

Estradiol Increases the Frequency of Multiple Synapse Boutons in the Hippocampal CA1 Region of the Adult Female Rat

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ABSTRACT

The effect of estradiol to increase the density of dendritic spines and axospinous synapses on hippocampal CA1 pyramidal cells in the adult female rat has been well-documented. However, presynaptic involvement in this process of synapse elimination and formation in the adult is unknown. To address this issue, we have reconstructed 410 complete presynaptic boutons through coded serial electron micrographs of CA1 stratum radiatum to determine the: (1) frequency of multiple (MSB) vs. single (SSB) synapse boutons; (2) number of synaptic contacts per MSB; (3) bouton volume and surface area; and (4) types of spines in synaptic contact with MSBs and SSBs in ovariectomized, estradiol-treated animals (OVX + E) versus ovariectomized oil-treated controls (OVX + O). Quantitative analysis of this tissue revealed that, in OVX + E animals, 45.0% of presynaptic boutons form multiple synaptic contacts with dendritic spines compared to 27.3% in controls ($P < 0.01$); the average number of synapses per MSB was 2.7 in OVX + E animals compared to 2.3 in controls ($P < 0.05$). This represents a 25.5% increase in the number of synapses formed by a given number of presynaptic boutons in estradiol-treated animals ($P < 0.01$) which largely accounts for the previously observed estradiol-induced increase in axospinous synapse density. There was no treatment effect on bouton size; however, because MSBs are larger than SSBs, the increased frequency of MSBs in estradiol-treated tissue results in a trend toward an estradiol-induced increase in average bouton size. Additionally, MSBs were found to be more irregular in shape, i.e., significantly less spherical, than SSBs. Our results indicate that estradiol-induced dendritic spines form synapses primarily with preexisting boutons in stratum radiatum and that these boutons enlarge and change shape as they accommodate new synapses. Such findings suggest a relatively active role for dendrites in the process of adult synapse formation. © 1996 Wiley-Liss, Inc.

Indexing terms: dendritic spines, Schaffer collaterals, synaptic plasticity, electron microscopy, serial reconstruction

Dendritic spines are the postsynaptic sites of excitatory input to a wide variety of neurons in the mammalian brain. Because of their integral role in synaptic machinery, spines are thought to be very important in the modulation of neuronal physiology. Indeed, structural differences in dendritic spines have been proposed as a neural basis of differences in behavior, such as learning and memory (Horner, 1993; Harris and Kater, 1994). Changes in spines have been shown to occur under numerous developmental, hormonal, and pathological conditions (Horner, 1993).

Previous studies have shown that dendritic spines on hippocampal CA1 pyramidal cells in the adult female rat are sensitive to the ovarian hormone, estradiol. Ovariectomy for 6 days results in a decrease in the density of dendritic spines in CA1 stratum radiatum which can either be

prevented (Gould et al., 1990) or reversed (Woolley and McEwen, 1993) by estradiol treatment. Subsequent treatment with progesterone initially augments the effect of estradiol and then results in a decrease in dendritic spine density (Woolley and McEwen, 1993). The hormonal sensitivity of CA1 pyramidal cell dendritic spines leads to a naturally occurring fluctuation in spine density on these cells as estradiol and progesterone levels rise and fall during the estrous cycle (Woolley et al., 1990). It is important to note that estradiol-induced changes in dendritic spine

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density occur without changes in the dimensions or branching pattern of the dendritic tree (Woolley and McEwen, 1994), indicating that differences in the density of spines do not result from expansion and shrinkage of the dendritic tree or loss of dendrites, but rather reflect differences in the number of spines present on CA1 pyramidal cell dendrites. Additionally, both in the case of estradiol treatment and during the estrous cycle, the density of axospinous synapses fluctuates in parallel with dendritic spine density, indicating that newly formed spines make anatomically normal synaptic contacts (Woolley and McEwen, 1992). These hormone-induced differences in synapse density are observed specifically in the density of synapses formed on spines; the density of synapses formed on dendritic shafts is unchanged (Woolley and McEwen, 1992). The specificity of this effect for one synapse type is evidence that hormone effects on dendritic spines and synapses are not the result of generalized changes in hippocampal volume, but rather an effect on the formation and elimination of axospinous synapses specifically.

Although the effect of estradiol on CA1 pyramidal cell dendritic spines has been well-documented, the presynaptic events which correspond to the process of spine/synapse formation and elimination remain unknown. Thus, it is unclear whether the newly formed spines in estradiol-treated animals make synapses with preexisting boutons in stratum radiatum or alternatively, whether formation of new presynaptic boutons is required to produce presynaptic partners for the new dendritic spines induced by estradiol.

The majority of boutons presynaptic to dendritic spines in the CA1 stratum radiatum belong to Schaffer collateral fibers which arise from ipsilateral CA3 pyramidal cells and commissural axons from CA3 pyramidal cells of the contralateral hippocampus. These axons make glutamatergic en passant synapses with CA1 pyramidal cell spines throughout stratum radiatum. Sorra and Harris (1993) have described a subpopulation of presynaptic boutons in the CA1 stratum radiatum of male rats that is in synaptic contact with multiple CA1 pyramidal cell dendritic spines; these boutons are referred to as multiple synapse boutons (MSBs) as opposed to single synapse boutons (SSBs). We hypothesized that estradiol-induced dendritic spines in CA1 form synapses with preexisting boutons, resulting in an increase in the average number of synapses per bouton. Such an increase could result from: (1) an increase in the proportion of boutons forming multiple synapses, i.e., an increase in the proportion of MSBs, (2) an increase in the number of synapses formed by MSBs or, (3) a combination of both. Alternatively, if new dendritic spines involve new presynaptic boutons as synaptic partners, one would expect no change in either the percentage of MSBs or the average number of synapses per bouton (possibly even a decrease in these parameters if new spines preferentially contact SSBs).

In order to test our hypothesis, we have examined 410 complete presynaptic boutons reconstructed from serial electron micrographs of CA1 stratum radiatum. This tissue was taken from adult female rats that were ovariectomized and treated either with estradiol or with sesame oil vehicle. Using this tissue, we have analyzed the effect of estrogen on: (1) the percentage of boutons that are MSBs, (2) the average number of synapses per MSB, (3) volumes and surface areas of MSBs and SSBs, and (4) the types of spines in synaptic contact with MSBs and SSBs.

