Genome and Hormones: Gender Differences in Physiology
Invited Review: Estrogens effects on the brain: multiple sites and molecular mechanisms

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McEwen, Bruce S. Invited Review: Estrogens effects on the brain: multiple sites and molecular mechanisms. *J Appl Physiol* 91: 2785–2801, 2001.—Besides their well-established actions on reproductive functions, estrogens exert a variety of actions on many regions of the nervous system that influence higher cognitive function, pain mechanisms, fine motor skills, mood, and susceptibility to seizures; they also appear to have neuroprotective actions in relation to stroke damage and Alzheimer’s disease. Estrogen actions are now recognized to occur via two different intracellular estrogen receptors, ER-α and ER-β, that reside in the cell nuclei of some nerve cells, as well as by some less well-characterized mechanisms. In the hippocampus, such nerve cells are sparse in number and yet appear to exert a powerful influence on synapse formation by neurons that do not have high levels of nuclear estrogen receptors. However, we also find nonnuclear estrogen receptors outside of the cell nuclei in dendrites, presynaptic terminals, and glial cells, where estrogen receptors may couple to second messenger systems to regulate a variety of cellular events and signal to the nuclear via transcriptional regulators such as CREB. Sex differences exist in many of the actions of estrogens in the brain, and the process of sexual differentiation appears to affect many brain regions outside of the traditional brain areas involved in reproductive functions. Finally, the aging brain is responsive to actions of estrogens, which have neuroprotective effects both in vivo and in vitro. However, in an animal model, the actions of estrogens on the hippocampus appear to be somewhat attenuated with age. In the future, estrogen actions over puberty and in pregnancy and lactation should be further explored and should be studied in both the hypothalamus and the extrahypothalamic regions.

Throughout the life span, the brain continues to be shaped and modified by the external world acting through the release and actions of circulating hormones and endogenous growth factors and neurotransmitters. Receptors for steroid hormones and thyroid hormone were the first transcription regulators discovered for eukaryotic cells (84). Besides helping to catalyze the discovery of other transcription regulators (134), the steroid-thyroid hormone family of receptors has provided an important tool for elucidating the sites and cellular mechanisms by which circulating hormones exert permanent developmental effects (e.g., sexual differentiation) and reversible and often cyclic effects on the mature brain (9).

Recent research is showing that the brain is more widely responsive to gonadal hormones than previously thought. That is, not only is the hypothalamus affected by circulating estrogens and androgens but...
also structures like the hippocampus, which undergo sexual differentiation and are hormone responsive in maturity (126). Even the cerebellum is sensitive to estrogens (12, 165, 194, 195). Moreover, major projecting neurons such as cholinergic, serotonergic, noradrenergic, and dopaminergic systems are responsive to gonadal hormones (18, 30, 126). In addition, one of the most striking effects of ovarian steroids is the cyclic induction of synapses not only in the hypothalamus but also in the hippocampus of female rats. Studies of this process have revealed new aspects of steroid hormone action involving the interactions between cells and between steroid hormones and neurotransmitters. Finally, there are developmentally programmed sex differences in many of these systems; therefore, it is not so surprising that a variety of nervous and mental disorders and recovery of the brain from damage are subject to sex differences and to gonadal hormone regulation (30, 126).

This review traces the development of studies on nonreproductive actions of ovarian hormones as an outgrowth of the studies of steroid hormone receptors in brain and their role on reproductive actions of estrogens and progestins. An overview of the nonreproductive actions of ovarian steroids in three brain areas in the context of our emerging knowledge of cellular mechanisms of steroid hormone actions and sexual differentiation of the brain and in relation to the impact of stress on cognitive function is also provided.

HISTORICAL PERSPECTIVE ON HORMONE ACTION IN BRAIN AND ROLE OF GENE EXPRESSION

A German zoologist, A. A. Berthold, published in 1849 the first experiment in the field of endocrinology. He transplanted the testes of a domestic rooster into the abdominal cavity of a capon and observed the return of secondary sex characteristics as well as the aggressive, copulatory, and vocalization behaviors of a typical barnyard cock (15). More than a century later, Ron Barfield demonstrated that crystals of testosterone implanted in the hypothalamus of a capon would reactivate sexual, aggressive, and vocal behaviors (7). At about the same time, tritium-labeled steroids were used to demonstrate intracellular steroid hormone receptors in endocrine target organs throughout the body (83). These receptors affect gene expression by acting on transcriptional events in the cell nucleus (8, 156, 222).

Studies of the mechanism of steroid hormone action have provided important insights into the control of eukaryotic gene expression, and studies of steroid receptors in brain showed that the brain responds by the same mechanism as the nonneural tissues. There have been three phases of investigation of steroid hormone effects on the brain. First, steroid and thyroid hormone receptors in the brain have been characterized and mapped, establishing that the nervous system has receptors for all six classes of steroid hormones (estrogens, androgens, glucocorticoids, mineralocorticoids, progestins, and vitamin D) as well as thyroid hormone and retinoic acid. Each receptor class is expressed in its own unique, developmentally regulated regional pattern in the brain (122, 161, 190, 197). Second, the role of steroid receptors in behavioral and neuroendocrine events has been elucidated by pharmacological means, including brain sexual differentiation. These studies have used agonists and antagonists applied systemically or locally into brain regions having receptors and known roles in behavioral or neuroendocrine events (64, 118, 213). Third, specific cellular and molecular processes have been identified in brain regions linked to specific functions. These processes include hormonal regulation of neuropeptide gene expression (2, 36, 75, 189) and aspects of signal transduction (98). Studies of structural plasticity, involving synaptogenesis (29), the retraction and expansion of dendrites (43), and neuronal cell death and neurogenesis (60) are also included in the area of investigation.

Besides the hypothalamus (98) and bulbocavernous nucleus of the spinal cord (21), one of the brain regions that has emerged as having considerable hormone-regulated plasticity is the hippocampus. This brain region has special relevance to human cognitive process and neurological disorders. Moreover, studies of the hippocampus have made it apparent that some steroid actions involve the coparticipation of certain neurotransmitters, such as N-methyl-D-aspartate (NMDA) receptors in the actions of estrogens and glucocorticoids. In addition, steroids alter the structure of cells that do not appear to have the genomic steroid receptors inside of them; rather, the effects may be transmitted via other steroid-sensitive neurons, as in the case of the hippocampus. Finally, the notion of a morphologically stable adult brain is contradicted by findings that steroids alter structure of the adult brain, including remodeling of synapses, changes in dendritic structure, and neurogenesis. These topics will be discussed later in this review.

