Brief Bursts of High-Frequency Stimulation Produce Two Types of Structural Change in Rat Hippocampus

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SUMMARY AND CONCLUSIONS

1. Electrophysiological and electron-microscopic techniques were used to investigate possible structural modifications associated with the induction of long-term synaptic potentiation in the hippocampal formation. Stimulation and recording were carried out using the Schaffer collateral-commissural projections from field CA3 to field CA1 of the rostral hippocampus of anesthetized rats.

2. In one group of animals repetitive stimulation was administered at a frequency of 100 s⁻¹ for 1 s (potentiated), while another group was stimulated at 0.2 s⁻¹ for 3 min (control). The first paradigm produced a marked increase in the strength of the postsynaptic potentials, which persisted without decrement for the 15-min periods of control testing, while the repetitive low-frequency activation had no detectable effects on synaptic transmission.

3. Following testing, the rats were perfused and prepared for electron microscopy. The dendritic zone adjacent to the recording microelectrode tip was found and measurements made of the numbers of synapses as well as of the area and length of various constituents of the neuropil.

4. The number of synaptic contacts on dendritic spines was not different between the two groups, but the incidence of synapses onto dendritic shafts was 33% higher in the animals that received high-frequency stimulation (potentiated group).

5. No statistically significant changes were observed in the mean size of any of the following measures: 1) area of synaptic boutons contacting dendritic spines, 2) area of dendritic spines, 3) width of dendritic spine stalks, 4) length of postsynaptic densities (PSDs) on dendritic spines, 5) area of synaptic boutons contacting dendritic shafts, 6) length of PSDs on dendritic shafts.

6. However, there were distinct changes in the within-animal variance and distribution of the dendritic spine measures. Specifically, in the potentiated group there was a reduction in the coefficient of variation in 1) the area of dendritic spines, 2) the length of PSDs on dendritic spines, and 3) the width of spine stalks. The extent to which the within-animal distributions of each of these measures were positively skewed was also reduced in the potentiated group.

7. It appears then that brief bursts of high-frequency stimulation produce two very different types of structural change: 1) an apparent increase in the number of shaft synapses, and 2) a decrease in the variability of the dendritic spines. The possible relationship of these morphological effects to long-term potentiation of synaptic responses is discussed.

INTRODUCTION

Long-term potentiation (LTP) is an unusual form of synaptic facilitation that occurs in any of several hippocampal afferent systems following brief trains of high-frequency repetitive stimulation (e.g., Ref. 5). It is distinguished from other forms of synaptic facilitation by its extreme duration—once induced, the LTP effect is quite stable and persists for days or even weeks (4, 6). Neurophysiological studies have, by a process of exclusion, indicated that the changes respon-
sible for LTP are to be found in some constituent of the synaptic complex. Three lines of evidence suggest that the repetitively activated axons are not significantly changed by the brief, high-frequency stimulation trains used to produce the effect: 1) The antidromic spikes produced by single-pulse stimulation are not increased (17). 2) The presynaptic potentials are not detectably changed after the induction of LTP (3, 5). 3) Repetitive stimulation in the absence of synaptic transmission (e.g., with low calcium, high magnesium) does not result in the appearance of LTP when normal medium conditions are restored (9).

It also appears that the target dendrites do not undergo generalized changes with the appearance of long-term potentiation. This was shown in experiments in which a high-frequency train was delivered to one of two populations of fibers terminating in the same dendritic zones under these conditions only the repetitively stimulated input exhibited potentiation and the responses to the second afferent were not increased (8, 13). Thus it seems likely that LTP involves a change in terminals, synapses, or spines of the “potentiated” pathway (see Ref. 14 for a review).

The extreme duration of LTP suggests that it may be due to structural changes, presumably in one or more of the above-mentioned constituents of the synapse. Van Harreveld and Fifkova (18) have reported that extended stimulation of the entorhinal cortex caused a swelling of dendritic spines in the middle molecular layer of the dentate gyrus. These studies did not make use of physiological recording and it is not known if long-term potentiation was actually induced in the regions selected for analysis. Beyond this, there is a possibility that the lengthy trains of stimulation may have caused profound physiological disturbances (e.g., seizures) and that these were responsible for the observed effects. In a recent experiment using the in vitro hippocampal slice method, we found that the induction of long-term potentiation by brief trains of high-frequency stimulation delivered to the Schaffer collateral-commissural projections to the region superior was accompanied by an increase in the number of synaptic contacts on the shafts of dendrites (11). However, while the percentage increase in this category of synapses was considerable (50%), the shaft synapses themselves are only a very small part of the total synaptic population; thus there is reason to question whether the shaft synapse effect could be responsible for the robust long-term potentiation effect.