MATERIALS AND METHODS

Animal surgery and hormone treatments

The estradiol treatment regimen that was employed in this experiment has previously been shown to result in differences in CA1 pyramidal cell dendritic spine (Gould et al., 1990) and synapse (Woolley and McEwen, 1992) density. Eight adult female Sprague-Dawley rats (250–270 g) were group-housed on a 14-hour light: 10-hour dark cycle with constant access to food and water. Ovariectomy was performed under Metofane anesthesia using aseptic surgical procedure. Three days following surgery, half the animals (OVX + E) were treated with 2 injections (s.c.) of 10 μ g 17 β -estradiol benzoate in sesame oil vehicle, 24 hours apart. The other half (OVX + O) received vehicle at the same times. Forty-eight hours after the second estradiol injection, each animal was deeply anesthetized with 100 mg/kg pentobarbital (i.p.) and its brain was fixed by transcardial perfusion. The rats were initially perfused with isotonic saline with heparin (100 units heparin/ml saline), followed by 200 ml of a solution of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (PB) pH 7.4. Brains were quickly removed from the cranium, hemidissected and postfixed in the perfusate solution for 24 hours at 4°C on a shaker.

Tissue processing for electron microscopy

Following rinses in 0.1 M PB (pH 7.4), coronal sections (100 μ m) from the left hemisphere were cut on a Vibratome. The hippocampus was dissected from each section and postfixed in 1% osmium tetroxide in 0.15 M PB (pH 7.4) for 1 hour at room temperature, dehydrated in alcohols and flat-embedded in Medcast resin. Serial thin sections (silver to light yellow interference color) which contained the CA1 region were cut. Ultrathin sections were stained with uranyl acetate and Reynold's lead citrate and then were examined on a Philips 410 electron microscope.

Data analysis

For each animal in the study, a series of electron micrographs (average 24 sections each) was taken in the CA1 stratum radiatum 150–200 μ m from the cell body layer at a magnification of 8,100 \times . These micrographs were printed at a final magnification of 19,500 \times and coded for analysis. For each series, the middle section was used as a reference section. An area of 95 μ m² was randomly defined in this reference section. All boutons, identified as membrane bound profiles containing at least 3 vesicles, that were transected within this area were numbered. Each numbered bouton (45–65 per series; total of 410) was then followed through serial sections to completion in both directions. All profiles of each bouton and the dendritic elements in synaptic contact with each bouton were traced onto acetate sheets. In this way, the percentage of boutons forming multiple synaptic contacts, the number of synaptic contacts each MSB formed, and the postsynaptic partner(s) of each bouton could be analyzed. Boutons which did not form any synaptic contacts (approximately 10% in each group) and boutons which were not completely contained within the series (6% on OVX + O and 8% in OVX + E) were excluded. Boutons contained within the series, but which were not visible in the reference section, were not included in the analysis.

Using the drawings of each bouton profile traced through serial sections, both bouton volume and surface area were

estimated by a modified version of the Cavalieri method. The area and perimeter of each profile were measured using NIH IMAGE software, summed and multiplied by section thickness to estimate volume and surface area, respectively. Section thickness for each series was determined two ways: First, section thickness was analyzed by the method of Harris and Stevens (1989) in which, (1) the diameter of a longitudinally sectioned mitochondrion was measured on the section in which it was maximal, (2) the number of sections through which the mitochondrion was longitudinally sectioned was counted, and (3) the maximum diameter was divided by the number of sections to obtain section thickness. Because a mitochondrion is cylindrical, the diameter measured on a single section is equal to the diameter measured through sections in a series. Second, section thickness was also estimated on the basis of the interference color of the newly cut section. Values obtained with these two methods were found to be in good accordance, consistent with Harris and Stevens (1989). Section thickness ranged from 60 to 84 nm. Measurements of bouton volume and surface area were used to calculate an index of sphericity with the following formula (Chicurel and Harris, 1992): sphericity index (SI) = $6(\pi^{1/2} \text{Volume})/\text{Surface Area}^{2/3}$ in which a value of 1 indicates a spherical shape; the lower the value, the greater the irregularity of shape.

The types of dendritic spines in synaptic contact with each bouton were also recorded. Spines were divided into three shape categories: mushroom, thin, and stubby (Peters and Kaiser-Abramof, 1970; Harris et al., 1992). Spines were determined to be mushroom if they had a maximum head diameter at least $5\times$ the maximum neck diameter or had a maximum head diameter of at least $0.6\ \mu\text{m}$; spines were determined to be thin if their maximum head diameter was less than $5\times$ maximum neck diameter and their maximum head diameter was less than $0.6\ \mu\text{m}$; spines were determined to be stubby if they had no obvious head and were approximately as long as they were wide. Using these criteria, spines could be categorized without overlap. The spine head size requirement was necessary when spine necks could not be followed because they were not contained within the series. Furthermore, without the spine head size requirement there was a subpopulation of spines that qualitatively appeared to be mushroom-shaped, but because they had a wide neck (often due to the presence of spine apparatus) would have been categorized as thin. In addition to the types of spines in contact with MSBs and SSBs, the frequencies of spines with spine apparatus or perforated postsynaptic densities were also determined. Perforated postsynaptic densities were those that appeared discontinuous in at least one section.

Means calculated per animal were subjected to unpaired, two-tailed Student's *t* tests. A Bonferroni correction for multiple comparisons within a scientific issue was applied to determine the statistical significance of the results; however all *P* values are reported. Four scientific issues are addressed by this study: (1) whether the increase in CA1 pyramidal cell synapse number can be accounted for on the basis of contacts with preexisting presynaptic boutons; (2) the effect of estradiol on presynaptic bouton size; (3) the effect of estradiol on presynaptic bouton shape; and (4) the effect of estradiol on the frequency of types of dendritic spines in synaptic contact with MSBs versus SSBs.

RESULTS

Quantitative analysis of 410 reconstructed presynaptic boutons in series of coded electron micrographs from OVX + O (Figs. 1, 2) and OVX + E (Figs. 3, 4) animals revealed that estradiol treatment results in a significant increase in the percentage of boutons forming multiple synaptic contacts in the CA1 stratum radiatum. The mean %MSBs in OVX + O animals was found to be 27.3% compared to 45.0% in OVX + E animals ($T = 5.455$, $P < 0.01$), an increase of 40% (Fig. 5A). Additionally, the mean number of synapses per MSB was found to be increased in OVX + E compared to OVX + O animals. MSBs in OVX + O animals formed an average of 2.3 synapses each compared to 2.7 synapses per MSB in OVX + E animals ($T = 2.868$, $P < 0.05$), an increase of 15% (Fig. 5B). Taken together, these data indicate a significant 25.5% increase in the number of synapses formed by presynaptic boutons in estrogen-treated animals ($T = 5.513$, $P < 0.01$; Fig. 5C). This increase in synapse number is highly consistent with the 28% increase in axospinous synapse density observed using the same estrogen treatment paradigm in a previous study (Woolley and McEwen, 1992). The increased occurrence of MSBs in estrogen-treated animals alters the distribution of synapses in stratum radiatum between MSBs and SSBs. In OVX + O animals, 46.3% of all axospinous synapses are formed with an MSB as the presynaptic partner, whereas in OVX + E animals, 69.3% of axospinous synapses are formed with an MSB ($T = 6.049$, $P < 0.01$; Table 1).

