Identification of pyramidal cells as the critical elements in hippocampal neuronal plasticity during learning

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ABSTRACT  The activity of single neurons recorded from rabbit hippocampus during classical conditioning of the nictitating membrane reflex was studied. All cells were first categorized according to their responses after fornix stimulation—i.e., antidromic activation, orthodromic activation, or no activation. The majority of cells that were antidromically activated—pyramidal cells—showed a highly positive correlation between the pattern of unit discharge and the topography of the nictitating membrane response within trial periods. Units that were orthodromically driven by fornix stimulation tended to inhibit during the presentation of trial stimuli, whereas most non-activated cells maintained low spontaneous levels of activity at all times. Thus, the major output neurons of the hippocampus appear to be the neuroanatomical substrate for the large and rapidly developing neuronal plasticity induced by this classical conditioning paradigm.

Identification of neuronal elements that exhibit learning-related changes in activity is a fundamental problem in analysis of neurobiological substrates of learning and memory in higher animals. Particular neurons or classes of neurons in the brain must be shown to exhibit specific changes dependent upon and predictive of the associative aspects of behavioral learning, in contrast to activity that is simply evoked by the sensory stimuli or related to behavioral movements. Putative mechanisms, synaptic or otherwise, cannot be characterized or analyzed until they have been localized. In this paper we report unequivocal identification of a class of neurons in the mammalian brain that show highly significant learning-dependent alterations in activity.

The mammalian hippocampus has been implicated in learning and memory in a wide variety of experimental and clinical conditions (1–4). However, the precise role of the hippocampus in learning and the particular hippocampal elements involved have not been clear. We have used a paradigm developed by Gormezano and associates (5)—classical conditioning of the nictitating membrane (NM) response in the rabbit—which permits characterization of neuronal changes that relate to the learning aspect of behavior. Recently, we reported that a large increase in unit activity (multiple unit recordings) develops in the hippocampus over the course of classical conditioning of the NM response (6, 7). These increases begin early in training and are predictive of subsequent behavioral learning. Increased unit activity does not develop in control animals given unpaired presentations of the stimuli.

A substantial increase in multiple unit activity recorded from a brain structure during learning indicates that a significant portion of the neurons participate. However, it is not possible to identify the types of neurons involved. The present study was undertaken to identify the particular class or classes of hippocampal neurons responsible for this large, learning-dependent increase in activity over the course of classical conditioning.

METHODS

Unit Recording. A total of eight New Zealand White rabbits were used. A week prior to training, a small, lightweight micromanipulator was implanted surgically (using halothane anesthesia) in the skull overlying the left hippocampus. At the same time, a bipolar, low-impedance stimulating electrode was permanently implanted in a region of the left fimbria–fornix, anterior to the most septal pole of the hippocampus. This area had earlier been determined to be an effective site for antidromic activation of hippocampal pyramidal cells. At the beginning of each training/recording session, the animal was placed in a restraining box inside a recording chamber, and a microelectrode (1–3 μm tip diameter; 500 KΩ to a MΩ impedance) was inserted in the micromanipulator. The electrode was advanced until a spontaneously active cell could be satisfactorily isolated by using the usual criteria (spike amplitude at least 3 times greater than background activity; amplitude and waveform constant; see Fig. 2) prior to any stimulation. Each cell was then physiologically identified by electrical stimulation of the fimbria–fornix with single 0.1-msec bipolar square-wave pulses. After this, the animal was given standard behavioral training (see below) for at least three blocks of eight paired trials or until the unit was lost. Training was then stopped, another unit was isolated, and the procedure was repeated.

An animal was given no more than 135 paired trials per day. Training was continued daily for as long as units could be recorded, the maximum being 7 days. All animals reached the behavioral criterion (7, 8) by the second day of training. Consequently, the great majority of units were recorded from animals that already had acquired significant learning. In few cases, larger multiple-unit microelectrodes were inserted to record from small populations of neurons, both to replicate the earlier findings (6, 7) and to provide direct comparisons with isolated single unit data here.

Behavioral Training. During all phases of training, animals were restrained and wore headgear mounted to a permanently implanted head-stage. The conditioned stimulus (CS) was a 1-KHz tone, calibrated at 85 db SPL. An air puff to the cornea served as the unconditioned stimulus (UCS) (6, 7).

