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Variations of Synaptotagmin I, Synaptotagmin IV, and Synaptophysin mRNA Levels in Rat Hippocampus during the Estrous Cycle

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Periodic changes in ovarian steroid levels during fertility cycles affect learning both in humans and in rats in parallel with electrophysiological and morphological fluctuations in selective neuronal populations. In particular, during the estrous cycle of the female rat, hippocampal CA1 region undergoes cyclic modifications in synaptic density. To investigate the molecular mechanisms involved in synaptic remodeling during the estrous cycle, we analyzed the expression of three presynaptic markers, synaptotagmin I, synaptotagmin IV, and synaptophysin, in the female adult rat brain by *in situ* hybridization. Relative abundance in mRNA for these three markers was quantified at four phases of the estrous cycle: diestrus, proestrus (AM and PM), and estrus. mRNA levels for syt1 exhibited cyclic variations in pyramidal neurons of the CA3 region of hippocampus during the estrous cycle, while mRNA levels for syt4 and SYN were relatively invariant in this or other regions of the hippocampus. Because CA3 pyramidal neurons make synaptic contacts in CA1, modulation of syt1 expression in CA3 may participate in the changes in synaptic density observed in CA1 during the estrous cycle. Furthermore, both syt1 and SYN mRNA varied cyclically in layer II, but not in layer III of entorhinal cortex, while syt4 remained unchanged throughout the cycle. These data suggest that regular variations in steroid hormone levels during fertility cycles may alter the properties of several networks involved in information processing and learning and memory through altered levels of presynaptic proteins.

Key Words: **synaptotagmin; synaptophysin; estrous cycle; mRNA; *in situ* hybridization.**

INTRODUCTION

Neuronal circuits undergo constant remodeling in adult brains under normal physiological conditions, as well as in response to challenges from pathological lesions (9, 23). Substantial evidence shows that structural modifications in dendrites and in synaptic connectivity of adult neurons are fundamental to ongoing reorganization of neuronal networks (31, 38, 63). During the 4- to 5-day estrous cycle of female rats (45), a cyclic pattern of synaptic remodeling occurs in the hypothalamic arcuate nucleus. In this key neuroendocrine locus, the number of synapses per length of perikaryal membrane transiently decreases; e.g., the number of axosomatic synapses is reduced by 30% within the 24-h period between proestrus and estrus, followed by return of synaptic density to proestrus level within the next 24 h (36). Synaptic changes in the arcuate nucleus are dependent on the preovulatory estradiol surge, as immunoneutralization of circulating estradiol with a specific monoclonal antibody abolished synaptic variations (32).

The rat hippocampus also shows physiological cycles of synaptic reorganization and plasticity across the estrus cycle (30, 65): the density of dendritic spines in CA1 increases during diestrus, with a peak at proestrus when estradiol is highest, and declines during estrus when estradiol decreases and progesterone is elevated (65, 66). Other *in vivo* experiments with ovariectomized rats showed direct effects of estradiol on dendritic spine density and the importance of progesterone in the precipitant decrease that follows proestrus (16). Moreover, the effect of estradiol on dendritic spines required the activation of NMDA receptors (67). Furthermore, changes in density of dendritic spines on CA1 neurons reflected a parallel cyclic fluctuation of synaptic connectivity between CA1 pyramidal cells and their afferents. Thus, increased spine numbers were accompanied with increased number of spine synapses (66). This finding indicates that estradiol coordinates the regulation of genes coding for proteins participating

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[1] Both authors contributed equally and share first authorship.

[2] Abbreviations used: syt1, synaptotagmin I; syt4, synaptotagmin IV; SYN, synaptophysin; LH, luteinizing hormone; SSC, sodium chloride/sodium citrate; PBS, phosphate-buffered saline.

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in the structure/functions of both pre- and postsynaptic elements of the synapses.

Little is known about the role of gene activity in the ovarian-driven changes in synaptic structure and function in the hippocampus. To investigate possible genes involved in the regulation of synapse number by estradiol, we examined the expression of three presynaptic markers, synaptophysin (SYN),[2] synaptotagmin I (syt1), and synaptotagmin IV (syt4), in the adult female rat hippocampus and related structures at different phases of the estrous cycle. SYN mRNA is induced in hippocampus during responses to lesioning of the perforant pathway from the entorhinal cortex in an estradiol-dependent manner (48), but changes during the estrous cycle have not been not examined. Our results suggest that ovarian steroid hormones regulate the expression of critical components of presynaptic terminals.

MATERIALS AND METHODS

Animals

Experiments were performed according to the NIH rules and regulations and following review by the USC institutional animal care committee. The minimum number of animals was used to obtain statistically significant results. Three-month-old female F344 rats ($n = 50$) were kept on a 12-h light/dark schedule (7:00 AM/7:00 PM). Vaginal cytology in daily lavages (10:00 AM) were monitored for 1 month to select regularly cycling animals ($N = 36$) for study. Animals were sacrificed across the estrous cycle on the morning (10:00–12:00) (“AM”) of each of three main cycle stages (diestrus, proestrus, estrus) and on proestrus evening (17:30–18:30) (“PM”), when ovulation occurs. The comparison of proestrus AM and PM was chosen to examine mRNA before the preovulatory surge of gonadotropins (LH, FSH) on proestrus PM. The brain sections used in the present study were from rats in which we observed a strong preovulatory LH surge with 30-fold elevations of blood LH at proestrus PM above blood levels at proestrus AM or estrus AM (49). We note that the studies of hippocampal synaptic changes during the normal estrous cycle (65, 66) compared proestrus PM and estrus PM, whereas another study of ovariectomized rats examined the time course after treatment with estradiol or progesterone, but did not specify the exact hour of day at sacrifice (67). Studies of synaptic organization in the hypothalamus during the estrous cycle have compared proestrus and estrus AM and PM (32). However, we chose to include a diestrus AM sample to better establish our baseline AM values. After sacrifice by decapitation under nembutal anesthesia, brains were frozen in isopentane (−18°C) and stored at −70°C until sectioning (16 μm). Coronal sections at 2.8 and 7.0 mm posterior to bregma were used for examination of the hippocampus and entorhinal cortex, respectively.

Preparation of Probes

Synaptophysin. ^{35}S-labeled antisense cRNA was transcribed from the pBluescript transcription vector containing the 2.96-kb fragment of human synaptophysin gene (ATCC, Rockville, MD) with 90% homology to the rat sequence; in the following, synaptophysin refers to synaptophysin I.

