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Remodeling of synaptic architecture during hippocampal "kindling"

(synaptic plasticity/synapse quantitation)

YURI GEINISMAN*, FRANK MORRELL†, AND LEYLA DETOLEDO-MORRELL†‡

*Department of Cell Biology and Anatomy, Northwestern University Medical School, Chicago, IL 60611; and Departments of †Neurological Sciences and ‡Psychology, Rush Medical College, Chicago, IL 60612

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ABSTRACT **The "kindling" phenomenon is associated with long-lasting facilitation of synaptic transmission. A possible mechanism of such facilitation could involve changes in the number of synaptic contacts. However, previous attempts to demonstrate a synaptic morphological alteration that could account for the long-term effects of kindling had failed, possibly due to the unavailability, at the time, of unbiased methods for synapse quantitation. Using the unbiased stereological disector technique, we estimated the number of synapses per neuron in the middle molecular layer of the hippocampal dentate gyrus in rats kindled by electrical stimulation of the medial perforant path with implanted electrodes. Unkindled but stimulated (coulombic control) and unstimulated but implanted rats served as controls. Animals were coded and killed 4 weeks after reaching the kindling criterion of five generalized seizures. The most important results were obtained when axospinous synapses with continuous or discontinuous postsynaptic densities ("nonperforated" or "perforated" synapses) were differentially analyzed. Kindling resulted in a selective loss of nonperforated synaptic contacts in contrast to preservation of perforated ones. Furthermore, the ratio of perforated to nonperforated synapses was increased by 45% or 40% in kindled rats relative to unstimulated or coulombic controls, respectively. These findings suggest that synaptic efficacy may depend on a balance of the two synaptic types; selective elimination of nonperforated synapses may augment the potency of remaining synaptic contacts, a process reminiscent of synaptic remodeling during development.**

"Kindling" may be viewed as an experimental model of the formation of an engram in the brain (1, 2). It differs from all other models of synaptic plasticity (or neuronal models of memory) by reason of its extraordinary duration. After only a few exposures to a low-level, localized electrical stimulus, the synaptic responsiveness of the stimulated circuit undergoes an augmentation that persists, without further reinforcement, for many months.

In the kindling paradigm (3, 4), a subthreshold electrical stimulus is repeatedly delivered to a local brain area once or twice a day for a duration of 1–2 sec. When first applied, the electrical stimulation results in only brief afterdischarge (AD) and no behavioral alteration. Without any change in stimulation parameters, the AD gradually increases in duration and spreads from the stimulated area to increasingly distant, though synaptically connected, brain regions. A progressive alteration of behavior is also seen, beginning with momentary arrest of ongoing locomotor activity and proceeding through localized twitching to a generalized seizure. Once generalized seizures have occurred, cessation of stimulation for weeks, months, or even years does not result in loss of the newly acquired, electrophysiologically and behaviorally defined change (2, 5, 6). Reintroduction of the original stimulus, which was behaviorally ineffective to begin with, reliably causes a generalized seizure.

This enduring enhancement of synaptic efficacy, its extension only along synaptically linked stations of the stimulated circuit (7), and its strict dependence on protein synthesis (8, 9) and axonal transport (7, 10) have suggested that kindling should be associated with some morphological alteration of the synapse itself. It seemed reasonable to assume that a possible mechanism of long-lasting facilitation of synaptic transmission during kindling might be an increase in the number of synaptic contacts. Yet previous attempts to document morphological modifications associated with kindling (1, 11) have yielded negative results. These earlier studies, however, were hampered by the unavailability of unbiased methods for synapse quantitation at the time they were carried out. With the aid of such a stereological technique, we have reexamined the issue and now report that kindling is, indeed, associated with a remodeling of synaptic architecture. Because kindling is an extremely robust and easily reproducible model of a persistent, experimentally induced modification of synaptic responsiveness, we regard these morphological observations as relevant to the neural substrate of memory. Some of the results reported here have been published in abstract form (12).