In the present studies we have used acute anesthetized rats to reinvestigate the possibility that the induction of long-term potentiation is accompanied by changes in the numbers of various types of synapses and/or in the size and configuration of dendritic spines. The results indicate first, that short bursts of high-frequency stimulation produce a selective increase in the incidence of “shaft” synapses and second, that such stimulation alters the shape of the dendritic spines and their postsynaptic densities.

METHODS

Neurophysiology

Experiments were conducted on adult male Sprague-Dawley rats weighing 190–300 g. Animals were anesthetized using sodium pentobarbital (Nembutal, 50 mg/ml, 0.1 ml/100 g body wt), given 0.15 ml atropine methyl nitrate, and placed in a stereotaxic apparatus. Further anesthesia, 0.06 ml chloral hydrate (350 mg/ml), was given as needed. A glass micropipette recording electrode, filled with 2 M NaCl (2- to 6-μm tip diameter; 1- to 5-MΩ resistance), was placed into the CA1 pyramidal cell layer of the dorsal hippocampus in the region where complex spike cell discharges could be observed. A bipolar stimulating electrode (twisted 120-μm nichrome wire) was then slowly lowered into the stratum radiatum near the junction of CA2 and CA3. This placement lies in the trajectory of the Schaffer collateral and commissural axons, which synapse on the apical dendrites of CA1 pyramidal cells. As the stimulating electrode was lowered, test pulses were presented periodically until the characteristic two-component wave indicative of the CA1 population EPSP and population spike was observed (1, 2). The recording electrode was then lowered approximately 100 μm into the apical dendritic region while test stimuli were continuously presented (0.2 s⁻¹). Both the sharp reversal point and the negative monophasic potential of the CA1 apical dendrites were used as indicators of the Schaffer collateral/commissural-induced CA1 response (1). Following this verification, the recording electrode was returned to the CA1 pyramidal cell body layer.
and the stimulating electrode was shifted slightly along the dorsal/ventral axis in order to maximize the amplitude of the evoked response. The intensity of stimulation voltage was adjusted to produce a small, negative-going deflection ("population spike") in the monophasic positive cell body response (see Fig. 1).

Base-line responses were tested for stability by giving three stimulation pulses (0.2 s⁻¹) at 1-min intervals for 10–15 min. If the evoked responses were stable across the test period, then repetitive stimulation was administered. This consisted of three widely spaced trains of either high- or low-frequency stimulation. In the high-frequency (potentiated) group, the first 1-s burst of 100 s⁻¹ pulses produced a marked potentiation in the amplitude of the evoked population spike response (Fig. 1B). If this potentiation was found to be nondecremental over the 10- to 15-min test period, two additional periods of high-frequency stimulation were delivered, each followed by a 10- to 15-min period of test stimulation. Multiple stimulation periods were employed because, as previously reported (5, 6), the second and third trains usually resulted in further, though lesser, potentiation of the amplitude of the population spike. After the final 10- to 15-min test period, which followed the third stimulation train, stimulating and recording electrodes were withdrawn and the rats were coded and then given to a second experimenter who carried out perfusion-fixation for electron microscopy.

In the low-frequency-stimulated animals (control group), 0.2 s⁻¹ stimulation was administered for 3 min following the testing of base-line responses. Responses were then tested for 10–15 min, as in the potentiated group. It was found that the 0.2 s⁻¹ stimulation produced little or no change in the amplitude of the population spike (Fig. 1A). A second and third train of low-frequency stimulation were then delivered with an intervening 10- to 15-min period of response testing. While the amplitude of the evoked responses fluctuated slightly in some animals, in 13 of 15 rats these changes were within the range of base-line responses observed over 30–40 min in several animals that received only test stimulation.

In both the control and potentiated groups, only a single electrode penetration was used for a given animal and if appropriate physiology was not obtained during this penetration, the experiment was terminated. There were two reasons for utilizing a single electrode "drop." First, it was of considerable importance to maximize the likelihood that the tissue sampled for electron-microscopic analysis contained synapses whose axons had been activated by the stimulation electrode. With a single penetration, the recording electrode tract could be unequivocally identified and the adjacent area could be sampled. Second, single electrode penetrations minimized the damage to local cellular and vascular components in the hippocampus (see DISCUSSION).

