Published in final edited form as:

Eur J Neurosci. 2007 November ; 26(9): 2595–2612.

Changes in hippocampal function of ovariectomized rats after sequential low doses of estradiol to simulate the preovulatory estrogen surge

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Abstract

In adult female rats, robust hippocampal changes occur when estradiol rises on the morning of proestrus. Whether estradiol mediates these changes, however, remains unknown. To address this issue, we used sequential injections of estradiol to simulate two key components of the preovulatory surge: the rapid rise in estradiol on proestrous morning, and the slower rise during the preceding day, diestrus 2. Animals were examined mid-morning of simulated proestrus, and compared to vehicle-treated or intact rats. In both simulated and intact rats, CA1-evoked responses were potentiated in hippocampal slices, and presynaptic mechanisms appeared to contribute. In CA3, multiple population spikes were evoked in response to mossy fiber stimuli, and expression of brain-derived neurotrophic factor was increased. Simulation of proestrous morning also improved performance on object and place recognition tests, in comparison to vehicle treatment. Surprisingly, effects on CA1-evoked responses showed a dependence on estradiol during simulated diestrus 2, as well as a dependence on proestrous morning. Increasing estradiol above the physiological range on proestrous morning paradoxically decreased evoked responses in CA1. However, CA3 pyramidal cell activity increased further, and became synchronized. Together, the results confirm that physiological estradiol levels are sufficient to profoundly affect hippocampal function. In addition: (i) changes on proestrous morning appear to depend on slow increases in estradiol during the preceding day; (ii) effects are extremely sensitive to the peak serum level on proestrous morning; and (iii) there are striking subfield differences within the hippocampus.

Keywords

BDNF; estrous cycle; mossy fibers; object placement; object recognition; potentiation; proestrus

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Introduction

Robust structural and functional changes occur in the hippocampus of the adult female rat on the morning of proestrus, just before ovulation (Woolley, 2007; Scharfman *et al.*, 2003). In area CA1, the activation of pyramidal cells by glutamatergic inputs is potentiated and spine synapse density is increased, and in area CA3, excitability is increased (Woolley, 2007; Scharfman *et al.*, 2003). Hippocampal-dependent learning is enhanced during the day of proestrus, although not under all conditions (Rubinow *et al.*, 2004), and probably not at all times of the day, because progesterone begins to rise in the middle of the day of proestrus, and has profound effects (Sandstrom & Williams, 2001). Hippocampal brain-derived neurotrophic factor (BDNF) synthesis rises during proestrous morning, and may be an important factor in the resultant effects, because BDNF induces long-term potentiation in area CA1 (Tyler *et al.*, 2002; Pang & Lu, 2004), improves hippocampal memory (Tyler *et al.*, 2002; Pang & Lu, 2004), and increases spine synapse density in CA1 (Murphy *et al.*, 1998a; Ji *et al.*, 2005).

The hippocampal changes that have been reported prior to ovulation have been attributed to estradiol, because circulating estradiol peaks on the morning of proestrus, and administration of estradiol to ovariectomized (Ovx) rats mimics some of the effects (Singh *et al.*, 1995; Sohrabji *et al.*, 1995; Foy, 2001; Sandstrom & Williams, 2004; Smith & Woolley, 2004; Woolley, 2007). However, there are concurrent changes in a large number of other hormones and neuromodulators during proestrous morning, including many that are known to have effects on hippocampal function (Rush & Blake, 1982; Barraclough *et al.*, 1984; Bohler *et al.*, 1991; Knobil & Neill, 1994; Haim *et al.*, 2003). Moreover, estradiol administration to Ovx rats has been inconsistent in reproducing the responses observed on proestrus. For example, some hippocampal slice studies have not found potentiation in area CA1 following acute or chronic exposure to estradiol (Barraclough *et al.*, 1999; Ito *et al.*, 1999). Estradiol does not elevate BDNF after chronic administration (Cavus & Duman, 2003). In Ovx rats, both hippocampal-dependent memory and increased CA1 synapse density seem to require circulating levels of estradiol that greatly exceed those observed on the morning of proestrus (Luine *et al.*, 2003; MacLusky *et al.*, 2005). Therefore, it remains unclear whether physiological levels of estradiol are sufficient to induce the changes in hippocampal function observed on proestrus, or whether other factors may also play a role.

One potential reason for the inconsistent results from studies of estradiol action in Ovx rats is that the effects of estradiol may depend on the temporal patterning of hormone exposure. In the intact rat, there is a slow rise in circulating estradiol on the day before ovulation (diestrus 2), followed by a rapid increase on proestrous morning that represents the preovulatory surge (Smith *et al.*, 1975; Haim *et al.*, 2003). Estradiol may have important effects during its rise on diestrus 2, because in the hypothalamus, brief periods of estrogen stimulation profoundly affect subsequent estrogen responses (Krey & Parsons, 1982; Parsons *et al.*, 1982). Estradiol action in the hippocampus could be similarly dependent on the pattern of hormone exposure.

In the present study, we have tested the hypothesis that precise simulation of the time- and dose-dependent rise of estradiol during diestrus 2 and proestrous morning is required for increased hippocampal function on proestrus morning. Therefore, Ovx rats were treated with estradiol to mimic diestrus 2 and the morning of proestrus. Comparisons were also made with rats treated with nonphysiological doses. Indices of hippocampal function included electrophysiological recordings from CA1 and CA3, BDNF immunohistochemistry, and tests of hippocampal-dependent memory. Our results suggest that estradiol treatment of Ovx rats can indeed reproduce hippocampal changes that have been associated with the proestrous morning, but the effects are critically dependent on both the absolute level and temporal sequence of estradiol exposure.

Materials and methods

Animal care and housing

Animal care and use met the guidelines of the National Institutes of Health. Procedures were approved by the Institutional Animal Care and Use Committees of Helen Hayes Hospital and Hunter College. Adult female Sprague-Dawley rats (Taconic Farms, Germantown, NY, USA) were housed two or three per cage, and provided with food (Purina chow) and tap water *ad libitum*. A 12 h light/dark cycle was used (lights on: 7:00 a.m.). The temperature was approximately 21 °C, and humidity ranged from 40% to 70%. All chemicals mentioned below were from Sigma-Aldrich Chemical Co. (St Louis, MO, USA), unless otherwise specified.

For electrophysiological experiments, animals were Ovx after ketamine-xylazine anesthesia (Scharfman *et al.*, 2003), housed in a quiet room near to the laboratories, and acclimated to handling (lifted with the hand, cradled in the arm, and stroked) twice per day. A quiet site for housing, close to the laboratory, as well as acclimatization to handling, were adopted after noticing that animals transported from a remote housing area just before being killed, or animals that had not been handled, showed signs of anxiety (running around in their cage when investigators approached). In slices from these animals, there were often poor responses to mossy fiber stimulation in area CA3, suggesting a confounding influence of stress, consistent with previous reports of acute stress-induced changes to CA3 (McEwen, 1994; Sunanda *et al.*, 1995). Because CA3 neurons project to area CA1 pyramidal cells, and this projection was to be evaluated, and because stress appears to reverse changes that occur on proestrus (Shors *et al.*, 2001), care was taken to avoid stress to the subjects.

Vaginal cytology, ovariectomy, and estradiol treatment

Vaginal cytology was examined as previously described (Edwards *et al.*, 1999; Scharfman *et al.*, 2003). In brief, 2–3 month old animals were examined daily between 10:00 a.m. and 12:00 p.m., using a 30 µL sample from the vaginal cavity to evaluate the relative abundance of leukocytes, epithelial cells, or cornified epithelial cells. A cycle was defined by four successive days as follows: (i) primarily epithelial cells (proestrus); (ii) primarily cornified epithelial cells (estrus); (iii) mostly leukocytes (metestrus, the first day of diestrus); and (iv) a mixture of cell types (diestrus 2). Previous studies confirmed the ability of vaginal cytology to predict the cycle stage using these methods (Scharfman *et al.*, 2003). Only animals with at least three consecutive 4 day estrous cycles were used for experiments. Ovx was conducted as previously described (Scharfman *et al.*, 2003).

Estradiol benzoate (EB) or 17β-estradiol (E2) stock solutions were made by diluting a concentrated stock solution (1 mg of EB or E2 in 1 mL of 100% ethanol) in corn oil (Mazola brand, AH Food Co., Cordova, TN, USA). Diluted stock solutions were stored in scintillation vials within a desiccant-filled plastic container, in the dark at room temperature. Doses were administered subcutaneously in the back, using a 25 gauge 3/4 inch needle and 1 mL syringe with accuracy to 0.05 mL. To avoid stress during injection, animals were gently held with one hand, swung back and forth, and then placed on a table for the injection. The injection volume (approximately 0.3 mL) was kept constant by using stock solutions containing different concentrations of the estrogens (3 µg/mL for a 3 µg/kg dose, 4 µg/mL for a 4 µg/kg dose, etc.).

Blood collection and estradiol radioimmunoassay (RIA)

Following CO₂ anesthesia and decapitation, trunk blood was collected and centrifuged within 1 h; serum was stored at –20 °C. Radioimmunoassays for estradiol, progesterone and testosterone were conducted by an investigator who was blind to treatment, using kits provided by Diagnostic Products Corporation (Los Angeles, CA, USA), as previously described (Scharfman *et al.*, 2003). Serum corticosterone was assayed by enzyme-linked immunosorbent

assay (ELISA) using a kit purchased from Cayman Chemicals Inc. (Ann Arbor, MI, USA). For the eight individual estradiol assays used in obtaining the data reported in this study, the interassay coefficients of variation were 16.7% at 20 pg/mL, 9.2% at 50 pg/mL, and 7.5% at 90 pg/mL. Mean intra-assay coefficients of variation were 16.1% at 20 pg/mL, 9.2% at 50 pg/mL and 7.3% at 90 pg/mL.

Slice preparation and maintenance

Subjects were either Ovx rats or intact rats that were killed mid-morning (approximately 10:30 a.m.) of proestrus. Ovx occurred at approximately 7 weeks of age (7.1 ± 0.36 weeks, range 6–11 weeks, $n = 20$). The first injection was approximately 2 weeks after Ovx (mean 17.3 ± 1.2 days, range 11–27 days, $n = 20$).