Synaptotagmins. Antisense oligonucleotides specific for syt1 mRNA (5′-ATCTGACTGCGGATGTTGGTTGCTGAAGCACTTTC-3′; nucleotides 393 to 359 of the 5′-untranslated region (49)) and syt4 mRNA (5′-TGGGGTTGGTTTTGGGGAAATTGCCATTGAGG-3′; nucleotides 570 to 539 corresponding to amino acids 99–110 (56)) were end-labeled using terminal deoxynucleotidyl transferase (Promega, Madison, WI) in the presence of [α-^{35}S]thio-dATP (NEN, Boston, MA; sp act 1250 Ci/mmol) at 37°C for 1 h. The incorporation ratio was about 80%.

In Situ Hybridization

Synaptophysin. Brain sections fixed in 4% paraformaldehyde (0.1 M phosphate, pH 7.4) were washed in phosphate-buffered saline (PBS) and dehydrated in an ethanol series (30–100%). Sections were prehybridized for 1 h at 55°C (prehybridization buffer, 0.75 M NaCl, 50% formamide, 10% dextran sulfate, 0.05 M phosphate, pH 7.4) and hybridized with the ^{35}S-labeled cRNA probe for 3 h at 55°C. Slides were then covered with NTB2 emulsion (Kodak) and exposed for 5 days for cellular analysis. After development, slides were counterstained with cresyl violet (49).

Synaptotagmins. Frozen sections were briefly thawed and fixed in 4% (w/v) paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature (RT). After three washes in PBS (10 min each), sections were dehydrated in increasing concentrations of ethanol and air-dried. Probes were diluted to 5000 cpm/μl in hybridization buffer (50% formamide, 4× SSC, 5× Denhardt’s solution, 1% SDS, 10% dextran sulfate, 0.1 M DTT, 25 μg/ml poly(A), 25 μg/ml poly(C), and 0.25 mg/ml tRNA). Each slide received 60 μl of radioactive hybridization solution and was covered with a parafilm strip. Hybridization was performed overnight at 44°C in a humidified chamber. Sections were washed in 4× SSC at RT for 20 min, 2× SSC at RT for 3–4 h, and a high-stringency wash was carried out in 0.2× SSC at 55°C for 30 min. Sections were dehydrated through graded ethanol concentration containing 0.3 M ammonium acetate, air-dried, and exposed to Hyperfilm βmax (Amersham, Arlington Heights, IL). After 10 days, the film was developed and the sections were dipped in photographic emulsion (NTB2, Kodak, Rochester, NY), developed after 30 days, and subsequently counterstained with hematoxylin–eosin.

Image Analysis and Statistics

Although both film and emulsion autoradiography were performed, only the data from the emulsion autoradiography are presented. Grain density was measured with IPLab Spectrum image analysis software (Signal Analytics Corporation) for three fields (>10 cells/field) for the hippocampus and the entorhinal cortex layers II and III for each brain. All slides were coded to ensure blind measurement. Unlabeled cells had signal intensity less than 1% of that of labeled cells.

Data were analyzed by two-way ANOVA on SuperANOVA statistical software (Abacus Concepts, Berkeley, CA).

RESULTS

The structures selected for this study are diagramed in Fig. 1: hippocampus, entorhinal cortex, and their interconnections (Fig. 1). The CA3 projections to the CA1 field (Schaffer collaterals) are topographically organized such that CA3 pyramidal cells located near the dentate gyrus (proximal CA3) tend to project more heavily to the distal portions of CA1 (near the subicular border), while CA3 cells located more distal from the dentate gyrus project more heavily to the CA1 cells near the CA2 border (1). In layer II of entorhinal cortex, the majority of neurons are stellate cells, and their axons constitute the principal source of fibers for the perforant pathway projection to the dentate gyrus and CA3. In layer III of the entorhinal cortex, the most common cell type is the pyramidal cell which projects to CA1 and subiculum, although some stellate cells are also present in this layer and contribute to the perforant pathway to the dentate gyrus (1).

The levels of the mRNAs encoding SYN, syt1, and syt4 were assayed in hippocampal and entorhinal cortex regions by *in situ* hybridization across the estrous cycle, including the time of the preovulatory LH surge on proestrus PM (Figs. 2 and 4). In the proximal CA3 region of the hippocampus, syt1 mRNA levels were about 30% higher on diestrus than on other cycle days (Figs. 2A, 2B, and 3). In the other hippocampal regions analyzed, including CA3 distal from the dentate gyrus, syt1 mRNA levels remained the same throughout the estrous cycle. Syt4 and SYN mRNA levels did not change in hippocampus during the estrous cycle, as was evident in the representative hybridization images of animals sacrificed on diestrus (or estrus) and the morning of proestrus (Figs. 2C–2F and 3).

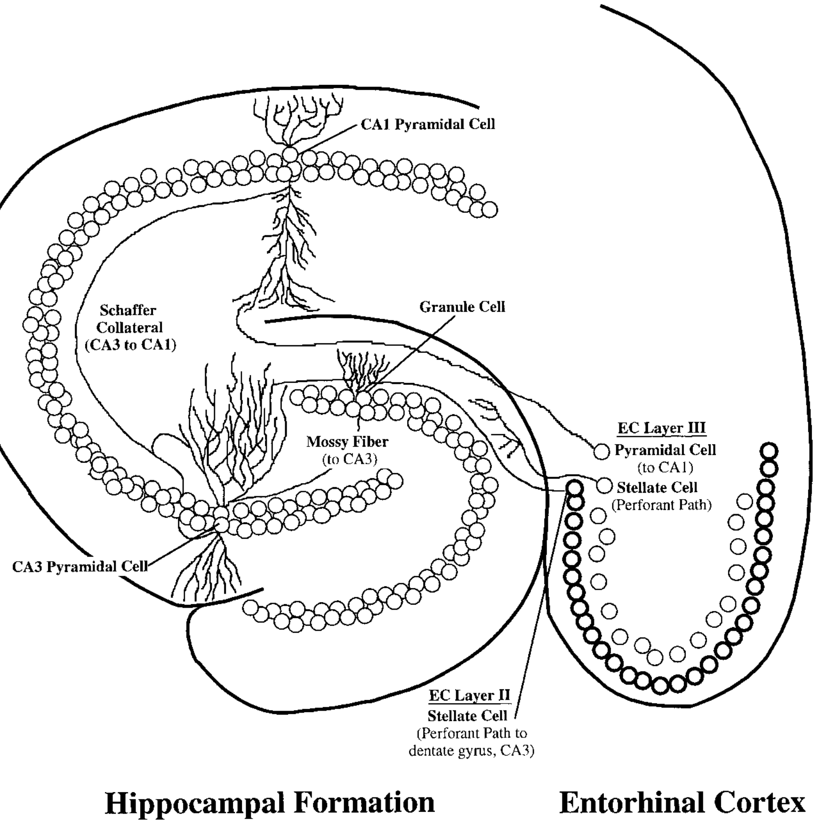

FIG. 1. Schematic diagram of hippocampus, entorhinal cortex, and their cellular connections. A horizontal section located 4.74 mm ventral to bregma shows both hippocampus and entorhinal cortex in the same plane.

