Interactive report

Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats ¹

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Abstract

In order to test the hypothesis that circulating levels of estrogen modulate synaptic plasticity in the hippocampus, we have studied the induction of long term potentiation (LTP) in awake rats. Ovariectomized animals, chronically implanted with a recording electrode in the cell body layer of CA1 and a stimulating electrode in stratum radiatum, were used to record evoked field potentials (population spike (PS) and summed EPSP) daily for at least 4 days before injection of sesame oil or 100 µg of estradiol benzoate per kg b.w. (E2). Basal levels of response to single square pulses (0.01 ms pulse width) delivered at 0.05 Hz through the stimulating electrode were recorded daily for 2 days after injection. To induce LTP a high-frequency ‘theta pattern’ stimulation was administered. Basal recordings at low-frequency stimulation did not change after injection. After high-frequency stimulation all 7/7 E2 injected animals showed LTP whereas only 1/6 oil injected controls did so; the mean increase in amplitude of the PS and slope of the EPSP after high-frequency stimulation were significantly greater in E2 treated rats. Input/output curves did not change significantly after E2 administration. These results show that at low-frequency stimulation, transynaptic responses of pyramidal neurones in CA1 are not affected by changes in levels of circulating estrogen, while synaptic plasticity — which is at the basis of proposed hebbian associative memory — is facilitated by estrogen treatment. © 1997 Elsevier Science B.V.

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1. Introduction

Levels of circulating estrogen influence sexual behavior and other forms of behavior that are not directly associated with reproduction, such as body temperature, running activity, food ingestion, aggression, sensory sensitivity, mood and performance in memory tests (for review see [42,31]). Although there has been considerable progress in the understanding of the cellular and systemic mechanisms responsible for these changes, many aspects of the neurobiology of steroid action remain unresolved. In this respect, the structural changes of neuronal assemblies responsible for instrumental control of specific responses have been the subject of considerable interest [35]. Previous work indicates that administration of estradiol to ovariectomized rats induces synaptic remodeling and growth in the ventromedial [7] and arcuate [29,30] hypothalamic nuclei. A series of reports from McEwen’s laboratory have demonstrated that this phenomenon is produced by an increase in the number of dendritic spines [14], extending these observations to the hippocampus [16] and showing that this also occurs as a consequence of the normal waxing and waning of estradiol levels along the reproductive cycle of rats [54]. More recently, data from the same laboratory have demonstrated that in the CA1 of estradiol treated rats, pyramidal neurons harbor increased levels of NMDAR1 mRNA and protein [15], findings that explain the greater sensitivity of these neurons to NMDA, but not to AMPA, receptor-mediated synaptic input [55].

An inquiry into the functional consequences of these changes using intracellular recording showed that estradiol administration increased synaptic excitability and prolonged the EPSP in 20% of pyramidal neurons in CA1 [53], in coincidence with pioneering work by Terasawa and Timiras [46], who showed that estradiol treatment decreases hippocampal seizure threshold. At a greater level of complexity provided by the study of transynaptically evoked field potentials, Warren et al. [50] have found that induction of long term potentiation (LTP), which depends

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on synaptic plasticity, is maximal in female rats during the afternoon of proestrus. Accumulating evidence from a variety of experimental human [39,22] and animal [37,27,49,12,36,23] data indicate that estradiol may affect memory and learning processes. Since LTP is generally accepted as an electrophysiological model of memory, it should be useful to probe the functional variations generated by different levels of estradiol. This approach is particularly appealing because induction of LTP depends on the activation of NMDA receptors [5], which are increased by treatment with E2. Present experiments were designed to test the hypothesis that estradiol administration to ovariectomized females facilitates the induction of LTP.