Slice preparation—An animal was transferred within its home cage to a tabletop near the area used for slice preparation, and the entire cage was covered by a clear plexiglas container. CO₂ was vented into the chamber for approximately 30 s, at which point the animal lost postural tone and was unresponsive when the foot was pinched. The animal was removed from the cage and decapitated immediately. Trunk blood was collected at this time by one investigator, who also confirmed the absence of ovaries, and removed the uterus for weight determination. The uterus was severed from the cervix, blotted on tissue paper to remove luminal fluid, and weighed immediately.

Another investigator removed the brain and immediately immersed it in ice-cold artificial cerebrospinal fluid (ACSF) containing sucrose instead of NaCl (in mM: 126 sucrose, 5.0 KCl, 2.0 CaCl₂, 2.0 MgSO₄, 1.25 NaH₂PO₄, 26.0 NaHCO₃, 10.0 D-glucose, pH 7.4). After removal of a section of the dorsal surface to provide a flat base, cyanoacrylate was used to glue the dorsal surface to a prechilled Teflon-coated holding tray. The tray was immersed in ice-cold ACSF, and horizontal sections (400 μ m thick) were cut with a Vibroslice (Stoelting Co., Wood Dale, IL, USA). Sections were immediately trimmed, while still immersed in ice-cold ACSF, so that they contained the hippocampus and overlying parahippocampal cortex. Slices were transferred in sucrose–ACSF with a wide-bore Pasteur pipette (so that they were transferred in fluid, which would inflict the least injury on the slice) to the base of a beaker with approximately 100 mL of oxygenated (95% O₂, 5% CO₂) sucrose–ACSF at room temperature. After all slices from one hemisphere were sectioned, slices were transferred to a recording chamber. The recording chamber was similar to the chamber available from Fine Science Tools, with modifications to increase humidification to the inner well, where slices were placed (Scharfman *et al.*, 2001). Slices were positioned on a nylon net in this inner well, with perfusion from below with prewarmed (31–32 °C), oxygenated (95% O₂/5% CO₂) sucrose–ACSF, so that they were submerged except for their upper surface. Warm, humidified air was vented over the slices from a compartment surrounding the area where slices were positioned. After 30 min, sucrose–ACSF was switched to ACSF that included NaCl, substituted in an equimolar amount for sucrose (NaCl–ACSF). Recordings were started 30 min later. Recordings were made only for 3 h thereafter, because in some estradiol-treated animals, evoked responses appeared to decline after that time, although they did not in vehicle-treated rats or in previous studies of male rats. To avoid the potential for this possible decline to confound the results, the recording period was restricted. The flow rate was approximately 1 mL/min. For each experiment, slices were sequentially evaluated to assess viability. Only those slices that passed the criteria for viability (see below) are included in Results.

Recording and stimulation—Extracellular recording electrodes were made from borosilicate capillary-filled glass (inner diameter 0.6 mm, outer diameter 1.0 mm; World Precision Instruments, Sarasota, FL, USA) pulled horizontally (Flaming-Brown Model P97; Sutter Instruments, Novato, CA, USA) and filled with NaCl–ACSF (resistance, 5–10 M Ω).

For stimulation of afferents, a monopolar stimulating electrode made from Teflon-coated stainless steel wire (75 μm diameter; A&M Systems, Carlsborg, WA, USA) was used. Rectangular current pulses were used to stimulate; they were triggered digitally using an interval generator (Pulsemaster; World Precision Instruments, Sarasota, FL, USA) and stimulus isolator (AMPI Instruments, Jerusalem, Israel). Stimulus strength was changed by varying pulse duration (10–300 μs) while keeping pulse amplitude constant (100 μA). Signals were amplified (Axoclamp 2B; Molecular Devices, Sunnyvale, CA, USA), recorded digitally (Pro 10 oscilloscope; Nicolet Instruments, Madison, WI, USA), and analysed offline using Nicolet software and Origin 7.5 (OriginLabs, Northampton, MA, USA).

Stimulation and recording sites are shown in Fig. 1. In all experiments, the recording electrodes were placed at the depth in the slice that produced a maximal response, usually 50–100 μm below the surface. The stimulating electrode was placed gently upon the slice surface so that it rested upon it but did not depress it. For area CA1 recordings, the Schaffer collaterals were stimulated at the border of CA1 and CA2, approximately 200 μm from the pyramidal cell layer, as verified by an ocular micrometer in the dissecting microscope used to visualize the slice. This position was near the end of the white fiber bundle of the Schaffer collaterals, which could be discriminated by transillumination of the slice. The recording electrode was placed 400–500 μm away in CA1b. To record population spikes, the recording electrode was placed on the border of the pyramidal cell layer and the stratum oriens, and a depth profile was conducted between the stratum radiatum and the stratum oriens (Fig. 1). A site in the cell layer was chosen that provided the largest population spike but also elicited a spike that was superimposed on a large positivity (Fig. 1B, PCL). Population spikes that were recorded closer to stratum radiatum were not completely superimposed on a large positivity, reflecting the fact that they were recorded too close to the site of synaptic input (Fig. 1B, PCL/RAD), and therefore were a suboptimal representation of the somatic response to stimulation. To standardize population spikes across slices, efforts were made to avoid these locations. In addition, population spikes that were too close to stratum oriens were avoided by rejecting locations that demonstrated a relatively small population spike superimposed on a very large positivity (Fig. 1B, PCL/OR).

Extracellular recordings of excitatory postsynaptic potentials (EP-SPs) (field EPSPs) were made in stratum radiatum at the point that corresponded to the Schaffer collateral input to apical dendrites of the cells used to record the population spike (Fig. 1A). In each slice, several sites were tested in stratum radiatum, so that the site where the field EPSP had the most rapid rise could be identified (Fig. 1).

For area CA3 recordings, the stimulating electrode was positioned in the subgranular zone of the dentate gyrus at the junction between the two blades (the ‘apex’; Fig. 1A). Mossy fiber input to CA3 pyramidal cells is best stimulated at this site, because other sites in the mossy fiber pathway usually activate CA3 pyramidal cells directly or by the CA3 recurrent collaterals (Claiborne *et al.*, 1993). To confirm preferential mossy fiber activation, recordings were made in stratum radiatum, stratum lucidum, and the pyramidal cell layer of CA3b. This allowed confirmation that the field EPSP evoked by subgranular zone stimulation was maximal in stratum lucidum, and had reversed polarity in stratum radiatum (Scharfman *et al.*, 2003). The site chosen to record the CA3 population spike was between the pyramidal cell layer and stratum oriens, i.e. where population spike amplitude was maximal (Fig. 1C) (Scharfman *et al.*, 2003).

At the onset of each experiment, slices were screened to determine which were viable. Slices that were accepted for study had a maximal population spike in areas CA1 and CA3 that was at least 5 mV, measured as described below (see Data analysis). These criteria were based on previous studies, which showed that numerous, healthy pyramidal neurons were impaired by

intracellular electrodes in slices with such field potentials, but were rare in slices with lesser field potentials.

For any given slice, the choice of recording first in area CA1 (vs. area CA3) was made randomly, in order to avoid any effects of CA3 recordings on CA1 responses, or vice versa. In addition, responses to stimulation in area CA1 and area CA3 were tested at least 5 min apart. There was no evidence of an influence of recording order.

BDNF immunohistochemistry

Methods for BDNF histochemistry have been described previously (Scharfman *et al.*, 2003). In brief, animals were anesthetized with an overdose of urethane (2.5 g/kg, intraperitoneal) and perfused transcardially with saline for 3 min, followed by 2% paraformaldehyde (in 0.1 M phosphate buffer; pH 7.6) for 10 min. Brains were removed after 1 h, postfixed in 2% paraformaldehyde for 4 h on a rotator at 4 °C, and transferred to 30% sucrose in 0.1 M phosphate buffer. They were placed on a rotator at 4 °C overnight, and cut on a cryostat the next day (50 µm thick sections). One hemisphere was cut coronally and the other in the horizontal plane.

Free-floating sections were incubated in a series of solutions, using approximately 5 mL of solution per five sections, in trays with wells that were pre-rinsed in 0.05% bovine serum albumin (BSA). Sections from different animals were processed concurrently. Initially, sections were washed in Tris buffer (pH 7.4; 3 × 5 min), and they were then transferred to 0.3% H₂O₂ in Tris for 15 min. Following washing in Tris buffer (3 × 5 min), sections were incubated in 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) in Tris-x (0.3% Triton X-100, 0.75% BSA in Tris), washed (Tris-x, 3 × 5 min), and then placed overnight on a rotator at 4 °C in primary antibody (1 : 10 000; rabbit polyclonal, a kind gift of Amgen Regeneron Partners, Tarrytown, NY, USA) (Scharfman *et al.*, 2003) in Tris-x. The next day, sections were washed in Tris-y (0.05% Triton X-100, 0.25% BSA in Tris; 3 × 5 min), and this was followed by incubation for 1 h in secondary antibody (biotinylated anti-rabbit IgG raised in goat; 1 : 400; Vector), washing in Tris-y (3 × 5 min), and 2 h of incubation in ABC (1 : 1000; Vector Elite kit) in Tris-z (0.75% BSA in Tris). Sections were washed in Tris (3 × 5 min) and developed using nickel-intensified (5 mM NiCl₂) diaminobenzidine (1 mg/2 mL Tris). Sections were mounted on gelatin-coated slides, dehydrated in a graded series of ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA). Micrographs were taken using a digital camera (Spot camera Model 2.2.1; Diagnostic Instruments, Sterling Heights, MI, USA) and an Olympus BH-2 microscope (Olympus America, Center Valley, PA, USA), and Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA, USA) was used to compose figures. The same illumination was used when photographs were taken with the digital camera, and identical brightness and contrast settings were used when compiling images into a single figure using Photoshop.

In separate experiments, a different antibody to BDNF (mouse monoclonal; Sigma) was used with slightly different procedures. Blocking serum was normal horse serum (10%; Vector) and the primary antibody concentration was 1 : 1000. The secondary antibody was biotinylated anti-mouse IgG raised in horse (1 : 400; Vector). Use of a second antibody allowed an opportunity to confirm the results from the first antibody.