The elapsed time for the electrophysiological

![FIG. 1. Traces of evoked potentials recorded from the stratum pyramidale of CA1 in response to stimulation of the combined Schaffer collateral and commissural pathways. In A, traces were taken prior to and following a train of low-frequency stimulation (0.2/s for 3 min). In this case traces were virtually identical, illustrating that the low-frequency stimulation yielded no effect on subsequent evoked potentials. In B, responses were taken before (pre) and after (post) the delivery of a high-frequency stimulation train (100/s for 1 s). As can be seen the evoked potential was sizably enhanced, resulting in an increased slope in the initial positivity and the amplitude of a population spike.](image-url)
phase of the experiments (i.e., from the administration of anesthesia to perfusion of the animal) averaged 2.0 h. Single day stimulation and recording sessions usually involved groups of three or five rats, with the control animal(s) selected by coin flip. We had anticipated that a substantial number of rats would "seize" or fail to show the nondecremental LTP effect (see Ref. 5), and consequently designed the study to include two "high-frequency-stimulation" rats for each control. In fact, the great majority (24 of 26) of the rats receiving the high-frequency trains did exhibit stable potentiation over the test period and, hence, the potentiated group was larger than the control group.

**Electron microscopy**

Rats were perfused intracardially with the following buffered solutions: 1) rinse—0.06 M sodium phosphate (pH 7.35); 2) concentrated fixative—7% paraformaldehyde, 9% glutaraldehyde in 0.06 M sodium phosphate buffer; 3) diluted fixative—2.3% paraformaldehyde, 3% glutaraldehyde, in 0.06 M phosphate buffer. The flow rate of the perfusion solutions was approximately 60 ml/min preinsertion. Concentrated fixative was introduced only briefly (1–2 min). After this initial fixation, diluted fixative was perfused for approximately 15 min. The brain was then removed and placed in dilute fixative overnight.

Thick sections, 120 μm in width, were cut in a coronal plane through the rostral hippocampus and were inspected with a dissecting microscope to localize the recording electrode tract. In most cases, this tract was clearly evident in only one thick section. The section containing the electrode tract was subsequently stained with osmium tetroxide and embedded in Epon-Araldite. In a few rats the recording electrode tract could not be located, and these animals were discarded from the study.

Ultrathin sections were cut through the CA1 region of the hippocampus at a point approximately 50 μm lateral (i.e., toward the CA3 region) to the recording electrode tract. These were stained in 2% uranyl acetate and lead citrate, then mounted on 1 x 2 mm (Formvar coated) slot grids. In a few cases, serial sections were taken from this zone for qualitative examination. Ultrathin sections were viewed on either a Zeiss 9S2 or JEOL JEM 100C electron microscope. Three animals were rejected at this point on the basis of poor fixation or edema (one in the control group and two in the potentiated group).

Sections with satisfactory fixation were photographed at an initial magnification of 6,500× (on the Zeiss) or 6,600× (on the JEOL). Electron micrographs were taken across the width of each section in a region located one-third of the distance from the pyramidal cell body layer to the hippocampal fissure, in the apical dendritic zone (stratum radiatum). In order to standardize magnification factors, carbon grating replicas (54,000 lines per inch) were photographed with each specimen. Electron micrographs of these grids, coupled with measurements of the print size, were employed to normalize the area analyzed for each animal. Approximately 2,000 μm² (ca. 40 micrographs) were photographed from each animal, and in every case, all of the synapses and synaptic boutons were counted and classified. The criteria for the identification of a synapse were 1) a recognizable postsynaptic specialization on a dendrite (either on a shaft or a spine), and 2) a presynaptic element with at least three vesicles proximal to the specialized contact zone. Dendritic shaft synapses were identified according to strict criteria to avoid possible confusion with large spine processes. Specifically, to be included in the shaft category, a postsynaptic element: 1) could not possess any protrusion along the postsynaptic membrane adjacent to the synaptic apposition; and 2) microtubules, oriented parallel to the PSD, had to be seen in proximity to the synaptic specialization. While these criteria were probably sufficient to prevent the inclusion of any spine synapses in the shaft category, it is possible that a small number of shaft synapses were included in the spine synapse category as a result of section angle.