2. Materials and methods

Adult (2–3 months old) Wistar derived female albino rats from our own colony, weighing 250–300 g, were ovariectomized (OVX) under ether anesthesia and maintained in a room with a reversed light-dark cycle (lights on from 21:00 to 09:00 h). At least 2 weeks later the animals were anesthetized with chloral hydrate (100 mg/kg) and placed in a stereotaxic frame with the incisor bar set 3.3 mm below the plane of the interaural line, after Paxinos and Watson [38]. The electrodes consisted of two twisted stainless steel wires, 50 μm diameter, isolated except for the cut end. The stimulating electrode was placed in the stratum radiatum of the dorsal hippocampus (AP −3.8; L 3; V 3) and the recording electrode at the cell body layer in CA1 (AP −3.8; L 1.4; V 2.4), using as a guide the laminar profile of field potentials elicited by pulses to the ipsilateral Schaffer collaterals. A stainless steel screw positioned over the nasal bone served as a grounded reference and another screw over the occipital bone served as a point of fixation. At the position where the population spike (PS) evoked by Schaffer collateral stimulation was maximal, the electrodes were permanently fixed to the skull and screws with dental acrylic. Amphenol pins attached to the upper end of the electrodes were inserted into a miniature plastic connector which was also cemented in place. The animal was then returned to its home cage for a one-week recovery period.

All stimulating and recording sessions were performed

Fig. 1. Recordings obtained in CA1 in one animal using 150 μA stimulation pulses applied to stratum radiatum. (A) E2 was administered on Day 0. (B) On Day 2, after a control recording had been obtained, theta pattern stimulation was applied and recordings obtained 20, 60 and 90 min after tetanization. Calibration marks 1 mV, 8 ms. (C) Tetanization produced potentiation of PS amplitude and increased EPSP slope.
between 10:00 and 13:00 h, with the animal inside its acrylic home cage (20 × 22 × 24 cm) which was placed in an 38 × 45 × 70 cm ventilated isolation chamber. The rats received handling and exposure to the recording chamber for three days, 2–3 h per day, before the first session. The recording signals were fed from the connector on the animal’s head, into a high input impedance differential amplifier set to a gain of 1000 and a band pass of 0.1 Hz to 3 kHz. The output from the amplifier was observed on a digital oscilloscope and stored on the hard disk of a PC type computer after sampling at 10 kHz with a 16 bits analog to digital converter. The freely moving animals were observed under dim red lighting through a VHS camcorder connected to a closed circuit monitor. Every day, after the animal was placed in the recording chamber, 10–20 min were allowed before the recordings began. During this time the animal explored the cage until it came to rest quietly and awake, the head held against gravity with eyes open and no apparent gross body movement. At this time the EEG usually showed large amplitude slow waves, but often would change among various states of arousal when stimulation began. For this reason no systematic effort was made to assure that recordings were obtained during time periods with identical states of EEG arousal. Recordings were temporarily interrupted if the animal resumed walking, groomed or made any movement that was perceived in the monitoring oscilloscope as cable or muscle artifacts. During a session single square pulses (0.01 ms pulse width) were delivered at 0.05 Hz to the stimulating electrode using a stimulator equipped with a

![Figure 2](image_url)

Fig. 2. (A) I/O curves for the amplitude of the evoked PS with respect to control records obtained before injection; E2 or oil vehicle were injected on Day 0. (B) Effect of theta pattern stimulation applied on Day 2; PS amplitude is expressed as percent of control record obtained in the same animal before tetanization. Graphs show means and S.E.M.; * P < 0.02; ** P ≤ 0.001 vs. basal records.
constant current isolation unit; this pulse duration was adopted to minimize the stimulus artifact. Input/output (I/O) relationships for 4 different stimulation intensities (150, 250, 350 and 450 μA) were calculated daily, based on the average of 16 responses per point. These stimulus intensities were chosen because they covered the range of just above threshold to maximum amplitude response for the PS. The changes in the excitability of the response after a stimulus was assessed using paired-pulse stimulation at variable (20, 40, 100, 200 and 400 ms) interpulse time intervals using the lowest stimulation intensity adopted. This paradigm is used to evaluate feedback and feedforward facilitation and inhibition in CA1 (see for example [25]). The extracellular postsynaptic potential (EPSP) was analyzed by its slope measured using linear regression of the first 0.5–2.0 ms interval near its onset, clearly before the onset of the PS. The PS amplitude was measured as the distance from the maximal negative-going peak of the PS to a line tangent to the lower and upper shoulders. The amplitude (mV) of the population spike (PS), slope (mV/ms) of the summed EPSP and paired-pulse facilitation index for PS amplitude (Test PS/Conditioning PS × 100) were analyzed using custom software. Baseline reference records for the I/O curves and for all paired-pulse facilitation intervals were obtained for at least 4 days or until the PS amplitude remained within a ±30% variation in 2 successive days. This was necessary because in some animals during the first days of recording there were large variations in PS amplitude and high levels of exploratory activity made it difficult to obtain noise free