Our estimates of the volumes and surface areas of presynaptic boutons indicate that both of these parameters are increased in MSBs compared to SSBs, consistent with the observations of Sorra and Harris (1993). The average volume estimate of MSBs was $0.245\ \mu\text{m}^3$ compared to the average volume estimate of $0.132\ \mu\text{m}^3$ for SSBs (Table 1). The average MSB surface area estimate was $2.08\ \mu\text{m}^2$ compared to the average SSB surface area estimate of $1.23\ \mu\text{m}^2$. No differences, however, were observed between treatment groups in the volumes or surface areas of MSBs or SSBs. While there was no treatment effect on either MSB or SSB volume calculated separately, the increased frequency of the larger MSBs in estrogen-treated animals results in a 16.5% increase in average volume ($T = 2.288$, $P = 0.062$; Table 1) and a 20% increase in average surface area ($T = 2.736$, $P < 0.05$; Table 1) of all presynaptic boutons in the estradiol-treated group.

Calculation of a sphericity index (SI) indicated that MSBs are significantly less spherical than SSBs. The mean SI for MSBs was 0.88 ± 0.03 , whereas for SSBs the mean SI was 1.01 ± 0.03 ($T = 3.327$, $P < 0.01$). Additionally, we observed trends toward estradiol-induced decreases in both the SI for all boutons ($T = 2.526$, $P < 0.05$) and for SSBs specifically ($T = 2.545$, $P < 0.05$); each decrease was approximately 15%. No differences or trends toward differences were observed in the shape of MSBs in estradiol-treated versus control animals.

In general, our observations of the relative proportions of different types of dendritic spines in the CA1 stratum radiatum of female rats are consistent with the those reported by Harris et al. (1992) for male rats. We found the majority (73%) of dendritic spines were thin. Mushroom spines made up most of the remaining contacts (25%), whereas stubby spines were relatively rare (2%). Quantitative analysis revealed no differences in the relative percent-

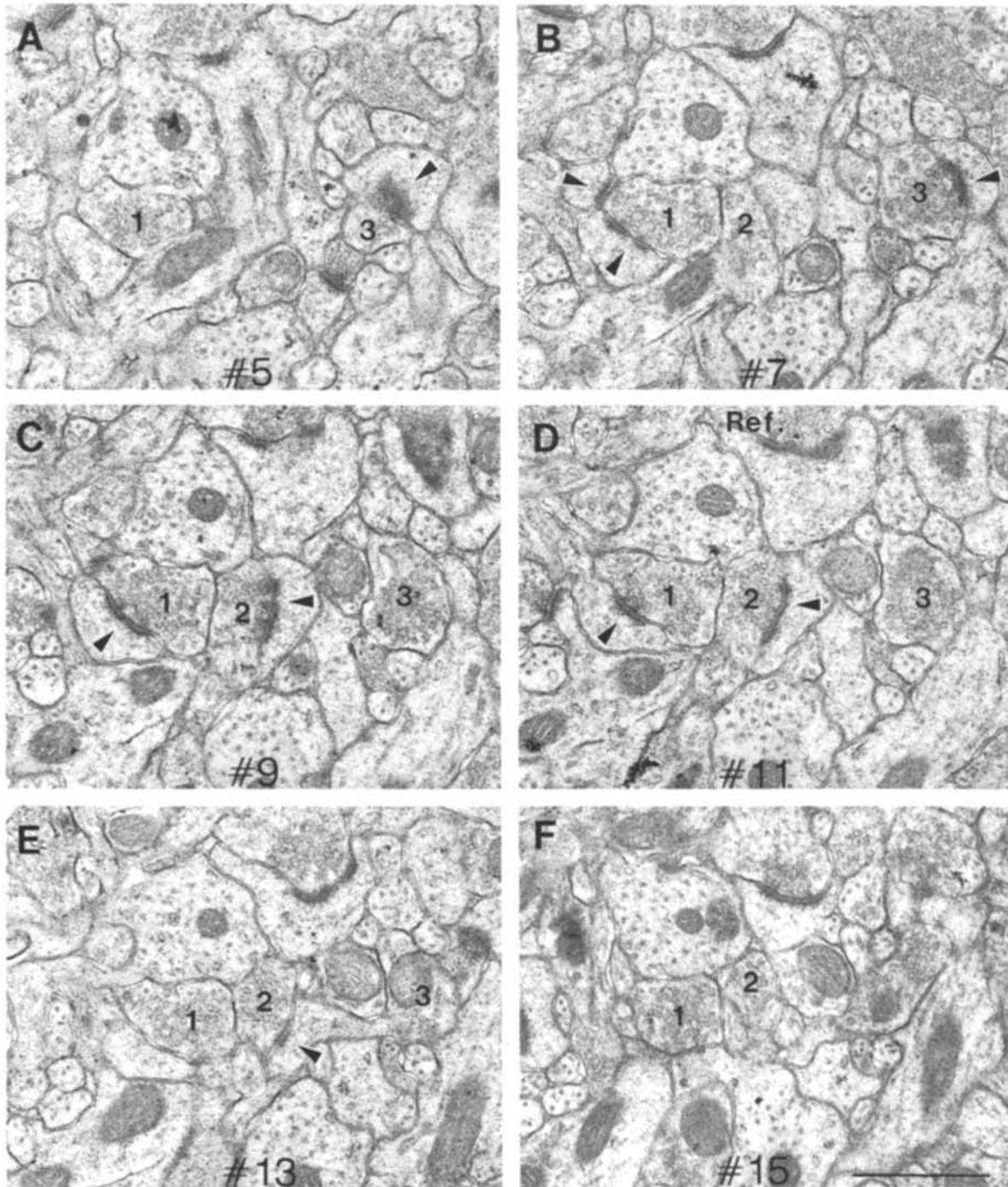


Fig. 1. **A–F:** Electron micrographs depicting every other section, in order, through the middle of a series of electron micrographs taken from the CA1 stratum radiatum of an ovariectomized control rat which received sesame oil vehicle (OVX + O). The middle, or reference (ref), section is shown in D. Section numbers are indicated at the bottom center of each panel. Three presynaptic boutons present in the reference section and completely contained within the series are indicated by

the numbers 1, 2, 3 (see D). Axospinous synaptic contacts formed with these boutons are indicated by arrowheads. Bouton 1 is a multiple synapse bouton (MSB) with two synaptic contacts (see B); boutons 2 and 3 are single synapse boutons (SSBs). A complete series of drawings showing every section through bouton 1 is shown in Figure 2. Scale bar = 1 μ m and applies to all panels. These micrographs contain a small amount of fine particulate artifact.