During conditioning, animals were given up to 15 blocks of training per day, with eight CS-UCS paired trials and one CS-alone test trial constituting each block. The intertrial interval randomly varied from 50 to 70 sec, with an average of 60 sec. The tone-conditioned stimulus had a duration of 350 msec. The corneal air puff UCS occurred 250 msec after CS onset and had

Abbreviations: NM, nictitating membrane; CS, conditioned stimulus; UCS, unconditioned stimulus.

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shown early and associated multiple ramidal cell reported positive left from the lesions (100 be to the 750 the ratio of paired on Fig. correlation with positive Such responses-i.e., classes of responses were modulated and stored potential for the UCS-onset course. The standard pulse of the discriminator was analyzed on a PDP-12 computer system which counted the number of pulses in successive 3-msec time bins throughout individual trials. The counts were cumulated over the succession of eight paired-trial blocks for which a given unit was held. Data were collected for 250 msec prior to CS onset (preCS period), for the CS period (250 msec), and for 250 msec of the post-UCS-onset period (UCS period). Analysis consisted of the creation and subsequent statistical treatment of poststimulus histograms for the total 750-msec trial duration, using 3- or 15-msec time bins.

Prior to each training session, the arm of a minitorque potentiometer was connected to a nylon loop sutured to the NM during the initial surgical preparation. This allowed the exact amplitude-time course of each NM response to be recorded as a potential change across the potentiometer, which was FM-modulated and stored on magnetic tape. At the time of analysis, the NM recording was demodulated and collected through an analog-to-digital converter of the PDP-12. The digitized NM responses were stored singly and also averaged for the total number of trials over which a given unit was held, for comparison with neural unit poststimulus histograms.

The major purpose of this study was to determine which classes of neural elements acted as did the multiple unit measures—i.e., were predictive of the behavioral conditioned response. Such individual units would be expected to have a positive correlation with the form of the behavioral response (see Fig. 1). To identify such neurons, a correlation ratio (η) was computed on data collected and cumulated for the total number of paired trials over which the unit was recorded. The correlation ratio estimated the strength of the relationship between the amplitude-time course of the mean behavioral NM response and the number of unit discharges in each 3-msec epoch over the 750 msec of the cumulated trials. The ratio was required to be positive and statistically significant (P < 0.05) for the unit to be categorized as exhibiting the learned response.

Histological Procedures. At the completion of training, the animal was anesthetized, a microelectrode was inserted to a location where one of the units had been recorded, and electrolytic lesions (100 μA, 1 sec) were made through recording and stimulating electrodes. The animal was then perfused through the heart with 0.9% saline followed by 10% formalin. Verification of all electrode placements was made with enlarged photographs and standard stains of frozen sections of the brain in conjunction with a Prussian blue reaction for iron deposit left from lesioning through the electrode tip. For all animals reported here, electrode tips were in or very near the pyramidal cell layer of regions CA1 and CA3.

RESULTS

Multiple Unit Response. A characteristic example of a hippocampal multiple unit recording from the present series of experiments is given in Fig. 1. The averaged NM response and associated histogram of multiple unit neural activity are shown early in training, before any behavioral learning had occurred (Fig. 1A) and late in training the second day, after behavioral conditioning had developed (Fig. 1B) Note that there is an appreciable hippocampal unit response in the UCS period early in training (Fig. 1A). This response grows in magnitude over the course of training and moves into the CS period (see Fig. 1B), always preceding the behavioral NM response. Control animals given unpaired tone and air puff presentations showed no hippocampal (or behavioral) responses to tone. Although, of course, they give reflex responses to air puff, there is little and variable associated activity in the hippocampus (6–8). The data of Fig. 1 simply replicate the observations in earlier studies and provide a typical multiple unit response pattern for comparison with the single unit data given below.