Fig. 3. (A) I/O curves for the slope of the summed EPSP with respect to control records obtained before injection; E2 or oil vehicle were injected on Day 0. (B) Effect of theta pattern stimulation applied on Day 2; EPSP slope is expressed as percent of control record obtained in the same animal before tetanization. Graphs show means and S.E.M.; * P < 0.001 vs basal records.
records. During recording sessions the EEG from CA1 was visually monitored on a storage oscilloscope for evidence of seizure activity. The experimental animals \( (n = 7) \) were injected s.c. between 09:00 and 10:00 h on day 0, with estradiol benzoate dissolved in sesame oil, 100 µg/kg b.w. (E2) and the daily recordings continued as before injection. Two days later, in the course of the recording session, the animals received high-frequency stimulation through the electrodes in stratum radiatum, using theta pattern stimulation as described by Staubli and Lynch [44]. The pattern adopted to induce LTP consisted of 2 trains of 5 bursts, 4 pulses at 100 Hz in each burst, with an interburst interval of 200 ms; the intertrain interval was 30 s. Patterned stimulation was adopted because it has been shown to be more sensitive to modulatory influences [34, 11, 45] avoiding the ceiling effects produced by unpatterned trains of high-frequency stimulation. To induce LTP we used the stimulation intensity that evoked 50% of maximum PS response. To evaluate potentiation, recordings were repeated 20, 60 and 90 min post tetanization using the lowest stimulation intensity (just above threshold for PS before tetanization). The following day animals were sacrificed and their brains removed, fixed in formalin and processed for histological control as indicated by Guzman-Flores et al. [17]. Control animals \( (n = 6) \) were submitted to the same schedule for injection and recording but received sesame oil vehicle instead of E2.

For each animal the raw data for amplitude of PS and slope of summed EPSP of the potentiated response were transformed into percentage of the control records, normalized to a natural log and compared statistically using Duncan’s post hoc test. Records obtained at Days 0, 1 and 2 before theta patterned stimulation were referred to the mean of preinjection control records in each animal; records obtained on Day 2 at 20, 60 and 90 min after theta patterned stimulation were referred to prestimulation records obtained the same day in each animal. The data for the paired-pulse facilitation and coefficient of I/O curve were normalized to a natural log and a multiple analysis of variance (MANOVA) was applied, followed by Duncan’s post hoc test where appropriate. In all cases a difference was considered to be significant when \( P \leq 0.05 \).

### 3. Results

As previously described [3] a single pulse at the Schaffer collaterals evoked a positive going field EPSP in CA1. At higher stimulation intensity (defined as just above threshold), starting from the rising phase of the summed EPSP (Fig. 1), there appeared a single negative-going PS generated by synchronous firing of CA1 pyramidal neurons.