Another aspect of the growing sophistication in our understanding of steroid hormone action concerns sexual differentiation of the brain. Sexual differentiation involves programming of the structure and wiring of the brain (22, 58, 64, 214). There are also sex differences in gene expression, in which the same hormone results in different responses in the male and female brain (119). For example, estradiol has a double role, as an ovarian steroid in females and as the product of the aromatization of testosterone in males. Therefore, it is not surprising that estradiol produces somewhat different effects on the male and female brain, for example, inducing prodynorphin mRNA in the female rat anterioventral periventricular nucleus but not in the male rat (189). Moreover, some of these sex differences are known to be reversed by the hormonal conditions during early life that reverse the sex differences in sexual behavior. For example, the anterioventral periventricular nucleus in the male rat expresses more preproenkephalin mRNA than that in the female rat, whereas the reverse is true for prodynorphin mRNA;
females that are androgen sterilized at birth show male patterns of neuropeptide gene expression (189).

With this background in mind, how knowledge of cellular and molecular mechanisms of steroid hormone action in brain and other tissues has evolved, leading to increased sophistication about the subtleties of differentiation and regulation of gene expression, will be considered.

MECHANISMS OF STEROID HORMONE ACTION

Steroid hormone actions on gene expression. The identification and mapping of cells expressing the genomic steroid receptors by binding, immunocytochemistry, and in situ hybridization has provided the target sites for investigation of hormonal control of gene expression. Nevertheless, it is only a starting point because hormonal regulation of gene expression cannot be predicted with any certainty from one brain region to another. For example, vasopressin is an important neuropeptide system that is subject to gonadal hormone regulation, and vasopressin mRNA levels are induced by androgens in the bed nucleus of the stria terminalis (132) and are suppressed by glucocorticoids in the paraventricular nuclei (177). Corticotrophin releasing hormone (CRH) gene expression is suppressed by glucocorticoids in paraventricular nuclei (81) but is induced in placenta (170) and central nucleus of the amygdala (180); however, there are many brain areas that contain glucocorticoid receptors in which there is no glucocorticoid regulation of CRH gene expression (81). With this in mind, what is known about estrogen receptors and their expression and distribution will be summarized.

Intracellular estrogen receptors. Beginning in the early 1960s, radioactive estrogens were used to identify estrogen receptors inside of the cell nuclei. The identification and mapping of cells that contain estrogen receptors extended from the uterus and mammary glands to the brain and pituitary gland (see Ref. 161). At first, only estrogen receptors in the hypothalamus and pituitary gland were studied because they were the most obvious and also the most obviously related to estrogen actions on reproduction. Eventually, however, nerve cells containing estrogen receptors were recognized in brain regions like the hippocampus, cerebral cortex, midbrain, and brain stem. We are now aware of two types of intracellular estrogen receptors, ER-α and ER-β (see Ref. 124 for summary).

ER-α shows a characteristic distribution, with high levels in the pituitary, hypothalamus, the hypothalamic preoptic area and amygdala and with much lower levels, and more scattered distribution, in other brain regions. The discovery and cloning of ER-β (101, 160, 205) provided a basis for understanding how the knockout of ER-α (αERKO) (97, 192) could have resulted in a viable organism and in the continued actions of estrogens on some tissues. However, after knockout of ER-β, βERKO mice appear quite normal and are able to reproduce, although they show some reduction in litter size (99). This is in contrast to the αERKO mice, which are sterile and show altered sexual and other behaviors (see Ref. 124).

Measurements of mRNA for ER-α and ER-β reveal distributions in the body that differ quite markedly from each other, with moderate to high expression of ER-α in pituitary, kidney, epididymus, and adrenal glands, moderate to high expression of ER-β in prostate, lung, bladder, and brain, and overlapping high expression in ovary, testis, and uterus (100). Isoforms of ER-β have been identified (see Ref. 124). The best characterized of these splice variants is ER-β2, as opposed to the originally identified isoform ER-β1. ER-β2 appears to have a lower affinity for estrogens than ER-β1 (31), presumably due to a 18-amino acid insertion in the ligand binding domain (115). There are also other splice variants of ER-β with differential expression in brain and other tissues, including a variant missing exon 4 that does not bind estradiol in the hippocampus (166). Estrogen receptors and actions in hippocampus will be discussed below. The fact that not all actions of estrogens and other steroids are delayed in onset and prolonged in duration, implying another fundamental mechanism of action, will be considered next.

Genomic and nongenomic mechanisms. It has been customary to distinguish between steroid hormone actions that are delayed in onset and prolonged in duration, called “genomic” effects, and actions that are rapid in onset and short in duration, called “nongenomic” effects (127) (see Fig. 1). This is because the discovery of intracellular steroid hormone receptors in the early 1960s led to a focus on the long-lasting effects of steroids on cell function, even though rapid actions of steroids were known from anesthetic effects of progesterone (182). Although rapid and delayed effects of steroids are clearly distinguishable from each other at their extremes in terms of mechanism, there is a gray area of uncertainty for actions that have onset times of minutes, as to whether genomic or nongenomic mechanisms apply. Genomic actions of glucocorticoids on lymphocytes were reported with onset latencies of 20 min (127). However, changes in neural activity recorded in vivo after systemic administration of steroids could have onset latencies of minutes, leading to uncertainty as to whether the lag was due to a delay in steroid reaching the tissue or to an intrinsic delay in the mechanism of action (161). Still further uncertainty about mechanisms of action was provided by demonstrations of rapid, but apparently genomic, actions of steroids affecting neuronal excitability and promoting or suppressing long-term potentiation (85, 157). On the other hand, at least several steroid actions on membranes involve either a demonstrated coupling to G proteins or result in the generation of a second messenger (149), as will be discussed below (90, 91). This raised the possibility that a membrane steroid receptor may regulate gene expression indirectly via a second messenger-regulated DNA binding protein such as a member of the CREB family (131). Second messenger-regulated DNA binding proteins such as CREB...
represent an important class of transcriptional regulators that parallel the actions of steroid hormones. In addition, the phosphorylation of steroid hormone receptors by second messenger systems has been shown in a rodent study to activate their transcriptional activity, resulting in changes in female sexual behavior in rodents (112, 164).