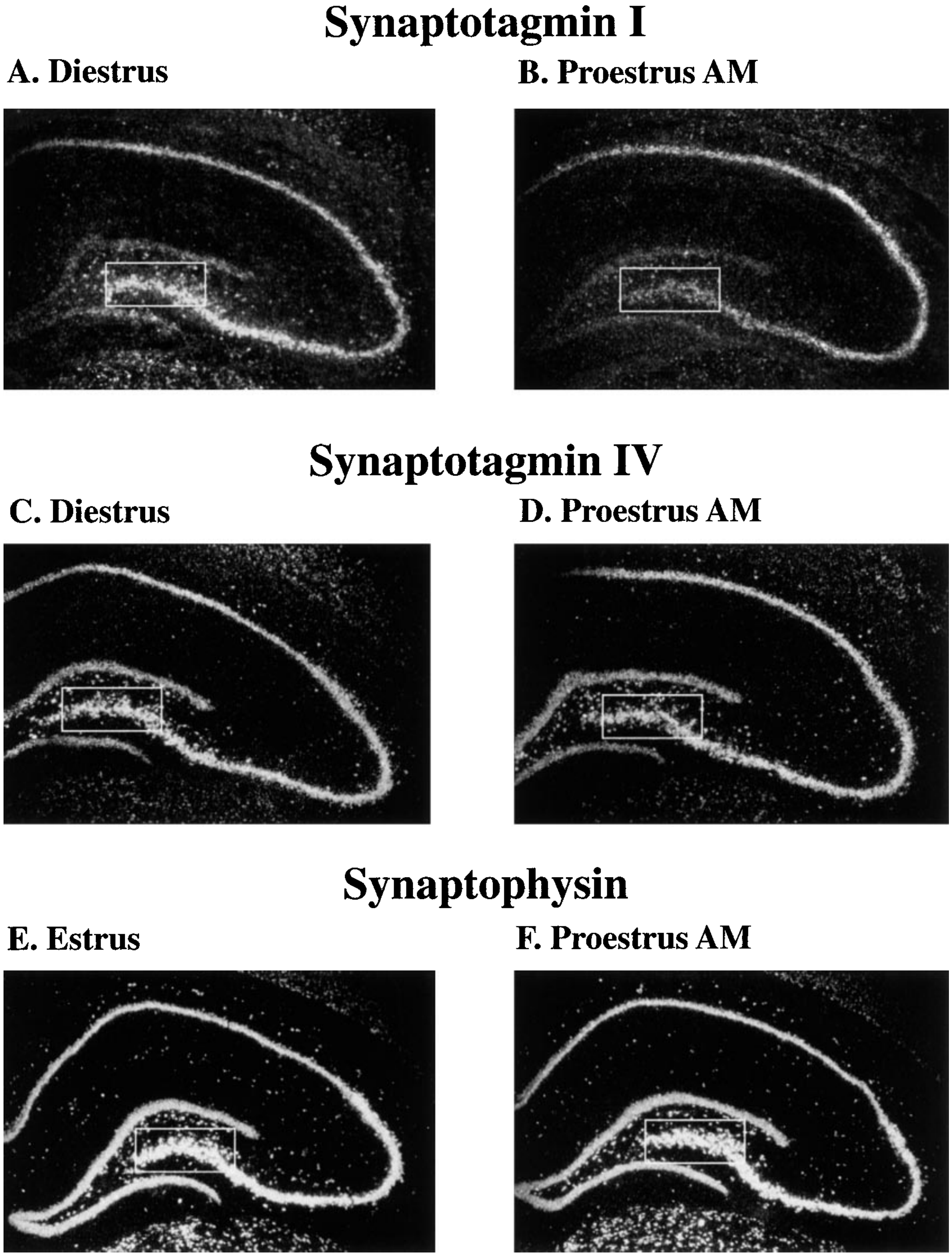

FIG. 2. Representative dark-field images of *in situ* hybridization signals in the hippocampus of female rats at different phases of the estrous cycle. *In situ* hybridization for the different presynaptic markers was performed as described under Materials and Methods. (A and B) ^{35}S-oligonucleotide probe specific for syt1; (C and D) ^{35}S-oligonucleotide probe specific for syt4; (E and F) ^{35}S-riboprobe for SYN. A statistically significant difference in grain density was observed in the proximal CA3 region (box). Magnification, ×200.

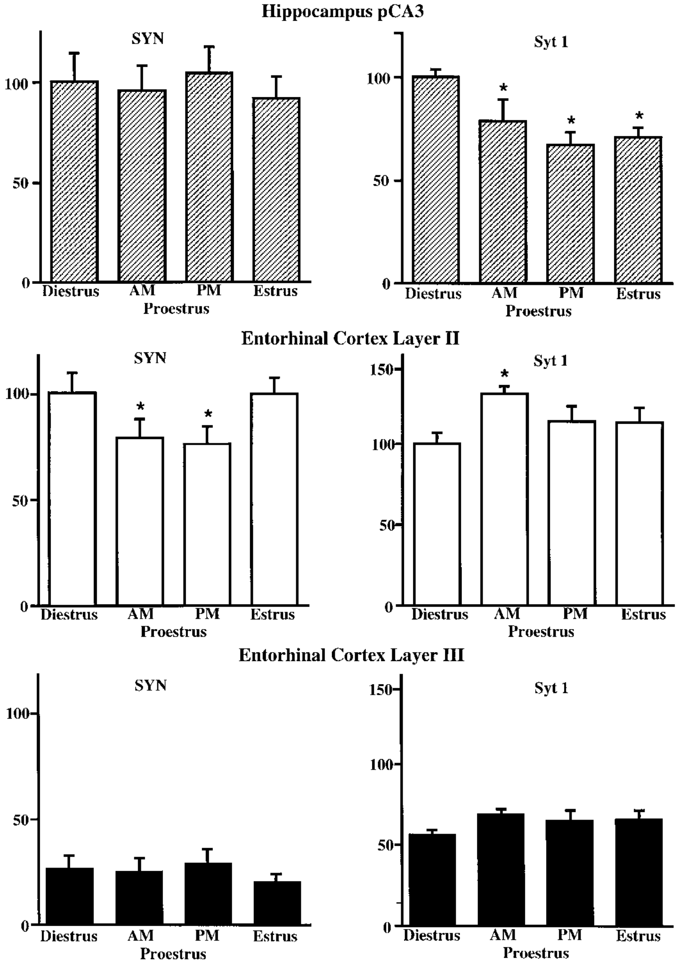

FIG. 3. *In situ* hybridization analysis of SYN and syt1 mRNA in CA3 and in layers II and III of the entorhinal cortex during the rat estrous cycle. The proximal CA3 region (pCA3) is the area indicated by a box in Fig. 2. In other hippocampal regions analyzed, no significant difference was observed throughout the estrous cycle. For hippocampus and entorhinal cortex layer II, data (mean ± SEM; $n = 9$/group) are expressed as a percentage of the values measured on diestrus. For entorhinal cortex layer III, data are expressed as percentage of the values measured on diestrus in layer II, in order to represent the differences in expression between the two cortical layers. $^*P < 0.05$.