ages of each dendritic spine type between treatment groups. However, we did observe a significant difference in the percentage of mushroom spines in contact with MSBs versus SSBs; whereas 46.7% of MSBs were in synaptic

contact with at least one mushroom dendritic spine, only 29.7% of SSBs contacted a mushroom spine ($T = 2.923$, $P < 0.025$). Generally, MSBs were observed to be in synaptic contact with no more than one mushroom spine; in only

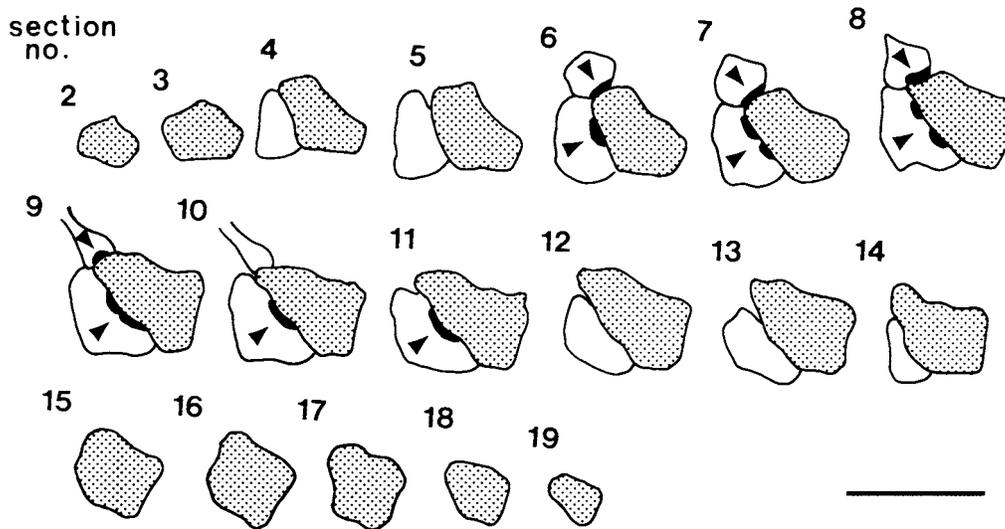


Fig. 2. A complete series of drawings showing every section through bouton 1 from Figure 1 including associated postsynaptic dendritic spines. Section numbers are indicated by numbers at the upper left of

each drawing. The bouton profiles are stippled; spine profiles are open. Synaptic contacts are indicated by arrowheads. This bouton is an MSB with two synaptic contacts. Scale bar = 1 μ m.

3 out of 141 MSBs was synaptic contact with more than one mushroom spine observed. Although a greater percentage of MSBs than SSBs contact a mushroom spine, each MSB forms a greater number of synaptic contacts than an SSB. Therefore, when all MSB synapses are taken into account, a significantly lower percentage of MSB synapses are formed with mushroom spines; of all synaptic contacts formed on MSBs, 19.7% were with mushroom spines, whereas 29.9% of contacts on SSBs were with mushroom spines ($T = 3.806$, $P < 0.01$). Finally, no significant differences or trends toward differences were observed in the presence of perforated synapses or spine apparatus in dendritic spines in synaptic contact with MSBs versus SSBs or between treatment groups.

DISCUSSION

Structural findings

The results of this study demonstrate that the frequency of presynaptic boutons forming multiple synaptic contacts with dendritic spines in the CA1 stratum radiatum of the female rat hippocampus is significantly increased by estradiol treatment. Furthermore, there is a trend toward an increase in the average number of synapses formed by multiple synapse boutons in estradiol-treated animals. Taken together, these differences produce a 25.5% increase in the number of axospinous synapses formed in this region in estradiol-treated compared to control animals.

This observation serves as both a confirmation and an extension of previous findings regarding the effects of estradiol on CA1 pyramidal cell connectivity. It has been shown that estradiol treatment induces new dendritic spines to form on CA1 pyramidal cell apical dendrites (Gould et al., 1990) and that these spines form asymmetric synaptic contacts (Woolley and McEwen, 1992). However, it was not known whether the new spines contact preexisting boutons in the stratum radiatum or whether new presynaptic boutons form in parallel with new dendritic spines. We have observed changes in MSB frequency and MSB synapse

number that produce a percent increase in total synapse number (25.5%) very similar to the previously observed percent increase in synapse density (28%; Woolley and McEwen, 1992). This result indicates that the new CA1 pyramidal cell synapses are formed with preexisting boutons; that is, no new boutons are required. Using the same hormone treatment schedule used in the previous experiments, but employing a different method of analysis, we can account for the previously observed increase in synapse number on the basis of increased MSB contacts. It should be noted that due to necessarily limited sampling of the CA1 stratum radiatum using serial electron microscopic (EM) reconstruction, the possibility of regional differences in morphological parameters within the sampled region of CA1 stratum radiatum (150–200 μ m from the cell body layer) cannot be excluded in our analysis.

Our analysis detected no effect of estradiol treatment on MSB or SSB volume, surface area or types of dendritic spines contacted by either of these bouton types. As these results indicate no basis upon which to distinguish MSBs in estradiol-treated animals versus MSBs in control animals, it appears that the physical characteristics of the MSB population are the same in each treatment group. However, since MSBs are more frequent in estradiol-treated animals and MSBs are larger in volume and surface area than SSBs, there are trends toward an estradiol-induced increase in average bouton surface area and volume in estradiol-treated animals. These results, in turn, indicate that although no new boutons are formed in the estradiol-treated tissue, the preexisting boutons which form synapses with new spines must enlarge as they take on new synaptic contacts. It is not yet clear whether this bouton enlargement is a cause or an effect of increased numbers of synaptic contacts.