Synaptic boutons were classified according to the type of postsynaptic element they contacted as well as the number of synaptic sites with which they were associated (Fig. 2). Those synaptic boutons in contact with more than one postsynaptic density were further grouped according to whether or not the multiple synaptic sites were located on the same ("doublet" or "perforated" synapses) or different postsynaptic elements (multiple synaptic bouton). Axoaxonal synapses were only rarely encountered and were not counted.

Of the approximately 40 electron micrographs that were analyzed for synapse and bouton counts, 10 micrographs with synaptic counts closest to the mean synaptic count of the 40 total micrographs were selected from each animal for more detailed analysis. For these micrographs the following measurements were taken: 1) the length of postsynaptic densities (PSDs), 2) the area of synaptic boutons, and 3) the area of dendritic spines (Fig. 3). Area measurements were taken with a digital planimeter and were converted into square micrometers (μm²). Both PSD length and synaptic bouton area were further classified with respect to the type of postsynaptic element with which they were associated.

**Sampling procedures**

Each animal was coded following the electrophysiological phase of the study, and during sub-
sequent phases of analysis the treatment group to which the animal belonged was unknown to the experimenters. As a further control against experimenter bias, the set of micrographs from one animal was shuffled together with at least one other set into a stack of micrographs such that minimally two and usually three animals were analyzed at the same time. Finally, each stack of micro-

FIG. 2. An electron micrograph showing types of synaptic interactions that are observed in the stratum radiatum of CA1. s, synaptic boutons contacting a dendritic shaft; m, synaptic bouton synapsing with two different postsynaptic spines; and p, bouton apposing a spine on which the PSD is perforated.
FIG. 3. An electron micrograph of a dendrite with an attached dendritic spine and spine stalk. An asymmetric synapse can be observed on the dendritic spine, apposed by a presynaptic element containing spherical vesicles. In the accompanying line drawing the manner in which measures were taken for PSD length, 1, spine area, 2, crossed-hatched; and spine neck width, 3, are depicted. The area of presynaptic elements for both spine synapses and shaft synapses and the length of PSDs on dendritic shafts were also measured but are not shown in this illustration.
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The values presented for synaptic counts in these studies are all expressed per unit area (i.e., per 100 μm²). It should be noted however, that the manner in which the micrographs were taken resulted in somewhat elevated numbers from those that would be derived from a purely random sample. In an effort to maximize the number of synaptic sites analyzed, the central portion of large dendritic shafts were not photographed. Rather, a long dendritic membrane was included as an edge region in the framing of a given micrograph, causing the dendritic cytoplasm, microtubules, etc., to subtend less area in the micrographs than they truly represent in the neuropil.

RESULTS

The variance for the different measures was greater than we have observed in experiments involving this sample region, which did not employ recording and stimulating electrodes. (Note standard error in Table 1.) There are two reasons for assuming that much of this variability was due to differential amounts of swelling: 1) the standard deviations for each of the measures was linearly related across rats to the mean for the measure, an effect that would be anticipated if the means were influenced by swelling (or shrinkage); and 2) the various parameters were correlated with each other and in a direction that would be predicted by swelling. Thus we found that the length of the postsynaptic densities was negatively correlated with synapse number (Y = -0.85) but positively correlated with spine (Y = +0.70) and bouton (Y = +0.65) area.

The effects of repetitive stimulation on the number of spine, multiple, and perforated synapses are summarized in Table 1. As is evident, for control (n = 11) and potentiated (n = 20) animals, the total number of synapses as well as the number of spine synapses were virtually identical for the two groups. The density of perforated synapses also appeared not to have been markedly affected by repetitive stimulation. The incidence of multiple synaptic boutons tended to be higher in the potentiated group and when expressed for each rat as a percentage of the spine synapse population, the difference between groups was marginally significant.

Shaft synapses were commonly found concentrated on long dendritic processes that were peculiar in that they possessed no dendritic spines in the plane of section (Fig. 4).

Table 2 provides a summary of the numbers of synapses onto dendritic shafts in the control and experimental groups and, as is evident, this type of contact was considerably more numerous in potentiated animals. This was true whether the data were expressed in terms of synapses per 100 μm² (+33%, P < 0.01) or as a percentage of the spine synapse population (+40%, P < 0.01).