Data analysis of BDNF immunoreactivity in the mossy fibers was conducted using ImageJ software. Before analysis, sections were digitally photographed using the same camera and light settings. For a given section, a mean gray scale value was calculated from the last 50 µm of the terminal field of the mossy fiber bundle at the CA2/CA3 border. To establish this value, a viewing frame within this area was outlined. This was done three times and the values were averaged. The terminal field was chosen because it is the location where BDNF is most concentrated in the mossy fiber pathway. Next, three viewing frames were sampled from an

area of the hippocampus that does not contain BDNF, the fimbria. This sample was measured as an estimate of nonspecific (background) staining. The final gray scale value was calculated by subtracting the background value from the mean gray scale value. The values for maximal (0; black) and minimal (255; white) darkness were set by the computer, and manually verified. The analysis used only sections from a selected part of the dorsal hippocampus (approximately 2–2.5 mm posterior to Bregma) (Paxinos & Watson, 1986), because BDNF in mossy fibers varies along the septotemporal axis of the hippocampus.

Behavior

For animal care and housing, the methods used were similar to those described above but were conducted at a different laboratory (V.N.L.). Animals were Ovx at the vendor (Harlan Sprague Dawley, Indianapolis, IN, USA) and shipped 1 week later. The light/dark cycle was 14 : 10 (14 h lights on and 10 h lights off; lights on at 7 a.m.). One week after shipment, animals began 2 weeks of habituation, consisting of placement in an open field and then habituation to objects on the field and movement of the objects, as in the recognition memory tasks; see Luine *et al.* (2003) for details. In addition, animals were habituated to subcutaneous injections by giving two saline injections on 2 days during the 2 week habituation period. Habituation and injections were done to avoid stress prior to, and during, the behavioral tests.

At the end of the 2 weeks, estradiol or vehicle was administered, and 2 h after the last injection, testing began with the sample trial (T1) according to previously published protocols (see Luine *et al.* (2003) and Wallace *et al.* (2006) for details. Animals were placed in an open field with two identical, novel objects). The number of seconds spent exploring objects was measured for 3 min. A retention trial (T2) was administered 4 h later. During the retention trial, one object was placed in a new position (for the object placement test) or one was exchanged for a new one (for the object recognition test). The time spent exploring either the old (familiar) object or the new object (or the object placed in the new location) was measured for 3 min.

Object placement and object recognition were chosen to test memory performance because these tests do not stress the animal (in contrast to food deprivation and forced swimming as used in the radial arm or Morris water maze tests) and trials can be completed within a few hours. Another important aspect of object recognition and object placement tests is that both involve the hippocampus, but object placement demonstrates greater hippocampal dependence than object recognition (Ennaceur *et al.*, 1997; Broadbent *et al.*, 2004; Moses *et al.*, 2005).

Data analysis

Electrophysiology—Population spike amplitude was defined as the average of the negative and positive voltage deflections that comprise the population spike; for CA1, see Alger & Teyler (1976) and Scharfman *et al.* (2003); for CA3, see Scharfman (1997). The amplitude of the fiber volley and field EPSP were defined as the voltage deflections from the pre-stimulus potential to the peak of either the volley or field EPSP (Scharfman, 1997). Field EPSP slope was defined as the maximal slope during the rising phase of the field EPSP. It was calculated offline, from digitized field EPSPs, and defined as the maximal voltage deflection per 50 μ s during the maximal rising phase.

Input–output curves were constructed from responses to increasing stimulus durations, using a 100 μ A rectangular stimulus that was increased by steps of 10–20 μ s from 10 to 300 μ s. Stimulus frequency was ≥ 0.03 Hz. Stimuli greater than 300 μ s were not used, because increasing stimulus strength above 300 μ s did not lead to a further increase in amplitude in any of the slices that were evaluated.

Paired pulse inhibition of the population spike was examined using 7, 10, 20 and 40 ms interstimulus intervals, because these provided a comprehensive overview of the time course of inhibition. Thus, population spikes were typically inhibited completely when 7 ms interstimulus intervals were used, but slowly decayed so that at the 40 ms interstimulus interval some facilitation occurred. This provided a means to examine the hypothesis that estradiol might influence the maximum, or decay, of paired pulse inhibition (or both). Paired pulses were tested using stimulus intensities that were approximately 25–35% of the maximum, half-maximal, and 80–90% of the maximum, to examine the possibility that inhibition would be influenced differentially if afferent input was weak vs. strong.

Statistics—Means ($\pm$ SEM), sample sizes and statistics are listed in Results or legends to tables and figures. Programs used for statistical analysis included Microsoft Excel 2000 (Microsoft, Redmond, WA, USA) and Statview (SAS Institute, Cary, NC, USA). Student's *t*-tests assumed unequal variance, and were two-tailed. The criterion for statistical significance was set at $P < 0.05$.

For electrophysiology, all slices from one animal were placed in a recording chamber immediately after dissection and evaluated in random order. Slices reaching criteria for viability (as discussed above) were selected for study. For an individual animal, 3–6 slices reached criteria. The following numbers of animals were used: V-V-V, $n = 4$; V-V-3, $n = 4$; 3-4-V, $n = 4$; 3-4-3, $n = 4$; 3-4-5, $n = 3$; 2-2-5, $n = 3$; proestrous morning, $n = 3$. More than one slice was used from each animal. Data from slices of animals within the same treatment group were pooled because there were no detectable differences in the variance of responses among slices, whether they were from the same animal or from different animals. There also was no evidence that effects varied as a function of dorsal vs. ventral location. For different measurements, the sample sizes differ because every test was not conducted in every slice.

Data relating CA1 population spike amplitude to different stimulus durations were analysed by computer-assisted nonlinear least squares curve fitting, as previously described (Scharfman *et al.*, 2003), using the programs developed by DeLean *et al.* (1978). The individual curves describing the relationship between output spike amplitude (y) and stimulus duration (x) at each stage of the cycle were fitted to a sigmoidal four-parameter logistic function $y = D + (A - D/[1 + (x/C)^B])$, where A and D are the expected maximum and minimum responses, respectively, B is the Hill slope coefficient, and C is the stimulus duration expected to generate a half-maximal response (abbreviated for the purposes of this study as ES_{50}). As CA1 population spikes were never observed when stimulus durations were less than 20 μ s, the expected minimum response was fixed at zero in all cases. Constrained curve fitting and analysis of variance (DeLean *et al.*, 1978) were utilized to test the significance of differences between the calculated coefficients in hippocampal slices from animals treated with different doses of estrogen. Analysis of residual variance was used to compare the variance of the data points around the 'best fit' curves for each dataset.

Results

Estradiol treatment

A hormone replacement regimen was developed to mimic the slow rise in estradiol during diestrus 2, and the sharp surge in estradiol levels on the morning of proestrus (Smith *et al.*, 1975; Haim *et al.*, 2003). To mimic diestrus 2, we used two injections, 12 h apart, of increasing amounts of EB. Initial studies using 1–2 μ g/kg EB demonstrated that this dose did not elevate serum estradiol above 5–10 pg/mL ($n = 3$), so the effects of higher doses were examined. Therefore, 3 μ g/kg EB was injected, followed 12 h later by a slightly higher dose, 4 μ g/kg. As shown in Fig. 2, this sequence of hormone administration led to a change in serum estradiol

that closely mimicked the slow rise in estradiol in intact female rats during diestrus 2 (Smith *et al.*, 1975).

Because the surge in estradiol on the morning of proestrus is rapid, an injection of E2 was used instead of EB to simulate the proestrous morning. We initially tested the serum levels of estradiol in response to 15 µg/kg E2, because previous studies have demonstrated that this dose is close to the threshold for influencing spine synapses in CA1 (MacLusky *et al.*, 2005), and is sufficient to enhance some, but not all, components of estrogen-sensitive cognitive behavior in the female rat (Luine *et al.*, 2003). The results indicated that serum estradiol levels had risen sharply by 1–2 h, and then subsequently declined by 4–6 h (Fig. 2A). Even at this relatively low dose, however, serum hormone levels at 1–2 h were still approximately five-fold higher than those observed on the morning of proestrus in intact rats (Smith *et al.*, 1975; Scharfman *et al.*, 2003). Therefore, lower doses, ranging between 1 and 5 µg/kg, were tested. The serum estradiol level obtained 2 h after injection of 3 µg/kg E2 (61.6 ± 4.9 pg/mL, $n = 7$) was similar to the peak on proestrous morning (Smith *et al.*, 1975), whereas 1 µg/kg was insufficient (29.0 ± 3.2 pg/mL, $n = 4$), and 5 µg/kg led to supraphysiological levels (89.0 ± 14.3 pg/mL, $n = 6$; Fig. 2B). The 2 h delay was chosen because this is the time when serum estradiol levels are maximal after subcutaneous injection in oil (Hilke *et al.*, 2005). In addition, if the injection was made at 8:30 a.m., 2 h later would be the time of day that is similar to the time when estradiol peaks in intact rats on proestrus. Time of day is potentially important because of the circadian influence on responses to gonadal hormones in the brain (Snabes *et al.*, 1977).

To evaluate the entire regimen (3 µg/kg EB at 8:30 a.m. on day 1, 4 µg/kg EB at 8:30 p.m. on day 1, 3 µg/kg E2 at 8:30 a.m. on day 2, followed by killing 2 h later; the entire protocol is abbreviated ‘3-4-3’ below, and shown schematically in Fig. 2C), 18 animals were divided into six groups to evaluate serum estradiol levels during the 26 h period. Animals were killed at the following times: 2 h after the first dose, 4 h later, 2 h after the second dose, 4 h after that time, and finally, 2 h after the third dose ($n = 3$ per group). Animals were also examined 2 h after vehicle injections, timed in exactly the same way as the estradiol injections (abbreviated ‘V-V-V’ below); this defined ‘baseline’ levels of estradiol in the Ovx rats that we used (<5 pg/mL). The results are shown in Fig. 2D. The 3-4-3 regimen led to serum estradiol levels that closely approximated the values of serum estradiol during diestrus 2 and proestrus morning that have been reported previously for the Sprague-Dawley rat (Smith *et al.*, 1975).

