the behaviour of CA_3 neurons during conditioning a controlled stimulus model was selected (a tone of 600 Hz for 6 secs followed and accompanied by a subcutaneous shock of 0.4V, 2 C/sec with pulse width 300 μsec for one sec). This CS+ could be repeated many times without any conceivable variations.

Results of these conditioning studies showed clearly that all the neurons studied from the CA_3 region, reacted to CS+ tonically. 64.5% of them were 'inhibitory' type of neurons

and 35.5% were 'excitatory' in nature. Similar observations were made by earlier workers using their conditioning techniques (4,7,9,19,21,24,27,28). However, because the CA_3 hippocampal neurons showed irregular, random frequency change spontaneously, it was difficult to comment on any specific change of frequency by the applied stimulus. Only 20% of these neurons which showed resting high irregular spike discharge, increased their firing rate significantly after CS+ (P value=<.001), (Fig. 5). In these cases frequency analysis

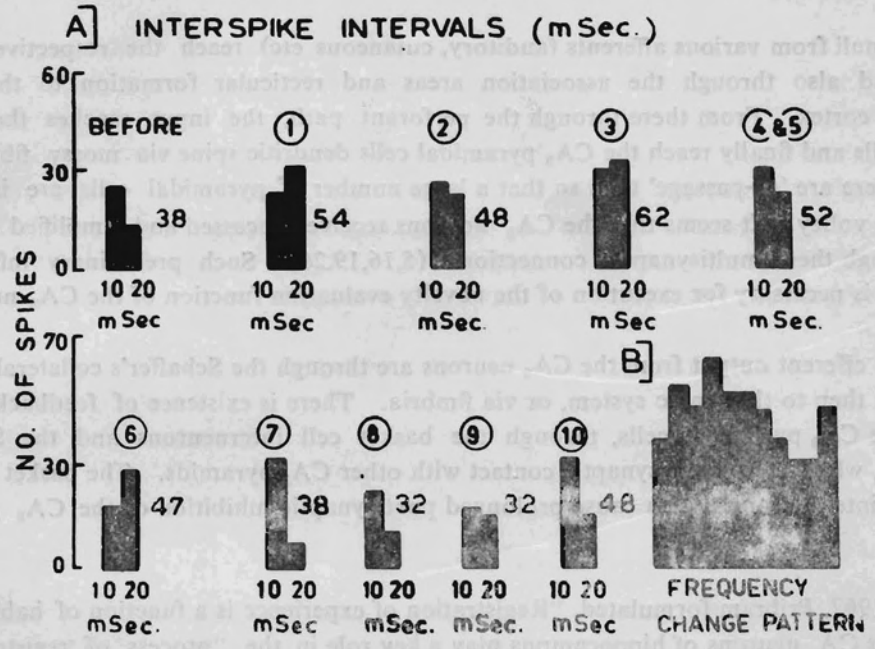

Fig. 5 : (A) Shows inter-spike intervals of the θ cells (with high spontaneous spike frequency) before and after CS+ applications. Number in the parenthesis over each of these histograms show the number of CS+ application.

(B) Combined frequency change pattern after each CS+ from the same neuron.

helped to interpret the data. In 80% of CA_3 neurons, plotting the histograms of the inter-spike intervals could represent the neuronal discharge pattern before, during and after conditioning, in a better way (Figs. 4A and 4B) Results analysed in this manner clearly depicted the habituation effect of the CA_3 neurons, after reported CS+. Initial three CS+ produced a significant change of pattern, by increasing the inter-spike intervals, in both θ and CSCS neurons (Fig. 4A). In 20% neurons described earlier did not show any significant pattern difference (Fig. 9A).

The most interesting observation was that after fourth CS+ the inter-spike interval pattern tended to come back to their original pattern (before CS+) and finally, during and at the end of the tenth CS+, the pattern was similar to the resting spontaneous level. So

the repeated application of CS+ somehow produced 'habituation' in the CA_3 neurons irrespective of their types, (θ cells or CSCS).

Earlier workers also observed a decreased reactivity of CA_3 neurons with repeated stimulation of dentate granules and reticular formation (1,2,3,5,12,27). The mechanism of habituation of these neurons could be explained by their afferent and efferent connections.

Stimuli from various afferents (auditory, cutaneous etc) reach the respective cortical regions and also through the association areas and recticular formation to the medial entorhinal cortex From there through the perforant path, the input reaches the dentate granule cells and finally reach the CA_3 pyramidal cells dendritic spine via mossy fibres. The synapses here are 'en-passage' type so that a large number of pyramidal cells are influenced by a single volley. It seems that the CA_3 neurons receive processed and simplified informations through these multisynaptic connections (5,16,19,26). Such preliminary information processing is necessary for execution of the novelty evaluation function of the CA_3 neurons.

