Acute Effects of Estrogen on Neuronal Physiology

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Key Words

hippocampus, hypothalamus, neurosteroid, estrogen receptor, synapse

Abstract

It has been known for more than 30 years that estrogen can alter the intrinsic and synaptic physiology of neurons within minutes. The physiological significance of these acute effects has been unclear, however, because some effects require higher concentrations of estrogen than are detected in plasma, and because estrogen secreted by the ovary rises and falls over a time course of days, not minutes. These concerns may be answered by new research demonstrating that estrogen is produced at high levels within the brain itself, and that production of estrogen in the brain may be regulated by neuronal activity. Additionally, recent studies indicate that classical estrogen receptor proteins are found not only in the nucleus where they regulate gene expression but also at extranuclear sites, including at synapses. These findings, together with evidence for new types of extranuclear estrogen receptors, suggest that estrogen might act directly at synapses to activate second messenger signaling, thereby rapidly altering neuronal excitability, synaptic transmission, and/or synaptic plasticity.

INTRODUCTION

Appreciation of estrogen's multiple and complex influences in the brain has increased considerably over the past 30 years. Some effects of estrogen arise from what is termed the classical (or genomic) mechanism, in which estrogen binds to nuclear transcription factor receptors to regulate gene expression; other effects occur too rapidly to require gene expression. Indeed, since Martin Kelly's discovery in the 1970s that estrogen can alter the firing rate of hypothalamic neurons within a few minutes, estrogen has been shown to acutely modulate various aspects of neuronal physiology in multiple types of neurons. Understanding the mechanisms of these acute effects of estrogen, along with their physiological significance, is currently an area of intense research.

The early 1990s marked the beginning of growing interest in estrogen's effects in the hippocampus, a brain region important in learning and memory. To date, approximately 80% of all the papers on estrogen effects in the hippocampus have been published since 1990, compared with fewer than 50% of those on estrogen effects in the hypothalamus. Multiple factors have contributed to this growth in interest. For example, evidence for estrogen receptors in the dorsal hippocampus, the region most commonly studied, was just beginning to emerge in the late 1980s (e.g., 1, 2). Additionally, in 1990, Gould, Woolley, and McEwen published the first in a series of papers showing that estrogen promotes the formation of new dendritic spines and excitatory synapses in the dorsal hippocampus (3–6). The estrogen-induced increase in spine synapse number, which requires several days to occur, provided a possible biological basis for estrogen's effects on hippocampus-dependent memory and seizure susceptibility, which occur with a similar time course. These delayed trophic effects of estrogen in the hippocampus, and elsewhere in the brain, as well as their consequences for neuronal physiology and behavior, have been reviewed previously (e.g., 7, 8), and so are not reiterated here.

At about the same time as the discovery that estrogen increases spine synapse number in the hippocampus, Robert Moss's group began to investigate acute electrophysiological effects of estrogen on the synaptic physiology of hippocampal neurons. Although these investigators made a number of intriguing and careful observations, two concerns about the physiological relevance of these acute effects were raised: First, the concentrations of estrogen necessary to induce these effects were greater than peak circulating estrogen levels during the estrous cycle, and second, the significance of neuronal responsiveness to estrogen on a timescale of minutes was unclear because levels of circulating estrogens produced by the ovary fluctuate on a timescale of days. These concerns may now be answered by new research showing that estrogen can be produced by the hippocampus, independent of the ovary. Furthermore, recent discoveries that estrogen receptors are found not only in the nucleus but also at distinct extranuclear sites, including at synapses, suggest that estrogen could act directly at these sites to acutely alter neuronal physiology.

This review traces the development of four lines of research, focusing primarily on the mammalian hippocampus: acute electrophysiological effects of estrogen, short-term effects of estrogen on memory that may be related to electrophysiological

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changes, estrogen as a neurosteroid, and estrogen receptor localization and function. Research in these areas has converged to suggest that estrogen synthesized within the hippocampus may act locally on extranuclear receptors to activate second messenger systems, which in turn induces rapid, nongenomic, electrophysiological changes that could facilitate memory retention. This scenario represents a considerable departure from the classical view that neuroactive estrogens come primarily from the ovary, diffuse into the CNS, and act through nuclear receptors to produce delayed effects through changes in gene expression. However, given recent findings, the acute effects of estrogen must be incorporated into an updated view of estrogen action in the brain that includes multiple modes and time courses.

ACUTE EFFECTS OF ESTROGEN ON HIPPOCAMPAL ELECTROPHYSIOLOGY

Intrinsic Excitability

The hippocampus in the rodent brain is a large, curved structure that extends from the septal nuclei rostrodorsally, wraps around the diencephalon, and ends caudoventrally in the temporal lobe (9; **Figure 1*a***). The well-organized cytoarchitecture of the hippocampus (**Figure 1*b***) makes its principal cells, the CA1 and CA3 pyramidal cells and dentate gyrus granule cells, easily identifiable in brain slice preparations, which greatly facilitates electrophysiological experiments.

Estrogen, usually in the form of estradiol (see below), has been shown to acutely alter the intrinsic excitability of neurons in a wide variety of brain regions, including the hypothalamus/preoptic area (10–12), amygdala (13), striatum (14), cerebellum (15), and hippocampus. In most of these experiments, estradiol was applied either in vivo or in vitro and found to rapidly alter neuronal firing rates and/or to modulate K^+ currents that control the resting membrane potential or limit action potentials. For example, in the hippocampal CA1 region, several minutes of exposure to 100 pM 17β-estradiol depolarizes some neurons and causes them to fire spontaneously (16). Similarly, 100 pM (17) or 100 nM (18) 17β-estradiol increases excitability of CA1 pyramidal cells within 5–10 min by suppressing the afterhyperpolarization (AHP) that follows an action potential. These effects are stereospecific in that the 17α enantiomer of estradiol affects neither firing nor the AHP. Estradiol-induced suppression of the AHP is paralleled by decreased Ca^{2+} influx and decreased amplitude of at least one of the calcium-activated K^+ currents that contributes to the AHP (18). Further, because estradiol occludes an effect of nifedipine to further suppress the AHP, it is likely that one of its proximal effects is to suppress Ca^{2+} influx through L-type Ca^{2+} channels (17). This suggestion is consistent with suppression of L-type Ca^{2+} channels by 100 pM 17β-estradiol in other cell types (14). Interestingly, the acute effects of 100 pM 17β-estradiol appear to be reversible (16, 17), whereas the effect of 100 nM 17β-estradiol on currents that underlie the AHP is not (18). Consistent with this, the acute effect of 100 nM 17β-estradiol on the AHP is occluded in hippocampal slices taken from animals pretreated with 17β-estradiol.