Progesterone and testosterone RIA confirmed that the levels of progesterone and testosterone were low in Ovx animals that were treated with vehicle (progesterone, 0.75 ± 0.13 ng/mL, $n = 3$; testosterone, <10 pg/mL, $n = 4$) or estradiol (progesterone, 0.99 ± 0.40 ng/mL, $n = 13$; testosterone, 19.25 ± 14.25 pg/mL, $n = 4$). Corticosterone ELISA in a subset of these animals showed that corticosterone was not elevated 2 h after the last injection (1.37 ± 0.90 ng/mL, $n = 3$). Corticosterone levels of other animals injected with the same schedule, and killed 2 h after the last dose, also showed that this procedure does not elevate corticosterone (3.97 ± 1.66 ng/mL, $n = 6$). Ovx animals that were age-matched, but did not receive injections of any kind, and were simply killed with methods similar to those used for the above cohort of 18 animals, also demonstrated mean values of corticosterone that were in the normal range (3.45 ± 0.81 ng/mL, $n = 18$).

The cohort of 18 animals discussed above were 20 weeks old at the time of Ovx, and the delay between Ovx and estradiol treatment was 18–19 days. To investigate whether age might influence the relationship between estradiol dose and resultant serum hormone levels, animals were examined at other ages. There were no significant differences in serum estradiol levels attained 2 h after the last injection between 6 and 10 week old rats (66.2 ± 5.1 pg/mL; $n = 5$) and the 20 week old rats (67.2 ± 6.5 pg/mL; $n = 4$; Student’s *t*-test, $t = 0.359$; d.f. 7; $P = 0.730$). Uterine weight was also examined with respect to the age of the animals at the time of Ovx.

Animals that were injected with estradiol after Ovx at a younger age (6–10 weeks old) were less variable than older animals (20 weeks; Table 1), suggesting that the younger animals might have a more consistent response to the estrogen. Previous data have demonstrated that estrogen responses, including those of the brain (Funabashi *et al.*, 2000), change with age in the rat. Therefore, relatively young animals (<10 weeks old at the time of Ovx) were used for the experiments described below.

Electrophysiological consequences of hormone replacement

Area CA1—Population spikes in area CA1 that were recorded from animals treated with the complete estradiol injection regimen (3-4-3) were significantly larger than those recorded from vehicle-treated rats, as shown in Fig. 3. The enhancement was evident as: (i) a larger maximum population spike; (ii) decreased stimulus intensity required for a half-maximal response; and (iii) decreased stimulus intensity required for a minimal response (Tables 2 and 3; mean values and statistics are provided in the tables). Results from 3-4-3 treatment were similar to those from intact rats that were examined on the morning of proestrus [Table 2; see also Scharfman *et al.* (2003)]. In addition, the positivity upon which the population spike was superimposed was larger in amplitude for animals treated with 3-4-3 (4.9 ± 0.4 mV, $n = 18$ slices) than for vehicle-treated rats (V-V-V, 3.0 ± 0.3 mV, $n = 12$ slices; Student's *t*-test, $t = 6.333$; d.f. 26; $P = 0.00059$), as shown in Fig. 3. The maximal slope and maximal amplitude of field EPSPs were greater in slices from animals that were treated with all three doses of estradiol (3-4-3) relative to vehicle-treated controls (V-V-V; Table 2). Field EPSPs were not significantly different when rats treated with 3-4-3 and proestrous rats (killed mid-morning of the day of proestrus) were compared (Table 2).

In animals treated with the doses that just simulated diestrus 2 (3-4-V) or only simulated the proestrous morning (V-V-3), effects appeared to synergize when combined (i.e. the entire 3-4-3 regimen). For these analyses, a four-parameter logistic model was used, based on previous studies showing that, in intact cycling female rats (Scharfman *et al.*, 2003), the relationship between stimulus intensity and population spike amplitude in CA1 can be fitted mathematically with such a model.

Figure 4A illustrates the 'best fit' four-parameter functions for slices from animals treated either with vehicle (V-V-V), V-V-3, 3-4-V or 3-4-3. The coefficients calculated from the curves, and statistics, are presented in Table 3. In all cases, 'Runs' tests demonstrated an approximately equal distribution of the individual data points either side of the four-parameter curves, indicating an acceptable goodness of fit ($P < 0.05$; Table 3). Analysis of the four-parameter curve fit coefficients revealed effects on both the predicted maximum population spike amplitude ($O_{\max}$) and ES_{50} (the stimulus strength required to elicit a half-maximal response; Table 3). Exposure to estradiol to mimic proestrous morning only (V-V-3) significantly increased $O_{\max}$, but did not alter ES_{50} (Fig. 4; Table 3). In contrast, simulation of diestrus 2 alone (3-4-V) led to a significant reduction in ES_{50} , but no change in $O_{\max}$ (Fig. 4; Table 3). Both $O_{\max}$ and ES_{50} were significantly increased in slices from animals treated with the complete regimen (3-4-3) as compared to the V-V-V control (Fig. 4; Table 3). $O_{\max}$ was significantly greater for 3-4-3 than for V-V-3 (Table 3).

Interestingly, all estrogen treatments significantly affected the variance of the data points around the regression lines. In all groups, residual variance was reduced as compared to the V-V-V-treated animals [analysis of residual variance, as described by Delean *et al.* (1978): V-V-3, $F = 2.51$, d.f. 10,42, $P = 0.048$; 3-4-V, $F = 0.28$, d.f. 7,42, $P = 0.010$; 3-4-3, $F = 0.29$, d.f. 11,42, $P = 0.006$]. A reduced variance may reflect an increase in the signal-to-noise ratio, improving network output, which may relate to the improved performance on hippocampal-dependent tasks described below.

It was surprising that the 3-4-V treatment had effects, because animals were examined over 12 h after the last EB dose. Uterine weight, a classic measure of estradiol action in the rat that is dependent on nuclear hormone action (Owens & Ashby, 2002), also showed a long-lasting effect, because it was significantly elevated relative to vehicle-treated rats (for means and statistical comparisons, see Table 1). In fact, uterine weights of rats treated with 3-4-V were not significantly different from those of animals treated with 3-4-3 (Table 1). In contrast, the uterine weights of animals that were treated with only one dose (V-V-3) were similar to those of vehicle-treated animals (Table 1).

Evidence for a presynaptic contribution to estradiol-induced potentiation in area CA1—In animals that were treated with the 3-4-3 sequence of injections, fiber volleys were smaller than those recorded from animals treated with vehicle (see Table 4 for means and statistics). In comparisons of treatment with vehicle vs. the 3-4-3 dose regimen, the 95% confidence intervals for the relationship between fiber volley amplitude and stimulus strength did not overlap (Fig. 5). The relationship between fiber volley amplitude and population spike amplitude shifted to the left in animals treated with 3-4-3 (Fig. 5), suggesting that 3-4-3 treatment led to an enhanced ability of afferent stimuli to evoke a population spike. Fiber volleys of rats treated with 3-4-3 were similar to those of intact rats examined on proestrous morning (Table 4).

Pairs of identical stimuli were used to evaluate the change in the amplitude of the population spike if an identical, ‘conditioning’ stimulus was triggered before it. Comparisons were made between vehicle treatment and estrogen treatment (3-4-3), and showed no difference in paired pulse ratios using any interstimulus interval (7, 10, 20, 40 ms), or when different stimulus strengths were used (Fig. 6, see Table 4 for means and statistical comparisons).

Pairs of stimuli were also tested with recordings in stratum radiatum. Paired pulse ratios using a 40 ms interval showed facilitation of the amplitude and maximal initial slope of the field EPSP, and this was increased in slices treated with estrogen (3-4-3) relative to vehicle (Table 4). Recordings were made using a weak stimulus (25–35% of the maximum) so that population spikes would not contaminate the field EPSP.

Area CA3—In the same slices that were used to evaluate area CA1, recordings were also made in area CA3. Mossy fiber stimuli were tested, because this input demonstrated changes on the morning of proestrus in the intact rat (Scharfman *et al.*, 2003). The same stimulation protocol was used as in past studies, pairs of half-maximal stimuli at 1 Hz, because this differentiated slices from rats with low and high serum levels of estradiol. Thus, in slices from rats with low serum estradiol (male rats, Ovx rats, or intact female rats on the morning of metestrus), pairs of half-maximal stimuli at 1 Hz normally elicit one population spike per stimulus, even if 10 pairs of stimuli are triggered in succession (Scharfman, 1997; Scharfman *et al.*, 2003). However, in slices from rats killed on the morning of proestrus, more than one population spike per stimulus developed after just 3–5 pairs of half-maximal stimuli at 1 Hz in 25% of slices (Scharfman *et al.*, 2003).

In animals treated with the complete regimen of hormone replacement (3-4-3), we found that 38% of slices (three slices of eight tested from three rats; one slice from each animal) demonstrated more than one population spike per stimulus after 3–5 pairs of stimuli (Fig. 7). This fraction (38%) is similar to the fraction reported in a study using slices from rats that were killed on proestrous morning (Scharfman *et al.*, 2003). Multiple population spikes did not occur in vehicle-treated controls (Fig. 7; $n = 0$ slices of 7 tested in rats) or slices from animals treated with 3-4-V or V-V-3 ($n = 0$ slices of 12 tested in six rats). These data suggest that proestrous morning-like effects developed in area CA3 in response to physiological hormone replacement (3-4-3).

Other consequences of physiological hormone replacement

BDNF—To compare BDNF expression, sections from three vehicle-treated animals (each administered V-V-V) were processed with sections from three animals treated with the 3-4-3 series of estradiol injections. As shown in Fig. 8, BDNF expression in vehicle-treated rats was relatively weak in comparison to expression in rats treated with 3-4-3. These data are similar to the relatively low levels of BDNF in untreated Ovx rats in a previous study (Scharfman *et al.*, 2003).