The efferent output from the CA_3 neurons are through the Schaffer's collaterals to CA_1 region and then to the limbic system, or via fimbria. There is existence of feedback mechanism to the CA_3 pyramidal cells, through the basket cell interneurons and the Schaffer's collaterals, which also make synaptic contact with other CA_3 pyramids. The basket cells are inhibitory interneurons, which cause prolonged post-synaptic inhibition of the CA_3 pyramids (2,3,5,12).

In 1967, Pribram formulated, "Registration of experience is a function of habituation" (20). Thus CA_3 neurons of hippocampus play a key role in the "process of registration of new information" by their definite property of 'habituation'. It makes 'new' information 'old'. This dynamic process of 'habituation' to a stimulus pattern, modulates the CA_3 neuronal influences on the activating system, which regulates the general orientation response to the sensory system.

As mentioned earlier, hippocampectomies do not affect short or long term memory or even the conditioned reflexes developed earlier, but it makes 'new' learning considerably difficult, by affecting "the process of registration of new information". Ir the present series of experiments, it clearly shows that CA_3 neurons play this vital role of hippocampus.

ACKNOWLEDGEMENTS

I am indebted to Professor K. N. Sharma for his constant inspiration and valuable criticism on this work. I especially thank Mr. Sahu for helping me during the experiments.

TABLE I

Type of neurons	*No. of neurons*	*Amplitude*	*Before CS+ (Frequency and inter-spike intervals)*	*During and after three CS+ exposure (Frequency and inter-spike intervals)*	*During and after 4 to 9th CS+ (Frequency and inter-spike intervals)*	*During and after 10th CS+ (Frequency and inter-spike intervals)*
Theta Cell (θ) cells	72 (77.5%)	2.5-15 mV (SD± 3.212)	a) Highly irregular in 72% of θ cells. b) In 28% of θ cells frequency high (Range 20-40 spikes/sec average 32 spikes/sec) c) In 72% of θ cells inter-spike intervals pattern is comparable	A) 39 neurons (54% of θ cells) :— 1) Show ↓ frequency 'inhibitory' type 2) Inter-spike interval shows significant increase in no. of spikes with 200 msec. (X^2 test=37.53 at 1 d.f. interval p=<.001) B) 33 neurons (46% of θ cells) :— 1) Show ↑ frequency* 'excitatory' neurons. 2) In 18 neurons frequency upto 62 spikes/sec (P value<.005). Inter-spike, interval did not show any significant difference. 3) In 15 neurons the inter-spike interval pattern was similar to A.	A) No significant change in frequency* or inter-spike intervals. X^2 test=3.4 at 1 d. f. P<0.05 (not significant)	A) No significant change in frequency or in intervals. Pattern is comparable to the pre CS+ condition.
Complex Spike cells (CSCS)	21 (22.5%)	10-15 mV SD± 3.314	Irregular with occasional 2-4 complex spikes with 2-7 msec. interspike interval. Frequency 2 to 8 spikes/sec	1) Frequency* 2) Inter-spike intervals pattern showed increase no. of spikes with 200 msec. interval change (same as θ cells)	No significant change in frequency or inter-spike intervals. X^2 test=2.71 at 1 d.f. P<0.10	No significant change in frequency or inter-spike intervals. X^2 test=0.45 at 1 d.f. P>0.5

*Frequency change was apparent visually but as the neurons had irregular spontaneous frequency of spike discharge, frequency changes after CS+ were not significant statistically (P value>0.05).

REFERENCES

1. Alger, B. E. and R. A. Nicoll. Spontaneous inhibitory post synaptic potentials in the hippocampus : Mechanism for tonic inhibition. *Brain. Res.*, **200** : 195-200, 1980.

2. Andersen, P., J. C. Eccles and Y. Löyning. Pathway of post synaptic innibitory synapses on hippocampal pyramids. *J. Neurophysiol.*, **27** : 592-607, 1964.

3. Andersen, P. and T. Lömo. Mode of control of hippocampal pyramidal cell discharge. In : Neural control of behaviour edited by Whalen, R. W., R. F. Thompson, M. Veerzeano and N. M. Weinberger, Academy Press, New York, 1970, pp. 3-26.

4. Andersen, P., T. V. P. Bliss, K. K. Skrede. Unit analysis of hippcampal population spikes. *Exp. Brain. Res.*, **13** : 208-221, 1971.

5. Andersen, P. Organization of hippocampal neurons and their inter-connections. The Hippocampus, Vol. I structure and development, edited by Isaacson R. L. and Karl H. Prioram, Plenum Press, New York and London, pp. 155-175, 1975.

6. Biscoe, T. J. and M. R. Duchen. An intracellular study of dentate, CA_1 and CA_3 neurons in the mouse hippocampal slice. *Quar. J. of Exp. Physiol.*, **70** : 189-202, 1985.