There was no effect of E2 treatment on the amplitude of the evoked PS (Fig. 2, A) through the recording period, up to 48 h after injection (E2 versus Oil; \( \text{df}_{3, 41}; F = 0.522; P = 0.66 \)). Also, in oil injected animals the response was remarkably stable. After tetanization there was a significant effect of treatment (E2 versus Oil; \( \text{df}_{1, 7}; F = 45.72; P < 0.001 \)) and time after tetanization (\( \text{df}_{3, 21}; F = 9.61; P = 0.03 \)) as well as a significant treatment by time interaction (\( \text{df}_{3, 21}; F = 10.03; P < 0.001 \)). Post hoc tests showed that PS amplitude was significantly larger at 20 min \( (P < 0.02) \), at 60 min \( (P = 0.001) \) and at 90 min \( (P < 0.001) \) after tetanization only in animals injected with E2 (Fig. 4). The excitability cycle of the evoked PS as demonstrated by the paired pulse paradigm on the day of injection (Day 0), 24 and 48 hs later. Graphs show means and S.E.M. There were no statistically significant interactions between controls and E2 injected animals (MANOVA).
E2. The criterion of 50% increase in PS amplitude for LTP induction was satisfied in 4 cases within 20 min and the remaining 3 within 60 min post tetanization. On the other hand, the group of vehicle treated control animals did not show potentiation of the PS amplitude (Fig. 2) and only 1/6 satisfied the criterion for LTP within 90 min. At no time during the recording period, before or after theta pattern stimulation, evidence of seizure activity was observed in the EEG recorded from CA1 in E2 or oil injected rats.

The slope coefficient of the EPSP showed a similar pattern of results: no effect of E2 up to 2 days after treatment (Fig. 3, A; df3, 4; F = 0.67; P = 0.57), but a significant effect of E2 treatment (df1.7; F = 15.09; P = 0.006) and time after tetanization (df1.21; F = 7.46; P = 0.001) as well as a significant treatment by time interaction (df3, 21; F = 7.31; P = 0.001). Post hoc tests showed that after tetanization the slope increased significantly at 20 min (P < 0.02), 60 and 90 min (P = 0.01 in both cases) only in animals injected with E2.

The overall analysis of variance showed no significant effect of hormonal treatment, days or pulse interval on paired-pulse facilitation index (df3, 42; F = 1.06; P = 0.39). Fig. 4 displays the effect on percentage of conditioning pulse amplitude on days 0, 1 and 2.

4. Discussion

Treatment of OVX rats with E2 facilitated the induction of LTP. As our results show, a stimulus paradigm that was subthreshold in OVX animals effectively induced LTP in E2-treated animals. Due to the fact that this finding was obtained in unanesthetized animals deprived of internal gonadal secretions, this effect can be directly ascribed to E2 and is in line with the well-documented excitatory, threshold-lowering capacity of estrogen [46] demonstrated in various experimental paradigms (see [21]). In our case, E2 increased the capacity of the CNS to develop a functional change, generally accepted as an electrophysiologically model of memory and learning. This mechanism provides an explanation for the positive effects of raised estrogen levels on cognitive performance on both humans [39,22,23] and rats [37,27,49,12,36,23].

In our experiments, both the PS of the EPSP and the slope of the summed EPSP recorded before high-frequency stimulation were not modified by E2 injection, indicating that the mechanisms involved in the facilitation of LTP induction do not depend on the improvement of responses to low-frequency or single-pulse stimulation. Rather, the obvious conclusion is that E2 specifically improved the mechanisms responsible for the long lasting increase in synaptic strength, which has been shown to depend on coaptaneous activation of pre- and postsynaptic neurons [6]. It would then follow that treatment with E2 facilitated synaptic plasticity in CA1.