Steroid hormone actions on putative receptors on membranes. Rapid membrane actions of steroids are not new. Anesthetic effects of progesterone derivatives (182) led, after many years, to the recognition of a unique membrane recognition site on many subunit combinations of the GABAA-benzodiazepine receptor system (53, 122). A-ring-reduced metabolites of progesterone and desoxycorticosterone are among the most active steroids affecting the GABAA receptor system, and such metabolites are produced in the body and in the brain from the parent steroid. The effects of these steroid metabolites include not only anesthetic effects but also antiepileptic, sedative-hypnotic, and anxiolytic actions (53). Their role in behavior is suggested by experiments in the hamster, in which it was found that local application of GABAA-active derivatives of progesterone to the ventral tegmental area of the estrogen-primed hamster was able to facilitate lordosis (48).

Nongenomic actions of estrogens. There is now a more widespread appreciation for nongenomic actions of steroids involving rapid actions of estrogen, progestins, and vitamin D and its metabolites (122, 142). Moreover, there are also rapid actions of glucocorticoids via G-protein-coupled receptors (150). In the newt Taricha granulosa, the corticosterone site has been shown to couple to a G protein and to have the characteristics expected of a site involved in the rapid...
inhibition of sexual behavior (148). Estrogen receptors also show coupling to second messenger systems, in some cases via G proteins (89, 90). In some instances, the intracellular receptors ER-α and ER-β appear to be involved, whereas in other cases the pharmacology of the effects favors a novel type of estrogen receptor (90).

Rapid estrogen effects on neuronal excitability (88, 141) have been known for a number of years. Only recently, however, has this topic emerged in full force as an alternative aspect of estrogen action that involves interactions of estrogen receptors with second messenger systems and potentially novel types of estrogen receptors (23, 90, 91). The variety of nonnongenic estrogen effects includes rapid actions on excitability of neuronal and pituitary cells and the activation of cAMP and mitogen-activated protein kinase pathways that affect activity of such targets as kainate and insulin-like growth factor-I receptors. Estrogen actions also involve modulation of G protein coupling and affect Ca²⁺ currents and gonadotropin-releasing hormone release, effects on Ca²⁺ channels and Ca²⁺ entry, rapid actions on neurite outgrowth and motility, and protection of neurons from damage by excitotoxins and free radicals (23, 90, 204).

For estrogen actions on some aspects of Ca²⁺ homoeostasis, certain aspects of second messenger systems, and some features of neuroprotection, a novel receptor mechanism is implicated, in which stereospecificity for 17β-estradiol over 17α-estradiol is replaced by a broader specificity for the 3-hydroxyl group on the A ring (104). On the other hand, there is also evidence that ER-α and ER-β are capable of participating in second messenger cascades involving second messenger activation and G protein coupling (90, 169). Membrane estrogen receptors have also been reported on pituitary, uterine, ovarian granulosa cell, spermatozoa, testes, and liver cell membranes, but these have been only partially characterized in binding studies and only in a few cases have been shown to be linked to signal transduction mechanisms (see Refs. 90 and 104 for review).

Finally, there are the novel ways in which estrogenic compounds protect nerve cells from damage by excitotoxins and free radicals (see Ref. 104). In this realm, there are neuroprotective effects that appear to be mediated via classical genomic receptor because they can be blocked by estrogen antagonists. There are also other actions that are not blocked by these antagonists, and these appear to involve a novel mechanism in which 17α-estradiol is as potent as 17β-estradiol (11, 67). These actions of estrogens, albeit from 100 nM to micromolar concentrations, reduce the production of or actions of free radicals in causing cell damage and promoting cell death through apoptosis. Mitochondria are major targets of estrogen action, and the estrogen effects stabilize mitochondrial membrane potentials, prevent ATP depletion, and reduce the generation of oxygen free radicals (116, 207).

OVARIAN STEROID EFFECTS ON THE NERVOUS SYSTEM

Effects on reproductive function have revealed basic principles of hormonal control of behavior. We have learned much about how steroid hormones regulate behavior from the study of ovarian hormone actions on neurons of the ventromedial hypothalamus, which are important for the regulation of sexual behavior in female rats (161). Hormone actions in ventromedial nucleus that are relevant to activation of lordosis behavior in female rats include regulation of neuropeptide gene expression (171) and second messenger systems (98) and the induction of oxytocin receptors and progestin receptors and the regulation of cyclic synaptogenesis (29, 45, 118, 121, 181). There are also developmentally programmed sex differences involving both neuronal wiring as well as programming of responses to hormonal activation of gene expression (118, 125). All of these actions occur in neurons that express high levels of estrogen and progestin receptors, in contrast to the hippocampus, which we shall discuss later, in which estrogen receptors are very scarce and are found in interneurons and in nonnuclear sites within pyramidal neurons.

Actions of ovarian hormones outside of the hypothalamus. Gonadal hormones have many effects on the nervous system that extend beyond their very important actions of regulating gonadotrophin and prolactin secretion and modulating sexual behavior. Perhaps the most prominent examples are the effects of estrogens and androgens on verbal fluency, performance on spatial tasks, verbal memory tests, and fine motor skills (72, 73, 185) and the actions of estrogens to influence locomotor activity in animals (195) and to affect symptoms of Parkinson’s disease and tardive dyskinesia in human subjects (10). The hormonal influences on memory processes appear to involve actions on brain structures such as hippocampus and basal forebrain, whereas the effects on normal and abnormal motor activity undoubtedly involve brain structures such as the caudate putamen, nucleus accumbens, and substantia nigra and ventral tegmental (A9 and A10, respectively, dopaminergic nuclei of the midbrain).

Many of these estrogen effects differ qualitatively or quantitatively between the sexes, suggesting that they may be influenced by the process of sexual differentiation during early prenatal or postnatal development; alternatively, circulating hormone levels may contribute differentially in male and female adults. Sex differences in brain function also include gender differences in the incidence of psychopathologies such as depressive illnesses (more common in women), substance abuse, and antisocial behaviors (more common in men), as well as pain sensitivity (124).

