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Review

Mechanisms underlying estrogen-induced sexual differentiation in the hypothalamus

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Summary. Estrogen plays critical roles in the sexual differentiation of the developing brain and gender-specific regulation of reproductive neuroendocrinology. Of the different regions of the brain, it is well known that hypothalamic areas contain key sexually differentiated neuronal circuits. Estrogen receptor (ER) proteins localized in the nucleus affect the expression of target genes when bound to their ligand estrogen. However, recent studies suggest that this may not be the only mechanism of estrogen action. Instead, estrogen can influence various cellular events through regulating different signaling pathways. Cross-talk between direct effects of estrogen on gene transcription and its effects on signaling pathways should be examined in future to elucidate mechanisms underlying sexual differentiation in the hypothalamus.

Key words: Estrogen, Estrogen receptor, Genomic, non-genomic

Introduction

Estrogen plays critical roles in the sexual differentiation of the developing brain (reviewed in McEwen, 2001). It is well established mainly from research on rodents, that sexual differentiation of the hypothalamus is an estrogen-dependent process. During the developmentally critical sensitive period, estrogen produces a permanent alteration in neural control of physiological functions persisting into adulthood. For example, excessive exposure of the neonatal female to estrogen permanently alters the brain and prevents the development of normal cyclicity (Arai, 1964). Accumulating evidence suggests an important role of estrogen receptors (ERs) in the hypothalamus for such sexual differentiation. For example, clear defects in estrogen-dependent male and female reproductive behavior are seen in ER knockout mice (Ogawa et al., 1998a,b, 2000) and neurochemical sex differences have been found to depend upon ER expression (reviewed in Gu and Simerly, 1994). Classically, effects of estrogen were thought to be mediated only through a gene transcriptional pathway. However, the importance of other rapid effects of estrogen is now becoming apparent in various cell types including neurons. It seems that the mechanisms underlying the effects of estrogen may be more complex than previously appreciated.

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Sexual differences in the hypothalamus

Functional sexual differences in the hypothalamus

Of different brain regions affected by estrogen, hypothalamic regions such as the medial preoptic area (mPOA) and the ventromedial nucleus (VMN) contain key sexually differentiated neuronal circuits responsible for controlling reproductive functions (reviewed in Flanagan-Cato, 2000; Flanagan-Cato et al., 2001; Simerly, 1998, 2005). Estrogen stimulates a massive increment in secretion of gonadotropin-releasing hormone (GnRH) from GnRH neurons in the mPOA to induce ovulation in the female mammal, but it has no comparable effect in males (Herbison, 1998). However, in males orchidectomized at birth, an LH surge can be induced by estrogen treatment in adulthood (Corbier, 1985). In addition, sex differences have been observed in the effects of estrogen on networks involved in regulating male and female sexual behavior (reviewed in McEwen et al., 1987; Flanagan-Cato, 2000). Lordosis, the primary female reproductive behavior is exquisitely dependent on estrogen (reviewed in Kow et al., 1994). In the ventromedial hypothalamus (VMH), estrogen imposes hormonal dependence on neurons that control descending efferent connections to the muscles in the lower back that allow for the arching of the back, a characteristic of lordosis (Kow et al., 1994; Calizo and Flanagan-Cato, 2003; Daniels et al., 2003).

Gender-dependent morphological differences in the hypothalamus

Sex differences have been observed morphologically mainly in mPOA and VMH, including differences in volume of nuclei and number of neurons. The volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) and VMH in the rat hypothalamus is larger, with greater numbers of cells present in the nuclei in males than females (reviewed in Gorski, 1985). The anteroventral periventricular nucleus (AVPV) of the rat POA is sexually dimorphic with greater numbers of dopaminergic neurons in the female rat compared with males (Simerly, 1989). Perinatal androgen or estrogen decreases the nuclear volume of AVPV-POA in female rats through enhanced apoptosis, whereas testosterone increases the nuclear volume of SDN-POA in females through suppressed apoptosis (Arai et al., 1994, 1996; Davis et al., 1996).