It has been known for some time that synaptic plasticity and LTP in CA1 depend on the availability of NMDA type glutamate receptors [10] and coincidentally, E2 increases the number of NMDA type binding sites in CA1 [52]. Wooley et al. [55] recently confirmed this finding, and proposed that this increased affinity may be explained by the greater concentration of the NMDAR1 receptor protein which is regulated by E2. These changes in NMDA binding result in greater sensitivity to NMDA receptor-mediated synaptic input. This effect was demonstrated in slices taken from E2-treated rats in which responses to AMPA receptor-mediated synaptic input and GABA-mediated inhibition were blocked [55]. The simplest explanation of our results would be that treatment with E2 induced in CA1 the synaptic growth and increased availability of NMDA receptors, providing the grounds for a facilitated potentiation of synaptic strength. In fact, both mechanisms seem to be inseparable, at least in CA1, as Wooley and McEwen [47] demonstrated that the increase in density of dendritic spines is blocked by NMDA receptor antagonists. The alternative possibility that LTP was facilitated by disinhibition of pyramidal cells instrumented through the action of E2 on inhibitory interneurons should also be considered, particularly since E2 is preferentially taken up by interneurons [26]. However, this possibility does not appear likely, because in our experiments the excitability cycle of pyramidal neurons measured by paired-pulse facilitation was not modified after E2 injection, at a time when the induction of LTP was facilitated. It is only reasonable to assume that the dynamic regulation of plasticity by E2 may also be operative in the ventromedial hypothalamic nucleus [7,14], arcuate nucleus [30], lateral septal nucleus [33], preoptic area [24] and midbrain central gray [8] where the steroid has been shown to modulate the number or size of synaptic contacts. Direct or transynaptic input from these or other structures containing estrogen-concentrating neurons may have significantly contributed to the lowered threshold for LTP found in CA1.

The observation that treatment with E2 facilitates the induction of LTP is reminiscent of the results of Warren et al. [50] who found that, in cycling rats recorded under urethane anesthesia, the greatest degree of potentiation after tetanic stimulation was obtained in the afternoon of estrus, after the brain had been exposed to peak levels of estrogen. Warren et al. [50] also found that high-frequency stimulation did not in fact induce LTP in cycling urethane anesthetized rats when estrogen levels are low, i.e. in diestrus or estrous (morning or afternoon), or even in males, when experiments were carried out during the afternoon. The intensity of stimuli used by these authors was necessarily low, to prevent the occurrence of seizures. LTP was not induced in recordings obtained during the morning or in other days of the cycle, results which are similar to our finding that LTP was not induced in OVX, oil treated animals. In any case, comparison between cycling and OVX animals should be done with
caution, as the administration of E2 by s.c. injection hardly mimics the continued and variable secretion of estrogen by the ovaries. Furthermore, in cycling rats during the afternoon of proestrus, progesterone is also secreted in a phasic pattern [42]. The effects of this intense and short lasting increase in the circulating levels of progesterone on LTP have not been analyzed. Our data should not be interpreted to mean that LTP induction is impossible in OVX rats. It is quite likely that stronger stimuli or different stimulation parameters could effectively induce LTP in these animals. In fact 1 out of 6 OVX animals injected with oil, did show an increase in response amplitude of over 50% of control, thus satisfying the criterion adopted for induction of LTP.

The question of circadian variability must also be considered, since our records were obtained during the first half of the dark period of the animals daily cycle. In hippocampal slices, pyramidal cells of area CA1 show more LTP when the animals are sacrificed during the light period [18]. Unfortunately this issue has not been studied in awake, chronically implanted animals, and moreover, there appears to be a sexual difference in circadian rhythms which further complicates direct comparisons between the results of the different laboratories. Presumably, the most appropriate time of day to study the function of a neural mapping system would be the time of increased exploratory activity, i.e. the night time in nocturnal animals like the rat.