The diversity of these effects implies that regions of the brain are involved outside of the hypothalamus. Indeed, mapping of intracellular receptors, which modulate genomic actions, has revealed the presence of ER-α and ER-β and progestin receptors in regions such as the amygdala, hippocampus, cingulate cortex, locus...
coeruleus, and midbrain raphe nuclei and central gray (124). Although the density of such receptors is far lower and more diffuse in many of these brain areas compared with the hypothalamus and amygdala, the existence of prominent estrogen and progestin effects in many of these brain areas requires a careful examination of the role of the cells that do express intracellular receptors in these brain regions, as well as a consideration of possible alternative mechanisms of steroid action. There are also indications that steroid receptor expression is developmentally regulated and that estrogen and progestin receptor expression is transient in some brain regions and stable in others (47, 118, 144). Three brain areas affected by ovarian hormones will now be discussed in more detail. Other brain areas and estrogen actions are discussed in Ref. 124.

ESTROGENS AND THE BASAL FOREBRAIN CHOLINERGIC SYSTEM

Estrogens affect the basal forebrain of the rat and, in particular, regulate the cholinergic neurons that project to cerebral cortex and hippocampus, where they play an important role in cognitive function. Studies of estrogen effects on the expression of cholinergic enzymes were among the first that pointed to nonreproductive actions of gonadal steroids (106, 107). Experiments with ovariectomy and estrogen replacement therapy revealed an induction of choline acetyltransferase (ChAT), the rate-limiting enzyme for acetylcholine formation, within 6–24 h in basal forebrain of female rats. In addition, there was evidence from measurements of increased ChAT activity in projection areas of the basal forebrain 10 days after hormone injection that estrogen-induced ChAT was transported from cell bodies to nerve endings. Estradiol also induced acetylcholinesterase, as well as ChAT activity, suggesting that a general trophic effect on the cholinergic neurons might occur (120).

A recent investigation of long-term (5–28 wk) ovariectomy and long-term estrogen replacement revealed a decline in high-affinity choline uptake and in ChAT activity in frontal cortex and hippocampus that was at least partially prevented by estrogen therapy (191). Along with these effects, long-term ovariectomy caused a decline in learned performance of active avoidance behavior that was prevented by estrogen replacement therapy (191). One possible candidate as a regulator of the cholinergic system of the basal forebrain are neurotrophins such as nerve growth factor and brain-derived neurotrophic factor. These are produced by the hippocampus and retrogradely transported to basal forebrain neurons to produce trophic effects (96) and are influenced by estrogen treatment (54, 55). Estrogen receptors were colocalized with low-affinity nerve growth factors receptors in cholinergic neurons of the basal forebrain of the rat (203).

The basal forebrain of male rats failed to show the same response to estradiol treatment as female rats, and postnatal estrogen treatment of females or blockade of aromatization in males failed to change this sex difference (107, 108, 120). The basal forebrain cholinergic system differs between male and female rats, with females having smaller and more densely packed cholinergic neurons compared with untreated males (211). Moreover, application of triiodothyronine (T3) to newborn male and female rats, creating transient hypothyroidism during the first postnatal week of life, revealed further indications of sexual differentiation of the basal forebrain cholinergic system, in which male rats responded to the treatment but female rats did not (211). For example, treatment with T3 increased cholinergic cell density and induced increased ChAT activity and muscarinic receptor binding in the septum/diagonal band region of male rats. Female rats did not respond to T3 in most respects, except in the medial septum, where they showed the opposite effect to males, namely, an increased cholinergic cell body area (211).

On the other hand, in another study of sex differences in the cholinergic system, female rats showed greater effects than males to the cholinergic lesions produced in the hippocampus by the specific cholinergic neurotoxin AF64A; female rats were particularly sensitive when the toxin was administered into the lateral ventricles on the day of proestrus (80). Taken together, these results point to a sexually dimorphic organization of the basal forebrain cholinergic system in the rat, involving a prenatally programmed difference in the neuroanatomic organization. In addition, there are sex differences in response to estradiol in cholinergic enzyme induction and the effects of T3 treatment within the first week of postnatal life. These differences may underlie, at least in part, the sex differences in spatial learning that are discussed below.

OVARIAN HORMONES AND THE SEROTONERGIC SYSTEM

The serotonin system projects widely to many brain regions and regulates many aspects of brain function, ranging from autonomic nervous system reactivity to mood, aggression, and cognitive function (38, 65, 78, 82, 113, 174, 176). Ovarian steroids regulate the serotonin system of rodents and primates (4, 18), but there are important differences in the relationship between detectable intracellular estrogen receptors and the serotonergic neurons.

In primates, both ER-α and ER-β are found in midbrain serotonin neurons, and estrogens regulate tryptophan hydroxylase as well as progestin receptor expression (17, 18, 159). In rats, ER-α is found in non-serotonin neurons, in which estrogen regulates expression of progestin receptors (4) but not expression of tryptophan hydroxylase (S. Alves, unpublished observations). Sex differences are found in the ability of estrogen treatment to induce progestin receptors (4). In mice, both ER-α and ER-β are present and functional in the midbrain. ER-α is expressed in serotonin neurons that also express progestin receptors (5). The
αERKO mouse, however, also shows estrogen induction of progestin receptors in midbrain raphe, implying that another estrogen receptor, most likely ER-β, is involved (5).

Estrogens also regulate other components of the serotonin system besides tryptophan hydroxylase. In midbrain raphe of primates, estrogen treatment decreased serotonin transporter mRNA expression (158). In macaque hypothalamus, estrogen treatment decreased expression of the 5HT2C serotonin receptor in a number of hypothalamic nuclei (70). In rat brain, 32 h of estrogen treatment increased levels of 5HT2A mRNA in dorsal raphe and 5HT2A receptor binding in frontal, cingulate, and primary olfactory cortex as well as in nucleus accumbens (198). There were no sex differences in this induction (198). Another study on rats reported that a 24-h estrogen treatment increased 5HT2A mRNA levels in amygdala, hippocampus, accumens, and a number of cortical areas but decreased 5HT1A mRNA levels in many of the same brain regions of several strains of rats differing in anxiety-related behaviors (152). Chronic (2 wk) estrogen treatment also decreased 5HT1A receptor binding in amygdala, hippocampus, and cerebral cortex (151). In this study, however, the effects of estrogen on 5HT1A mRNA levels, evident after acute estrogen treatment, disappeared with the chronic treatment that decreased 5HT1A receptor binding.