Our analysis of the types of dendritic spines in synaptic contact with presynaptic boutons suggests the possibility that thin spines, which make up the majority of postsynaptic contacts (73%), could constitute a more plastic population than mushroom or stubby dendritic spines. Mushroom

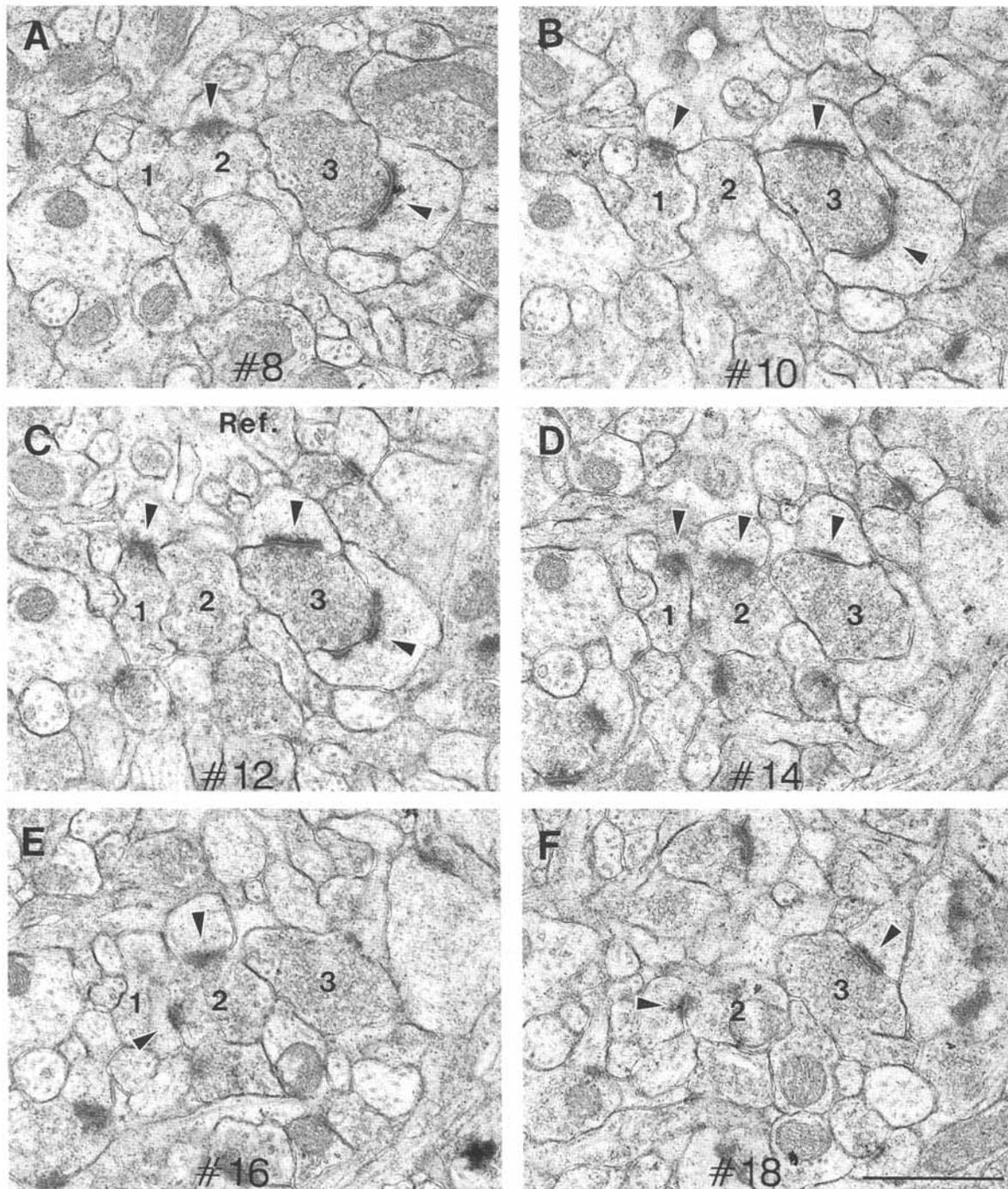


Fig. 3. **A–F:** Electron micrographs depicting every other section, in order, through the middle of a series of electron micrographs taken in the CA1 stratum radiatum of an ovariectomized, estradiol-treated (VX + E) rat. The middle, or reference, section is shown in C. Section numbers are indicated at the bottom center of each panel. Three presynaptic boutons present in the reference section and completely retained within the series are indicated by the numbers 1, 2, 3 (see C).

Axospinous synaptic contacts formed with these boutons are indicated by arrowheads. Bouton 1 is an SSB; boutons 2 and 3 are both MSBs with 3 synaptic contacts each. A complete series of drawings showing every section through bouton 3 is shown in Figure 4. Scale bar = 1 μ m and applies to all panels. These micrographs contain a small amount of fine particulate artifact.

ines are larger than other spine types, often possess perforated postsynaptic densities and sometimes contain the cytoskeletal apparatus. Interestingly, MSBs in both treatment groups were observed to be in synaptic contact with no

more than one mushroom dendritic spine. The observation of only one mushroom spine per bouton is consistent with the hypothesis that these larger spines are more structurally stable than thin or stubby spines. It seems plausible

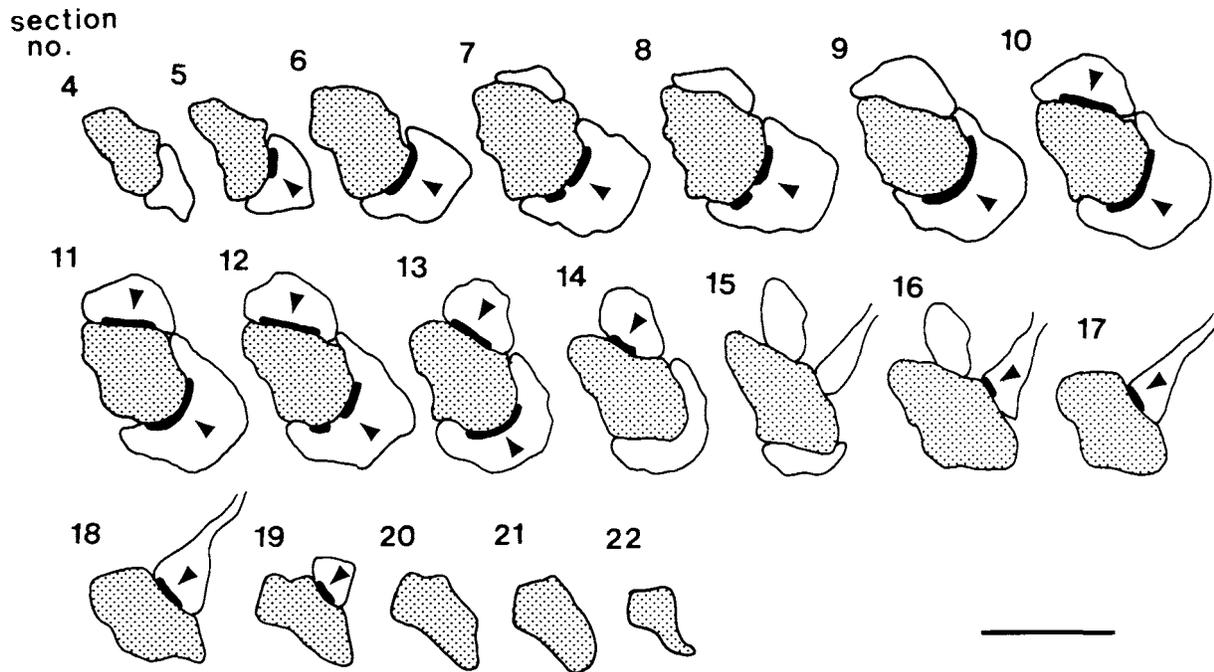


Fig. 4. A complete series of drawings showing every section through bouton 3 from Figure 3 including associated postsynaptic dendritic spines. Section numbers are indicated by numbers at the upper left of each drawing. The bouton profiles are stippled; spine profiles are open. Synaptic contacts indicated by arrowheads. This bouton is an MSB with three synaptic contacts. Scale bar = 1 μ m.