Wong and Moss [53] found that E2 priming 2 days before obtaining the hippocampal slices, increased synaptic excitability by prolonging EPSP and inducing repetitive firing in response to Schaffer collateral stimulation in a small percentage of CA1 neurons recorded in vitro with intracellular electrodes. However, in a similar preparation Wooley et al. [55] found no difference in the efficacy of synaptic input, unless the postsynaptic response had been stripped of AMPA dependent components. In coincidence with these results, Warren et al. [50] did not observe changes in basic excitability measured by I/O curves during the estrous cycle of chronically implanted rats. In our experiments baseline excitability remained unmodified after E2 administration as evidenced by stable amplitude, slope and I/O relationship of the extracellular field potential. No secondary evoked responses, indicative of repetitive firing, were observed after E2 administration. The reason for the different findings may be the different preparations and/or techniques employed, since recording of population field responses is not particularly sensitive to variations in the excitability of a small percentage of the responding population of neurons. Besides, NMDA and AMPA type receptors are colocalized in 70% of excitatory synapses and only 10% have pure NMDA type receptors [2]. Therefore, the response to synaptic input in CA1 pyramidal neurons is mediated in the majority of the cases by a mixture of AMPA type glutamate receptors (which are not modified by E2 treatment [55]) and NMDA type glutamate receptors, thus allowing a masking effect that precludes the demonstration of changes in amplitude when only NMDA type receptors are increased. Moreover, at low stimulation frequencies only the AMPA type receptors would be activated, given that NMDA type receptors are normally blocked by Mg$^{2+}$ at the hyperpolarized resting state [5]. The fact that binding of AMPA and sensitivity to AMPA-mediated synaptic input is not increased after E2 priming [55] may be the reason why we found no effect on normal low-frequency synaptic transmission, since AMPA receptor channel provides the majority of current responsible for generating synaptic responses at the resting membrane potential [28]. We may conclude that the amplitude of the baseline response to electrical stimulation of Schaffer collaterals is not affected by doses of E2 that increase the number of dendritic spines or augment the sensitivity to NMDA-mediated glutamate input. This proposition is in agreement with recent findings of Tsien et al. [48] using mice genetically deprived of NMDA receptors in CA1. They found that fast responses of pyramidal neurons, presumably mediated by AMPA type receptors, were normal, as were I/O relations and paired-pulse facilitation, whereas NMDA dependent slow responses and LTP were eliminated in knockout mice. In summary, the overall pattern of results obtained in awake or anesthetized animals as well as in hippocampal slices indicates that baseline responses in the majority of pyramidal neurons are not modified by treatment with E2, whereas synaptic plasticity is sensitive to priming with E2. A report by Isaac et al. [19] showed that in a proportion of synapses on CA1 pyramidal cells only NMDA receptors are active, but if LTP is induced in these synapses, AMPA receptors become functional (see also [20]). Our data and the evidence cited above, that NMDA binding is increased by E2, help sustain the hypothesis initially proposed by Warren et al. [50] that E2 facilitates plasticity in CA1 by increasing precisely that population of NMDA receptor-mediated ‘silent synapses’ in growing dendritic spines, whose AMPA receptor-mediated responses sustain the greater synaptic strength observed after patterned stimulation.

The argument about the relationship between LTP and memory has not been solved (for review see [13,1,9]). Recently however, a significant stride forward has been taken, as 2 independent groups demonstrated that in mice in which NMDA dependent neurotransmission was blocked specifically in CA1, LTP could not be induced and spatial memory was jeopardized [32,48,40]. Under the light of these and numerous previous studies [1] it is reasonable to argue that since the hippocampus is definitely involved in storage of spatial memory and E2 facilitates the induction of LTP in CA1, it may also facilitate the spatial performance of female rats. This question has been specifically addressed but the results are contradictory. In one study, administration of E2 to OVX rats improved performance in an active avoidance task but not in the Morris water maze [41], whereas in another study, performance in a water maze task [36] was improved. On the other hand,
Warren and Juraska [51] have studied the correlation between estrous cycle and performance in the Morris test, and determined that although there are variations along the estrous cycle, the results were not as predicted from the LTP data [50]: the best performers were estrous (not proestrous) rats. However, the question of cycle related variability was also addressed by Stackman et al. [43] and by Berry et al. [4], but neither found cycle-related variations in spatial learning and memory. Luine and Rodriguez [27] studied the effect of E2 administration to rats of both sexes and found that, in a radial maze task, the hormone improved the performance of males but not of females.

The pattern of results in this area is obviously conflicting. Here again, some of the divergence may be explained by the differences between OVX animals receiving injections of E2 and cycling animals. It may help solve the variabilities by comparing the differences between OVX animals receiving injection of E2 and ovulating animals or after hormone injection to OVX animals and determining that although there are variations along the estrous cycle, the results were not as predicted from the Morris test, but not long-term potentiation, Neurosci. Lett. 202 (1996) 204–208.

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