In the entorhinal cortex layer II, SYN mRNA was lower on proestrus (AM or PM) than on estrus or diestrus (Figs. 3, and 4E, 4F), whereas syt1 mRNA level was increased by about 30% on proestrus (AM) (Figs. 3, 4A, and 4B). In contrast, syt4 mRNA levels did not differ between cycle days (Figs. 4C and 4D). In entorhinal cortex layer III, no mRNA changes were detected during the estrous cycle.

We note several important regional differences in the expression of these different presynaptic proteins (Figs. 3, and 4): in entorhinal cortex layer III, SYN mRNA levels were 70% and syt1 mRNA levels were 40% lower than in layer II; in contrast, syt4 mRNA levels were relatively invariant between the two layers of entorhinal cortex.

DISCUSSION

In vertebrate animals, steroid hormones play a critical role in neuronal development and also greatly influence the fine tuning of neuronal circuitry. In addition, periodic changes in ovarian steroid levels during fertility cycles produce subtle modifications of specific behavioral and cognitive activities, including learning ability, both in humans and in rats (20, 21, 46, 60). Cyclic changes in behavioral patterns generally occur in parallel with electrophysiological and morphological changes in certain neurons during the female estrous cycle (30, 32, 59). In particular, the CA1 region of the hippocampal formation of female rats undergoes transient synaptic remodeling during the estrous cycle (65). Our results indicate that steroid hormone-mediated changes in the expression of different presynaptic proteins might participate in synaptic remodeling in the CA1 region of adult female rats.

Synaptotagmin I mRNA Levels Are Modulated during the Estrous Cycle in the CA3 Region of Hippocampus and in Enthorinal Cortex

Synaptotagmin belongs to a family of integral membrane proteins present in synaptic vesicles that includes at least 11 related genes (2, 51, 58), each of which encoding a single transmembrane region and two C2 domain repeats homologous to the regulatory domain of protein kinase C (51). Although several synaptotagmins are neuron specific, others are expressed outside the nervous system (25). Syt1 is the first identified integral membrane protein of synaptic vesicles and the best-characterized member of this family (29) (see Fig. 5 for syt1 structure). On the basis of its general properties and several *in vivo* studies (7, 14, 26, 35), syt1 has been proposed to function as a Ca^{2+} sensor that could trigger the fusion of synaptic vesicles with presynaptic plasma membranes in response to calcium influx (27). Other synaptotagmins may have different roles. In hippocampal neurons, many synaptotagmin genes are coexpressed with syt1, but the calcium-signaling defect phenotype resulting from the loss of syt1 in transgenic mice indicates that there is no compensation by other members of the family (14).

The majority of cell bodies of the neurons projecting to pyramidal cells of CA1 are located in the CA3 region. Thus, the mRNA encoding the proteins of presynaptic terminals in CA1 region is expected to be localized in the perikarya of CA3 neurons. Syt1 mRNA levels fluctuate in CA3 during the estrous cycle, with low levels at the estrus phase when estradiol level is decreasing from its peak at proestrus. This effect was clearest in the proximal portion of CA3. Thus, regula-