The data for the sizes of the constituents of the spine synapses are summarized in Table 3. The mean values for the areas of spines and boutons and the lengths of spine PSDs and spine neck widths were calculated for each rat and were not found to differ between the groups. In order to determine if stimulation had any effects on the within-rat variability of the spine measures, the coefficient of variation was calculated for each animal in the control and experimental groups (the coefficient of variation is the more appropriate statistic in this study because,

### Table 1. Numbers of spine synapses, perforated synapses, and multiple synaptic boutons

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 11)</th>
<th>Potentiated (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spine synapses</td>
<td>39.3 ± 1.1</td>
<td>39.6 ± 1.0</td>
</tr>
<tr>
<td>Multiple synaptic boutons (MSB)</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Percent of spine synapses*</td>
<td>3.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Perforated synapses</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Percent of spine synapses*</td>
<td>5.3</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Values are means ± SE per 100 μm². * The ratio of MSBs and perforated synapses to spine synapses was calculated for each rat. The values shown are the means for the groups.

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1 It is theoretically possible that an observer might be able to use some type of structural alteration produced by the stimulation to identify a micrograph from a potentiated animal from that of a control. While exceedingly remote, we nonetheless tested this idea by using a forced-choice paradigm with a trained observer and found no evidence that he could discriminate one group from the other. Thus, the potentiated selections contained as many micrographs from control animals as from rats that had received high-frequency stimulation.
as noted above, the size of the standard deviation correlated strongly with the size of the mean across rats). As shown in Table 3, the coefficient of variation was substantially reduced in the potentiated rats for each of the three postsynaptic spine indexes and in each case the difference between control and potentiated groups was statistically significant. Thus it appears from the measures of PSD length, neck width, and spine area that the variability of the spine population within an animal was reduced following the induction of long-term potentiation.

Examination of the within-animal distributions for the three spine parameters indicated that the population of observations was skewed by varying degrees to the right. Therefore, median values were determined for each rat and the results are shown in Table 3. With the exception of the spine neck widths, this tended to increase the difference between controls and potentiated animals, but only in the case of the postsynaptic den-
TABLE 3. Mean, median, and coefficient of variation for within animal distributions of four measures of spine synapses

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>Coef Var, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bouton area, µm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.242 ± 0.012</td>
<td>0.219</td>
<td>51.4</td>
</tr>
<tr>
<td>Potent</td>
<td>0.247 ± 0.007</td>
<td>0.231</td>
<td>49.7</td>
</tr>
<tr>
<td>Spine area, µm²</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.115 ± 0.004</td>
<td>0.095</td>
<td>62.6</td>
</tr>
<tr>
<td>Potent</td>
<td>0.116 ± 0.002</td>
<td>0.100</td>
<td>57.6</td>
</tr>
<tr>
<td>PSD length, µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.201 ± 0.006</td>
<td>0.182</td>
<td>42.1</td>
</tr>
<tr>
<td>Potent</td>
<td>0.206 ± 0.004</td>
<td>0.191*</td>
<td>37.9*</td>
</tr>
<tr>
<td>Neck width, µm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.140 ± 0.007</td>
<td>0.104</td>
<td>83.5</td>
</tr>
<tr>
<td>Potent</td>
<td>0.132 ± 0.005</td>
<td>0.108</td>
<td>64.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE of groups. * The median of the distribution within each animal was calculated—the value shown is the mean of the medians. Coefficient of variation (standard deviation/mean) was calculated for each animal—the values shown are the group means. e P < 0.01. d P < 0.005. c P < 0.10.

ses did these differences approach statistical significance. However, the greater proximity of the mean and median for the spine measures in the potentiated rats suggested the possibility that the degree of skewedness in the population of observations for each animal may have been smaller in this group than was the case for the controls. Tests for skewedness were done for each of the measures for each animal and this prediction was confirmed (Table 4). A reduction of skewedness in the potentiated animals could reflect, in part, a decrease in the number of extreme positive observations (i.e., the positive “tail” of the distribution). In accord with this we found that the number of postsynaptic densities above 0.45 µM was substantially less in the potentiated group than was the case for the controls (1.8 versus 3.2% of total observations; P < 0.01). Comparable reductions in the incidence of extreme observations were also found for the neck width (P < 0.03) and spine area (P < 0.05). It appears then that repetitive stimulation reduced the variability of spine measurements and decreased the skewedness of their distribution.