MATERIALS AND METHODS

Young adult (4 months old at the start of experiment) male rats of the Fischer 344 strain were stereotaxically implanted with bipolar electrodes into the medial perforant path on the right side. After a 2-week recovery period, they were randomly assigned to three groups. One group (kindled rats) was stimulated twice a day (with 1-msec pulses at 60 Hz for 2 sec) at a current level that initially produced an AD of 10 sec or less. The stimulation was terminated when a kindling criterion of five generalized seizures was reached. A second group of animals (coulombic controls) was stimulated with parameters (120 pulses at 2 Hz) that do not evoke kindling (2, 6). Each rat of this group was matched with a respective kindled rat according to the current level and the total amount of current delivered. The third group (unstimulated controls) consisted of animals that received the same handling and electrode implantation as the other two groups but were not stimulated. Three rats, one from each group, were coded and killed 4 weeks after reaching the kindling criterion, to allow the possible immediate effects of seizures on brain morphology to dissipate. The brains from each triplet were processed simultaneously for electron microscopy. Synapses were quantified in the middle third of the dentate gyrus molecular layer, since this is the site of termination of the medial perforant path (13–15).

The protocol of tissue preparation for electron microscopy was described by us in detail in previous publications (16, 17).

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Abbreviations: AD, afterdischarge; LTP, long-term potentiation; PSD, postsynaptic density.

In brief, rats were perfused intracardially with 1% paraformaldehyde/1.25% glutaraldehyde/0.002% $CaCl_2$/0.12 M phosphate buffer (pH 7.3), followed by the same fixative at twice the aldehyde concentration. The brain was postfixed overnight in the concentrated fixative, and the right hippocampal formation was dissected free and cut perpendicular to its septotemporal axis into blocks about 1 mm thick. Two blocks, with their rostral faces 1.5 or 3.5 mm caudal to the septal pole of the hippocampal formation, were treated with osmic acid, dehydrated, and embedded in Araldite. All blocks were assigned code numbers to be decoded after completion of morphological work. The rostral face of the blocks was trimmed down so as to include the whole width of the molecular and granule cell layer in the central (in the mediolateral direction) segment of the hidden blade of the dentate gyrus. From each block, two complete series of ultrathin sections were prepared and stained with uranyl acetate and lead citrate. Electron micrographs were obtained from the middle zone of the molecular layer and from the granule cell layer at a final magnification of ×20,000 or ×2500, respectively. A magnification standard (grating replica) was photographed and printed with each series of micrographs.

Synapses were identified in micrographs of serial sections by the presence of synaptic vesicles in a presynaptic axon terminal and a postsynaptic density (PSD) in a postsynaptic element. The number of synapses per neuron was estimated by means of the disector technique (18) using a two-step procedure (19, 20). In the first step, neurons were sampled in the granule cell layer, the neuronal nucleus being employed as a counting unit. The first and the last of $k + 1$ sections of a series were used, in turn, as a reference and a look-up section of disectors. Q^-, the number of neurons whose nuclei were seen in a reference but not in a look-up section, was counted in a sampling area A with the aid of the unbiased counting rule (21). In the next step, synapses were sampled in the middle third of the molecular layer, the PSD being employed as a counting unit. From each series, 12 reference sections were selected at random, a section immediately above a reference one being used as a look-up section of a disector. q^-, the number of synapses having a PSD observed in a reference but not in a look-up section, was counted in a sampling area a, the unbiased counting rule being followed. The unbiased estimate of the number of synapses per neuron n/N was obtained by the formula

$$n/N = [(\Sigma q^- \cdot \Sigma A \cdot k)/(\Sigma Q^- \cdot \Sigma a)] \cdot (w/W).$$

In this formula, the summation Σ is over all disectors of a series, and w/W is the ratio between the widths of the middle third of the molecular layer w and of the granule cell layer W. A final n/N value per animal was calculated by averaging n/N estimates derived from four section series. The data for individual animals presented here were obtained by analyzing series of 25–73 (mean = 42) sections in which a total number of 24–63 (mean = 46) axodendritic or 484–687 (mean = 579) axospinous synapses was sampled in a total area of 4392–4644 (mean = 4509) μm^2. Additionally, 53–96 (mean = 68) neurons were sampled in a total area of 14,155–41,412 (mean = 24,839) μm^2.

RESULTS

Synaptic contacts involving dendritic shafts, which are here referred to as axodendritic synapses, and those involving dendritic spines, or axospinous synapses, were differentially analyzed. Postsynaptic elements that contained mitochondria and arrays of parallel microtubules were identified as dendritic shafts. Those postsynaptic elements that were attached to a parent dendrite, contained a spine apparatus, or lacked mitochondria and microtubules were classified as dendritic spines.