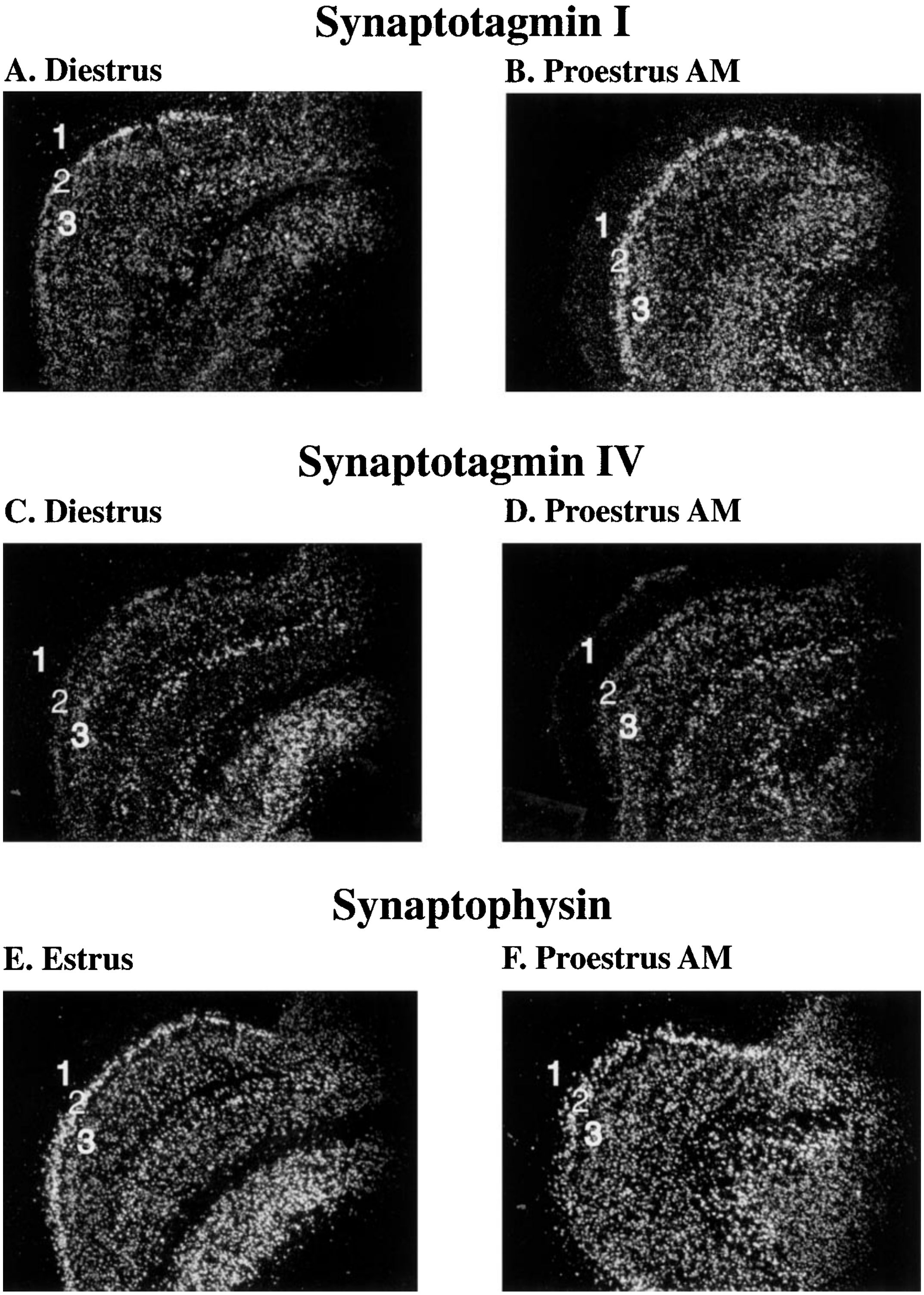

FIG. 4. Representative dark-field images of *in situ* hybridization signals in the entorhinal cortex of female rats at different phases of the estrous cycle. *In situ* hybridization for the different presynaptic markers was performed as described under Materials and Methods. (A and B) ^{35}S-oligonucleotide probe specific for syt1; (C and D) ^{35}S-oligonucleotide probe specific for syt4; (E and F) ^{35}S-riboprobe for SYN. 1, 2, and 3, layers I, II, and III of entorhinal cortex. Magnification, ×200.

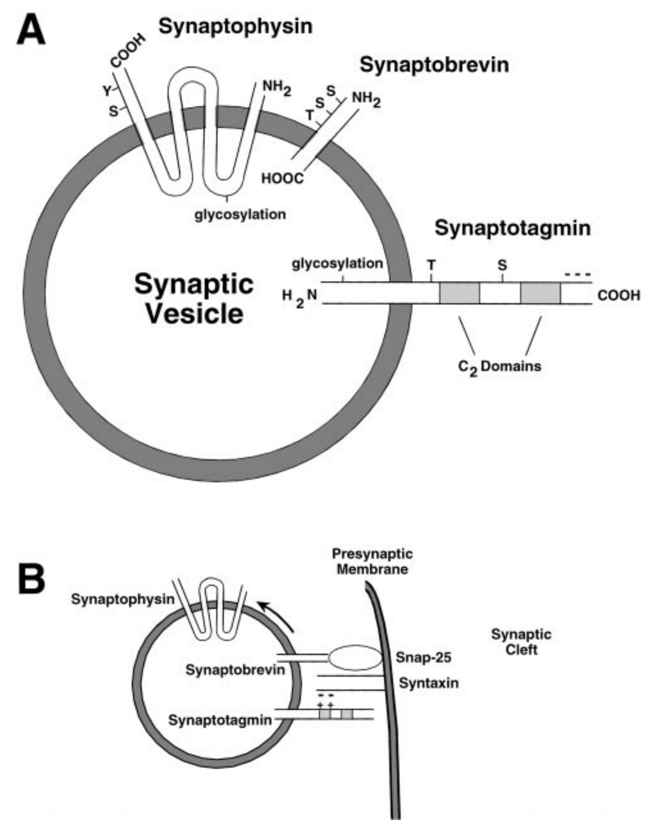

FIG. 5. Schematic diagram of various proteins associated with synaptic vesicles. (A) Topological organization of the proteins in the synaptic vesicle. The N-terminal domain of syt1 is glycosylated and is facing the vesicular lumen, whereas the C-terminal region faces the cytoplasmic side. The C-terminus contains two C_2-domains and a negatively charged short tail. The two C_2-domains bind Ca^{2+} and phospholipid in a Ca^{2+}-dependent manner (8, 10). The first C_2 domain (C_2A) of syt1 mediates Ca^{2+}-dependent binding to syntaxin, a synaptic plasma membrane protein essential for exocytosis (41). The serine (S) and threonine (T) residues are phosphorylated by CAMK II (37) and casein kinase II (4), respectively. Synaptophysin (SYN) contains four transmembrane region, and both amino and carboxyl termini are in the cytoplasmic compartment. The first intravesicular loop of SYN is glycosylated whereas the cytoplasmatic carboxy-terminus tail is phosphorylated on serine and tyrosine residues by CAMK II and pp60c-src, respectively (3, 39). (B) A model of neurotransmitter release that involves interaction of SYN and syt1 with other proteins in presynaptic terminals. Following depolarization and calcium influx, syt1 binds to syntaxin and SNAP-25, whereas synaptobrevin dissociates from SYN. Synaptobrevin subsequently interacts with the syt1–syntaxin–SNAP-25 complex, triggering a cascade of events leading to neurotransmitter release (53).

tion of syt1 gene expression by estradiol may be a mediator of synaptic changes in CA1. However, the levels of syt1 mRNA were highest at diestrus, whereas the highest density of CA1 synapses has been reported to occur at proestrus, i.e., 1 day later (65). This phase difference could arise from delays in translation of the mRNA and the subsequent modification and transport of the proteins. As synaptotagmins and synaptic vesicles are transported by fast axonal transport, our data indicate the possibility of a significant build-up of synaptotagmin before the increase in synaptic density. Thus, the changes in perikaryal syt1 mRNA levels may determine the timing of changes in protein levels and thereby participate in alterations in synaptic density in certain domains of the CA1 region.

We also determined syt1 mRNA levels in layers of the enthorinal cortex, which project to various hippocampal subfields. In particular, layer II of enthorinal cortex projects to CA3, while layer III projects to CA1. In layer II, syt1 mRNA levels increased by 30% in proestrus AM relative to other cycle phases. On the other hand, we detected no change in syt1 mRNA in layer III. These results suggest that cyclic fluctuations of steroid hormones may also influence synaptic density in the distal dendrites of CA3 by modulating presynaptic molecules in the connections between enthorinal cortex and CA3.

Synaptotagmin IV mRNA Levels Do Not Change during Estrous Cycle

Syt4 has unique features as an immediate early gene transiently induced in PC12 cells following depolarization and in hippocampal neurons following kainate-induced seizure activity (56). In contrast, syt1 mRNA is slightly depressed in hippocampus following seizure activity (54). Based on these observations, we hypothesized that syt4 may play a role as a transcription factor in the brain, while syt1 is more directly involved in exocytosis. The present study indicates that the level of syt4 mRNA is relatively invariant in the hippocampus or entorhinal cortex during the estrous cycle. However, this evidence should not discourage examination of levels of syt4 protein, because of the importance of posttranscriptional regulation in many other systems.

Synaptophysin mRNA Levels Are Modulated Only in the Enthorinal Cortex during the Estrous Cycle

Synaptophysins are among the most abundant synaptic vesicle proteins. Two different synaptophysins have been identified in the brain, synaptophysin I (22, 64) and synaptophysin II/synaptoporin (24). SYN is associated with another membrane protein of the synaptic vesicle, synaptobrevin (VAMP) (11, 61). A multimolecular complex of synaptophysin–synaptobrevin is implicated in the process of vesicle docking and fusion with the presynaptic membrane. SYN expression is increased in entorhinal neurons exhibiting sprouting following lesions (5, 48).