In all rats, BDNF was expressed mostly in the mossy fiber pathway, consistent with the original description of BDNF expression in the hippocampus of male rats (Conner *et al.*, 1997). In addition, 3-4-3-treated rats demonstrated increased BDNF immunoreactivity in the neuropil of area CA1 (see inset in Fig. 8), like rats examined mid-morning of proestrus (Scharfman *et al.*, 2003); this is presumably a reflection of increased synthesis, in CA3 pyramidal cells, of BDNF, which is anterogradely transported to Schaffer collateral nerve terminals. However, the BDNF expression in stratum radiatum is relatively weak in comparison to the mossy fiber pathway under all conditions (Conner *et al.*, 1997; Scharfman *et al.*, 2003). The data suggest an increase in BDNF expression in response to 3-4-3 treatment that was similar to that in the intact rat on the proestrous morning.

To confirm that the rise in BDNF expression was not dependent on the antibody that was used, the comparison of 3-4-3-treated rats with V-V-V-treated rats was repeated using a mouse monoclonal antibody to BDNF (Sigma; see Methods). Although the Sigma antibody had a weaker signal-to-noise ratio than the first antibody (Amgen-Regeneron Partners; compare Figs 8 and 9), both antibodies demonstrated that BDNF levels were higher in the mossy fibers in animals treated with 3-4-3 relative to vehicle-treated rats (Figs 8 and 9).

To clarify the potential contribution of diestrus 2 vs. the proestrous morning to BDNF expression, animals treated with V-V-V, V-V-3 and 3-4-V and 3-4-3 were compared. The comparison was conducted by processing sections from four rats concurrently, one being rat treated with V-V-V, the second rat with V-V-3, the third rat with 3-4-V, and the fourth rat with 3-4-3. This four-animal comparison was replicated four times. In three of those comparisons, sections from a fifth rat, perfused mid-morning of proestrus, were also included. This allowed a comparison of all four treatments with intact rats on the proestrous morning.

The diaminobenzidine reaction was truncated so that V-V-V-treated rats demonstrated little detectable mossy fiber BDNF, and when this was done, all 3-4-3-treated rats demonstrated robust mossy fiber BDNF by comparison (Fig. 9). BDNF expression was highest at the tip of the mossy fiber bundle near area CA2 (Fig. 9). Interestingly, there was some expression of BDNF in this region in two of the four rats treated with V-V-3. An example of a section from a V-V-3-treated rat with mossy fiber BDNF expression is shown in Fig. 9. Mossy fiber BDNF was robust in all rats tested mid-morning of proestrus, and was comparable to what was seen in 3-4-3-treated rats ($n = 3$; Fig. 9). When relative gray scale values at the tip of the mossy fiber bundle were compared among animals treated with V-V-V, 3-4-V, V-V-3, or 3-4-3, or tested on the proestrous morning, the groups were significantly different (one-way ANOVA: $F = 48.85$; d.f. 4,14; $P < 0.000001$), and *post hoc* tests showed that animals treated with the 3-4-3 regimen were significantly different from those treated with V-V-V, 3-4-V, or V-V-3 (Tukey-Kramer, $P < 0.05$), but not from those tested on the proestrous morning (Tukey-Kramer; $P > 0.05$; Fig. 9).

Behavior—The data described above suggest that the regimen that simulated the preovulatory surge optimally (3-4-3) might improve hippocampal function because synaptic transmission in two stages of the hippocampal trisynaptic circuit was enhanced *in vitro*. To test this hypothesis, animals treated with vehicle (V-V-V) or estradiol (3-4-3) were compared using

tests for object placement or object recognition ($n = 8$ per group; Fig. 10; for statistics, see legend to figure). Estrogen-treated animals spent more time with a familiar object that was placed in a novel location when the object placement task was conducted, suggesting improved memory for spatial location of an object (Fig. 10). In addition, estradiol-treated rats spent more time exploring novel objects when the object recognition task was administered (Fig. 10), suggesting improved memory for an object that was encountered previously. The results suggest improved performance in tasks that involve memory for spatial location and recognition of objects.

Effects of elevating the dose of estradiol above the normal range on the proestrous morning

Area CA1 recordings from animals treated with supraphysiological hormone replacement—To evaluate the effects of supraphysiological hormone levels, we changed the 3-4-3 regimen by increasing the final estradiol dose to 5 $\mu\text{g/kg}$ (3-4-5). The 5 $\mu\text{g/kg}$ dose would be predicted to elevate serum estradiol to approximately 90 pg/mL when tested 2 h after injection (Fig. 2B), which is well above the peak at mid-morning of proestrus in the Sprague-Dawley rat (Smith *et al.*, 1975).

Surprisingly, there was a smaller mean population spike amplitude in area CA1 of slices from animals treated with 3-4-5 relative to slices of animals treated with 3-4-3, reflected by the O_{max} (see Table 3 for statistical comparisons). Moreover, stimulation of the Schaffer collaterals did not always evoke a population spike with a maximal amplitude over 5 mV, our criterion for acceptance for this study. Curve-fit analysis of the input–output function for slices from animals treated with 3-4-5 demonstrated that the maximal population spike amplitude was depressed in comparison to vehicle-treated rats (Fig. 4B, Table 3). However, the Hill slope coefficient was not statistically distinct, arguing against a nonspecific depressive effect. Indeed, field EPSPs from animals treated with 3-4-5 demonstrated large amplitudes and increased slope in comparison to vehicle treatment, and were not statistically different from field EPSPs recorded in slices from animals that were injected with 3-4-3. Paired pulse inhibition of the population spike was not significantly different either (animals treated with 3-4-5 relative to 3-4-3; Table 4), suggesting that an increase in somatic inhibition could not explain the results.

Area CA3—Because of the paradoxically weaker evoked responses area CA1 in slices of animals treated with 3-4-5, compared to 3-4-3 we also examined CA3 in animals given 3-4-5. In these slices, excitability appeared to be enhanced relative to slices from animals treated with 3-4-3, the opposite of results in area CA1. Thus, multiple population spikes could be evoked even by one pair of stimuli to the mossy fibers (Fig. 7; $n = 4/8$ slices in three rats treated with 3-4-5; at least one slice per animal). In some slices, there were spontaneous field potentials (Fig. 7; $n = 5/8$ slices in three rats, at least one slice per animal). Spontaneous field potentials were large, irregular negative potentials that were largest in amplitude in stratum lucidum, suggesting synchronous field EPSPs (Fig. 7). Indeed, evoked responses demonstrated that multiple population spikes corresponded to field EPSPs in stratum lucidum (Fig. 7).

Spontaneous field potentials recorded in stratum lucidum decreased in amplitude when recording electrodes were placed in adjacent strata (stratum radiatum and stratum pyramidale; Fig. 7), suggesting that activity was generated in the stratum lucidum. The spontaneous field EPSPs were associated with bursts of unit activity recorded within the pyramidal cell layer (Fig. 7). However, they did not resemble epileptiform activity, because they were distinct from epileptiform bursts recorded after slices are exposed to convulsants (Wong & Traub, 1983; Swann *et al.*, 1986; Scharfman, 1994). In addition, we could not detect spontaneous field potentials in other subfields (area CA1 stratum radiatum, the dentate gyrus granule cell layer; data not shown). These data suggest an unusual, abnormal form of increased excitability in area CA3 in response to supraphysiological estradiol replacement.

Can effects of supraphysiological estradiol be compensated by decreasing prior estrogen exposure?—Because estrogen exposure can facilitate subsequent effects of estradiol administration on the brain (Krey & Parsons, 1982; Parsons *et al.*, 1984), we speculated that the decreased CA1 population spike amplitude in slices from animals treated with supraphysiological hormone levels might reflect sensitization of the hippocampus to the acute effects of estradiol. If so, then it followed that if we used less sensitization (by lowering the first two doses of the 3-4-3 treatment), we might be able to compensate for the effects of a supraphysiological dose on the simulated proestrous morning. Therefore, we examined the effects of 2-2-5 (substituting 2 µg/kg EB for the 3 and 4 µg/kg EB doses in the 3-4-3 protocol). The 2 µg/kg EB dose was chosen because it did not raise estradiol levels to the range normally observed during diestrus 2 (mean serum estradiol concentration, 2 h after 2 µg/kg EB, <10 pg/mL; $n = 3$).

The results of these experiments are shown in Fig. 4B. The maximal population spike amplitude was greater in slices from animals treated with 2-2-5 than in slices from animals given vehicle. Therefore, the effect of 2-2-5 was closer to the effects of 3-4-3 than those of 3-4-5 (Fig. 4B; Table 3). However, 2-2-5 was not as effective as 3-4-3 in potentiating population spike amplitude (Fig. 4B). The results suggest that the effects of the preovulatory surge in the intact rat are best modeled by estradiol doses that precisely simulate estradiol levels during diestrus 2 and proestrous morning, but that some compensation for a supraphysiological dose on proestrous morning can be made by reducing the doses administered during the previous 26 h.

Discussion

The effects of preovulatory changes in estradiol on hippocampal function

The results suggest that estradiol is necessary and sufficient for improved hippocampal function when it rises on the morning of proestrus in the adult female rat, but that simulating physiological levels of estradiol is critical. The hippocampal changes are evident *in vitro* and *in vivo*, at the level of electrophysiology, anatomy, and behavior. They demonstrate an unanticipated dependence on a slow rise in estradiol long before the estrogen surge, suggesting that diestrus 2 in the intact rat is critical for effects on the proestrous morning. They document subfield differences that have thus far not been reported, including an ability of supraphysiological estradiol to synchronize mossy fiber transmission.

The results have implications for understanding both normal and abnormal conditions. The results suggest that physiological levels of estradiol normally improve hippocampal function, but when estradiol is above the norm on the proestrous morning, CA1 function may be impaired. At the same time, the results predict an increase in synchronized activity in CA3. Taken together, the data suggest a potential explanation for cognitive changes associated with certain disease states that influence circulating levels of estradiol.

Although the results suggest that estradiol is necessary and sufficient for effects on proestrous morning, we cannot exclude a modulatory role of other hormones and neuromodulators that rise on proestrous morning. Local estrogen synthesis is potentially important, not just ovarian secretion (Rune *et al.*, 2006). We also cannot exclude the possibility that other aspects of hippocampal structure and function that were not studied here may be distinct, such as spine synapses.