The actions of estrogen on the 5HT1A receptor system illustrate the complexities of distinguishing between traditional genomic effects of estrogens and those involving a nonnuclear action. Estrogen treatment causes a rapid decrease in coupling to G proteins that reduce the inhibitory effect of 5HT1A agonists on lordosis behavior, hyperphagia, and oxytocin and adrenocorticotropic hormone responses (136, 167). Regarding the rapid estrogen-induced decrease in 5HT1A efficacy, this has been assessed by measuring binding of radiolabeled guanosine 5’-O-(3-thiotriphosphate) (GTPγS) binding (136) after treatment with estrogen in homogenates of hippocampus and frontal cortex. 17β-estradiol (EC50 = 25 nM) showed a dose-dependent ability to decrease GTPγS binding, and this effect was mimicked by diethylstilbestrol but not by the less potent estrogens, 17α-estradiol and estriol, and was blocked by the estrogen antagonist, ICI-182780 (136). These results are consistent with the involvement of a nonnuclear form of ER-α or ER-β.

ESTROGEN ACTIONS IN THE HIPPOCAMPUS IN RELATION TO COGNITIVE FUNCTION

One of the most surprising actions of estrogens concerns the regulation of synapse formation and breakdown in the female rat brain, and it also illustrates a convergence of some of the cellular and molecular mechanisms of estrogen action noted above, as well as the fact that a brain region does not have to have high levels of cell nuclear estrogen receptors to be responsive to circulating estrogens. Indeed, nonnuclear forms of estrogen receptors appear to play an important role in the hippocampus, as well as in other brain regions, and these have largely escaped detection until relatively recently.

The hippocampus is an important brain structure because it is implicated in spatial and explicit memory functions (37) and is part of the limbic system and therefore a participant in the affective and vegetative states (66). It is a brain region that provides “context” and cognitive meaning to many appetitive and aversive events (102, 103), and it is a major target for circulating adrenal steroids and/or for their actions during stress and in the diurnal sleep-waking cycle (128). The function of the hippocampus will be discussed further after first summarizing the story of the hippocampal synapse formation under the control of estrogens.

Ovarian hormones interact with NMDA receptors to induce new synapses. Estrogen treatment of ovarioctomized female rats increases dendritic spine density on CA1 pyramidal neurons. As observed by electron microscopy (EM), treatment of ovarioctomized adult rats with estrogen also induces new synapses on spines and not on dendritic shafts of CA1 neurons (217). There are no estrogen effects on dendritic length or branching (62, 216, 217). Progesterone treatment acutely enhances spine formation. However, over a 12- to 24-h period, progesterone caused the downregulation of estrogen-induced synapses (62, 218).

Estrogens do not act alone. Ongoing excitatory neurotransmission is required for synapse induction, as shown by the finding that antagonists of NMDA receptors block estrogen-induced synaptogenesis on dendritic spines in ovarioctomized female rats (219). Because estrogen treatment increases the density of NMDA receptors in the CA1 region of the hippocampus (209, 220), the activation of NMDA receptors by glutamate is an essential factor in causing new excitatory synapses to develop.

Spines are occupied by asymmetric, excitatory synapses and are sites of Ca2+ accumulation and contain NMDA receptors (79). NMDA receptors are expressed in large amounts in CA1 pyramidal neurons and can be imaged by conventional immunocytochemistry, as well as by confocal imaging, in which individual dendrites and spines can be studied for colocalization with other markers (51, 52, 188). Confocal microscopic imaging showed that estrogen treatment upregulates immunoreactivity for the largest NMDA receptor subunit, NR1, on dendrites and cell bodies of CA1 pyramidal neurons, whereas NR1 mRNA levels did not change after estrogen treatment that induces new synapses (52), suggesting the possibility that NR1 expression is regulated posttranscriptionally by estrogen.

Recent evidence indicates that, in young female rats, estrogen induction of NR1 is proportional to the induction of new spines, so that NMDA receptor density per spine is not increased; however, in the aging female rat, there is NR1 induction without an increase in dendritic spines (1). This might make the aging hippocampus more vulnerable to excitotoxic damage, for example, by stroke or seizures.
Cellular and molecular events associated with synapse formation. The estrogen-induced increase in dendritic spines on CA1 neurons parallels an increase in synapse density on spines without any decrease in shaft synapses (217), implying that new spine synapses are formed. Whereas synapse formation during development is considered to be a collaborative process involving growth of a presynaptic element on a site where a postsynaptic spine is either present or ready to form (79), the story for estrogen-regulated hippocampal synaptogenesis is somewhat different. Estrogens induce increased numbers of synapses on multiple synaptotagmins between neurons not previously connected (223). This is reminiscent of the finding in cultured hippocampal cells studied by time-lapse photography that filopodia extend from dendrites and reach out to establish contact with nearby axons (193, 224). This implies an active role for the dendrite in forming synaptic contacts.

As far as the time course, sequence of steps, and key gene products and events in synapse formation, individual synapses are reported to form in cell culture within 1–2 h (46, 168). During synapse formation, the cadherin/catenin and cadherin-like neuronal receptor systems are postulated to play a role in the recognition between presynaptic growth cones and dendritic filopodia (25). After the initial contact is established, recruitment of pre- and postsynaptic proteins leads to the formation of a synapse at the site of initial contact (25). The immediate early genes, Narp (42, 143), Arc (71), and synaptotagmin IV (206), are activated by synaptic firing and are candidates for the recruitment and localization of protein components of the synapse. The neuroligin-neurexin system is believed to play an important role in the recruitment and localization of pre- and postsynaptic components of the forming synapse (25). Neuroligin-1 and -2 can induce presynaptic differentiation in contacting axons, suggesting that the postsynaptic cell has a strong influence on presynaptic differentiation (178). However, it is presently not known what effects estrogen treatment may have on these gene products in the adult hippocampus.

There are a number of presynaptic molecular markers of mature synapses that can be used in studies of synapse formation. GAP43 is a marker of the growth cone and has been shown to increase in the hypothalamus after estrogen treatment (110); however, no studies of this type have been done on the hippocampus. Synaptosomal-associated protein SNAP25 is a marker of synaptic vesicles (154), as are syntaxin (13), synaptotagmins (16, 105), synaptobrevin (95, 114), syntrophin (212), and the synapsins (41, 68, 74). Although mRNAs for these proteins are most likely found in neuron cell bodies, growth cones of hippocampal neurons in culture have been reported to have mRNAs for proteins such as GAP43 and Arc and perhaps other presynaptic proteins; these can be translated in the growth cone (33).