As described above, the number of shaft synapses was substantially greater in the potentiated group than in controls both in terms of contacts per 100 µm² and as a percentage of total synapses. It was of interest, therefore, to repeat for shaft synapses the tests for variability and skewedness that had been conducted for spine contacts, and the results are shown in Table 5. It is apparent that repetitive stimulation did not produce the effects on the shaft PSDs that it had on spines and in fact may have had opposite effects. Thus the coefficient of variation tended to be greater for shaft PSDs in the potentiated group, although the effect did not achieve statistical significance. It was also evident that the positive skew of the distribution of shaft synapses was not decreased by stimulation.

TABLE 4. Mean skewedness of within-animal distributions for three spine measures

<table>
<thead>
<tr>
<th>Spine Area</th>
<th>PSD Length</th>
<th>Neck Width</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.878</td>
<td>0.630</td>
</tr>
<tr>
<td>Potentiated</td>
<td>0.721</td>
<td>0.570</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.025</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

All significance values on this table were calculated using the Mann-Whitney U test with direction of difference predicted.

TABLE 5. Mean, median, coefficient of variation, and skew of within-animal distributions of postsynaptic density lengths located on dendritic shafts

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>Coef Var, %</th>
<th>Skew</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.257 ± 0.008</td>
<td>0.258</td>
<td>28.8</td>
<td>-0.069</td>
</tr>
<tr>
<td>Potentiated</td>
<td>0.270 ± 0.005</td>
<td>0.261</td>
<td>31.4*</td>
<td>+0.307*</td>
</tr>
</tbody>
</table>

Values are means of groups ± SE. There were significantly more shaft PSDs in the potentiated group (see Table 2). * P < 0.10.
DISCUSSION

These results provide evidence that repetitive stimulation of the type that causes long-term potentiation produces two types of structural changes: 1) a reduction in variability in the postsynaptic constituents of the spine synapse, and 2) an increase in the incidence of synapses onto dendritic shafts.

The coefficient of variation for PSDs, spine necks, and spine areas were all somewhat smaller in the experimental animals than they were in the controls. Furthermore, there was a definite tendency for the positive skew of the distribution of each of these variables within an animal to be smaller in the potentiated animals. The fact that each of the spine measures was affected in the same direction greatly strengthens the conclusion that high-frequency stimulation decreased the variability of spine population. This effect might reflect an alteration in some population of spines with atypical shape such that they assume a more normal configuration. Alternatively, the increase in homogeneity could be due to a shape change in a significant percentage of the spines such that random sections through the spines are less variable. If, for example, the postsynaptic densities were to change from an oval toward a spherical shape, then random lines through them might well be more homogeneous. A shape change of this type would also be expected to produce a reduction in the skew of the distribution of the lines through the PSDs as well as a reduction in the number of extreme observations, two effects that were in fact obtained in the present studies. Comparable shape changes (i.e., from ovoid to spherical) could also account for the observed effects of repetitive stimulation on spine and spine neck measurements.\(^2\)

It should be noted that the stimulating electrode presumably activated something less than the entire population of synapses and spines in the dendritic region analyzed in these experiments. Neurophysiological experiments have shown that stimulation paradigms of the type used in the present study do not produce generalized changes in the physiology of the target dendritic zone and, in particular, do not affect the synaptic responses in those zones generated by fibers that have not received high-frequency stimulation (8). Therefore, it seems reasonable to assume that the effects described above were restricted to those spines innervated by the potentiated synapses. If so, then it follows that the observed changes were likely to have occurred in subgroups of the total population of spines. This, in turn, suggests that the actual magnitude of the effects in terms of individual spines, PSDs, and necks was to some degree greater than that indicated by the means for the total spine population.

Fifkova and van Harreveld (10), using a very rapid freeze substitution method for fixation, have reported that spines exhibit marked swelling following intense stimulation of the perforant path. While the physiological consequences of such stimulation are unclear, their studies do demonstrate that spines can undergo lasting increases in their volume. However, it does not appear that the brief, high-frequency bursts used in the present experiments produced general swelling of spines. Swelling would cause an increase in mean area and standard deviation and no necessary effect on the coefficient of variation; we found no detectable effects on the mean size of the spines and lengths of the PSDs and a decrease in both standard deviation and coefficient of variation. Beyond this, repetitive stimulation reduced the skewness of the populations as well as the incidence of extreme observations, effects opposite to those anticipated for generalized swelling.