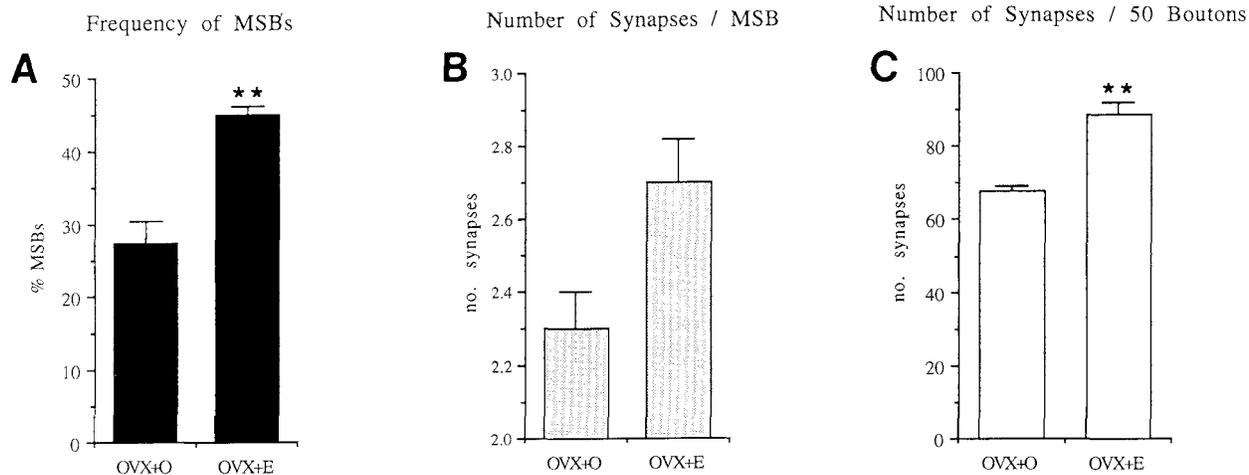


Fig. 5. Bar graphs depicting quantitative differences in presynaptic boutons in hippocampal CA1 stratum radiatum of ovariectomized control rats (OVX + O) and ovariectomized estradiol-treated rats (OVX + E). **A:** Estradiol treatment increases the percentage of presynaptic boutons forming multiple synaptic contacts from 27.3% to 45%. **B:** Estradiol treatment increases the average number of synaptic contacts

formed by an MSB from 2.3 to 2.7. **C:** The estradiol-induced increase in both frequency of MSBs and in the average number of synapses formed by an MSB produces a 25.5% increase in the number of synaptic contacts formed by the same number of presynaptic boutons in each treatment group (expressed per 50 boutons). Double asterisk indicates $P < 0.01$.

that synapses formed with the large mushroom spines remain intact during fluctuation in synapse number, and that thin spines are the population which fluctuates with changing hormone levels. A summary of the structural changes predicted to occur with fluctuating estradiol levels is shown in Figure 6. That smaller, thin spines are a plastic population is consistent with results from learning experiments in which the two-dimensional density of spines with

small heads ($< 0.25 \mu$ m) was found to increase following training of rats (Wenzel et al., 1980).

Changes in the number of dendritic spines and the size of presynaptic boutons in CA1 stratum radiatum are likely to result in changes in the volume distribution of various components of the neuropil. As hormone levels decrease, dendritic spines are eliminated and average bouton size decreases. As these changes occur, either the volume of

TABLE 1. Quantitative Aspects of Presynaptic Boutons in the CA1 Region of the Hippocampus of Estradiol-Treated and Control Female Rats¹

	OVX + O	OVX + E	
average volume of all boutons (μm^3)	0.157 \pm 0.007	0.188 \pm 0.012	$P = 0.062$
average volume of SSBs (μm^3)	0.133 \pm 0.002	0.131 \pm 0.001	
average volume of MSBs (μm^3)	0.230 \pm 0.014	0.259 \pm 0.026	
average surface area of all boutons (μm^2)	1.38 \pm 0.06	1.72 \pm 0.11	$P < 0.05$
average surface area of SSBs (μm^2)	1.20 \pm 0.04	1.26 \pm 0.05	
average surface area of MSBs (μm^2)	1.94 \pm 0.11	2.22 \pm 0.24	
percentage of synapses formed on MSBs	46.2%	69.3**	$P < 0.01$

Values represent mean \pm S.E.M. of quantitative parameters of presynaptic boutons in varicectomized control rats which received sesame oil vehicle (OVX + O) and ovariectomized rats which received estradiol (OVX + E).

*Indicates a significant difference detected using an unpaired, two-tailed, Student's *t* test. $P < 0.01$.

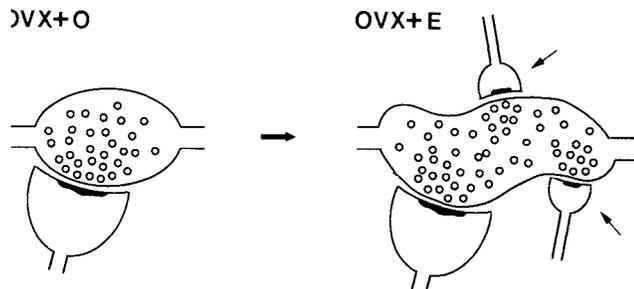


Fig. 6. A summary diagram of the changes in presynaptic boutons proposed to occur with estradiol treatment. In the ovariectomized control (OVX + O), an SSB is shown forming a synaptic contact with a large, mushroom dendritic spine. Estradiol treatment (OVX + E) results in the following changes: (1) new CA1 pyramidal cell dendritic spines (arrows) extend and form synaptic contacts with preexisting boutons transforming some SSBs into MSBs and increasing the average number of synapses formed per MSB; (2) the boutons that become MSBs enlarge as they accommodate additional synapses; and (3) boutons transformed into MSBs become more irregular in shape. Additionally, because the percentage of boutons forming synapses with mushroom spines is roughly the same between OVX + O and OVX + E hippocampi, and since only one mushroom spine is observed per MSB, we hypothesize that the new spines are smaller, thin spines.

extracellular space must increase or another neuropil component must increase to fill in the vacated space. Klintsova et al. (1995) have demonstrated that the volume of glia in the CA1 stratum radiatum fluctuates during the estrous cycle in a manner complementary to dendritic spines and presynaptic boutons. That is, during the proestrus phase of the cycle when dendritic spine density is high and average bouton volume would be increased, the volume fraction occupied by glia is decreased. In contrast, during the estrus phase when spine density is low and average bouton volume would be decreased, the volume of glia is increased. These results are consistent with the interpretation that glia fill in vacated space when dendritic spines are eliminated and presynaptic boutons diminish in size as hormone levels decrease. This interpretation is supported by previous studies of steroid- and activity-dependent synaptic remodeling in the hypothalamus in which decreased synaptic innervation has been shown to correlate with increased glial-neuronal apposition (reviewed in Theodosis and Poulin, 1993).