SYN mRNA levels changed in layer II of enthorinal cortex during the estrous cycle, becoming lowest on proestrus. In contrast to syt1, however, no change was observed in hippocampus. Thus, SYN, syt1, and syt4 displayed different patterns of expression during the estrous cycle. Although each protein is present in presynaptic terminals, they are structurally different and have distinct roles in synaptic physiology.

We briefly note the regulation of other genes by estradiol which are pertinent to synaptic remodeling in the hippocampus. In previous studies, we observed changes during the estrous cycle in expression of apolipoprotein E (apoE); apoE is secreted by astrocytes and is a transporter of lipids used in synaptic remodeling (47). In CA3 neuropil, apoE mRNA is highest at diestrus, which is in parallel with syt1 mRNA as described above. In CA3, apoE mRNA shows a reciprocal pattern, with a peak at proestrus. Further studies are needed to define, at the cellular level in different hippocampal circuits, how apoE and other astrocyte genes may be coordinately regulated with neuronal genes during localized synaptic remodeling. Other genes of potential interest which are induced by estradiol in the hippocampus or other neuronal systems include the presynaptic protein GAP-43 (neuromodulin) (43), glutamatergic system components NMDAR1 (13) and GluR1 (19), glutamic acid decarboxylase (62), and neurotrophin system components NGF (15, 44), BDNF (40), and trkA (15).

Mechanisms of Synaptic Transmission

A large number of vesicle-associated proteins have been identified and incorporated in models of neurotransmitter release (45). One of the most recent models suggests that synaptobrevin and SYN are bound to each other before synaptic vesicles fuse with the plasma membrane. Following an influx of calcium, syt1 (the calcium sensor of synaptic vesicles) interacts with syntaxin and SNAP-25. Synaptobrevin dissociates from SYN and interacts with the syt1–syntaxin–SNAP-25 complex. The arrival of SNAP dislodges syt1, and a new intermediate complex is formed. Subsequent steps leading to the fusion of the synaptic vesicle with the plasma membrane have not yet been completely identified (53).

In addition to the different roles played by SYN and syt1 in transmitter release, these two presynaptic proteins are present in different synaptic vesicle populations. Neurons contain at least two types of secretory organelles: the smaller vesicles (synaptic vesicle, SV) store classical neurotransmitters, whereas neuropeptides are stored in larger heterogeneous vesicles with an electron-dense core (large dense-core vesicles, LDVC). Although both organelles share functions of Ca^{2+}-binding, targeting, and membrane fusion, they differ in their biogenesis, release mechanism (28, 55), site of release (52), and composition of membrane proteins. In particular, syt1 is a constituent of both SV and LDVC, while SYN is largely confined to SV (33, 34, 58). The selective presence of syt1 and SYN in different populations of synaptic vesicles suggests that they may be differentially expressed in different neurons and as a consequence, differentially regulated during the estrous cycle. In addition, SYN and syt are implicated in brain pathology and are both decreased in Alzheimer disease, SYN by about 30% and syt1 by 10% (42). The decrease of these presynaptic proteins could be a factor in the progressive deterioration of neuronal circuitry in AD.

The mechanisms underlying the steroid regulation of these presynaptic markers are not known. Their 5′-upstream promoters do not contain canonical estrogen response elements (ERE sequences) (Stone, unpublished). However, there is increasing evidence for membrane actions of estradiol in the brain, which could be mediated by second messenger systems (12, 17, 18).

The structural modifications described above occur in parallel with electrophysiological modifications during the estrous cycle. In the CA1 region of the hippocampus, neurons exhibit long-term potentiation (LTP) of synaptic transmission, a long-lasting form of synaptic plasticity that is generally considered to be a cellular model of learning and memory. When female rats were tested for LTP in the Schaffer collateral pathway at each phase of the estrous cycle, they showed the greatest degree of potentiation during the afternoon of proestrus when the number of synaptic contacts is the highest (59). The behavioral implications of these changes have been examined by testing female rats in the Morris water maze during the various phases of the estrous cycle. In the spatial version of the task, the best performance of female rats occurred during the estrus phase and the worst during proestrus, while opposite results were obtained in nonhippocampal version of the task (60). On the other hand, when a different protocol was used in the water maze task, the performance of female rats was indistinguishable across the estrous cycle (6). Similarly, no significant alterations in the performance was observed during the cycle in a radial arm task (46). These apparently controversial results may suggest that the hormone-dependent cyclic changes in hippocampal plasticity affect restricted aspects of memory-based task performance.

In conclusion, our data demonstrate that cyclic modulations of syt1 mRNA levels in CA3 could participate in periodic changes in synaptic densities in the CA1 region of hippocampus during the estrous cycle. Fluctuations of syt1 and SYN mRNA levels in the entorhinal cortex suggest that the upstream projection to the hippocampus is also affected by circulating levels of steroid hormones. Furthermore, transcriptional regulation of these key molecules for neurotransmission supports the idea that the presynaptic endings remodeled during the estrous cycle are functional. In contrast to syt1 and SYN, syt4 did not exhibit any changes in hippocampus or entorhinal cortex during the estrous cycle. This observation may imply that third messengers, such as syt4, are less essential to steroid-dependent synaptic remodeling. Thus, the differential regulation of the presynaptic proteins may reflect their distinct roles in the physiology of the synapse and in the plasticity of the nervous system.