Synergism between short-term and long-term effects of estradiol

The data suggest that there are specific effects of the rise in serum estradiol on diestrus 2. This finding is surprising, because diestrus 2 is often considered to be a control condition. However, we have shown here that there is increased sensitivity of CA1 pyramidal cells to Schaffer

collateral input that is induced simply by simulation of diestrus 2. The surge of estradiol on proestrous morning, in contrast, appears to have a very different effect: it leads to greater output (population spike amplitude). There also appears to be a small – albeit inconsistent – effect on BDNF expression in mossy fibers after simulation of proestrous morning alone.

An interaction between diestrus 2 and the proestrous morning has not previously been suggested in the hippocampus, although a need for ‘priming’ for estrogen activation of gonadotrophin release and sex behavior has been proposed (Krey & Parsons, 1982; Parsons *et al.*, 1982). Our results suggest that there is a synergism between diestrus 2 and proestrous morning, because the increase in evoked population spike amplitude that occurred in response to simulation of proestrous morning was significantly greater when it was preceded by simulation of diestrus 2. In addition, BDNF expression was most consistent and robust after both diestrus 2 and proestrous morning were simulated in sequence, rather than proestrous morning only.

A potential explanation for the apparent synergism between diestrus 2 and the proestrous morning is induction of transcriptional estrogen responses on diestrus 2, consistent with the uterotrophic data. On the basis of the evidence of rapid changes in CA1-evoked responses after simulation of proestrus, it is likely that rapid (possibly membrane receptor-mediated) effects occur on the proestrous morning. In support of the rapid effects observed here are studies showing that CA1 population spikes can be rapidly potentiated by adding estradiol to hippocampal slices (Foy, 2001). This sequence of transcriptional actions, followed by rapid actions, would be consistent with previous suggestions that estradiol exerts both genomic and nongenomic actions in the hippocampus (Adams *et al.*, 2002; McEwen, 2002; Foster, 2005).

Changes in hippocampal function: CA1 population spike potentiation

Changes in the fiber volley, field EPSP and paired pulse facilitation suggest potential presynaptic mechanisms, which is supported by previous studies showing estrogen receptors on presynaptic profiles in the area CA1 stratum radiatum (Milner *et al.*, 2001, 2005). In addition, proteins involved in transmitter release increase on the proestrous morning, such as BDNF (Scharfman *et al.*, 2003), or BDNF mRNA is high during late diestrus, suggesting elevation of protein on the proestrous morning (Crispino *et al.*, 1999). The results of the current study support a role of BDNF, which would have been predicted, because BDNF is known to potentiate population spikes in area CA1 (Pang & Lu, 2004), and there is an estrogen-sensitive response element on the BDNF gene (Sohrabji *et al.*, 1995; Scharfman & MacLusky, 2006).

GABAergic mechanisms may play a role (Rudick & Woolley, 2001), possibly mediated by BDNF, because BDNF influences GABAergic neurons (Murphy *et al.*, 1998b). However, GABAergic inhibition – at least as reflected by paired pulse tests – did not appear to change in our experiments. In addition, GABA receptor antagonists do not reproduce the effects that we observed (Wong & Traub, 1983; Scharfman, 2005). However, changes in inhibition may have occurred at specific times, possibly before or after those used here (Rudick & Woolley, 2001). Postsynaptic changes in the spines of CA1 pyramidal cells are a potential factor, because previous studies have shown dendritic spine changes on proestrus (Woolley, 2007). Changes in *N*-methyl-D-aspartate receptors (Woolley *et al.*, 1997; Smith & McMahon, 2005), or activation of signal transduction cascades [e.g. mitogen-activated protein (MAP) kinase/extracellular regulated kinase (ERK) (Bi *et al.*, 2001) and Akt (Znamensky *et al.*, 2003)] suggest postsynaptic mechanisms. Glial effects should be considered, because glia express estrogen receptors in area CA1 (Milner *et al.*, 2001, 2005), glia change during the estrous cycle (Klintsova *et al.*, 1995), and glial function regulates synaptic transmission in area CA1 (Keyser & Pellmar, 1994).

Changes in hippocampal function: area CA3 excitability

To our knowledge, few studies have examined relative changes in area CA1 and CA3 in response to estradiol. One study found that there c-fos was expressed by both CA1 and CA3 pyramidal cells after estradiol treatment of Ovx rats, with CA1 showing a greater effect, depending on the time after estradiol administration when animals were examined (Rudick & Woolley, 2000). The results of the current study, using a different estrogen treatment paradigm, would suggest that there are also increases in the ability to activate CA1 and CA3 neurons, and that there are some differences between CA1 and CA3.

Remarkably, the effects in CA3 after the 3-4-3 regimen, which was used to simulate the preovulatory estradiol surge, were similar to the effects described in a previous study using intact, female rats that were examined immediately after the morning surge in estradiol on proestrus (Scharfman *et al.*, 2003). Because the mossy fibers express BDNF, and the effects in CA3 were reduced by a neurotrophin receptor antagonist (Scharfman *et al.*, 2003), a role of BDNF is likely (Croll *et al.*, 1999; Scharfman *et al.*, 2003; Scharfman, 2005; Scharfman & MacLusky, 2005). The greater expression of BDNF in mossy fibers than in Schaffer collaterals might relate to the subfield differences that were observed in the current study. Future experiments would be required to fully elaborate the subfield differences between CA1 and CA3 that develop in response to estradiol exposure.

Changes in hippocampal function: behavior

Simulation of the preovulatory changes in estradiol levels using the 3-4-3 paradigm increased object recognition and object placement performance, suggesting enhanced learning and memory. These data are consistent with reports that proestrous rats navigate their environment better (Marcondes *et al.*, 2001), and spatial learning is improved on proestrus (Warren & Juraska, 2005), although there are discrepancies in past studies (Berry *et al.*, 1997; Stackman *et al.*, 1997; Tropp *et al.*, 2005; Warren & Juraska, 2005; Luine & Dohanich, in press), possibly due to different estradiol treatments (Sava & Markus, 2005), or the time of day when tests were conducted. Time of day is critical because in the afternoon of proestrus, progesterone rises, and it is known that progesterone has profound effects on the response to estradiol (Sandstrom & Williams, 2001).

One mechanism for improved performance in response to estradiol, suggested by past studies [see Woolley (2007) for a review] and the present study, is potentiation in CA1. In addition, the present study provides intriguing evidence that there is a decreased variance in CA1, which could more finely 'tune' CA1. It is also possible that the increased activation of CA3 pyramidal cells by mossy fibers plays a role.

The results provide an answer to previous questions about the need for high doses of estradiol to improve hippocampal function. Regarding the effects of estradiol after a single injection, place memory was improved, but only if the dose elevated estradiol well above the physiological range (Luine *et al.*, 2003; MacLusky *et al.*, 2005; Luine & Dohanich, in press). In the present study, we have shown that extremely low doses are surprisingly effective, if they are precisely timed to mimic ovarian estradiol release. Therefore, the potency of the effect of estradiol on hippocampal-dependent behavior may depend on the sequencing of hormone administration, not only the absolute levels.

Differential effects of 3-4-3 and 3-4-5

The results were different when the 3-4-3 and 3-4-5 paradigms were compared. In CA1, responses to Schaffer collateral stimulation increased after 3-4-3, but decreased in rats treated with 3-4-5. In contrast, the effects of 3-4-3 in CA3 appeared to increase when rats treated with 3-4-5 were examined, because there was an increase in the ability to evoke repetitive population

spikes in response to mossy fiber stimuli. Furthermore, spontaneous, synchronized potentials emerged, a novel response that, to our knowledge, has not yet been reported.

The decrease in responses in CA1 in response to the 3-4-5 treatment paradigm might be explained by recruitment of inhibition, given the presence of estrogen receptors on hippocampal interneurons (Hart *et al.*, 2001; Blurton-Jones & Tuszynski, 2002; Nakamura & McEwen, 2005), but paired pulse inhibition was not increased in rats treated with 3-4-5. High levels of CA3 activity are an alternative explanation, because heightened activity in CA3 can decrease the ability of Schaffer collateral transmission to activate CA1 (Sastry *et al.*, 1985).

Differential effects of 3-4-3 and 3-4-5 regimens could also arise through activation of membrane receptor-regulated signaling pathways. In cerebellar neurons, phosphorylation of ERK via membrane-associated estrogen receptors exhibits a bimodal dose-response curve, similar to the results described here (Zsarnovszky *et al.*, 2005). The ERK/MAP kinase pathway may play a role in the hippocampal effects of estrogen (Jover-Mengual *et al.*, 2006).

The reason why supraphysiological estradiol treatment led to an even greater increase in the activity of pyramidal cells in CA3 than physiological dosing may be due to the elevation in BDNF, which was primarily in the mossy fiber pathway. This may be relevant to reports that estrogen can be proconvulsant, as can BDNF, under some circumstances (Scharfman & MacLusky, 2006). The data from animals treated with 3-4-5 indicate a dramatic shift in the pattern of CA1 and CA3 activity, suggesting that even relatively small variations in estrogen doses, at least during mid-morning of proestrus, could profoundly influence hippocampus.

Acknowledgements

This work was supported by grants NS37562, GM60654, and GM60655.

Abbreviations

ACSF	artificial cerebrospinal fluid
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
EB	estradiol benzoate
EPSP	excitatory postsynaptic potential
ERK	extracellular regulated kinase
ES₅₀	stimulus strength required to elicit a half-maximal response
E2	17- β estradiol
GABA	γ -aminobutyric acid

MAP kinase

mitogen-activated protein kinase

 $O_{\max}$

maximum population spike amplitude

Ovx

ovariectomized

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Fig. 1.