Regarding the postsynaptic side, gene products characterizing dendritic spines include microtubule-associated protein-2, actin, and spinophilin (3, 28, 40, 117). Spinophilin, a protein that helps to bundle actin filaments in the dendritic spine, regulates many of the properties of spines (3, 40). The calcium/calmodulin kinase II (CaMKII) is a major protein of the postsynaptic density (14, 92, 153, 172, 183) that plays an important role in long-term potentiation and synaptic differentiation. Recent evidence indicates that CaMKII plays a key role in the formation of synapses and localization of receptors in synapses (77, 92, 172). Glutamatergic synapses contain other key proteins in the postsynaptic density besides CaMKII; these include postsynaptic density (PSD)-95, densin-180, and citron, a Rac/Rho effector protein (92). PSD-95 plays a key role in the anchoring of the NMDA receptor within the synapse (92). NRI is one of those proteins that may be translated from mRNA located in the dendrites (50).

Application of radioimmunocytochemistry to study synapse formation and maturation. The methods used to assess synapse formation, namely, Golgi staining, dye filling of cells, and EM, are all labor intensive and time consuming, and they do not provide information about the underlying molecular events. Radioimmunocytochemistry is a method for assessing the locally expressed levels of synaptic and spine proteins that uses a primary antibody and a radioactive secondary antibody and then assesses levels of radioactivity using quantitative autoradiography. This procedure has not only confirmed the anatomic methods for assessing spine synapse formation but it has also added a new dimension by providing insights into estrogen-induced increases in proteins that characterize presynaptic terminals and spines.

The first study used synaptophysin and syntaxin as presynaptic markers and spinophilin as a spine marker (20). Estrogen treatment was conducted exactly as in previous studies using the Golgi method, and the estrogen antagonist, CI-628, was used to block estrogen actions (129). Estrogen treatment induced both pre- and postsynaptic markers in the stratum oriens and stratum radiatum of the CA1 region, the location of the spine synapses (20), and the magnitude of the increases corresponded very well to the magnitude of the changes in spine density seen with the Golgi method (62, 129, 220). These estrogen effects were blocked by CI-628, which had no agonist effects by itself, and this finding agreed with the Golgi results (129).

Location of estrogen receptors and mechanisms of estrogen action. Adult CA1 pyramidal cells of the dorsal hippocampus do not express detectable cell nuclear estrogen receptors by tritium autoradiography and light microscopic immunocytochemistry (210), whereas they express low levels of ER-α and ER-β mRNA by in situ hybridization (see Refs. 123 and 186). Instead, immunocytochemistry for ER-α showed cell nuclear estrogen receptors in sparsely distributed interneurons in the CA1 region as well as other regions of Ammon’s horn and dentate gyrus, with greater density in the ventral than dorsal hippocampus (see Ref. 124). Regarding ER-β immunoreactivity, an antibody generated to the COOH-terminal end of the receptor re-
revealed only weak labeling of cell nuclei, particularly in the ventral hippocampus, and some staining of dendrites of pyramidal cells (187). Autoradiography with $^{125}$I-labeled estrogen to label estrogen receptors with a higher specific radioactivity showed binding sites not previously detected in hippocampus using [$^{3}$H]estradiol (186). Besides the interneurons previously seen to contain ER-α by other methods, labeling with $^{125}$I-estrogen was found in CA1-CA3 pyramidal cell nuclei in ventral hippocampus. A similar dorsal-to-ventral gradient was seen for ER-β in ventral hippocampus. A similar dorsal-to-ventral gradient was seen for ER-α and ER-β mRNA and $^{125}$I-estrogen binding, and the ER-α signal appeared to be stronger than that for ER-β (186). Thus the greater sensitivity of $^{125}$I-estrogen labeling of estrogen receptors reveals sites that may indicate locations of estrogen actions in hippocampal pyramidal cells, particularly in the ventral hippocampus. A recent developmental study of ER-α in the rat hippocampus is consistent with this and suggests that, early in development, more pyramidal cells may have nuclear ER-α than in the adult (196). Recent data with radioimmunocytochemistry have shown much stronger estrogen effects on synapse and spine protein levels in ventral compared with dorsal hippocampus (27).

The importance of cell nuclear ER-α in interneurons for synapse formation has been strongly supported by studies demonstrating estrogen-induced synapse formation in cell cultures of hippocampal neurons. Estrogen induces spines on dendrites of dissociated hippocampal neurons in cell culture by a process that is blocked by an NMDA receptor antagonist and not by an AMPA-kainate receptor blocker (139). Furthermore, estrogen treatment increased expression of phosphorylated CREB, and a specific antisense to CREB prevented both the formation of dendritic spines and the elevation in phospho-CREB immunoreactivity (140). The cellular location of ER-α in the cultures, resembling the in vivo localization, was in putative inhibitory interneurons, i.e., glutamic acid decarboxylase (GAD)-immunoreactive cells that constituted ~20% of total neuronal population. Estrogen treatment caused decreases in GAD content and the number of neurons expressing GAD; when this decrease was mimicked with an inhibitor of GABA synthesis, mepatropipionic acid, an upregulation of dendritic spine density occurred, paralleling the effects of estrogen (138). Thus estrogen-induced synapse formation may involve the suppression of GABA inhibitory input to the pyramidal neurons where the synapses are being generated.

As compelling as the role of estrogen-regulated GABA input appears to be to the mechanism of synapse formation, we believed that there was an additional role for estrogen actions in the pyramidal neurons themselves. Given the increasing evidence for such a mechanism, it seemed plausible that, besides the indirect, transynaptic mechanism described above, local signaling by estrogen might also be involved. This hypothesis was stimulated by a seminal study in Chinese hamster ovarian cells in which it was found that both ER-α and ER-β are expressed in a form that couples to second messenger systems that are stimulated by estrogen and blocked at least partially by nonsteroidal estrogen antagonists (169). Previous studies had indicated that nonnuclear estrogen receptors can be seen at the light microscopic level in cultured cells (32) and also at the EM level in hypothalamus (19). The proliferation of articles on nonnuclear actions of estrogen via membrane estrogen receptors and membrane-associated estrogen receptors (90) has reinforced the importance of investigating nonnuclear actions of estrogens in the hippocampus.