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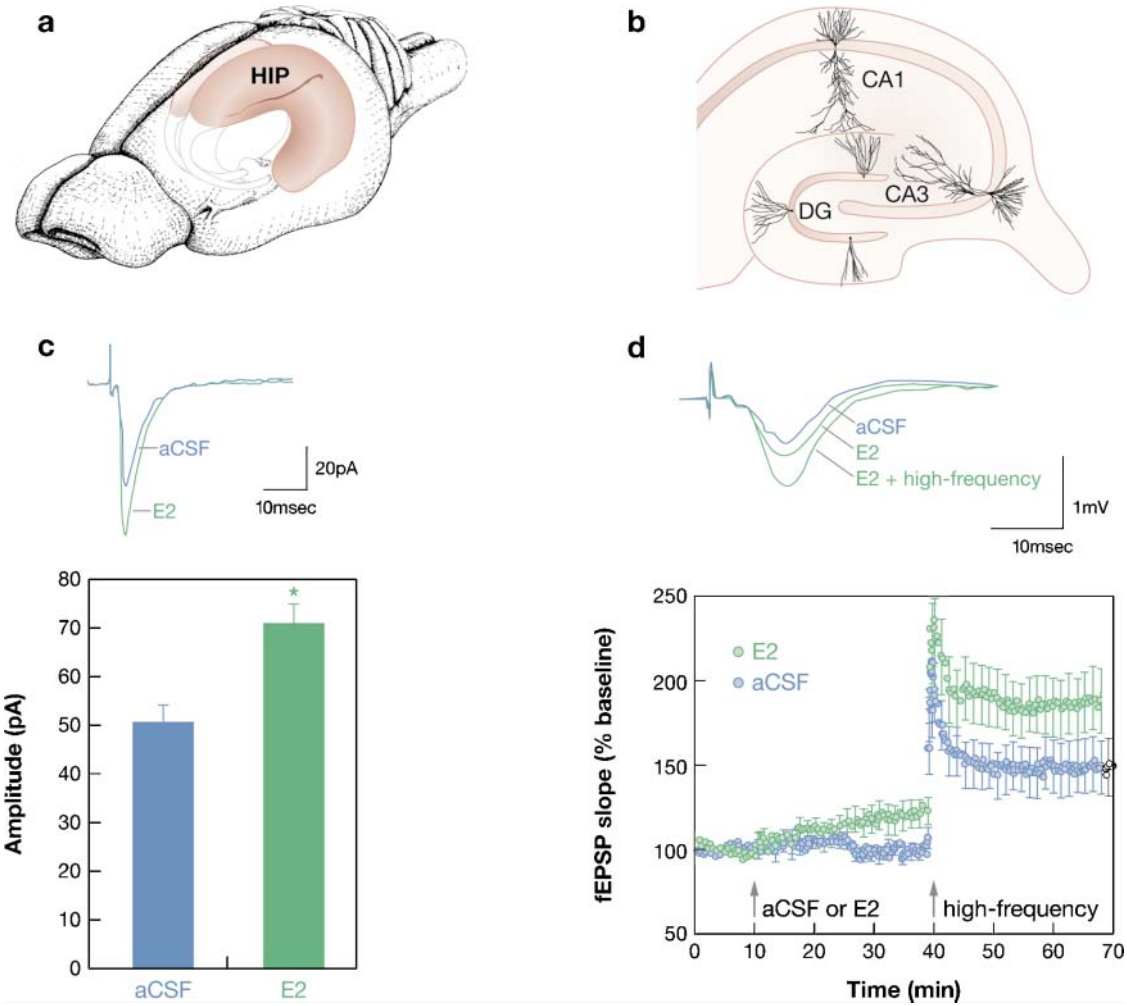

Figure 1

(*a*) Drawing of the rat brain, showing the location of the hippocampus (HIP; *shaded*). (*b*) Drawing of a cross-section through the rat hippocampus, showing the arrangement of principal cells: CA1 and CA3 pyramidal cells and dentate gyrus (DG) granule cells. Panels A and B modified from Reference 9. (*c*) Top: Examples of excitatory postsynaptic currents (EPSCs) in a CA1 pyramidal cell recorded in artificial cerebrospinal fluid (aCSF) and 3 min following addition of 100 pM 17β-estradiol (E2); note that E2 increases EPSC amplitude. Bottom: Group data showing the acute E2-induced enhancement of EPSCs averaged from 34 E2-responsive CA1 pyramidal cells ($p < 0.05$). Panel C modified from Reference 26. (*d*) Top: Examples of dendritic field excitatory postsynaptic potentials (fEPSPs) recorded in aCSF, E2, and E2 followed by high-frequency stimulation; note that E2 increases fEPSP slope and amplitude and these are further increased by high-frequency stimulation. Bottom: Group data showing the E2-induced increase in fEPSP slope and further increase following high-frequency stimulation; note that the relative increase in fEPSP slope induced by high-frequency stimulation is greater in E2. Panel D modified from Reference 20.

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Synaptic Physiology and Plasticity

In addition to its effects on membrane excitability, 17β-estradiol also acutely modulates synaptic physiology in the hippocampus. This was suggested first by Teyler et al. (19), who reported that application of 100 pM 17β-estradiol to hippocampal slices increased the amplitude of the synaptically evoked, extracellularly recorded population spike in CA1 within 5–10 min, and selectively in slices from males (but see below). The principal excitatory input to CA1 pyramidal cells comes from CA3 pyramidal cells, which form glutamatergic synapses with CA1 pyramidal cell dendritic spines. The population spike in CA1 reflects the concerted action potentials fired by many cells together in response to activation of these synapses; an increase in population spike amplitude indicates a greater number of postsynaptic cells firing in response to stimulation of the same presynaptic axons. Although Teyler et al. (19) described their findings as an increase in excitability, inspection of the traces shown in this paper suggests that the slope of the field excitatory postsynaptic potential (fEPSP), which is an indirect measure of dendritic depolarization, was increased as well. Consistent with this interpretation, 17β-estradiol subsequently was shown to increase the dendritic fEPSP in CA1 in slices from males (20–22; **Figure 1*d***) and females (22). Recently, this effect has been extended to the hippocampal CA3 region and dentate gyrus in addition to CA1 (23).