7. Black, A. H., L. Nadel and J. O. Keefe. Hippocampal function in avoidance learning and punishment. *Psychol. Bull.*, **84** : 1107-1129, 1977.

8. Bland, B. H. and I. Q. Whishaw. Generations and topography of hippocampal theta in the anaesthetized and freely moving rat. *Brain Res.*, **118** : 259-280, 1976.

9. Feder, R. and J. B. Ranck Jr. Studies on single neurons in dorsal hippocampal formation and septum in unrestrained rats. Part II Hippocampal slow waves and theta cell firing during bar pressing and other behaviours. *Exp. Neurol.*, **41** : 532-555, 1973.

10. Fox, S. E. and J. B. Ranck Jr. Localization and anatomical identification of theta cells and complex spike cells in dorsal hippocampal formation of rats. *Exp. Neurol.*, **49** : 299-313, 1975.

11. Gralweicz, K. Facilitatory effects of electrical stimulation of the hippocampus on a one way active avoidance response in rats. *Acta. Neurobiol., (Warsz)* **43** (2) : 89-102, 1983.

12. Hass, H L. and G. Rose. Long term potentiation of excitatory synaptic transmission in the rat hippocampus : the role of inhibitory processess. *J. Physiol. (London.)*, **329** : 541-552, 1982.

13. Hyden, H. and P. W. Lange. Protein synthesis in the hippocampal pyramidal cells of rats during a behavioural test. *Science*, **159** : 1370-1373, 1968.

14. Hyden, H. and P. W. Lange. Brain cell protein synthesis specifically related to learning. *Proc. of the Nat. Acad. Sci. USA.*, **65** : 898-904, 1970.

15. Johnson, D., J. J. Hablitz and W. A. Wilson. Voltage camp discloses slow inward current in hippocampal burst firing neurons. *Nature*, **286** : 391-393, 1980.

16. Jones, E. G. and T. P. S. Powerll. An anatomical study coverging sensory pathways within the cerebral cortex of the money. *Brain*, **93** : 793-826, 1970.

17. O'Keefe, J. Place units in the hippocampus of the freely moving rat. *Exp. Neurol.*, **51** : 89-109, 1976.

18. O'Keefe and A. H. Black. Single unit and leison experiments on the sensory inputs to the hippocampal cognitive map. In : functions of the septo-hippocampal system. *CIBA Foundation Symp.* 58 (New series) pp. 179-198, 1978.

19. Olds, J. The behaviour of hippocampal neurons during conditions experiments. In : The Neural control of Behaviour" edited by R. E. Whalen *et al.*, New York Acad. Press, 257-293, 1970.

20. Pribram, K. H. Memory and the organization of attention. In : Brain functions and learning, edited by Lindsley, D. B. and A. A. Lunsdaine, pp. 79-333, 1967.

21. Ranck, J. B. (Jr.). Studies on single neurons in dorsal hippocampal formation and septum in unrestrained rats. *Exp. Neurol.*, **41** : 461-555, 1973.

22. Ranck James, B. (Jr.). Behavioural correlates and firing repartoures of neurons in the dorsal hippocampal formtions and septum of unrestrained rats. In : Hippocampus, Vol. 2 : Neurophysiology and Behaviour, edited by Robert L. Isacson and Karl N. Pribrahm, Plenum Press, New York and London, pp. 207-244, 1975.

23. Simonov, P. V. On the role of the hippocampus in the integrative activity of the brain. *Acta Neurobiol. Exp.*, **24** : 33-41, 1974.

24. Stafekhina, V. S. Characteristics of unit activity in hippocampal structure (the subiculum). *Neurosci. Behav. Physiol.* **12 (1)** : 37-43, 1982.

25. Vanderwolf, C H., R. Kramis, L. A. Cillespie and B. Bland. Hippocampal rhythemic slow activity and neocortical low-voltage fast activity : relations to behaviour. In : The Hippocampus, Vol. 2, Neurophysiology and Behaviour, Edited by R. L. Isacson and Karl, H. Pribrum, Plenum Press, pp. 101-128, 1975.

26. Van Horsen, H. and D. N Pandya and N. Butters. Some connections of the entorbinal (area 28) and perirhinal (area 35) cortics of the rhesus monkey. II. Frontal lobe afferents. *Brain Res.*, **95** : 25-38, 1975.

27. Vinogradova, O. S. Functional organization of the limbic system in the process of registration of information : facts and hypothesis : In : The Hippocampus, Vol. 2 : Neurophysiology and Behaviour, Edited by Robert L. Isaacson and Karl, H. Pribrum, New York and London, Plenum Press, pp. 3-69, 1975.

28. Vinogradova, O. S. and E. S. Brazhnik. Neuronol aspects of septohippocampal relations. In : Functions of the Septohippocampal system. *CIBA Foundation Symp.* 58 (New series) pp. 145-177, 1978.