Kindled rats did not differ significantly from either unstimulated or coulombic controls with respect to the number of axodendritic synapses per neuron (Table 1); the difference between the two control groups on this measure was not statistically significant ($P > 0.2$). However, the number of axospinous synapses per neuron was significantly decreased by 18% and 19% in animals from the kindled group compared with unstimulated and coulombic controls, respectively (Table 1).

In order to determine whether this loss of synapses selectively involved only a certain synaptic type, all axospinous synaptic contacts were further subdivided into two types according to the appearance of their PSD. Axospinous synapses exhibiting a discontinuous or perforated PSD profile in at least one serial section were attributed to the first type, and those without PSD discontinuities to the second type (Fig. 1). These will be referred to as "perforated" and "nonperforated" synapses, respectively.

Examination of perforated synaptic contacts showed that their number per neuron did not change significantly in kindled animals, although a trend towards an increase was observed (Table 2). Analysis of nonperforated synapses, however, demonstrated a 21% and 22% reduction in their number per neuron for the group of kindled rats relative to unstimulated and coulombic controls, respectively (Table 2).

Additionally, the ratio of perforated to nonperforated synapses was estimated. This parameter can be calculated from the number of perforated and nonperforated synapses per neuron (as well as directly assessed from raw counts of synapses in the same disectors); it represents a measure of the relative quantities of the two synaptic types in the neuropil. A pronounced and highly significant increase of 45% and 40% in the ratio of perforated to nonperforated

Table 1. Number of axodendritic and axospinous synapses per neuron in control and kindled rats

Rat	No. of synapses per neuron (n/N)		
	Unstimulated controls (group I)	Coulombic controls (group II)	Kindled animals (group III)
	Axodendritic synapses		
1	192	194	129
2	115	109	112
3	117	118	211
4	96	131	211
5	122	205	161
6	271	284	142
7	113	158	146
Group mean	147	171 (ΔII/I, +16.3%)	159 (ΔIII/I, +8.2%; ΔIII/II, −7.0%)
	Axospinous synapses		
1	1966	2410	1532
2	2032	2290	1668
3	1854	1999	2021
4	2313	1888	1864
5	2087	2119	2018
6	2541	2530	1324
7	2090	1971	1832
Group mean	2126	2172 (ΔII/I, +2.2%)	1751 (ΔIII/I, −17.6%*; ΔIII/II, −19.4%*)

Values in parentheses are differences between group means.
*$P < 0.02$, two-tailed Mann–Whitney U test.