We used EM to examine ER-α localization in rat hippocampal formation (133), utilizing four antibodies to different parts of the ER-α structure (two polyclonal and two monoclonal). The specificity of these antibodies was determined by preabsorption with the full-length estrogen receptor protein, which abolished labeling in all sites examined, both nuclear and nonnuclear. We were able to see the cell nuclear labeling at the EM level, which has been seen by light microscopy in some GABA interneurons. In addition, some pyramidal and granule neuron perikarya have small amounts of ER-α immunoreactivity in the nuclear membrane, which is consistent with a recent report that $^{125}$I-estradiol labels a small number of estrogen binding sites in cell nuclei of hippocampal principal cells (186).

In stratum radiatum of CA1, ~50% of the ER-α-immunoreactive profiles were found in unmyelinated axons and axon terminals containing small synaptic vesicles. This is of potential functional relevance, given findings that estrogen can influence neurotransmitter release (see Ref. 123 for references). The synaptic ER-α immunoreactivity was found in terminals that formed both asymmetric and symmetric synapses on dendritic shafts and spines, suggesting that both excitatory and inhibitory transmitter systems are associated with ER-α (133).

Around 25% of the ER-α immunoreactivity was found in dendritic spines of principal cells, where it was often associated with spine apparatus and/or postsynaptic densities, suggesting that estrogen might act locally to regulate Ca$^{2+}$ availability, phosphorylation, or protein synthesis. Finally, the remaining 25% of ER-α immunoreactivity was found in astrocytic profiles, often located near the spines of principal cells.

Although these findings corroborate existing evidence for an indirect GABAergic mediation of estrogen actions (138, 175), the close association between the ER-α immunoreactivity and dendritic spines supports a possible local, nongenomic role for this estrogen receptor in regulation of dendritic spine density via second messenger systems. Initial in vivo and in vitro studies in hippocampus of one second messenger pathway, the phosphorylation of CREB, have indicated that estrogen has rapid effects, evident within as little as 15 min, to increase phospho-CREB immunoreactivity in cell nuclei of hippocampal pyramidal neurons (S. Lee, S. Alves, and B. McEwen, unpublished observations). One pathway by which CREB phosphorylation may occur involves the phosphatidylinositol 3-kinase (PI3-kinase), or Akt, system (35). Further studies are...
needed to connect these events together in the early actions of estrogen on hippocampal neurons that precede the induction of synapse formation.

**A model of estrogen action involving genomic and nongenomic estrogen receptors.** The results summarized above have led us to propose a testable, working model that delineates possible sites of estrogen action in relation to the location of nuclear and nonnuclear estrogen receptors. Although this model pertains to ER-α because we know more about its distribution, further studies of ER-β may reveal that it is also present in nonnuclear as well as cell nuclear sites within the hippocampus and may participate in synapse formation. According to our model, estrogen receptors in the dendritic spine may be associated with the activation of mRNA translation from polyribosomes (202) or endomembrane structures found in spines (162). In addition, other second messenger signaling effects might include the phosphorylation of neurotransmitter receptors or ion channels. Estrogen receptors in certain presynaptic terminals might modulate neurotransmitter release or reuptake (see Ref. 123 for references). Moreover, estrogen receptor-mediated activation of second messenger systems in dendritic spines and presynaptic endings might lead to retrograde signal transduction back to the cell nucleus, perhaps via Akt or CREB, providing another pathway through which estrogen could regulate gene expression. These postulated actions of estrogen are considered to operate synergistically with the actions of estrogen via nuclear receptors in interneurons, discussed above, that modulate the inhibitory tone on the CA1 pyramidal neurons where synapse formation occurs.

**Functional significance of estrogen actions in hippocampus.** The functional significance of estrogen actions in the hippocampal CA1 region is evident from studies of hippocampal-dependent learning and memory. In a recent examination of the natural estrous cycle of the female rat, a delayed matching-to-place task in female rats was used to show a close parallel between the temporal conditions by which estrogen improves memory and the conditions for estrogen to induce new excitatory synaptic connections in the hippocampus (123). Moreover, estrogen treatment of ovariectomized female rats has been reported to improve acquisition on a radial maze task as well as in a reinforced T-maze alternation task (34, 39). Furthermore, sustained estrogen treatment is reported to improve performance in a working memory task (146), as well as in the radial arm maze (34, 109). Finally, the effects of estrogen replacement in rats are reminiscent of the effects of estrogen treatment in women whose estrogen levels have been suppressed by a gonadotrophin-releasing hormone agonist used to shrink the size of fibroids before surgery (185).

Besides the behavioral studies of estrogen action on learning and memory, electrophysiological studies have shown that estrogen treatment of ovariectomized rats produces a delayed facilitation of synaptic transmission in CA1 neurons that is NMDA mediated and leads to an enhancement of voltage-gated Ca\(^{2+}\)-cur- rents (215). By using biocytin injection after recording from CA1 pyramidal neurons to visualize estrogen induction of dendritic spines, Woolley et al. (220) found that spine density correlated negatively with input resistance and input-output curves showed an increased slope under conditions where NMDA receptor-mediated currents predominated, whereas there was no increased slope where AMPA receptor currents predominated. Other studies have shown that long-term potentiation sensitivity peaks on the afternoon of proestrus in intact female rats at exactly the time when excitatory synapse density has reached its peak (208). Proestrus is also the time of the estrous cycle when seizure thresholds in dorsal hippocampus are the lowest (200).

In addition to the delayed effects of estrogens in hippocampus, estrogens and some form of estrogen receptors are involved in local signaling within neurons. Among the possible targets of local signaling is the translation of RNAs found in dendrites of hippocampal and other neurons. Other targets for local signaling by estrogens include the rapid activation of kainate-induced ion currents via a G-protein-coupled estrogen receptor that is present in αERKO mice and is insensitive to nonsteroidal estrogen antagonists (69) and the suppression of Ca\(^{2+}\) currents that is mimicked by a nonsteroidal estrogen antagonist (130), as well as rapid actions of estradiol on NMDA receptor activity and long-term potentiation (44, 201).