Sexual differences and their hormonal dependence were also reported in the number of synapses in VMH and the dorsal part of mPOA (Field and Raisman, 1971; Raisman and Field, 1971, 1973; Matsumoto and Arai, 1986). Gender-dependent differences in the number of axo-dendritic synapses were observed in the central component of the medial preoptic nucleus (MPNc) (Larriva-Sahd, 1991). Furthermore, sexual differences and hormonal effects on the number of axo-dendritic and axo-somatic synapses were found in the arcuate nucleus (ARN) of the hypothalamus from early postnatal period (Matsumoto and Arai, 1981; Perez et al., 1990): neurons from females showed a greater number of presynaptic inputs than males. Quantitative evaluation of freeze-fracture replicas of the arcuate neuronal perikarya revealed that the sexual differentiation of the plasma membrane in the neuronal soma precedes the establishment of sex differences in axo-somatic synapses (Perez et al., 1990). In addition to neurons in the developing rat POA and ARN, sexual differentiation of astrocyte morphology induced by sex steroids was observed (Amateau and McCarthy, 2002; Mong and McCarthy, 2002).

Binding of aromatized estrogen to ERs

The precise mechanisms underlying these sexually dimorphic effects of estrogen are still unclear. Since steroid receptor proteins were discovered and the field of research on nuclear steroid receptors progressed, a number of investigators have shown that steroid receptor proteins, which are transcription factors localized in the nucleus, affect the expression of target genes when bound to a steroid hormone.

Concentrations of testosterone in the male rat are high during the late fetal and immediate postnatal periods, whereas estradiol-17 beta (E_2) is consistently low (Slob et al., 1980; Gogan et al., 1981). Accumulating evidence has shown that androgen produced in the male fetus or given to the newborn female rat becomes effective after being converted into estrogen by aromatase present in the neonatal brain (McEwen et al., 1977). Thus, it has been suggested that, during the neonatal period, estrogen produced from androgen by aromatization binds to estrogen receptors (ER) in the target neurons and exerts a defeminizing effect (McEwen et al., 1977; MacLusky et al., 1985; Naftolin, 1994). Areas with intense aromatase immunoreactivity are present in the ventrolateral part of the VMH (VMHvl) and the medial preoptic nucleus (MPN) of the mPOA, where there is strong ERα-immunoreactivity during postnatal development (Yokosuka et al., 1997). Indeed, studies using binding assays and autoradiography revealed the presence of estrogen binding to ER during the critical period of sexual differentiation of the rat brain (MacLusky et al., 1979a,b; Vito and Fox, 1981; Kuhnemann et al., 1994, 1995).

Subtypes and contributions of ERs to sexual differentiation in the hypothalamus

Subtypes and distributions of the ERs, ERα and ERß

Two different subtypes of ER have been identified so far: ERα and ERß (the latter existing in five isoforms: ER ß1-ß5) (Green et al., 1986; Kuiper et al., 1996; Petersen et al., 1998; Price et al., 2000). α and ß forms of ER have been shown to possess different binding affinities for a variety of estrogenic compounds (Kuiper et al., 1998; reviewed in Katzenellenbogen et al., 2000), differential subtype affinities and responses to a subset of estrogen responsive elements (EREs) (Hyder et al., 1999; Klinge et al, 2004), as well as different transactivation activities on estrogen-regulated gene constructs (Pennie et al., 1998; Yi et al., 2002).

Furthermore, ERα and ERß have distinct distribution patterns in the brain (Orikasa et al., 2000; Kamegai et al., 2001; Zhang et al., 2002; Ikeda et al., 2003). A significantly larger number of ERß mRNA-positive cells are present in the female rat anteroventral periventricular nucleus (AVPV) of the POA and the VMN than in the male (Orikasa et al., 2002, Orikasa and Sakuma, 2004). Orchidectomy of male neonates or estrogen treatment of female pups reverses this brain phenotype, suggesting that gender-specific expression of ERß is patterned by perinatal hormone exposure. Similarly, receptor expression in VMHvl was also sexually dimorphic with females having significantly more ERß-immunoreactive cells than males during the postnatal period. Neonatal treatment with E_2 down-regulated ERß mRNA in the female rat VMHvl. In contrast, no differences in expression of ERß mRNA or protein were detected between controls and E_2-treated males. These results show that estrogen is involved in regulating the sexually dimorphic expression of ERß in the VMH during early postnatal development in the rat (Ikeda et al., 2003). Gender-determined differences in the number of ERα-immunoreactive cells and ERα

mRNA-positive cells were also observed in the POA, MPN and VMHvl during postnatal development (DonCarlos and Handa, 1994; DonCarlos, 1996; Yokosuka et al., 1997).