Excitatory synaptic responses in CA1 are mediated by three types of ionotropic glutamate receptors (reviewed in 24): α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), kainate receptors, and N-methyl-D-aspartate receptors (NMDARs). AMPARs and kainate receptors together are referred to as non-NMDARs. Most excitatory synapses in CA1 contain both non-NMDARs and NMDARs, but in varying numbers at individual synapses; differences between synapses in the number, density, and/or phosphorylation state of these receptors confer differential sensitivity to glutamate and differential capacity for synaptic plasticity. In a series of papers from Robert Moss and colleagues, 10 nM 17β-estradiol bath-applied to hippocampal slices from female rats first was shown to increase the amplitude of intracellularly recorded EPSPs in CA1 pyramidal cells (25); this effect occurred within minutes, was reversible, and similar to effects on excitability, was stereospecific. Moss's group found that the ability of 17β-estradiol to potentiate EPSPs was selective for non-NMDAR-mediated responses; potentiation was preserved in the presence of APV, an NMDAR antagonist, but blocked in the presence of CNQX, an antagonist of non-NMDARs. In our own studies, we have used whole-cell voltage-clamp recording to show a similar rapid and reversible effect of 100 pM 17β-estradiol to potentiate synaptically evoked non-NMDAR-mediated excitatory postsynaptic currents (EPSCs) in CA1 pyramidal cells by ~25% (26; **Figure 1*c***).

In subsequent studies using acutely dissociated CA1 cells, Gu & Moss (27, 28) found that 10–100 nM 17β-estradiol potentiated currents evoked by brief puffs of kainate by 30%–40%. Kainate is an agonist for both types of non-NMDAR, and so this was interpreted to reflect the previously observed increase in non-NMDAR-mediated synaptic transmission. Estradiol-induced potentiation of kainate-evoked currents was mimicked and occluded by 8-bromo-cAMP, enhanced by IBMX, a

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phosphodiesterase inhibitor, and blocked by an inhibitor of protein kinase A (PKA) and by GDP-γ-S. These results together suggested that estradiol increases non-NMDAR-mediated postsynaptic responses through cAMP/PKA-dependent phosphorylation in the postsynaptic cell. Although these studies from Moss's group clearly point to an effect of estradiol selectively on non-NMDAR-mediated synaptic responses, another group reported a similar effect of 1 nM 17β-estradiol on both non-NMDAR- and NMDAR-mediated EPSPs in slices (20). Because both non-NMDA and NMDA receptors can be positively modulated by PKA-dependent phosphorylation (29, 24), it is possible that potentiation of both types of glutamate receptor-mediated synaptic responses share a common PKA-dependent mechanism.

Interestingly, in each of the studies discussed above that used single cell recording, only a subset of CA1 cells was responsive to estradiol. In some studies, the proportion of responsive cells was increased by pretreating animals with estradiol for several days. We have confirmed that pretreatment with 17β-estradiol increases the proportion of CA1 pyramidal cells that are responsive to acute estradiol modulation of non-NMDAR-mediated EPSCs, from 33% cells in ovariectomized controls to 73% cells in estradiol pretreated animals (26). These observations suggest that delayed, possibly genomic, effects of estradiol increase neuronal sensitivity to subsequent acute estradiol modulation of synaptic transmission.

In addition to its effects on baseline synaptic responses, estradiol can acutely increase the capacity for synaptic plasticity in CA1. Long-term potentiation (LTP; 30, reviewed in 24) is a well-established model of activity-dependent enhancement in synaptic efficacy that is thought to underlie learning and memory formation. LTP is often induced using brief, high-frequency trains of presynaptic stimuli delivered to hippocampal slices or in vivo. The initiation of LTP at excitatory synapses in CA1 requires NMDAR activation, whereas the resulting potentiation of synaptic strength occurs largely through modifications in the number and/or function (e.g., phosphorylation) of AMPARs. The degree of potentiation is related to the level of postsynaptic Ca^{2+} achieved during the train (31). As would be predicted from acute estradiol-induced enhancement of NMDAR-mediated synaptic responses, high-frequency trains delivered in the presence of 17β-estradiol (100 pM) produced greater LTP than in control (20; **Figure 1*d***). Subsequently, it was shown, using hippocampal slice cultures, that both the increase in baseline synaptic transmission and in LTP induced by 17β-estradiol were blocked by the tyrosine kinase inhibitor, PP2, which also inhibited estradiol-induced phosphorylation of NMDARs (21). Together, these findings demonstrated that acute estradiol activation of intracellular signaling pathways in hippocampal neurons leads to enhancements of neuronal excitability, glutamatergic synaptic transmission, and synaptic plasticity that could acutely facilitate hippocampus-dependent memory.

SHORT-TERM ESTROGEN EFFECTS ON MEMORY

Do the acute effects of estradiol on intrinsic and/or synaptic physiology in the hippocampus contribute to improved hippocampus-dependent memory? Interestingly,

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both reduction of CA1 pyramidal cell AHPs (32) and increased capacity for LTP in CA1 (compare References 33 and 34 to References 35 and 36) correlate positively with improved performance in memory tasks that depend on the hippocampus. Consistent with a role for acute estrogen modulation of hippocampal physiology in memory, four studies have reported time-limited effects of estrogen to improve retention of recently learned information.

Using a Morris water maze, Mark Packard and colleagues were the first to demonstrate a short-term effect of estradiol on retention of hippocampus-dependent spatial memory. The water maze is a large, circular pool of water containing a platform submerged just below the surface. The maze is located in a room with a variety of extramaze cues that rats (or mice) placed into the water can use for spatial orientation. Rats swim to find the platform and thus escape from the water, which is aversive to them. The time required to find the platform decreases as animals learn its location, and the ability to do this is impaired by lesions of the dorsal hippocampus (37). Packard's group showed first in male rats (38), and subsequently in females (39), that an intrahippocampal injection of 1–5 μg estradiol (in water-soluble form, estradiol-hydroxypropyl-β-cyclodextrin) given immediately following training in the water maze improved memory for the platform location tested 24 h later. Interestingly, in both studies, intrahippocampal estradiol given 2 h after training was ineffective. Thus, these results showed that estradiol has a time-limited effect, likely within the hippocampus itself, to improve retention of a recently formed hippocampus-dependent memory.

Two additional studies have confirmed time-limited effects of various estrogens to improve memory; these studies used object recognition/place memory or inhibitory avoidance tasks. Both of these tasks have a spatial component and so depend, in part, on the hippocampus. In one study, 17β-estradiol, 17α-estradiol, or a synthetic estrogen, diethylstilbystrol (DES), was given systemically to rats 30 min before training on object recognition or place memory tasks; each of these estrogens facilitated recall tested 4 h later (40). Interestingly, in this experiment, 17α-estradiol enhanced place memory at a lower dose (15 μg kg^{-1}) than 17β-estradiol (60 μg kg^{-1}), indicating that the 17α enantiomer was more effective. In a second part of the same study, DES (15 μg kg^{-1}) was shown to improve memory in both tasks if given immediately after training, but not 2 h later (post-training 17α- or 17β-estradiol were not tested). Similar to these results with DES, a second study (41) found that systemic 17β-estradiol (10 μg per rat) given immediately post-training, but not 2 h later, improved retention in an inhibitory avoidance task tested 24 h later. In this second study, and in contrast to Reference 40, 17α-estradiol was ineffective in enhancing memory retention.