Repetitive stimulation produced very different effects on the shaft synapses than were observed for spine contacts. The number of contacts on shafts was substantially increased, a result that is in accord with our previous findings using the in vitro slice method (11). We also found, in marked contrast to the spine synapses, that the variability of shaft postsynaptic density lengths tended to be greater in the experimental rats than was

\(^2\) It would be expected that a "rounding" of the spines would also decrease the incidence of very small measurements. Tests of this prediction were confounded by the fact that extremely small elements could not be measured, and so the estimates of the size of the tail at the low end of the distribution were likely to be less accurate than those of the high end. The number of spines with areas smaller than 0.04 \(\mu m^2\) was less in the potentiated group (controls, 9.1% of total observations, potentiated 6.5%) but this was not statistically significant. The number of observations in the smallest category was also less for spine PSDs (5.4% for controls, 4.2% for potentiated) and spine neck widths (11.3% for controls, 10.1% for potentiated), but again these results were not statistically significant.
the case for the controls. There are at least three possible explanations for this pattern of results: 1) a shape and/or size change in the shaft PSDs made it more likely that this category of contacts would be encountered in the electron microscope, 2) a transformation of a small number of spine contacts into shaft synapses, 3) new synapses were formed and these did not have the same size or shape as the already-present group.

In support of the first suggestion there did appear to be a slight tendency for shaft PSDs to be longer in the potentiated group, but the effect was small and did not approach statistical significance. On the other hand, estimates of synaptic density per unit volume, correcting synapse numbers for PSD length using Dubin’s (7) formulas, had no appreciable effect on the differences in numbers of shaft contacts between potentiated and controls. Serial sectioning electron microscopy will be required to settle definitively the question of whether changes in shape might explain the increase in synapse numbers. However, given the relative magnitudes of the changes in the coefficient of variation and spine numbers, it seems unlikely that the former is responsible for the latter.

The increase in the number of shaft synapses could also be accounted for by a “retraction” of a small number of dendritic spines into the shaft following the stimulation train. The number of lost spines required by such an explanation is sufficiently small such that it would not necessarily be detected in our counts of spine synapses. However, the spine postsynaptic densities are 20–25% shorter than their shaft counterparts and the addition of large numbers of spine contacts to the shaft synapse category should certainly have reduced the mean length of the shaft PSDs. As mentioned, the length of shaft synapse PSDs were slightly longer rather than shorter in the potentiated group.

Finally, it is possible that the increase in numbers of shaft synapses were due to the formation of entirely new synaptic contacts and that these did not have the same size or shape as the existing population. Recent studies using adult rats have shown that the associational projections to the dentate gyrus enter into synaptogenesis with the granule cells following removal of the other afferents of those cells and that this involves the generation of new boutons and postsynaptic densities (15). But this “sprouting” response is triggered by a drastic manipulation (deafferentation) and occurs only after a delay of several days and requires weeks to restore a normal complement of synapses. It is possible, however, that the rate-limiting step in sprouting is not at the level of synapse formation, but in the glial and degenerative activities that are closely correlated with denervation and reinnervation. In any event, it appears that axons and dendrites in adult rat hippocampus have the capacity to generate new synapses.

In the in vitro experiments the slices were removed 15 min after a single brief period of high-frequency stimulation and immersed in dilute, cold fixative. Thus, beyond confirming the pattern of results found in the present experiments, the “slice” studies indicate that the observed changes occur quite quickly.

In summary, brief bursts of high-frequency stimulation produce two very different types of effects in the zones in which the “driven” afferents form synapses. The spine changes provide a candidate for the substrate of the extremely persistent long-term potentiation effect. An increased or more symmetrical zone of apposition between the pre- and postsynaptic elements could serve to increase the efficacy of the transmission process, while shape change in the body and neck of the spine might well act to improve the “coupling” between the initial components of the postsynaptic potential and depolarization of the shaft. Interpretation of
the significance of the apparent increase in the numbers of shaft contacts is made difficult by the uncertainty surrounding the identity of the cells on which these elements are found. As noted, we routinely find shaft synapses concentrated on long dendritic processes that have few if any spines. There is a possibility that such processes do not belong to the pyramidal cell but instead are part of the dendritic tree of an interneuron, possibly one of the basket types known to have long, straight dendrites coursing through the stratum radiatum (12, Figs. 8 and 16). Tests of the relationship of the observed structural changes to the long-term potentiation effect will necessarily involve attempts to correlate the two across time and various experimental manipulations. In any event, the results provide further evidence of the anatomical plasticity of the hippocampus and raise the possibility that this is induced by physiological events.

REFERENCES