Single Neuron Results: Responses to Fornix Stimulation.
To date we have studied a total of 36 units, 19 recorded from CA1 and 17 from CA3. In terms of response to single shock stimulation of the fornix, spontaneously active units fell into three classes: very short latency response (n = 20; range, 2.4–3.8 msec), longer latency response (n = 8; range 5.2–12.0 msec), and no response (n = 8; see Fig. 2 for examples). Units in the first class met the usual criteria for antidromic response: short latency, very low variability in latency, and ability to follow high-frequency stimulation (e.g., up to 100/sec). The last test was done only on a few of these units at the end of recording because of the possibility of potentiation effects. All units so tested followed up to 80–100/sec. Pyramidal cells are the only class of hippocampal neurons that send axons out the fimbria and through the fornix (9). Therefore, all cells demonstrating antidromic spikes after fornix stimulation were identified as pyramidal cells. Previous intracellular investigations of hippocampal physiology have demonstrated that fornix stimulation-induced antidromic spikes are often followed by a long lasting IPSP (10–13). Most cells identified as pyramidal in the present study also were inhibited after antidromic activation.

Fourteen of the total 20 antidromically activated cells were recorded from the CA3 pyramidal layer. The remainder were localized to CA1. Andersen et al. (14) have reported difficulty in activating CA1 cells antidromically with fimbria stimulation in the anesthetized preparation (urethane/chloralose). Although we found such activation possible in the unanesthetized rabbit, the majority of activated cells recorded from CA1 were orthodromically driven.

The second group of cells met the usual criteria for orthodromic activation, presumably either mono- or polysynaptic. Response latencies occurred in the appropriate range (5.2–12 msec), exhibited considerable variation, and, for the units tested, failed to follow stimulus frequencies above about 40–50/sec. It may be presumed that the shortest latency neurons were monosynaptically activated, but this is not a certainty. Virtually all (seven of eight) orthodromically driven units were recorded from the CA1 pyramidal layer.

Little can be said about the third class of units. They did not respond, even at relatively high stimulus intensities. Five were recorded from CA1 and three from CA3. Cells in this category tended to have very low spontaneous rates, sometimes showing interspike intervals as long as 60–120 sec. Such cells were monitored closely to ensure that successive spikes were generated by the same neuron.

**Single Neuron Results: Responses During Conditioning.**

Three major response patterns were found for single hippocampal cells recorded during NM conditioning. Each spike pattern correlated closely with one of the above categories of responses induced by fornix stimulation.

Most cells in category 1—identified as pyramidal cells—behaved as the multiple unit response shown in Fig. 1. Of the 20 neurons so identified, 15 showed statistically significant positive correlation ratios (P < 0.05) with the corresponding NM response. One other unit appeared to resemble these but did not reach statistical significance. Only two units showed inhibition, and the remaining two exhibited no change relative to spontaneous rates.

An example of a typical pyramidal cell is shown in Fig. 3, averaged for 13 blocks of trials. As indicated by the averaged behavioral NM response, the animal had achieved stable conditioned responding. The unit histogram indicates an increase in the activity of the unit that preceded the onset of the behavioral conditioned response. Furthermore, the temporal form of the unit histogram predicts surprisingly well the amplitude-time course of the averaged behavioral NM response. The correlation ratio between the NM and cell discharges was 0.78 for this cell, highly significant (P < 0.01). These same features are characteristic of multiple unit responses recorded from the hippocampus (Fig. 1; refs. 6–8).

Eight of the total 36 cells were orthodromically activated by fornix stimulation (category 2). Of these eight, only one cell revealed an excitatory response paralleling the NM curve. Five showed apparent inhibitory responses, and the two remaining neurons maintained spontaneous firing levels during trial periods. An example of the characteristic response pattern of a cell
in the category of orthodromic activation held for 10 blocks is shown in Fig. 4. Note the pronounced inhibition that develops shortly after onset of the tone CS and persists throughout most of the trial period. The correlation ratio computed between the NM and the unit activity for this cell was negative and highly significant ($\eta = -0.39, P < 0.01$). As noted above, five of the eight cells in this category showed this pattern. Although only two reached statistical significance, all five had negative correlation ratios.

Eight of the total 36 cells could not be activated in any manner by fornix stimulation. None of these cells showed patterns of activation similar to antidromically activated cells. Four nonactivated cells exhibited no change in spontaneous rates during paired trials; three neurons appeared to show a small inhibitory response. One cell showed a tone-evoked excitatory response which, of course, did not correspond to the NM amplitude–time curve. An example of the characteristic response pattern of a neuron in category 3 recorded for seven blocks—not activatable by fornix stimulation—is shown in Fig. 5. This cell shows no relationship to the behavioral NM response and is typical of four of the eight cells in this category.