Functional implications

The hormone-dependent changes in CA1 pyramidal cell connectivity reported here are likely to have important consequences for hippocampal physiology. There has been

considerable speculation regarding the implications of multiple synaptic contacts between CA1 pyramidal cells and their excitatory afferents (e.g., Sorra and Harris, 1993; Lisman and Harris, 1993). In adult male rats, the postsynaptic partners of MSBs have been traced through serial sections to both the same and different CA1 pyramidal cell dendrites (Sorra and Harris, 1993). In the current studies, it is still unclear whether new synaptic contacts in estradiol-treated female rats are formed preferentially between an axonal bouton and dendritic spines from the same or different CA1 pyramidal cells (or if there is an equal distribution of contacts between the same and different postsynaptic cells).

Whether the estradiol-induced synapses serve to increase the coupling of multiple CA1 pyramidal cells to a single presynaptic bouton or whether they mediate an increase in the number of postsynaptic partners contacted by an individual presynaptic bouton is an important issue in predicting the electrophysiological consequences of estradiol treatment. It is more conservative to predict that new synapses are formed primarily between a presynaptic bouton and different postsynaptic cells since this scenario would not invoke specific pre- and postsynaptic interactions. If this prediction were the case, one would expect estradiol treatment to increase the number of postsynaptic cells affected by depolarization of a single presynaptic bouton. The alternative, that an MSB forms multiple synapses with an individual postsynaptic cell, leads to the prediction that depolarization of an MSB would result in a greater effect on an individual postsynaptic neuron than would depolarization of an SSB.

Harris (1995) has suggested that MSBs forming synapses with multiple CA1 pyramidal cells could serve to mediate the spread of long-term potentiation (LTP) between a potentiated cell and its neighbors while still retaining the input specificity that is a hallmark of LTP. Indeed, several groups have demonstrated that LTP can spread between neighboring pyramidal cells in the CA1 region of the hippocampus (Bonhoeffer et al., 1989; Schuman and Madison, 1994). Harris' proposal, which presumes that LTP is initiated postsynaptically and then maintained, at least in part, by a presynaptic change is that: (1) LTP is initiated in a postsynaptic cell, (2) a retrograde signal is transmitted back to the presynaptic bouton of potentiated synapses and then, (3) all postsynaptic cells sharing that presynaptic bouton are exposed to the presynaptic maintenance factor and thus the shared synapses become potentiated as well. This model would predict that by increasing the frequency of multiple synaptic contacts between CA1 pyramidal cells and their excitatory afferents, estradiol treatment would facilitate the spread of LTP in CA1. This hypothesis is consistent with recent studies of LTP during the estrous cycle of the female rat. The threshold for induction of LTP in vivo has been shown to fluctuate across the estrous cycle in a pattern complementary to that of changing dendritic spine and synapse density (Warren et al., 1995). Thus, during the proestrus phase of the cycle, in which spine and synapse density are high, LTP threshold is decreased compared to the estrus phase of the cycle when spine and synapse density are low.

The changes in synaptic configuration in CA1 reported here may also be important for modulating seizure activity in the hippocampus. Several electrophysiological studies have demonstrated a facilitatory effect of estradiol on seizure activity in general and on hippocampal seizures

specifically. Estrogen treatment decreases the threshold and/or increases the severity of electroshock seizures in the rat (Woolley and Timiras, 1962a), mouse (Blackham and Spencer, 1970), rabbit (Logothetis and Harner, 1960) and cat (Marcus et al., 1966). Furthermore, in the rat, electroshock seizure threshold fluctuates during the estrous cycle such that when estrogen levels are high, seizure threshold is decreased (Woolley and Timiras, 1962b). In the female rat, estrogen treatment has been shown to facilitate acquisition of kindled seizures in the hippocampus (Buterbaugh and Hudson, 1991) and to increase the severity of kainic acid-induced seizures (Nicoletti et al., 1985) which involve the hippocampus. Additionally, the threshold for localized seizure activity in the hippocampus is decreased by treatment with exogenous estrogen and also during the phase of the estrous cycle in which estrogen levels are elevated (Terasawa and Timiras, 1968). These effects of estrogen on hippocampal seizure threshold have two different time courses; the estrous cycle effect occurs over a period of approximately 24 hours, whereas the effect of estrogen treatment requires several days. In each case, the timing of decreased seizure threshold is very well-correlated with changes in synaptic configuration in CA1; when dendritic spine, synapse and MSB number are high, seizure threshold is low.

The correlation of estrogen-associated spine/synapse plasticity with enhanced excitatory synaptic function suggests an interesting possibility: that a particularly unstable subpopulation of hippocampal synapses containing primarily N-methyl-D-aspartate (NMDA) receptors exists and is responsible for increased excitatory function. Several lines of evidence support the hypothesis that estrogen induces a subpopulation of NMDA receptor containing synapses: (1) Liao et al. (1995) have recently demonstrated a subpopulation of synapses in the CA1 region of the hippocampus that is activated primarily by NMDA rather than α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors. This subpopulation appears to be involved in initiating LTP in this region. Due to the voltage-dependent Mg^{++} block of the NMDA receptor, these synapses are not activated by low level stimulation; however, they are activated by LTP-inducing stimuli. Activation of these synapses results in induction of AMPA receptors which then support LTP; (2) the threshold for induction of LTP in CA1 (which is NMDA receptor-dependent) fluctuates during the estrous cycle, although the pre-LTP input/output curve generated with low level test stimuli (primarily AMPA receptor-dependent) remains unchanged (Warren et al., 1995). The threshold for LTP induction is decreased during the phase of the estrous cycle in which spine and synapse density are highest; and (3) estradiol specifically increases NMDA receptor binding without affecting AMPA receptor binding. NMDA-displaceable 3H -glutamate binding is increased in CA1 stratum radiatum in parallel with increased spine and synapse density with no change in binding of 3H -AMPA on adjacent sections (Woolley, 1993).