Together, these behavioral studies show that exogenous estrogens can act within a relatively short time to facilitate memory processes that involve the hippocampus. Although the time course of these short-term effects of estradiol in memory studies (<2 h) is longer than in electrophysiological studies (several minutes), and could involve genomic effects, the results are consistent with the possibility that acute estradiol-induced increases in excitability and/or excitatory synaptic transmission and LTP in the hippocampus contribute to improved memory retention.

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ESTRADIOL AS A NEUROSTEROID

Although the acute electrophysiological effects of exogenous estrogens are intriguing, there are two potential concerns about their physiological relevance: whether the concentrations of estradiol that produce acute effects are achieved physiologically, and whether hippocampal neurons in vivo are exposed to changing levels of endogenous estrogens on a timescale that corresponds to the acute effects that have been observed in brain slices or dissociated cells. In nonprimate mammals, levels of estradiol secreted by the ovary fluctuate across the estrous cycle, which, in the rat, lasts 4–5 days. Blood levels of estradiol are lowest, ~5 pg ml^{-1}, during the estrus stage of the cycle, increase gradually across 2–3 days of diestrus, and then peak at ~40 pg ml^{-1} on the night of proestrus. Female rats are sexually receptive at proestrus ("behavioral estrus"). However, barring pregnancy, the cycle begins again with the estrus stage the following day (42). The proestrus peak of circulating estradiol, at 40 pg ml^{-1}, corresponds to 109 pM. Although some acute effects of 17β-estradiol have been observed with 100 pM, others, such as acute potentiation of kainate-evoked currents in CA1 cells, apparently require a greater concentration. Dose-response studies show that EC_{50} for estradiol potentiation of kainate-evoked currents is 16.4 nM, and this effect is not observed below 10 nM (27). Thus, if during the estrous cycle, estradiol levels rise on a timescale of days, and are, at their peak, two orders of magnitude lower than is required to detect some acute effects, are those effects meaningful? If the ovaries were the only physiological source of estradiol, the answer might be no. However, recent studies indicate that estradiol should be added to the growing list of steroids termed neurosteroids, and that the hippocampus is a site of estradiol synthesis.

The term neurosteroids was used first by Etienne-Emile Baulieu to identify steroids that are produced and act within the brain itself. Baulieu's group observed that levels of dehydroepiandrosterone (DHEA) in the brains of adult male and female rats were up to 20 times greater than in plasma (43). Similar results were subsequently obtained for a precursor of DHEA, pregnenolone (44). In both cases, high brain levels of these steroids persisted even after prolonged removal of peripheral endocrine glands by gonadectomy and adrenalectomy. This raised the possibility that neurons and/or glial cells in the brain produce steroids that could then act locally. However, at the time, the possibility that brain tissue concentrated and retained peripherally generated steroids could not formally be ruled out.

Cholesterol is the precursor of each of the five classes of steroids, including estrogens. There are three biologically relevant estrogens: estradiol, estrone, and estriol, of which estradiol is the most potent in many assays, which is why it is used frequently in studies of estrogen action. The initial step in the biosynthesis of estradiol (**Figure 2**) is conversion of cholesterol to pregnenolone, which is catalyzed by the enzyme cytochrome P450 side-chain cleavage (P450scc). In 1987, Le Gascogne et al. (45) showed immunoreactivity for P450scc in white matter and some neuronal cell bodies in various regions of the adult rat brain. A few months later, Hu et al. (46) demonstrated that mitochondria isolated from rat oligodendrocytes and primary cultures of rat glia were capable of converting cholesterol to pregnenolone. These fundamental discoveries confirmed that the first necessary step for production of steroid hormones

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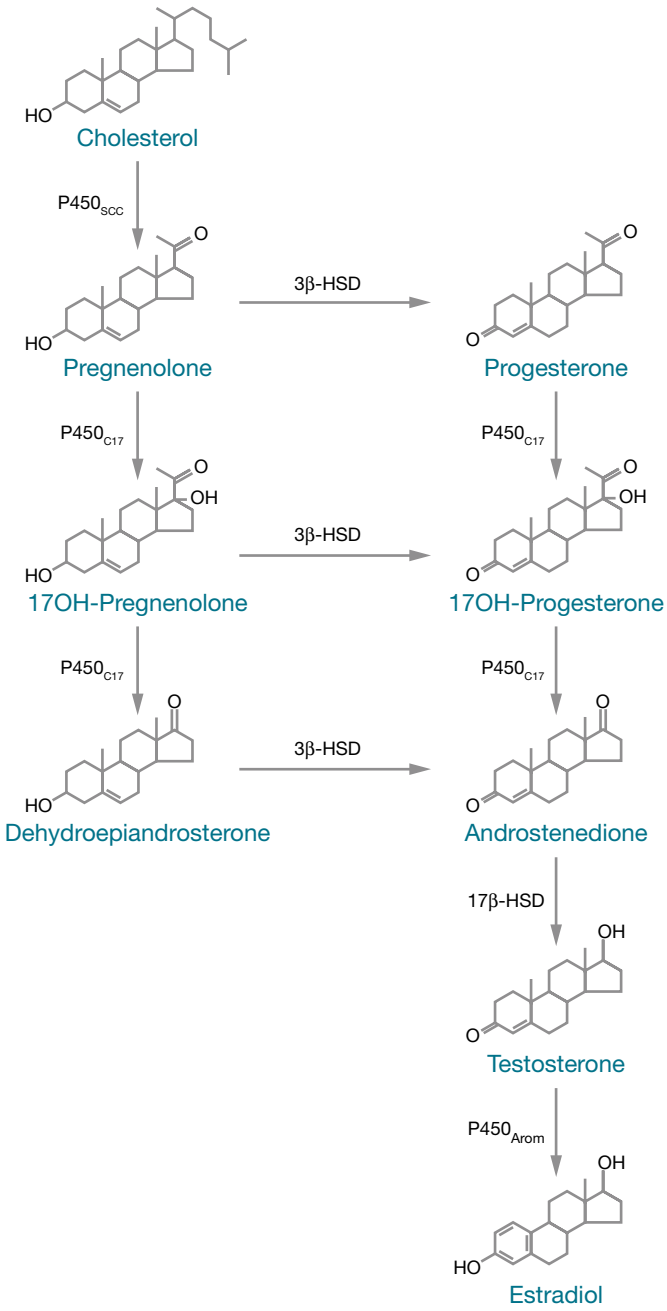

Figure 2

Biosynthesis of estradiol from cholesterol. Cytochrome P450 side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD).

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from cholesterol was present in the brain and set the stage for the next 20 years of research on neurosteroids.