Recording and stimulation sites used to evaluate evoked responses in area CA1 or area CA3. (A) CA1 population spikes were recorded in CA1b (site 1) with a stimulus site in CA2 stratum radiatum to activate Schaffer collateral axons of CA3 pyramidal cells (black circle). CA1 field excitatory postsynaptic potential (EPSPs) were recorded in stratum radiatum, at a location corresponding to the dendrites of the cells recorded at site 1. CA3 field EPSPs were recorded in stratum lucidum of CA3b (site 3); population spikes were recorded at a site (site 4) corresponding to the cell bodies of the dendrites at site 3. The black circle in the subgranular zone was the site for stimulation for CA3 recordings, chosen because it is a position that preferentially activates the mossy fiber (mf) axons of dentate granule cells, which innervate CA3 pyramidal cells in stratum lucidum. OR, stratum oriens; PCL, pyramidal cell layer; RAD, stratum radiatum; LUC, stratum lucidum. (B) Sample recordings in area CA1b are shown to illustrate the way in which the site for cell layer recordings was chosen. For each slice, sites were initially tested by moving the recording site along an axis perpendicular to the PCL, from the RAD to the OR. Sites were rejected if a maximal stimulus evoked a spike that was less than twice the amplitude of the positivity upon which it was superimposed (top). Sites were also rejected if they were too close to RAD (bottom), reflected by a population spike that was not completely superimposed on a positive potential. The center trace shows the optimal recording. (C) Sample recordings of field EPSPs illustrate the evaluation of the optimal site for field EPSP recordings: steepest and largest responses reflected the optimal site (top) relative to sites too close to stratum lacunosum-moleculare (RAD/LM; bottom). (D) Sample recordings are shown to illustrate typical responses recorded in the CA3b PCL and LUC in response to mossy fiber stimulation.

Fig. 2.

Hormone replacement that simulates the preovulatory estradiol surge in ovariectomized (Ovx) rats. (A) Serum estradiol levels in OvX rats that were given a subcutaneous injection of 17 β -estradiol (E2) and killed at different times thereafter. There was a rapid rise in serum estradiol at 1–2 h, and the level subsequently fell by 4–6 h after the injection. The dose used (15 μ g/kg), based on previous studies (MacLusky *et al.*, 2005), exceeded the physiological range, and therefore lower doses were subsequently tested. (B) Serum estradiol levels are shown for OvX animals that were injected with E2 (1, 3 or 5 μ g/kg) and killed 2 h later. There was a dose-dependent rise in serum estradiol, and 3 μ g/kg led to a dose that was similar to the peak serum levels on proestrus morning in the Sprague-Dawley rat (Smith *et al.*, 1975). (C) A schematic illustrating the timing of estradiol injections used to simulate preovulatory estradiol secretion on proestrous morning. White bars indicate day, and dark bars denote night. At 8:30 a.m., estradiol benzoate (EB) was injected (3 μ g/kg), and 12 h later, it was injected again at a slightly higher dose (4 μ g/kg); this was followed by an injection of E2 (3 μ g/kg) on the next morning at 8:30 a.m. This treatment is called 3-4-3 in subsequent figures. Two hours after the third dose, at a time of day when the proestrous surge would be expected to peak in the intact rat (Smith *et al.*, 1975), animals were tested. (D) Mean serum estradiol levels in OvX rats, given the doses of estradiol illustrated in C, show that the mean levels obtained throughout the time of hormone replacement matched those of the intact female rat. The line is adapted from published values for the Sprague-Dawley rat (Smith *et al.*, 1975). Mean $\pm$ SEM are plotted; n = 3 animals per group.

Fig. 3.

Comparison of CA1 responses to Schaffer collateral stimulation in slices from animals pretreated with vehicle or estradiol. (A and B) Population spikes evoked in a slice from a rat treated with three vehicle injections (V-V-V) or three estradiol injections (3-4-3). Population spikes were recorded in response to increasing stimulus durations. Slices were made 2 h after the last injection. Stimulus artifacts are marked by the dots and are truncated for this and subsequent figures. Stimulus intensity is expressed as duration and is indicated to the right of each evoked response; stimulus current was 100 μ A throughout.

Fig. 4.

Input–output relationships for the population spike of hormone-replaced and vehicle-treated rats. (A) Mean population spike amplitudes are plotted as a function of stimulus duration for animals treated with vehicle (V-V-V) or estradiol (3-4-V, V-V-3, or 3-4-3). Curve-fit analysis was used to fit the data (DeLean *et al.*, 1978). There was a decrease in the ES_{50} (stimulus required for a half-maximal response) and increase in the O_{max} (maximal population spike amplitude) for 3-4-3 relative to vehicle. Partial doses either decreased the ES_{50} (3-4-V) or increased the O_{max} (V-V-3). For A and B, values and statistics are presented in Table 3. (B) A comparison of input–output relationships for physiological hormone replacement (3-4-3) and nonphysiological hormone treatment (3-4-5, 2-2-5) relative to vehicle treatment (V-V-V). Raising the last dose from 3 to 5 $\mu\text{g/kg}$ (3-4-5) depressed the O_{max} , and decreasing the first two doses (2-2-5) partly reversed this effect.

Fig. 5.

Fiber volley amplitude in slices from vehicle-treated or hormone-replaced rats. (A) Fiber volley amplitude is plotted as a function of stimulus intensity. The 95% confidence limits (dotted lines) of the input–output functions for 3-4-3 (white circles) and vehicle (V-V-V; black circles) did not overlap. (B) The mean fiber volley amplitude is plotted as a function of the mean population spike amplitude for each stimulus duration tested (3-4-3, white circles; V-V-V, black circles). Error bars reflect the largest standard error of the mean for either the measure of fiber volley amplitude or population spike amplitude. (C) Left: For vehicle-treated, estrogen-treated (3-4-3) and intact rats (Pro, proestrous morning), the mean fiber volley amplitude for the maximal stimulus strength is expressed as a percentage of the mean maximal population spike amplitude. Bars represent standard errors of the mean. N (slices) is listed at the base of each bar. Asterisks indicate statistical significance (Mann–Whitney U -test, V-V-V relative to 3-4-3 or proestrous morning, $P < 0.05$). Right: The mean fiber volley amplitudes for a maximal stimulus strength are shown for the same groups as on the left. Asterisks indicate statistical significance of 3-4-3 or proestrous morning relative to vehicle (one-way ANOVA: $F = 3.35$; d.f. 2,27, $P < 0.0001$ followed by Tukey–Kramer *post hoc* test, $P < 0.05$).

Fig. 6.

Paired pulse inhibition in vehicle-treated and hormone-replaced rats. (A) Sample responses from a vehicle-treated rat illustrate the normal pattern of responses to pairs of identical stimuli. A preceding stimulus typically inhibits the population spike elicited by a subsequent stimulus in a graded manner, depending on the interstimulus interval. When the interstimulus interval is minimal, maximal inhibition of the population spike typically occurs, and this inhibition decreases as the interstimulus interval increases. (B) Comparison of paired pulse ratios for vehicle-treated rats (V-V-V; black circles) and estradiol-treated rats (3-4-3; white circles). Tests were conducted using stimulus intensities that were weak (25–35% of the maximum), intermediate (50% of the maximum) and strong (80–90% of the maximum). For statistical comparisons, see Table 4.

Fig. 7.

Effects of estradiol replacement on evoked responses of area CA3 pyramidal cells to mossy fiber stimulation. (A) Recordings from a slice of a vehicle-treated control rat show normal responses to mossy fiber stimulation using paired half-maximal stimuli (40 ms interstimulus interval) triggered 10 times at 1 Hz, because there was only one population spike following each stimulus. The responses to the first and tenth pairs of stimuli are shown. (B) In a slice from a rat treated with estradiol (3-4-3), the responses to the first and third pair are shown. More than one population spike was evoked per stimulus (arrows). (C) Top: In a slice from a rat treated with 3-4-5, recordings in the pyramidal cell layer (PCL) showed that a single pair of stimuli evoked more than one population spike per stimulus. The asterisk marks discharge of a single unit. Bottom: The recording site was moved to the stratum lucidum (LUC) and more than one field EPSP was evoked per stimulus (arrows). The secondary field potentials demonstrated a maximal negative amplitude in the LUC (data not shown), consistent with generation at the mossy fiber synapse. (D) Spontaneous extracellular recordings from the PCL or LUC from a slice of a rat treated with 3-4-5. Bursts of unit firing in the PCL occurred, and they were synchronized to spontaneous field EPSPs recorded from the LUC, which were largest in the LUC relative to other layers tested (expanded at arrows). Different rat from C. (E) For the same slice in D, recording sites are diagrammed to indicate where potentials on the right were recorded.

Fig. 8.

Physiological hormone replacement increases BDNF expression in Ovex rats. (A) BDNF immunoreactivity in an Ovex rat treated with vehicle (V-V-V) using a rabbit polyclonal antibody provided by Amgen-Regeneron Partners. (A1) Coronal section through the dorsal hippocampus. (A2) Horizontal section through the temporal hippocampus of the same rat. Insets shows area CA1 at higher magnification. BDNF immunoreactivity in the neuropil of area CA1 stratum radiatum was greater after estrogen treatment (asterisk) in comparison to vehicle. (B) BDNF immunoreactivity in a section from an estrogen-treated animal (3-4-3) that was processed concurrently with the section shown in A. The estrogen-treated rat demonstrated increased BDNF expression in hippocampal mossy fibers (MFs; arrows) and the inner molecular layer (IML, single arrow in B). DG, dentate gyrus; SO, stratum oriens, SP, stratum pyramidale, SR, stratum radiatum; SL-M, stratum lacunosum-moleculare. Calibration (in B2) = 200 μ m for low-magnification images; 400 μ m for insets.

Fig. 9.