These previous studies showing E_2-induced sexual differentiation in the distribution of ERs suggest an important role of ERs in mediating the establishment of gender-dependent differences in the hypothalamus. The following two results support the requirement for ERs in functional sexual differentiation during the early postnatal period. First, treatment of neonatal females with tamoxifen, an ER antagonist, disrupts the estrous cycle (Dohler et al., 1984, 1986). Second, antisense oligonucleotides specific for the region encompassing the translation start codon of ER mRNA during the critical period significantly interferes with the masculinization of the brain induced in females by exogenous androgen treatment (McCarthy et al., 1993).

Transcriptional regulation of estrogen mediated by ER

It is known that estrogen can regulate gene expression through several different modes including direct DNA binding by ERs (as homodimers or as heterodimers of ERα and ERß) or through tethering to other transcription factors such as activating protein (AP-1) and stimulating protein 1 (Sp1) (Paech et al., 1997). Moreover, transcriptional activities of estrogen are also affected by steroid receptor coactivators (SRCs) and corepressors that interact with DNA-bound ERs to potentiate/suppress their transcriptional activity (reviewed in Matthews and Gustafsson, 2003). Apostolakis et al. (2002) reported a significant inhibitory effect of antisense to SRC-1 and SRC-2, but not SRC-3, on hormone-induced reproductive behavior, suggesting that the biological activities of hypothalamic steroid receptors are selectively regulated by regional distribution of specific SRCs. Thus, estrogen-regulated gene expression is mediated through transcriptional complexes, the constituents of which, such as ERs, coactivators, corepressors and transcription factors, may be subject to regional, hormonal and/or temporal regulation in their expression and/or activation.

Sexual behaviors of ER knockout mice

As mentioned above, many studies revealed developmental as well as sexual differences in the distribution of ERα and ERß. The functional consequences of these differences have been investigated recently using ER-knockout (ERKO) mice and distinct phenotypes of gene-null animals and wild-type animals have been found (reviewed in Matthews and Gustafsson, 2003). Using ERα-knockouts (αERKO), it was shown that ERα gene expression plays a key role, not only for sexual behavior but also for other interrelated behaviors, such as parental and aggressive behaviors in female mice (Ogawa et al., 1998b). In contrast, ERß-knockout (ßERKO) females showed normal lordosis and courtship behaviors, though extension of these beyond the day of behavioral estrus was observed in some cases (Ogawa et al., 1999). αERKO males, although they rarely ejaculated and were infertile, showed almost normal levels of mounts and only slightly reduced levels of intromissions (Ogawa et al., 1998a). In contrast, all three components of sexual behaviors were present in ßERKO males (Ogawa et al., 1999). However, double αßERKO male mice did not show any components of sexual behavior, including simple mounting behavior (Ogawa et al., 2000). These studies suggested that sexually different reproduction-related behaviors require different and/or co-operative patterns of activation of ERs.

E_2-induced rapid response mediated by intracellular ERs

In addition to direct genomic responses triggered through binding to ER, estrogen can induce other responses. For example, it can promote phosphorylation of serine residues of the ER and enhance its gene activation function (Arnold et al., 1995; Kato et al., 1995). On the other hand, estrogen regulates the activity of transcriptional factors like CREB (Zhou et al., 1998, 2002; Abraham et al., 2003) as well as the interaction of nuclear E_2/ER with transcription factors like AP-1 (Kushner et al., 2000). Recently, Abraham and Herbison (2005) showed that estrogen treatment significantly increased the numbers of phosphorylated cAMP responsive element binding protein (pCREB)-immunoreactive cells in the mPOA and VMHvl of female mice, but not male mice. In addition, estrogen significantly increased the numbers of GnRH neurons expressing pCREB only in females. These results document the existence of a marked gender difference in the effects of estrogen on signaling pathways in the brain in vivo. They also show that the rapid E_2-induced increase in pCREB was not mediated by ER located in the membrane but by intracellular ERß, because membrane impermeable E_2 conjugated to bovine serum albumin (E_2-BSA) was not effective and the effect of estrogen on CREB phosphorylation in GnRH neurons was normal in αERKO mice but absent in ßERKO mice (Abraham et al., 2003).