In addition to P450scc, the four critical enzymes in the pathway from cholesterol to estradiol, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), P450-C17, and P450-aromatase, now have been confirmed in at least some regions of the adult mammalian brain. Activities of 3β-HSD (47) and 17β-HSD (48, 49) were detected in brain tissue in the late 1970s. Some years later, mRNA (50, 51) and/or protein (52–54) for these enzymes were also reported. Whether P450-C17 and P450-aromatase are active in the adult brain was more controversial because several studies indicated that these enzymes might be expressed only transiently during brain development (e.g., 55–57). Other studies, however, found activity (58, 59), mRNA (60, 61), and/or protein (59, 62, 63) for each in mature brain.

For locally synthesized estradiol to be involved in producing acute effects on the intrinsic and/or synaptic physiology of hippocampal neurons, the relevant enzymes must be expressed in the hippocampus. Two recent studies (59, 63) confirm that this is the case. Hojo et al. (59) showed that immunoreactivity for both P450-C17 and P450-aromatase is present in hippocampal pyramidal and granule neurons from adult rats. These investigators confirmed that their antisera recognize single bands in western blots from hippocampal tissue and that mRNA for both enzymes in hippocampus is detectable by RT-PCR. Interestingly, although light microscopic analysis showed the most intense labeling in neuronal cell body layers, electron microscopic immunocytochemistry revealed labeling for both enzymes pre- and postsynaptically in the dendritic layers of the CA1 region. To corroborate immunoreactivity, Hojo et al. also demonstrated activity of steriodogenic enzymes in hippocampal tissue ex vivo. First, they showed that hippocampal slices can produce estradiol when stimulated with NMDA, and second, they showed that hippocampal tissue can produce each of the key metabolites in the pathway from pregnenolone to estradiol from their relevant precursors.

These findings are particularly exciting because they suggest that estradiol could be synthesized by adult hippocampal neurons in vivo. Locally synthesized estradiol might then act in a paracrine or autocrine fashion to acutely alter the function of nearby neurons and even synapses. The observation that NMDA can stimulate hippocampal estradiol synthesis suggests that estradiol synthesis might be dependent on neuronal activity, and therefore estradiol levels could be regulated on the timescale of minutes, corresponding to acute electrophysiological effects. Additionally, it is plausible that local concentrations of estradiol achieved through hippocampal synthesis are much higher than those measured in plasma across the estrous cycle, perhaps high enough to produce the acute electrophysiological effects that are observed only with nanomolar concentrations. Consistent with this idea, a recent analysis of estradiol measured by liquid chromatography/tandem mass spectrometry in adult brain tissue from mice that were adrenalectomized and gonadectomized confirms high concentrations in the hippocampus (64). In this study, 17β-estradiol was detectable in some samples, but interestingly, concentrations of 17α-estradiol were much greater, being as high as ~900 pg mg^{-1} protein in males.

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ESTROGEN RECEPTOR LOCALIZATION AND FUNCTION

Nuclear and Extranuclear Estrogen Receptors

What receptors might be involved in mediating effects of estradiol produced in the hippocampus? The classical mechanism of estrogen action is through one of two estrogen receptors (ERs), ERα (65) and ERβ (66), which act as nuclear transcription factors. ERα and ERβ each are composed of several domains (67): (*a*) a highly variable N-terminal domain containing a transactivation region that activates expression of target genes by interacting with core transcriptional machinery; (*b*) a DNA binding domain that contains two zinc fingers and is involved in receptor dimerization and specific DNA binding; and (*c*) a large and complex ligand-binding domain that is also involved in nuclear localization, receptor dimerization, and interactions with other transcriptional regulatory factors. ERα and ERβ share high sequence homology in their DNA and ligand-binding domains (95.5% and 59.7%, respectively, in the rat), but differ in other domains (<30% homology in the rat; 66). When ERs bind estradiol, they can form homo- or heterodimers, which then bind to estrogen response elements (EREs) in DNA and recruit other components of transcription machinery to promote gene expression. Estradiol-bound ERα and ERβ also can bind the Fos/Jun complex to regulate gene expression through activated protein-1 sites in a manner that is independent of EREs (68).

Binding assays with in vitro translated ERs (69) indicate that estradiol has similar binding affinity for ERα ($K_d = 0.1$ nM) and ERβ ($K_d = 0.4$ nM). The 17β enantiomer of estradiol is capable of stimulating ER-mediated transcription, whereas 17α-estradiol can promote DNA binding, but only for a short time and is generally transcriptionally inactive (70). Historically, the pharmacology of estrogen receptors has been puzzling. Some selective estrogen receptor modulators (SERMs), such as tamoxifen, can act as either antagonists or agonists based on cell type, whereas other agents, such as ICI 182780, are so-called pure antagonists. SERMs and the ICI drugs both bind to the same site on the receptor as estradiol. SERMs generally promote receptor dimerization and DNA binding, but block some protein-protein interactions; ICI drugs block receptor dimerization and ER transcriptional activity. It is important to note, however, that the activity of SERMs and antagonists in transcriptional assays cannot necessarily be used to predict their effects on nongenomic estrogen action(s).

Early studies aimed at localizing estrogen-responsive neurons employed in vivo autoradiography (71) in which animals were ovariectomized to remove endogenous ligand, injected with ^{3}H-estradiol, and their brains subsequently were processed to visualize estradiol-concentrating nuclei. More recently, the same approach has been used with ^{125}I-estrogen (72). Theoretically, autoradiography should detect all estrogen-concentrating cells, regardless of which nuclear receptor they contain. In vivo autoradiography indicates that nuclear ERs are found largely in hypothalamic and limbic structures, consistent with the known effects of estrogen on reproductive physiology and behavior.

In the hippocampus, the density of cells containing nuclear ERs depends heavily on the rostrodorsal to caudoventral level examined. In the rostrodorsal hippocampus, in which most electrophysiological and anatomical effects of estrogen have been