**Hippocampus as a sexually differentiated target of hormones in relation to cognitive function.** Sex differences have been described in hippocampal morphology involving the size of the dentate gyrus (61, 86, 173), and spatial learning with global spatial cues is faster in males than in females (213). This trait can be reduced in newborn male rats by castration, and it is enhanced in newborn female rats by neonatal treatment with estrogens (213); this may be the pathway for sexual differentiation, since the hippocampus transiently expresses estrogen receptors and aromatizing enzymes during the first 2 wk of neonatal life (111, 144, 145). Androgen receptors are expressed in the hippocampus of adult male and female rats (93). The dentate gyrus is larger in males than in females, due in part to sexual differentiation (173, 184), and there is some preliminary evidence for sex differences in neurogenesis and granule cell death in adult voles captured in the wild (49).

Thyroid hormone treatment immediately after birth has specific effects on the basal forebrain, dentate gyrus, and CA3 region of the hippocampus that last into adult life. Transient neonatal hyperthyroidism enhances basal forebrain cholinergic markers and increases the size of the dentate gyrus and branching of dendrites of CA3 pyramidal neurons (59, 63, 211). There are sex differences, in that the developing male cholinergic system is much more enhanced by the neonatal hyperthyroid state (211). Moreover, the direction of the thyroid hormone effect in the hippocampal formation is very much like that of testosterone, namely,
to increase the size of the dentate gyrus and increase innervation of the CA3 pyramidal neurons (173).

**ESTROGENS AND NEUROPROTECTION**

Estrogens are reported to have cognitive enhancing and neuroprotective effects. Loss of estrogens as a result of suppression of ovarian function with gonadotropin-releasing hormone agonists, or the loss of ovarian hormones as a result of surgical and natural menopause, leads to generally reversible decreases in declarative memory and motor coordination that respond to estrogen replacement therapy (94, 185). Estrogen actions in hippocampus are suspected of underlying the declarative memory deficits, whereas estrogen actions on the nigrostriatal dopaminergic system are likely to explain the effects on motor coordination. Mood is also sensitive to estrogen effects but differ among individuals. Perimenopausal depression is benefited by estrogen treatment in women who do not suffer from premenstrual syndrome, in whom ovarian hormones seem to exacerbate symptoms of PMS (174, 179).

After menopause, estrogen replacement therapy is used in the treatment of hot flashes and to reduce loss of bone calcium leading to osteoporosis. Likewise, cardiovascular protection is another reason that has led to postmenopausal estrogen replacement therapy. Another long-term consequence of estrogen loss at menopause is increased risk for Alzheimer’s disease; some studies have shown that this risk can be reduced by estrogen replacement therapy (87, 155, 199), although recent evidence suggests that treatment with estrogens once the disease is clearly established has no beneficial effect (137). There are at least two ways in which estrogens appear to be able to protect the brain from neurodegeneration. First, estrogens maintain function of key neural structures such as the hippocampus and basal forebrain and the widely projecting dopaminergic, serotonergic, and noradrenergic systems. As estrogen levels decline during menopause, these systems and the cognitive and other behavioral processes that depend on them also decline, as least functionally; however, these appear to respond to estrogen replacement. It is conceivable that estrogens not only maintain function but also confer resilience against neural damage by various agents due to their ability to maintain synaptic connections and promote the activity of these important neural systems.

The second type of neuroprotective effects of estrogens is a more direct involvement in blocking the actions of neurotoxic agents or inhibiting their generation. These effects also extend to protection from damage produced by strokes, as well as toxic effects of excitotoxic agents that may be related to Alzheimer’s disease (124). As noted earlier, the A ring of the estrogen molecule appears to have special properties with respect to the formation of free radicals and special protective effects on cells in culture that are deprived of serum or exposed to free radical generators (see Ref. 124). In this regard, in vivo studies of estrogen-mediated neuroprotection have reported successful reduction of lesion size by Silastic implants of 17β-estradiol in male rats subjected to middle cerebral artery occlusion (76). In another study, a single injection of 17β-estradiol reduced damage to hilar neurons in the hippocampal dentate gyrus of female rats caused by kainic acid treatment (6). In addition, estrogen treatment of cultured nerve decreases formation of the toxic form of the β-amyloid protein (221). Moreover, estrogen treatment interferes with the toxic effects of the β-amyloid protein (56) and the HIV coat protein, gp120 (24), both of which act via free radical generation. Nevertheless, a recent study showed that mice deficient in ER-α were not protected by physiological doses of 17β-estradiol from ischemic damage. This finding emphasized that ER-α may play a central role in neuroprotection by physiological levels of estradiol.

**CONCLUSIONS AND FUTURE CHALLENGES**

Besides the potentially important therapeutic implications that cognitive function, mood disorders, pain pathways, motor activity, and neuroprotection are modulated by estrogens, the studies of estrogen action on extrahypothalamic areas of the brain have highlighted our ignorance about basic cellular and molecular mechanisms and have revealed new potential brain sites and cellular and molecular mechanisms. These considerations apply to many neural systems, and they have been illustrated in this article by the review of studies on the hippocampus, serotonin system, and basal forebrain cholinergic system.

Besides sex differences in these actions of estrogens, another important and relatively unexplored topic is puberty. There are indications of mechanisms that operate in hypothalamus to bring about ovarian cyclicity [for example, small increases in intracellular estrogen receptor expression during puberty in rats (26), increased expression of estrogen-sensitive neuropeptides such as galanin in both sexes during puberty (163), increased expression of NMDA receptors on gonadotropin-releasing hormone neurons (57) and decreased inhibitory effects of GABA at the onset of puberty (135), and estrogen influences during puberty on neurotrophic factors produced in hypothalamus by glial cells (147)]. However, very little is known about the changes during puberty in other estrogen-sensitive brain regions, such as the hippocampus, brain stem, midbrain, and basal forebrain. Moreover, very little is known about what happens to estrogen receptors and effects during pregnancy and lactation, and this topic also needs to be investigated more closely.

Somewhat more is known about the loss of estrogens related to the loss of ovarian function and the aging process, but studies of neuroprotection in relation to aging and insults such as ischemia have once again revealed our ignorance about underlying cellular and molecular mechanisms of estrogen actions, particularly those that do not seem to involve classical intracellular estrogen receptors.
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