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REFERENCES

1. Amaral, D. G., and M. P. Witter. 1985. Hippocampal formation. In *The Rat Nervous System* (G. Paxinos, Ed.), pp. 443–493. Academic Press, San Diego, CA.
2. Babity, J. M., J. N. Armstrong, J. C. L. Plumier, R. W. Currie, and H. A. Robertson. 1997. A novel seizure-induced synaptotagmin gene identified by differential display. *Proc. Natl. Acad. Sci. USA* **94:** 2638–2641.
3. Barnekow, A., R. Jahn, and M. Schartl. 1990. Synaptophysin: A substrate for the protein tyrosine kinase pp60c-src in intact synaptic vesicles. *Oncogene* **5:** 1019–1024.
4. Bennet, M. K., K. G. Miller, and R. H. Scheller. 1993. Casein kinase II phosphorylates the synaptic vesicle protein p65. *J. Neurosci.* **13:** 1701–1707.
5. Bergmann, M., A. Post, I. Rittel, I. Bechmann, and R. Nitsch. 1997. Expression of synaptophysin in sprouting neurons after entorhinal lesion in the rat. *Exp. Brain Res.* **117:** 80–86.
6. Berry, B., R. McMahan, and M. Gallagher. 1997. Spatial learning and memory at defined points of the estrous cycle: effect on performance of a hippocampal-dependent task. *Behav. Neurosci.* **11:** 267–274.
7. Bommert, K., M. P. Charlton, W. Debello, G. J. Chin, H. Betz, and G. J. Augustine. 1993. Inhibition of neurotransmitter release by C2-domain peptides implicates synaptotagmin in exocytosis. *Nature* **363:** 163–165.
8. Brose, N., A. G. Petrenko, T. C. Sudhof, and R. Jahn. 1992. Synaptotagmin: A Ca2+ sensor on the synaptic vesicle surface. *Science* **256:** 1021–1025.
9. Cotman, C. W., and M. Nieto-Sampedro. 1984. Cell biology of synaptic plasticity. *Science* **225:** 1284–1294.
10. Davletov, B. A., and T. C. Sudhof. 1994. Ca^{2+}-dependent conformational changes in synaptotagmin I. *J. Biol. Chem.* **269:** 28547–28550.
11. Edelmann, L., P. I. Hanson, E. R. Chapman, and R. Jahn. 1995. Synaptobrevin binding to synaptophysin: A potential mechanism for controlling the exocytotic fusion machine. *EMBO J.* **14:** 224–231.
12. Foy, M. R., J. Xu, X. Xie, R. D. Brinton, R. F. Thompson, and T. W. Berger. 1999. 17β-Estradiol enhances NMDA receptor-mediated EPSPs and long-term potentiation. *J. Neurophysiol.* **81:** 925–929.
13. Gazzaley, A. H., N. G. Weiland, B. S. McEwen, and J. H. Morrison. 1996. Differential regulation of NMDAR1 mRNA and protein by estradiol in the rat hippocampus. *J. Neurosci.* **16:** 6830–6838.
14. Geppert, M., Y. Goda, R. E. Hammer, C. Li, T. W. Rosahl, C. F. Stevens, and T. C. Sudhof. 1994. Synaptotagmin I: A major Ca2+ sensor for transmitter release at a central synapse. *Cell* **79:** 717–727.
15. Gibbs, R. B., D. Wu, L. B. Hersh, and D. W. Pfaff. 1994. Effects of estrogen replacement on the relative levels of choline acetyltransferase, trkA, and nerve growth factor messenger RNAs in the basal forebrain and hippocampal formation of adult rats. *Exp. Neurol.* **129:** 70–80.
16. 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.
17. Gu, Q., and R. L. Moss. 1996. 17beta-Estradiol potentiates kainate-induced currents via activation of the cAMP cascade. *J. Neurosci.* **16:** 3620–3629.
18. Gu, Q., and R. L. Moss. 1998. Novel mechanism for non-genomic action of 17β-estradiol on kainate-induced currents in isolated rat CA1 hippocampal neurones. *J. Physiol.* **506:** 745–754.
19. Gu, G., F. Varoqueaux, and R. B. Simerly. 1999. Hormonal regulation of glutamate receptor gene expression in the anteroventral periventricular nucleus of the hypothalamus. *J. Neurosci.* **19:** 3213–3222.
20. Hampson, E. 1990. Estrogen-related variations in human spatial and articulatory-motor skills. *Psychoneuroendocrinology* **15:** 97–111.
21. Hampson, E. 1995. Spatial cognition in humans: Possible modulation by androgens and estrogens. *J. Psychiatry Neurosci.* **20:** 397–404.
22. Jahn, R., W. Schiebler, C. Ouimet, and P. Greengard. 1985. A 38,000 dalton membrane protein (p38) present in synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **82:** 4137–4141.
23. Jones, T. A., J. A. Kleim, and W. T. Greenough. 1996. Synaptogenesis and dendritic growth in the cortex opposite unilateral sensorimotor cortex damage in adult rats—A quantitative electron microscopic examination. *Brain Res.* **733:** 142–148.
24. Knaus, P., B. Marqueze-Pouey, H. Scherer, and H. Betz. 1990. Synaptoporin, a novel putative channel protein of synaptic vesicles. *Neuron* **5:** 453–462.
25. Li, C., B. Ullrich, J. Z. Zhang, R. G. W. Anderson, N. Brose, and T. C. Sudhof. 1995. Ca2+-dependent and -independent activities of neuronal and non-neuronal synaptotagmins. *Nature* **375:** 594–599.
26. Littleton, J. T., M. Stern, K. Schulze, M. Perin, and H. J. Bellen. 1993. Mutational analysis of *Drosophila* synaptotagmin demostrates its essential role in Ca++-activated neurotransmitter release. *Cell* **74:** 1125–1134.
27. Littleton, J. T., and H. J. Bellen. 1995. Synaptotagmin controls and modulates synaptic vesicle fusion in a Ca2+-dependent manner. *TINS* **18:** 177–183.
28. Matteoli, M., C. Haimann, F. Torri-Tarelli, J. M. Polak, B. Ceccarelli, and P. De Camilli. 1988. Differential effect of alphalatrotoxin on exocytosis from small synaptic vesicles and from large dense-core vesicles containing calcitonin gene-related peptide at the frog neuromuscular junction. *Proc. Natl. Acad. Sci. USA* **85:** 7366–7730.
29. Matthew, W. D., L. Tsavaler, and L. F. Reichardt. 1981. Identification of a synaptic vescicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. *J. Cell Biol.* **91:** 257–269.
30. McEwen, B. S., and C. S. Woolley. 1994. Estradiol and progesterone regulate neuronal structure and synaptic connectivity in adult as well as developing brain. *Exp. Gerontol.* **29:** 431–436.
31. Meyer, G., R. Ferres-Torres, and M. Mas. 1978. The effect of puberty and castration on hippocampal dendritic spines in mice. *Brain Res.* **155:** 108–112.
32. Naftolin, F., G. Mor, T. L. Horvath, S. Luquin, A. B. Fajer, F. Kohen, and L. M. Garcia-Segura. 1996. Synaptic remodeling in the arcuate nucleus during the estrous cycle is induced by estrogen and precedes the preovulatory gonadotropin surge. *Endocrinology* **137:** 5576–5580.
33. Navone, F., R. Jahn, G. Di Gioia, H. Stukenbrok, P. Greengard, and P. De Camilli. 1986. Protein p38: An integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. *J. Cell Biol.* **103:** 2511–2527.