CONCLUSION

In summary, we have demonstrated that estradiol treatment increases both the frequency of MSBs and the number of synapses per MSB in the CA1 region of the adult female rat. Taken together, these data indicate a 25.5% increase in the number of synapses formed in estradiol-treated animals. This difference largely accounts for the previously observed estradiol-induced increase in axospi-

nous synapse density on the basis of changes in MSBs. This finding suggests that new dendritic spines in the hippocampus of estradiol-treated rats form synapses primarily with preexisting boutons increasing the number of synapses per bouton rather than requiring formation of new presynaptic boutons.

The data presented here may also provide a means to further investigate the process of hippocampal synapse formation and elimination in the adult brain. Until now, there has been no way to morphologically distinguish the new, i.e., estradiol-induced, synapses from other synapses in hippocampal CA1. Our data indicate that the new synapses will comprise a subpopulation (approximately 40%) of contacts formed on MSBs in the CA1 region of estradiol-treated rats. Studies of the structural and biochemical properties of this subpopulation may lend insight into characteristics of putatively permanent versus temporary subpopulations of synapses in the adult brain.

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LITERATURE CITED

- Blackham, A., and P.S.J. Spencer (1970) Response of female mice to anticonvulsants after pretreatment with sex steroids. *J. Pharm. Pharmacol.* 22:304-305.
- Bonhoeffer, T., V. Staiger, and A. Aertsen (1989) Synaptic plasticity in hippocampal slice cultures: Local "Hebbian" conjunction of pre- and postsynaptic stimulation leads to distributed synaptic enhancement. *Proc. Natl. Acad. Sci. USA* 86:8113-8117.
- Buterbaugh, G.G., and G.M. Hudson (1991) Estradiol replacement to female rats facilitates dorsal hippocampal but not ventral hippocampal kindled seizure acquisition. *Exp. Neurol.* 11:55-64.
- Chicurel, M.E., and K.M. Harris (1992) Three-dimensional analysis of the structure and composition of CA3 branched dendritic spines and their synaptic relationships with mossy fiber boutons in the rat hippocampus. *J. Comp. Neurol.* 325:169-182.
- Gould, E., C.S. Woolley, M. Frankfurt, and B.S. McEwen (1990) Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. *J. Neurosci.* 10:1286-1291.
- Harris, K.M. (1995) How multiple synapse boutons could preserve input specificity during an interneuronal spread of LTP. *TINS* 18:365-369.
- Harris, K.M., and S.B. Kater (1994) Dendritic spines: Cellular specializations imparting both stability and flexibility to synaptic function. *Ann. Rev. Neurosci.* 17:341-371.
- Harris, K.M., and J.K. Stevens (1989) Dendritic spines of CA1 pyramidal cells in the hippocampus: Serial electron microscopy with reference to their biophysical characteristics. *J. Neurosci.* 9:2982-2997.
- Harris, K.M., F.E. Jensen, and B.H. Tsao (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: Implications for the maturation of synaptic physiology and long-term potentiation. *J. Neurosci.* 12:2685-2705.
- Horner, C.H. (1993) Plasticity of the dendritic spine. *Prog. Brain Res.* 41:281-321.
- Klintsova, A., W.B. Levy, and N.L. Desmond (1995) Astrocytic volume fluctuates in the hippocampal CA1 region across the estrous cycle. *Brain Res.* 690:269-274.
- Liao, D., N.A. Hessler, and R. Malinow (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in the CA1 region of the hippocampal slice. *Nature* 375:400-404.
- Lisman, J.L., and K.M. Harris (1993) Quantal analysis and synaptic anatomy—integrating two views of hippocampal plasticity. *TINS* 16:141-147.
- Logothetis, J., and R. Harner (1960) Electrocortical activation by estrogens. *Arch. Neurol.* 3:290-297.
- Marcus, C.M., C.W. Watson, and P.L. Goldman (1966) Effects of steroids on cerebral electrical activity. *Arch. Neurol.* 15:521-532.

- Nicoletti, F., C. Speciale, M.A. Sortino, G. Summa, G. Caruso, F. Patti, and P.L. Canonico (1985) Comparative effects of estradiol benzoate, the antiestrogen clomiphene citrate, and the progestin medroxyprogesterone acetate, on kainic acid induced seizures in male and female rats. *Epilepsia* 26:252–257.
- Peters, A., and I.R. Kaiserman-Abramof (1970) The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. *Am. J. Anat.* 127:321–355.
- Schuman, E.M., and D.V. Madison (1994) Locally distributed synaptic enhancement in the hippocampus. *Science* 263:532–536.
- Sorra, K.E., and K.M. Harris (1993) Occurrence and three-dimensional structure of multiple synapses between individual radiatum axons and their target pyramidal cells in hippocampal area CA1. *J. Neurosci.* 13:3736–3748.
- Terasawa, E., and P.S. Timiras (1968) Electrical activity during the estrous cycle in the rat: Cyclical changes in limbic structures. *Endocrinology* 83:207–216.
- Theodosios, D.T., and D.A. Poulain (1993) Activity-dependent neuronal-glia and synaptic plasticity in the adult mammalian hypothalamus. *Neuroscience* 57:501–535.
- Warren, S.G., A.G. Humphreys, J.M. Juraska, and W.T. Greenough (1995) LTP varies across the estrous cycle: Enhanced synaptic plasticity in proestrus rats. *Brain Res.* 703:26–40.
- Wenzel, H.J., E. Kammerer, W. Kirsche, H. Matthies, and M. Wenzel (1980) Electron microscopic and morphometric studies on synaptic plasticity in the hippocampus of the rat following conditioning. *J. Hirnforsch* 21:647–654.
- Woolley, C.S. (1993) Estradiol and progesterone mediate naturally-occurring fluctuation in adult hippocampal dendritic spine and synapse density via an NMDA receptor dependent mechanism. (Doctoral Dissertation, Rockefeller University).
- Woolley, C.S., and B.S. McEwen (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. *J. Neurosci.* 12:2549–2554.
- Woolley, C.S., and B.S. McEwen (1993) Roles of estradiol and progesterone in regulation of hippocampal dendritic spine density during the estrous cycle in the rat. *J. Comp. Neurol.* 336:293–306.
- Woolley, C.S., and B.S. McEwen (1994) Estradiol regulates hippocampal dendritic spine density via an NMDA receptor-dependent mechanism. *J. Neurosci.* 14:7680–7687.
- Woolley, C.S., E. Gould, M. Frankfurt, and B.S. McEwen (1990) Naturally-occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *J. Neurosci.* 10:4035–4039.
- Woolley, D.E., and P.S. Timiras (1962a) Estrous and circadian periodicity and electroshock convulsions in rats. *Am. J. Physiol.* 202:379–382.
- Woolley, D.E., and P.S. Timiras (1962b) The gonad-brain relationship: Effects of female sex hormones on electroshock convulsions in the rat. *Endocrinology* 70:196–209.