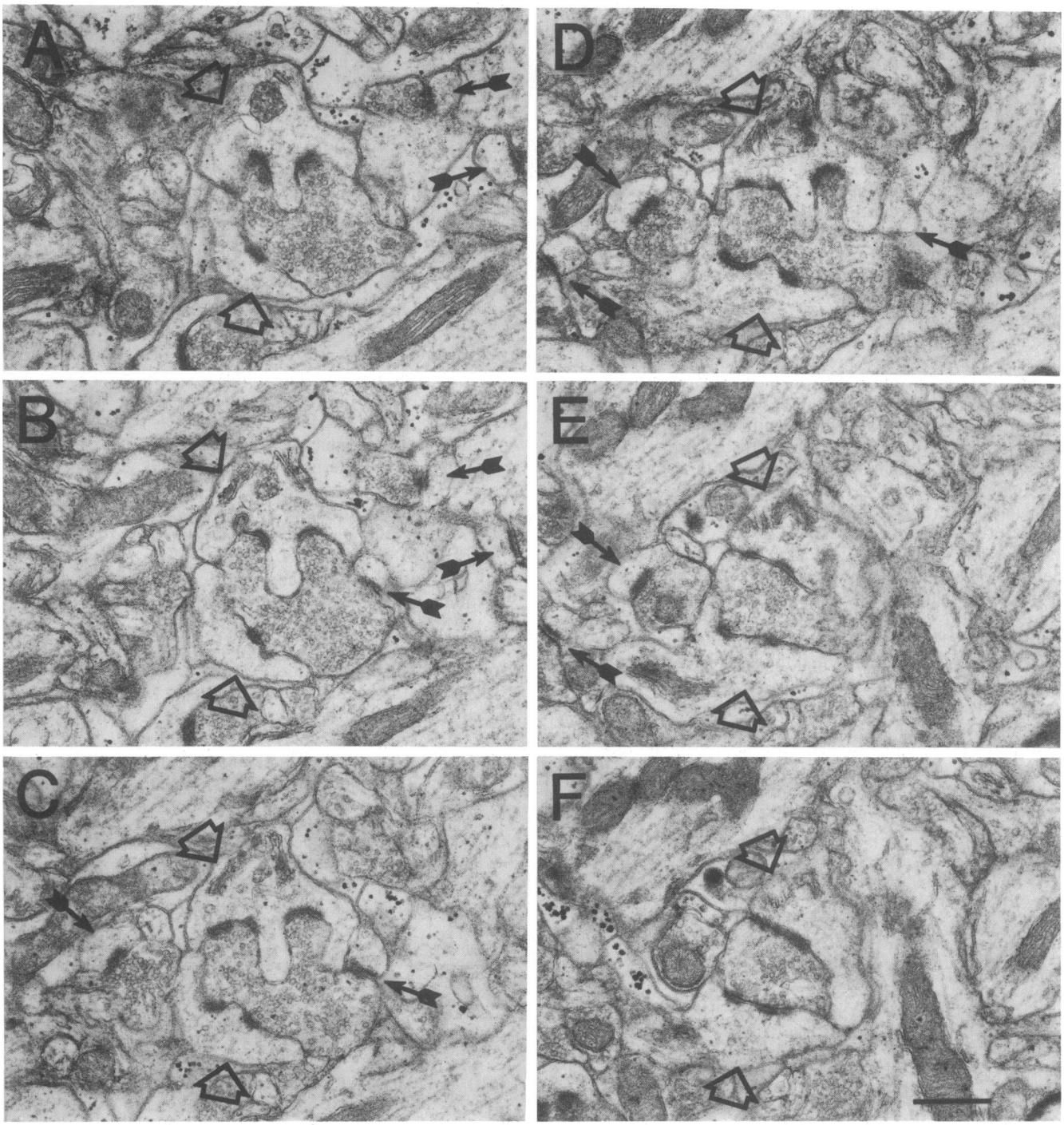

FIG. 1. Electron micrographs of consecutive serial sections (*A–F*) demonstrating two morphologically distinct types of axospinous synapses found in the middle molecular layer of the rat dentate gyrus. "Perforated" synapses (open arrowheads) show a PSD discontinuity(ies) in at least one serial section. "Nonperforated" synapses (black arrows) do not exhibit a discontinuous PSD profile in any consecutive section. (Bar = 0.5 μm.)

synapses was found to occur in kindled rats relative to unstimulated and coulombic controls, respectively (Table 3).

DISCUSSION

The major findings of this study are that hippocampal kindling results in a selective loss of nonperforated axospinous synapses and a marked increase in the ratio of perforated to nonperforated synaptic contacts in the terminal field of the stimulated axons. This was an unexpected result; our prediction was that the long-lasting facilitation of synaptic transmission that characterizes kindling would be associated with an increase in the number of synapses formed by the stimulated axons. However, the result is less surprising than it may seem at first glance. In the middle molecular layer of the rat dentate gyrus, the large majority of nonperforated and virtually all perforated axospinous synapses are formed by perforant-path axons (22). Although it is generally accepted that the major consequence of perforant-path activation is excitatory to dentate granule cells (23–26), it does not necessarily follow that all excitatory synaptic junctions are equally effective in their action. Perforated synapses are believed to be more efficacious than nonperforated ones, due to a closer apposition of pre- and postsynaptic membranes at

Table 2. Number of perforated and nonperforated axospinous synapses per neuron in control and kindled rats

Rat	No. of synapses per neuron (n/N)		
	Unstimulated controls (group I)	Coulombic controls (group II)	Kindled animals (group III)
	Perforated synapses		
1	174	250	227
2	220	250	203
3	187	177	243
4	150	187	231
5	198	182	212
6	264	251	184
7	170	149	256
Group mean	195	207 (ΔII/I, +6.2%)	222 (ΔIII/I, +13.8%; ΔIII/II, +7.2%)
	Nonperforated synapses		
1	1792	2160	1305
2	1812	2040	1465
3	1667	1822	1778
4	2163	1701	1633
5	1889	1937	1806
6	2277	2279	1140
7	1920	1822	1576
Group mean	1931	1966 (ΔII/I, +1.8%)	1529 (ΔIII/I, −20.8%*; ΔIII/II, −22.2%*)

Values in parentheses are differences between group means.
*$P < 0.005$, two-tailed Mann–Whitney U test.