BDNF expression after partial or complete simulation of the preovulatory estrogen surge on proestrous morning. (A and B) BDNF immunoreactivity using a mouse monoclonal antibody to BDNF (Sigma) demonstrated very little mossy fiber BDNF expression in vehicle-treated rats when reacted briefly (A), and the same was true in sections from a rat treated with 3-4-V that were processed at the same time (B). SO, stratum oriens; SP, stratum pyramidale; SL, stratum lucidum; SR, stratum radiatum. Calibration = 100 μ m. (C) Sections from a rat treated with V-V-3 showed mossy fiber BDNF expression (MFs; arrows). (D and E) Sections from a rat treated with 3-4-3 (D) and from a rat that was perfused mid-morning on proestrus (E) had the most robust BDNF expression in mossy fibers. Sections from A–E were processed in the same immunocytochemical assay, so the times of exposure to reagents were the same. Note that only two of four V-V-3-treated rats demonstrated BDNF expression, whereas other groups were internally consistent ($n = 4$ per group). (F) Mean gray scale values of the terminal field of the mossy fiber pathway (at the double arrows in C, D and E) were computed using digitized micrographs that were sampled using the same light settings. ImageJ software computed the darkest (0 = black) and lightest (255 = white) values. Sample sizes are listed at the base of each bar. Statistical analysis: because the data were not based on a continuously distributed variable, they were analysed nonparametrically. Groups were significantly different (Kruskal–Wallis ANOVA: $H = 14.4$; d.f. 4,14; $P = 0.006$). Animals treated with the 3-4-3 regimen, or rats that were killed on the proestrous morning, were significantly different from rats treated with V-V-V, 3-4-V, or V-V-3 (Mann–Whitney U -test, $P < 0.05$; asterisks), but there was no difference between 3-4-3 and proestrous morning (Mann–Whitney U -test; $P > 0.05$).

Fig. 10.

Physiological hormone replacement improves performance on object placement and object recognition tasks. (A) Object placement tests used vehicle-treated rats (V-V-V; white bars) compared to estrogen-treated rats (3-4-3; black bars; $n = 8$ per group). Two hours after the last injection, a sample trial (T1) was used to measure the time spent exploring two objects for a 3 min period. The mean time spent exploring both objects was not different for the vehicle and estrogen-treated rats (Student's t -test: $t = 0.324$; d.f. 14; $P = 0.755$). Four hours later, animals were tested for retention (T2) by measuring the time spent exploring the same objects for a 3 min period, but one was placed in a new location. The percentage of time spent exploring the object in the new location, relative to the total time spent exploring both objects, was significantly increased for estrogen-treated rats (asterisk; Mann-Whitney U -test, $P < 0.05$). The dotted line represents 50%, which would be expected by chance. (B) Object recognition tests were conducted using two additional groups of vehicle-treated or estrogen-treated rats ($n = 8$ per group). During the sample trial (T1), conducted 2 h after the last injection, there was no difference between groups in the total time spent exploring both objects (Student's t -test: $t = 0.9374$; d.f. 14; $P = 0.380$). During the retention trial (T2) 4 h later, a new object was substituted for one of the previously encountered objects. The total time exploring the new object relative to the total time exploring both the new and familiar object is expressed as a percentage, and was increased for estrogen-treated rats (asterisk; Mann-Whitney U -test, $P < 0.05$).

Table 1
Changes in uterine weight (mg) after hormone replacement or vehicle treatment

Group	Estradiol replacement regimen				
	V-V-V	V-V-3	3-4-V	3-4-3	3-4-5
6-10 weeks old (Slices, <i>n</i>)	91.0 ± 8.8 (4)	110.0 ± 6.0 (3)	177.0 ± 30.4* (3)	232.1 ± 21.6* (8)	216.4 ± 15.0* (7)
All ages (Slices, <i>n</i>)	161.2 ± 29.2 (9)	170.3 ± 38.5 (6)	282.2 ± 34.6* (9)	264.1 ± 18.3* (13)	219.8 ± 17.3* (6)
					225.2 ± 20.5* (5)

Data are presented as means ± SEM. Uterine weight is listed for those animals that were OvX between 6 and 10 weeks of age, and for all ages (range: 6-20 weeks). One-way ANOVA demonstrated differences among groups for young or for all ages (6-10 weeks, *F* = 3.76, d.f. 5,28, *P* = 0.01; all ages, *F* = 3.41, d.f. 5,44, *P* = 0.01).

* Animals that were treated with two initial doses of estradiol (3-4-V, 3-4-3, 3-4-5, 2-2-5) were different from animals treated with vehicle (Tukey-Kramer *post hoc* test, *P* < 0.05).

Changes in the population spike and field excitatory postsynaptic potential (field EPSP) in area CA1 in response to stimulation of Schaffer collateral axons after hormone replacement or vehicle treatment relative to intact rats

Table 2

	Estradiol replacement regimen					Intact rat, proestrous morning
	V-V-V	V-V-3	3-4-V	3-4-3	3-4-5	2-2-5
Minimal intensity, population spike (μ s) (Slices, <i>n</i>)	63.80 $\pm$ 5.06 (17)	57.50 $\pm$ 6.98 (12)	49.92 $\pm$ 7.44 (13)	37.14 $\pm$ 4.49* (21)	86.25 $\pm$ 25.11 (4)	52.22 $\pm$ 8.51 (9)
Maximum amplitude of field EPSP (mV) (Slices, <i>n</i>)	4.8 $\pm$ 0.6 (5)	7.4 $\pm$ 1.0 (10)	7.5 $\pm$ 0.5* (9)	8.1 $\pm$ 0.8* (6)	9.9 $\pm$ 0.7* (5)	7.4 $\pm$ 0.7* (5)
Maximum slope of field EPSP (V/s) (Slices, <i>n</i>)	3.7 $\pm$ 0.9 (3)	4.8 $\pm$ 1.0 (3)	11.7 $\pm$ 0.3* (3)	12.3 $\pm$ 0.1* (5)	12.0 $\pm$ 0.4* (4)	NT

Data are presented as means $\pm$ SEM. The minimal stimulus intensity needed to evoke a population spike in area CA1, using Schaffer collateral stimulation, is listed for all treatment groups. Minimal intensity was defined by the stimulus duration that evoked a 0.5–1.0 mV population spike, an amplitude that was chosen because lesser amplitudes were difficult to discriminate from the positivity upon which they were superimposed. Stimulus pulse amplitude was constant (100 μ A). One-way ANOVA demonstrated differences among groups ($F = 2.22$; d.f. 6,74; $P < 0.005$). Slices from animals treated with 3-4-3 or from proestrous rats were significantly different from slices from animals treated with vehicle (V-V-V; significant differences denoted by asterisks; Tukey–Kramer *post hoc* test, $P < 0.05$). The maximal field EPSP amplitude and maximal initial slope of the field EPSP are listed for all treatment groups. One-way ANOVA demonstrated group differences (amplitude, $F = 2.57$, d.f. 5,32; $P = 0.046$; slope, $F = 28.80$, d.f. 5,17, $P < 0.0001$). 3-4-V, 3-4-3, 3-4-5 and proestrous rats demonstrated increased amplitude and slope relative to V-V-V rats (Tukey–Kramer *post hoc* test, $P < 0.05$). NT, not tested.

Table 3
Properties estimated from curve-fit analysis of the CA1 population spike input-output relationship for vehicle-treated and hormone-treated rats

	Estradiol replacement regimen				
	V-V-V	V-V-3	3-4-V	3-4-3	2-2-5
Maximum population spike amplitude, O_{max} (mV) (Slices, n)	10.8 ± 1.7 (17)	16.4 ± 5.9* (13)	9.9 ± 0.9 (10)	17.0 ± 0.9* (14)	15.7 ± 1.8* (12)
Half-maximal stimulus, ES_{50} (μs) (Slices, n)	111.0 ± 20.0 (17)	132.0 ± 19.0 (13)	68.7 ± 7.4* (10)	65.1 ± 4.9* (14)	76.0 ± 12.1 (12)
Hill coefficient, C_{Hill} (Slices, n)	2.17 ± 0.57 (17)	1.19 ± 0.42* (13)	2.84 ± 0.78 (10)	2.20 ± 0.34 (14)	1.87 ± 0.40 (12)

Data are presented as means ± SEM. Stimulus strength required to generate a half-maximal response (ES_{50} ; intensity is expressed as the stimulus duration), Hill slope coefficients (C_{Hill}) and maximal population spike amplitudes (O_{max}) estimated from the CA1 input-output function data points shown in Fig. 4. Estimates were obtained using four-parameter logistic regression analysis of the raw data, using the ALLFIT computer program (DeLean *et al.*, 1978). Asterisks indicate significant differences, for the same parameter, relative to vehicle treatment (V-V-V). For O_{max} - V-V-3, $F = 3.58$, d.f. 9,66, $P = 0.001$; 3-4-3, $F = 4.86$, d.f. 9,66, $P < 0.001$; 3-4-5, $F = 2.49$, d.f. 9,66, $P = 0.015$; 2-2-5, $F = 11.69$, d.f. 9,66, $P < 0.001$. For ES_{50} - 3-4-V, $F = 2.02$, d.f. 9,66, $P = 0.049$; 3-4-3, $F = 2.25$, d.f. 9,66, $P = 0.027$.

Comparison of paired pulse inhibition and paired pulse facilitation after vehicle treatment and physiological hormone replacement

Table 4

Treatment	Population spike				Field EPSP			
	ISI 10 ms		ISI 20 ms		ISI 40 ms		ISI 40 ms	
	30% Amplitude	85% Amplitude	30% Amplitude	85% Amplitude	30% Amplitude	85% Amplitude	30% Amplitude	30% Slope
V-V-V (Slices, n)	2.5 ± 2.5 (10)	53.4 ± 10.6 (14)	58.0 ± 19.4 (5)	93.4 ± 6.7 (6)	163.3 ± 21.4 (12)	109.7 ± 5.4 (12)	109.6 ± 4.5 (5)	104.8 ± 3.7 (5)
3-4-3 (Slices, n)	9.6 ± 9.6 (7)	52.7 ± 11.5 (9)	62.9 ± 30.5 (6)	102.6 ± 3.6 (6)	157.8 ± 26.1 (7)	107.2 ± 3.9 (9)	146.6 ± 11.9* (5)	130.0 ± 4.3* (5)

Data are presented as means ± SEM. Comparisons of paired pulse inhibition and facilitation for vehicle-treated rats relative to animals treated with estradiol using the 3-4-3 treatment. Recordings of the population spike amplitude used stimulus strengths that were 30% or 85% of the maximum; field EPSPs used 30%. Interstimulus intervals (ISIs) were 10, 20 or 40 for population spikes, and 40 ms for field EPSPs. There were no significant differences in comparisons for population spikes (Fisher's exact test, $P > 0.05$), but there were differences for the field EPSP amplitudes and slopes (Fisher's exact test, $P < 0.05$), denoted by asterisks.