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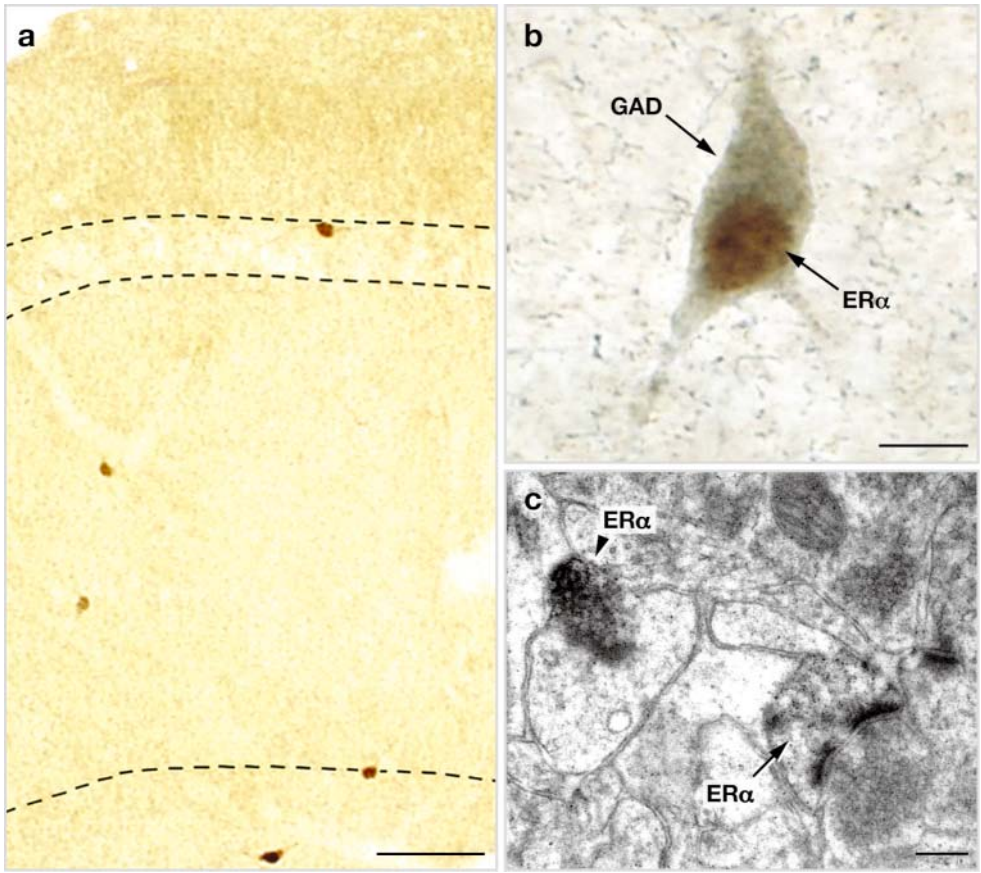

Figure 3

(*a*) Low-magnification view of immunolabeling for estrogen receptor-α (ERα) in the CA1 region of the dorsal hippocampus in the rat. Dotted lines mark boundaries of the pyramidal cell layer (*upper*) and two subregions of the apical dendritic layer (*lower*); note that nuclear labeling is sparse and found mostly in dendritic layers; scale = 50 μm. (*b*) High-magnification view of a neuron in the CA1 apical dendritic layer in tissue double-labeled for ERα and glutamic acid decarboxylase (GAD), which marks inhibitory neurons. The vast majority of nuclear ERα labeling in dorsal CA1 is found in inhibitory neurons; scale = 10 μm. *a* and *b* modified from Reference 73. (*c*) Electron micrograph showing extranuclear ERα immunolabeling in a presynaptic bouton (*arrowhead*) and a postsynaptic dendritic spine (*arrow*) in the CA1 apical dendritic layer; scale = 250 nm.

studied, cells expressing nuclear ERs are quite sparse (71–73; **Figure 3*a***); the low density of these cells and their location primarily in dendritic layers suggest that they are inhibitory interneurons rather than principal cells, and this was confirmed by double-label immunohistochemistry for ERα and glutamic acid decarboxylase, a marker of inhibitory neurons (73; **Figure 3*b***). The number of nuclear ER-containing cells increases through the middle and caudoventral levels of the hippocampus, where,

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in contrast to the dorsal hippocampus, the vast majority are CA1 and CA3 pyramidal cells (73). The pattern of hippocampal labeling observed with immunolabeling for ERα corresponds very closely to that observed with ^{125}I-estrogen autoradiography (72). Indeed, most studies have found very little nuclear ERβ in the hippocampus of either the rat (74) or the mouse (75, 76).

Interestingly, in situ hybridization studies of mRNA expression for ERα or ERβ indicate a weak but detectable signal in the pyramidal cell layers, even in the dorsal hippocampus (77, 78). Discrepancies between in situ hybridization and immunohistochemistry could be due to technical limitations of ER antibody sensitivity, or because mRNA for ERs is not translated into protein. However, an additional possibility is that a substantial fraction of ER protein is located outside the nucleus. Extranuclear ERs would likely have appeared as background in in vivo autoradiography studies, as these focused on estradiol-concentrating nuclei. Indeed, over the past several years, substantial evidence has accumulated for ERs localized in axons, dendrites, and at synapses.

The possibility of extranuclear ERs in neurons was suggested first by Blaustein and colleagues who showed ERα immunoreactivity in dendrites and axonal boutons in the hypothalamus (79). More recently, Milner and colleagues have shown immunoreactivity for ERα (80) and ERβ (81) in some axons, dendrites, and dendritic spines in the hippocampus, and also in glia. In our own studies, we have confirmed ERα immunoreactivity in both presynaptic boutons and postsynaptic dendritic spines in CA1 (**Figure 3*c***), and in glia. Interestingly, ERα immunoreactivity associated with synapses is rarely observed in both pre- and postsynaptic elements of the same synapse. If the ER immunoreactivity associated with synapses reflects functional ERs, then these observations suggest that estradiol could act directly at pre- and/or postsynaptic sites to alter neuronal physiology. Additionally, because these ERs appear to be expressed only in some boutons and spines, such estradiol effects could be synapse-specific rather than neuron-wide.

In addition to extranuclear localization of the classical nuclear ER proteins, a third extranuclear ER, termed ER-X, has also been reported (82). The gene for ER-X has not yet been cloned, but ER-X represents a saturable estradiol binding protein that appears to be distinct from ERα and ERβ, although it has homology to ERα and can be recognized by some C-terminal ERα antibodies. ER-X is found primarily in caveolar-like domains of neuronal plasma membranes and can mediate activation of the mitogen-activated protein kinase (MAPK) signaling cascade by 17β-estradiol, and interestingly also by 17α-estradiol. In fact, 17α-estradiol is the preferred ligand for ER-X. It is unlikely that many of the effects of estradiol in the normal adult brain are mediated by ER-X because ER-X is highly developmentally regulated and barely detectable in the adult. Interestingly, however, ER-X can be induced in the adult by ischemia or other insults (82).