PSD perforations (27) or to the presence of additional PSD "edges" around the perforations (28, 29). It is possible, therefore, that the observed shift in the preponderance of perforated over nonperforated synapses may account for the sustained enhancement of excitatory synaptic "gain" characteristic of kindling.

It is also conceivable that an augmentation of synaptic responsiveness during kindling could result from a diminution of inhibitory synaptic action (30–32). In the context of our experiment, the "inhibitory" explanation would require that some nonperforated axospinous synapses on dentate granule cells have an inhibitory action. At present, there is no direct evidence for or against an inhibitory role of this synaptic type. Most importantly, however, electrophysiological investigations have not revealed a loss of inhibition in the dentate gyrus with perforant-path kindling (33, 34).

Table 3. Ratio of perforated to nonperforated axospinous synapses in control and kindled rats

Rat	Perforated/nonperforated ratio		
	Unstimulated controls (group I)	Coulombic controls (group II)	Kindled animals (group III)
1	0.097	0.116	0.174
2	0.121	0.123	0.139
3	0.112	0.097	0.137
4	0.069	0.110	0.142
5	0.105	0.094	0.117
6	0.116	0.110	0.161
7	0.088	0.082	0.162
Group mean	0.101	0.105 (ΔII/I, +4.0%)	0.147 (ΔIII/I, +45.5%*; ΔIII/II, +40.0%*)

Values in parentheses are differences between group means.
*$P < 0.005$, two-tailed Mann–Whitney U test.

Kindling is related to another, less enduring neuronal model of memory, long-term potentiation (LTP). LTP can be elicited by repeated electrical stimulation of the perforant path (26, 35), using parameters identical to those that cause kindling. LTP appears to be an early and invariant feature of the kindling process (36), and its prior induction in a pathway subsequently stimulated to evoke kindling results in more rapid kindling of that circuit (37). One may consider kindling as an outgrowth of LTP, a manifestation of augmented synaptic efficacy that is not confined to the first synaptic relay (as is LTP) but that extends gradually and successively to other synaptic stations in the stimulated circuit (2). In fact, Siekevitz (38) had predicted, on theoretical grounds, that both kindling and LTP would be associated with similar alterations of synaptic morphology. Desmond and Levy (39) reported differential changes in the density of two synaptic types in the dentate molecular layer consequent to potentiation. They found an increase in the relative proportion of concave axospinous synapses, the majority of which are perforated. Although a different classification of synapses was used and unbiased stereological methods were not employed, we believe that the data are indicative of structural modifications very similar to those described here. Furthermore, other kinds of environmental stimulation, including visual discriminative training (40) or rearing in a complex environment (29), have been shown to result in an increase in the relative proportion of perforated axospinous synapses. These data support the concept that the rearrangement of synaptic architecture induced by kindling may be typical of various phenomena involving long-lasting facilitation of synaptic transmission.

There are also striking similarities between the patterns of structural synaptic alterations in the kindled state and in postnatal development. During development, a large fraction of the synaptic population is winnowed away so that only a smaller number of synapses persists into adult life (41). It is thought that the surviving synaptic junctions are those that have "won out" as a consequence of use and efficiency (42). The elimination of synapses is, therefore, "selective." Such a process may augment the potency of surviving synapses and lead to their functional stabilization (43). Interestingly, not only selective synapse elimination, but also a relative increase in the proportion of perforated axospinous synapses (29), has been shown to occur during development. These data, taken together with the observations reported here, suggest that the rearrangement of synaptic connectivity found in kindling (selective elimination of nonperforated axospinous synaptic contacts coupled with the pronounced increase in the ratio of perforated to nonperforated synapses) may represent a more generalized phenomenon. Such a remodeling of synaptic architecture may play a significant role in the fine tuning of synaptic drive to meet environmental contingencies not only in development, but also later in life.

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