A critical characteristic of multiple unit responses recorded in previous studies was the increase in discharge rate (relative to spontaneous counts) that occurred from onset of paired conditioning training. Almost all units reported in the present study were recorded after conditioned NM responding had reached asymptotic levels. This provided stable conditions under which unit correlations or noncorrelations with the NM response could be more easily identified.

To date we have been able to isolate and identify (as a pyramidal cell) one unit and hold it from the beginning of training until the animal was giving conditioned responses—i.e., over the course of learning (in this case, 45 trials). The results from this cell are shown in Fig. 6 for the total 40 paired trials the animal received. The growing pattern of unit response is clear. For the first four paired trials, counts in the CS and UCS periods remained at spontaneous firing rates. By the second four-trial block (paired trials 5–8, Fig. 6A), UCS period activity already clearly was increased over PreCS levels and it continued to grow with successive trials, to an average 300% increase over PreCS counts. CS period activity, on the other hand, differentiated more slowly from spontaneous discharge rates. Increased responsiveness to tone was not evident until the sixth or seventh four-trial block, about the time conditioned NM responding occurred for this animal.

FIG. 4. Data (see Fig. 3 legend) collected from an orthodromically activated hippocampal cell. Vertical calibration for lower histogram trace is equivalent to 21 counts per 15-msec time bin.

FIG. 5. Data (see Fig. 3 legend) collected from a hippocampal cell not activated by fornix stimulation. Vertical calibration for lower histogram trace is equivalent to 28 counts per 15-msec time bin.

FIG. 6. Mean number of unit spikes over the course of conditioning for a pyramidal cell recorded from the CA3 region of hippocampus. Data are averaged in successive four-trial blocks for paired conditioning trials. (A) UCS period (solid line) and PreCS period (broken line). (B) CS period (solid line) and PreCS period (broken line).
DISCUSSION

The major finding in this study is unambiguous. The neurons in areas CA1 and CA3 of the hippocampus that generate the learning-dependent multiple unit response highly correlated with the learned behavioral response are largely, if not entirely, pyramidal cells. Although our sample is small, it appears that at least 75% of recordable pyramidal cells exhibit this response.

In marked contrast, virtually no cells that can be activated only orthodromically show the learning-dependent increase in activity. The only cell in this category that did so could easily have been a pyramidal cell whose axon could not be activated by the fornix-stimulating electrode in this particular animal. Certainty exists only with the antidromic activation. However, most of these orthodromically activated cells did in fact show a significant relationship to the behavioral conditioned response, but it was a negative relationship—inhibition. The extent to which this inhibition develops as a result of training remains to be determined. It is tempting to speculate regarding the nature of these cells. If it is assumed that they are not pyramidal cells, they could be interneurons that are orthodromically activated by hippocampal afferent fibers in the fornix, either monosynaptically or polysynaptically. Alternatively, they could be interneurons activated by the fornix-stimulating electrode via recurrent collaterals of the antidromically activated hippocampal efferent fibers—i.e., basket cells, which are believed to be inhibitory to the pyramidal cells (11–13). In either case, the inhibition shown by these cells is of interest. If these cells are inhibitory interneurons, then some other mechanism or set of interneurons must exist that can inhibit these inhibitory interneurons during trial periods. These possibilities remain to be explored.

The third category of neurons show either no change in activity or inhibition. They may reflect two different sets of interneurons. As a group, they may be physiologically distinct from the first two categories. All these cells have two properties in common—they cannot be activated by fornix stimulation and they have very low spontaneous activity levels.

The one identified pyramidal cell that we have held over the course of learning showed a steady and consistent increase in activity over trial blocks (Fig. 6). From the beginning of training until conditioning developed, the unit increased its firing rate 148% in the combined CS plus UCS periods. This amount of increase and the time period over which it developed are at least consistent with the possibility that a process such as low-frequency, long-term potentiation could serve as the underlying mechanism (2, 7, 15–17). It could be that the specific patterns of overlapping conditioned and unconditioned stimulus activation of the hippocampal circuits establish conditions much like the brief trains of electrical stimuli that are used to induce long-term potentiation (7). This possibility is of course only speculation.

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