Axonal/Dendritic Estrogen Receptors and Synaptic Physiology

Could the ERs found in axonal boutons and/or dendritic spines mediate acute effects of estradiol on hippocampal physiology? A potential role for ERα in acute estrogen

potentiation of synaptic transmission has been addressed in two studies by comparing the acute effects of estradiol in wild-type versus ERα knockout (ERαKO) mice. Each of these studies so far has been done with the ERαKO mice generated by the Korach group. In one study, rapid potentiation of kainate-induced currents by 50 nM 17β-estradiol was indistinguishable between CA1 cells dissociated from wild-type and ERαKO mice (83), indicating that ERα is not required for this effect. A second study evaluated the effect of acute application of 100 pM 17β-estradiol on extracellularly recorded dendritic fEPSPs in hippocampal slices from wildtype and ERαKO mice (22). This study found that estradiol potentiation of fEPSPs was significantly attenuated in slices from male and female ERαKO mice, but was not completely eliminated. The disparate results of these two studies suggest that these two acute effects of 17β-estradiol may involve different mechanisms. In the case of kainate-evoked currents in dissociated cells, it should be noted that axons and dendrites are mostly eliminated from the preparation so the currents evoked by kainate bypass presynaptic neurotransmitter release mechanisms and arise from largely somatic ion channels. Thus, any acute effects mediated by ERs in dendritic spines and/or axonal boutons would not be detected with this approach. On the other hand, experiments in hippocampal slices leave neurons relatively intact and the electrophysiological responses measured arise from synaptically released neurotransmitter. It is these slice studies that support at least a partial role for ERα in mediating acute estradiol potentiation of synaptic transmission.

There is anatomical evidence that at least a portion of extranuclear ER in hippocampal neurons is located at or near the plasma membrane. For example, electron microscopic immunocytochemistry for ERα (80; S.A. Hart & C.S. Woolley, unpublished) and ERβ (81) show labeling associated with various intracellular organelles and the plasma membrane. How ERα or ERβ associates with the neuronal plasma membrane is not yet clear. These receptors contain neither a known membrane targeting sequence nor stretches of hydrophobic residues characteristic of an integral membrane protein. However, in breast cancer cells and CHO cells, ERα has been shown to be palmitoylated and to associate with other proteins, such as caveolin-1 and Shc, which facilitate its transport to the cytoplasmic face of the plasma membrane (84–86). In these cells, membrane-associated ERα forms a signaling complex with other proteins through which estradiol can rapidly increase cAMP and activate MAPK and phosphatidylinositol-3 kinase (PI3K; reviewed in 87). In non-neural cells, rapid estrogen signaling through MAPK and PI3K has been shown to require dimerization-competent ERα and to involve the ligand binding and distinct protein-protein interaction domains (88). In hippocampal neurons, acute electrophysiological effects of 17β-estradiol depend on increased cAMP levels (27) and/or on stimulation of MAPK activity (21). Thus, it is plausible that a plasma membrane–associated ER is at least partially responsible for these effects. Consistent with this suggestion, a recent study in cultured hippocampal neurons showed that 17β-estradiol can acutely stimulate MAPK-dependent phosphorylation of cAMP response element binding protein (CREB) and suppress L-type Ca^{2+} channel-dependent phosphorylation of CREB (89). Interestingly, 4OH-tamoxifen, which is the active metabolite of the SERM tamoxifen, mimicked both these effects of 17β-estradiol, whereas ICI 182780 blocked

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both effects. These findings suggest that ER dimerization is required for intracellular signaling events that also have been shown to underlie acute estradiol modulation of intrinsic and synaptic hippocampal physiology.

Alternative Estrogen Receptor Ligands: E2-BSA and Other Estrogenic Molecules

Two additional approaches have been used to investigate the nature of the ERs that mediate acute effects of estradiol on hippocampal physiology and/or behavior. One approach is to use estradiol conjugated to bovine serum albumin (E2-BSA), which is presumed to be membrane impermeant and therefore to interact only with extracellular binding sites. Interestingly, several studies have found that effects of 17β-estradiol are mimicked by E2-BSA. Among these are suppression of calcium-activated K^+ currents that contribute to the AHP in CA1 pyramidal cells (18), activation of MAPK (90), MAPK- and L-type Ca^{2+} channel-dependent phosphorylation of CREB (89), and suppression of L-type Ca^{2+} currents [in striatal neurons (14)]. The effects of E2-BSA on potentiation of kainate-evoked currents in dissociated CA1 cells are somewhat more complicated. Neither E2-BSA applied outside of cells nor E2-BSA introduced into cells via a pipette, alone, is capable of mimicking 17β-estradiol-induced potentiation of kainate-evoked currents; however, E2-BSA both intra- and extracellularly does mimic 17β-estradiol (28). Although this curious result remains to be replicated, it suggests that 17β-estradiol is required at multiple sites, inside and outside a cell, to potentiate kainate-evoked currents. Unfortunately, to date, no studies have investigated whether E2-BSA can mimic the acute effects of 17β-estradiol to facilitate synaptic transmission or LTP in hippocampal slices.

As mentioned above, effects of E2-BSA typically are interpreted to indicate estradiol action at the plasma membrane. However, there are potential technical and conceptual problems with this interpretation. From a technical point of view, estradiol can dissociate from the E2-BSA conjugate (91), and there is evidence from other cell types that E2-BSA (92) or other more durable E2-conjugates (93) can be endocytosed within 5 to 45 min. From a conceptual point of view, it is difficult to envision how an E2-BSA conjugate would interact with classical ERs at the plasma membrane. For example, some authors have depicted membrane-associated ERα as an integral membrane protein. However, as mentioned above, ERα and ERβ do not have the structural features of integral membrane proteins. Rather, ERα appears to be able to associate with the cytoplasmic face of the plasma membrane. Biophysically, it is implausible that the estradiol moiety in an intact E2-BSA conjugate could invade the plasma membrane bilayer sufficiently to interact with ERs associated with the cytoplasmic face. Thus, barring dissociation and/or endocytosis, effects of E2-BSA are likely to be mediated by other estradiol-binding proteins in the plasma membrane. Identification of these proteins will be a critical step forward in understanding the acute effects of estradiol on neuronal physiology.

Another approach to understanding the nature of the ERs that mediate acute effects of estradiol is to determine the effectiveness of other estrogenic molecules that have varying affinities for ERα or ERβ. One complication with this approach is

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that, although the relative binding affinity (RBA) of these molecules can be measured, their effectiveness as ER agonists typically is determined in transactivation assays, which may not relate well to nongenomic ER activities. Nevertheless, this approach has provided some insight into the potential involvement of ERα or ERβ in acute effects of estradiol on electrophysiology and behavior.

The most commonly tested alternative to 17β-estradiol is 17α-estradiol, which has a relative binding affinity of 58 for ERα and 11 for ERβ (17β-estradiol = 100; 69). Early reports of acute estradiol effects on neuronal excitability (11, 15) found that 17α-estradiol was unable to mimic 17β-estradiol, and this has held true in most studies (but see 14). In hippocampal neurons, 17α-estradiol was ineffective in mimicking the potentiating effects of 17β-estradiol either on intrinsic excitability (16, 18), synaptic transmission (25, 22), or kainate-evoked currents (27). The lack of effect of 17α-estradiol, the preferred ligand for ER-X, argues against involvement of ER-X in these effects.