There is another example showing a rapid effect of estrogen on hypothalamic neurons mediated by intracellular ER but not via membrane 'ERs'. Exposure of GnRH-1 cells to TTX plus E_2 increased the number of calcium peaks/cell, mean peak amplitude, and percentage of cells that contributed to each calcium pulse in explants compared with TTX alone (Temple et al., 2004). These effects were induced within 30 min but were not mimicked by E_2-BSA (17ß-etradiol 6-O-carboxymethloxime-BSA), although other types of E_2-BSA (1,3,5(10)-estratrien-3,16α, 17ß-triol-6-one 6-O-carboxymethyloxime-BSA and 1,3,(10)-estratrien-3,17ß-diol 17-hemisuccinate-BSA) did differentially alter them (Temple and Wray, 2005). In addition, these E_2-effects were inhibited by the ER antagonist ICI 182,780

(Temple et al., 2004). Therefore, E_2 may rapidly increase GnRH-1 neuronal activity and synchronization via intracellular ER.

Subtypes and contributions of membrane receptors bind to estrogen (membrane 'ERs')

Subtypes of membrane 'ERs'

Increasing evidence suggests that distinct pools of 'ERs' that localize to the plasma membrane also play important roles in estrogen-dependent rapid responses. Three types of membrane 'ERs' have been proposed so far; namely, the classical ERs (ERα and ERß), as well as ERx and the G protein coupled receptor (GPCR) 30, GPR30.

Transient transfection of ERα and ERß results in a small but significant pool of receptors localizing to the plasma membrane, suggesting that this pool of ERs arises from the same gene products as those localized to the nuclear compartment (Razandi et al., 1999). Recently, Ser^{522} of ERα has been shown to be necessary for the efficient translocation and function of ERα at plasma membrane; mutation of this residue to alanine significantly reduces ERα translocation to the plasma membrane (Razandi et al., 1999, 2003). E_2-BSA has similar effects as E_2 on neuronal events including neurite outgrowth, suggesting that the effects of E_2 are mediated via membrane ERs (Cambiasso and Carrer, 2001). Because contamination by free E_2 dissociated from E_2-BSA cannot be completely excluded, the effect of E_2-BSA has been controversial. However, the binding of E_2-BSA to purified ER and its localization to the cell membrane was recently confirmed by Taguchi et al. (2004). Furthermore, ERα-immunoreactivity was detected in the plasmalemma of interneurons in CA1 in the hippocampus by electron microscopy (Milner et al., 2001).

Second, Toran-Allerand et al. (2002) proposed a novel plasma membrane-associated putative ER that is neither ERα nor ERß, which they designated 'ER-X'. They showed that this receptor is enriched in caveolar-like microdomains of postnatal, but not adult, wild-type and ERKO neocortical and uterine plasma membranes. They further showed that it is re-expressed in the adult brain after ischemic stroke injury. The preferred ligand of ER-X is 17α-estradiol. Although ER-X shares some homology with the C-terminus of ERα, it is not an alternative splicing variant and may be the product of a new gene (Toran-Allerand et al., 2002, 2005).

Third, Thomas and co-workers reported high-affinity estradiol binding to the membranes of breast cancer cells, which lack ERα and ERß but express GPR30 (Thomas et al., 2005). Membranes from human embryonic kidney cells, which lack all three molecules, acquire estradiol-binding activity when induced to express GPR30. GPR30 binding is selective for estradiol and the ER antagonists tamoxifen and ICI 182,780. Although functions and locations of GPR30 in the brain have not yet been precisely determined, GPR30 has been detected in several areas of the human and rat central nervous system (CNS) (O'Dowd, et al., 1998), suggesting possible involvement in mediating estrogen effects in the brain.