34. Navone, F., G. Di Gioia, R. Jahn, M. Browning, P. Greengard, and P. De Camilli. 1989. Microvesicles of the neurohypophysis are biochemically related to small synaptic vesicles of presynaptic nerve terminals. *J. Cell Biol.* **109:** 3425–3433.
35. Nonet, M. L., K. Grundahl, B. J. Meyer, and J. B. Rand. 1993. Synaptic function is impaired, but not eliminated in C. elegans mutants lacking synaptotagmin. *Cell* **73:** 1291–1305.
36. Olmos, G., F. Naftolin, J. Perez, P. A. Tranque, and L. M. Garcia-Segura. 1989. Synaptic remodeling in the rat arcuate nucleus during the estrous cycle. *Neuroscience* **32:** 663–667.
37. Popoli, M. 1993. Synaptotagmin is endogenously phosphorylated by Ca2+/calmodulin protein kinase II in synaptic vesicles. *FEBS Lett.* **317:** 85–88.
38. Purves, D., R. D. Hadley, and J. T. Vodyvodic. 1986. Dynamic changes in the dendritic geometry of individual neurons visualized over period of up to three months in the superior cervical ganglion of living mice. *J. Neurosci.* **6:** 1051–1060.
39. Rubenstein, J. L., P. Greengard, and A. J. Czernik. 1993. Calcium-dependent serine phosphorylation of synaptophysin. *Synapse* **13:** 161–172.
40. Scully, J. L., and U. Otten. 1995. Neurotrophin expression modulated by glucocorticoids and estrogen in immortalized hippocampal neurons. *Mol. Brain Res.* **31:** 158–164.
41. Shao, X., C. Li, I. Fernandez, X. Zhang, T. C. Sudhof, and J. Rizo. 1997. Synaptotagmin–syntaxin interaction: The C2 domain as a Ca2+-dependent electrostatic switch. *Neuron* **18:** 133–142.
42. Shimohama, S., S. Kamiya, T. Taniguchi, K. Akagawa, and J. Kimura. 1997. Differential involvement of synaptic vesicle and presynaptic plasma membrane proteins in Alzheimer's disease. *Biochem. Biophys. Res. Commun.* **236:** 239–242.
43. Shugrue, P. J., and Dorsa. 1993. Estrogen modulates the growth-associated protein GAP-43 (Neuromodulin) mRNA in the rat preoptic area and basal hypothalamus. *Neuroendocrinology* **57:** 439–447.
44. Singh, M., E. M. Meyer, and J. W. Simpkins. 1995. The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague–Dawley rats. *Endocrinology* **136:** 2320–2324.
45. Smith, M. S., M. E. Freeman, and J. D. Neill. 1975. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: Prolactin, gonadotropin, and steroid levels associated with the rescue of the corpus luteum of pseudopregnancy. *Endocrinology* **96:** 219–226.
46. Stackman, R. W., M. E. Blasberg, C. J. Langan, and A. S. Clark. 1997. Stability of spatial working memory across the estrous cycle of Long–Evans rats. *Neurobiol. Learning Memory* **67:** 167–171.
47. Stone, D. J., I. Rozovsky, T. E. Morgan, C. P. Anderson, H. Hajian, and C. E. Finch. 1997. Astrocytes and microglia respond to estrogen with increased apoE mRNA in vivo and in vitro. *Exp. Neurol.* **143:** 313–318.
48. Stone, D. J., I. Rozovsky, T. E. Morgan, C. P. Anderson, and C. E. Finch. 1998. Increased synaptic sprouting in response to estrogen via an apolipoprotein E-dependent mechanism-implications for Alzheimer's disease. *J. Neurosci.* **9:** 3180–3185.
49. Stone, D. J., Y. Song, C. P. Anderson, K. K. Krohn, C. E. Finch, and I. Rozovsky. 1998. Bidirectional transcription regulation of glial fibrillary acidic protein by estradiol in vivo and in vitro. *Endocrinology* **139:** 3202–3209.
50. Sudhof, T. C. 1995. The synaptic vesicle cycle: A cascade of protein–protein interaction. *Nature* **375:** 645–653.
51. Sudhof, T. C., and J. Rizo. 1996. Synaptotagmin: C2-domain proteins that regulate membrane traffic. *Neuron* **17:** 379–388.
52. Thureson-Klein, A. K., and R. L. Klein. 1990. Exocytosis from neuronal large dense-cored vesicles. *Int. Rev. Cytol.* **121:** 67–126.
53. Ting, A. E., and R. H. Scheller. 1995. Insights into the molecular mechanism of synaptic vesicle docking and fusion. *CSH Symp. Quant. Biol.* **60:** 361–369.
54. Tocco, G., X. Bi, L. Vician, I. K. Lim, H. R. Herschman, and M. Baudry. 1996. Two synaptotagmin genes, Syt1 and Syt4, are differentially regulated in adult brain and during postnatal development following kainic acid-induced seizures. *Mol. Brain Res.* **40:** 229–239.
55. Verhage, M., H. T. McMahon, W. E. Ghijsen, F. Boomsma, G. Scholten, V. M. Wiegant, and D. G. Nicholls. 1991. Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. *Neuron* **6:** 517–524.
56. Vician, L., I. K. Lim, G. Ferguson, G. Tocco, M. Baudry, and H. R. Herschman. 1995. Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain. *Proc. Natl. Acad. Sci. USA* **92:** 2164–2168.
57. Von Poser, C., K. Ichtchenko, X. Shao, J. Rizo, and T. C. Sudhof. 1997. The evolutionary pressure to inactivate A subclass of synaptotagmins with an amino acid substitution that abolishes Ca2+ binding. *J. Biol. Chem.* **272:** 14314–14319.
58. Walch-Solimena, C., K. Takei, K. L. Marek, K. Midyett, T. C. Sudhof, P. De Camilli, and R. Jahn. 1993. Synaptotagmin: A membrane constituent of neuropeptide-containing large dense-core vesicles. *J. Neurosci.* **13:** 3895–3903.
59. 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:** 26–30.
60. Warren, S. G., and J. M. Juraska. 1997. Spatial and nonspatial learning across the rat estrous cycle. *Behav. Neurosci.* **111:** 259–266.
61. Washbourne, P., G. Schiavo, and C. Montecucco. 1995. Vesicle-associated membrane protein 2 (synaptobrevin-2) forms a complex with synaptophysin. *Biochem. J.* **305:** 721–724.
62. Weiland, N. G. 1992. Glutamic acid decarboxylase messenger ribonucleic acid is regulated by estradiol and progesterone in the hippocampus. *Endocrinology* **131:** 2697–202.
63. Weiler, I. J., N. Hawrylak, and W. T. Greenough. 1995. Morphogenesis in memory formation—synaptic and cellular mechanisms. *Behav. Brain Res.* **66:** 1–20.
64. Wiedenmann, B., and W. W. Franke. 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of Mr 38,000 charateristic of presynaptic vesicles. *Cell* **41:** 1071–1028.
65. 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.
66. 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.
67. Woolley, C. S., and B. S. McEwen. 1994. Estradiol regulates hippocampal dendritic spine density via an *N*-methyl-D-aspartate receptor-dependent mechanism. *J. Neurosci.* **14:** 7680–7687.