Additional estrogenic molecules have been tested for short-term effects on memory. Consistent with the lack of 17α-estradiol effects on hippocampal physiology, Rhodes et al. (41) found no effect of 17α-estradiol on memory retention in the inhibitory avoidance test. However, this is in contrast to results of Luine et al. (40), who found that 17α- and 17β-estradiol were similarly effective in facilitating object recognition, and that 17α-estradiol facilitated place memory at a lower dose than 17β-estradiol. Rhodes et al. also tested several ERα- and ERβ-selective ligands for facilitation of inhibitory avoidance and obtained equivocal results. The highly selective ERα ligand, propylpyrazole triol (PPT), which binds ERα with 410 times greater affinity than ERβ (94), was ineffective at facilitating inhibitory avoidance, pointing to a role for ERβ. Consistent with this idea, coumesterol, which has an RBA of 94 for ERα and 185 for ERβ, did facilitate retention of inhibitory avoidance. However, another more selective ERβ agonist, 2,3-bis(4-hydroxyphenyl)propionitrile (DPN; 95), had no effect. Results with the water maze task were more interpretable; 17β-estradiol, coumesterol, or DPN given immediately after training all appeared to facilitate recall of the platform location 24 h later, whereas 17α-estradiol and PPT were ineffective. Together, these results from behavioral studies with alternative ER ligands suggest that multiple estradiol-ER interactions underlie the various time-limited effects of estradiol on memory facilitation.

CONCLUSIONS

Thirty years of research aimed at understanding how estrogens influence neuronal physiology point to an important role for acute effects on intrinsic excitability and/or synaptic function. Although acute effects of estrogen originally were discovered in the hypothalamus, many studies since have focused on the hippocampus. At this point, all the pieces are in place to support a scenario in which the hippocampus can synthesize its own estradiol, which then could act locally at extranuclear ERs to acutely alter neuronal physiology. Most of the acute electrophysiological effects of estradiol in the hippocampus serve to facilitate neuronal firing, to increase excitatory synaptic transmission, and/or to increase the capacity for synaptic plasticity in the form of

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LTP. Behavioral studies using a variety of memory tasks show that estradiol given within a short time of training can facilitate recall tested 24 h later, consistent with the possibility that acute effects of estradiol on hippocampal physiology play a role in promoting the consolidation of hippocampus-dependent memory.

The literature on acute effects of estrogen makes it clear that a diverse set of cellular mechanisms is likely to be involved. The effects that have been studied vary considerably in their pharmacology, for example, whether or not they are reversible, the minimum effective concentration of estradiol, and to what degree ERα- and/or ERβ-selective ligands can mimic the effects of 17β-estradiol. Extranuclear ERs are found at both pre- and postsynaptic sites, which also suggests multiple types of acute, possibly synapse-specific, actions. The few studies that have investigated acute effects of estradiol on synaptic function in ERαKO mice suggest that ERα plays a role in at least some of these effects, but that other ERs also must be involved.

Much discussion has been devoted to whether acute effects of estradiol are mediated by plasma membrane ERs. Experiments in which estradiol-conjugates (e.g., E2-BSA) mimic estradiol's effects typically are interpreted to indicate that the plasma membrane is the site of estradiol binding. However, nuclear ER proteins do not have the structure of integral membrane proteins; instead, it appears that ERs, with ERα being the more studied, can associate with the cytoplasmic face of the plasma membrane. Because it is unlikely that estradiol in an E2-conjugate can gain access to the inner face of the membrane, the effects observed with E2-conjugates may be due instead to dissociation of the conjugate, rapid internalization, and subsequent estradiol interaction with intracellular ERs, or to the much more interesting possibility of interaction(s) with novel estradiol-binding proteins in the plasma membrane (see below).

There is evidence for interactions between long-term hormone exposure and acute estradiol effects. A number of acute effects are observed to a greater or lesser extent in males versus females, or in animals that have been pretreated with estradiol versus those that have not. An important aim of future research will be to investigate how the genomic actions of steroid hormones alter neuronal susceptibility to acute effects, as well as how acute effects lead to genomic activation. For example, acute estradiol activation of the MAPK signaling cascade and phosphorylation of CREB likely lead to changes in gene expression, even in cells that lack nuclear ERs.

Interestingly, mechanisms related to acute effects of estradiol may be involved in one of the phenomena that contributed to interest in estrogen effects in the hippocampus: the induction of new dendritic spines and synapses. Although spine synapse numbers are regulated by 17β-estradiol and fluctuate over the estrous cycle (e.g., 3, 4, 5, 6), MacLusky et al. (96) recently showed that treatment with 17β- or 17α-estradiol can increase dendritic spine synapse density in vivo as rapidly as 30 min, and furthermore, that 17α-estradiol is more potent than 17β-estradiol in producing this rapid effect. Additionally, while many studies have shown that treatments with exogenous hormones can induce spine synapses on hippocampal (and other) neurons in vivo or in vitro (7, 8), another study recently reported that letrazole, an inhibitor of P450-aromatase, decreases spine density in hippocampal slice cultures (97). This suggests

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a role for hippocampal estradiol synthesis in modulating dendritic spine number, at least in vitro.

Perhaps the best route to understanding the acute effects of estradiol on neuronal physiology will come from mapping the locations and understanding the functions of extranuclear ERs: ERα; ERβ; ER-X; newly recognized G protein–coupled receptors for estradiol, such as GPR30 (reviewed in 98) and others; or as yet unknown estrogen-binding proteins. Any of these may mediate estradiol activation of second messenger cascades. Interestingly, Martin Kelly's work on hypothalamic neurons may again lead the way. His group recently identified a novel G protein–coupled membrane ER in hypothalamic neurons (12). This ER is accessible to the extracellular space, is activated by 17β- but not 17α-estradiol, and acts through phospholipase C to activate PKCδ and PKA, which then modulate K^+ currents. The actions of estradiol on this ER are mimicked by 4OH-tamoxifen but blocked by ICI 182780, a profile that mirrors some effects of estradiol on hippocampal neurons. Thus, if this receptor is also expressed in the hippocampus, it may play a role in mediating similar acute effects of estradiol on hippocampal physiology.

It is now clear that the initial focus on nuclear ERs and genomic actions of estrogen underestimated the number of estrogen-responsive sites in the brain, the time courses of estrogen action, and the variety of cellular mechanisms through which estrogens can influence brain function. Indeed, over the next few years, we may come to recognize that acute effects mediated by extranuclear receptors represent an important, if not the principal, class of estrogen actions in brain, particularly in brain areas in which few cells express nuclear ERs.

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