Effects of estrogen mediated by membrane 'ERs'

Several groups have demonstrated that E_2, when it binds to membrane 'ERs', induces rapid generation of a number of signaling cascades including protein kinase A (PKA), inositol-1,4,5-triphosphate (IP3), phospholipase C, and members of the mitogen-activated protein kinase (MAPK) family in various cells including breast cancer cells and endothelial cells (reviewed in Boonyaratanakornkit and Edwards, 2004). There is an *in vitro* study showing that E_2 induces neurite outgrown of hypothalamic neurons from 16 day male rat fetuses (Cambiasso and Carrer, 2001). In this model two specific nuclear ER inhibitors (tamoxifen and ICI 182,780) were ineffective in blocking the neuritogenic effect of estrogen, and E_2-BSA was as effective as E_2. These results indicate that the axogenic effect of E_2 in the hypothalamus at embryonic day 16 is exerted through a membrane-mediated mechanism.

There are some other studies showing E_2 effects on neurons mediated via membrane 'ERs' in other part of the brain. For example, E_2 can potentiate kainate-induced currents in isolated hippocampal CA1 neurons. Under a whole cell voltage clamp recording configuration, E_2-induced potentiation was observed in both wild-type and αERKO mice. Studies with 8-bromo-cAMP, GDP-beta-S, GTP-gamma-S, ER antagonist ICI 182,780, an inhibitor of phosphodiesterase (IBMX) and a specific inhibitor of PKA (Rp-cAMPS) suggest that the potentiation of kainate-induced currents by E_2 was most likely a G-protein(s) coupled, cAMP-dependent phosphorylation event (Gu et al., 1999). Moreover, in neocortical explants, derived from developing wild-type and αERKO mice, both 17α- and 17ß-estradiol elicited the rapid and sustained phosphorylation and activation of the MAPK isoforms, the extracellular signal-regulated kinases (ERK1 and ERK2) (Singh et al., 2000). Toran-Allerand and colleagues (2002) proposed that the ER mediating activation of the MAPK cascade is ER-X, which may uniquely rapidly interact with kinases of the MAPK cascade and other signaling pathways (Toran-Allerand et al., 2002). Furthermore, it was recently demonstrated that estrogen binds to membrane 'ERs' and modulates the phosphorylation of synapsins by various protein kinases in the rat cerebral cortex and hippocampus in vivo and in vitro (Rebas et al., 2005), suggesting the involvement of membrane 'ERs' in regulation of synaptogenesis and/or synaptic transmission. Akama and McEwen (2003) showed in an in vitro model system of differentiated NG108-15 neurons that estrogen does not stimulate a rapid increase in post synaptic density (PSD)-95 mRNA levels, but does cause a rapid increase in new PSD-95 protein

synthesis via the Akt kinase (Akt, protein kinase B) pathway. They suggested the possibility that estrogen may be coupled to Akt at the cell membrane.

Membrane ERs are also known to affect certain channels in neurons. E_2 is capable of rapidly modulating physiological parameters of developing midbrain neurons by directly interacting with specific membrane binding sites coupled to a signal transduction mechanism that triggers calcium release from intracellular Ca^{2+} stores (Beyer and Raab, 1998). Chaban and Micevych (2005) showed that E_2 decreased ATP-induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse DRG neurons. The effect of E_2 was rapid (5-min exposure) and E_2-BSA had the same effect as E_2, suggesting that a membrane-associated ER mediated the response. In DRG neurons from ßERKO mice, E_2 attenuated the ATP-induced $[Ca^{2+}]_i$ flux as it did in wild type mice. However, in DRG neurons from αERKO mice, E_2 failed to inhibit this ATP-induced $[Ca^{2+}]_i$ increase. These results show that mouse DRG neurons express ERs and that the rapid attenuation of ATP-induced $[Ca^{2+}]_i$ signaling is mediated by membrane-associated ERα (Chaban and Micevych, 2005). Moreover, direct binding of E_2 induces activation of Slack (sequence like a calcium-activated potassium channel) channels; in lipid bilayers derived from rat cortical neuronal membranes, E_2 increases the probability of Slack being open and appears to decrease channel inactivation (Zhang et al., 2005).

The involvement of membrane 'ERs' in sexual differentiation still remains unclear, because only a few studies have been performed in this area. However, the examples shown in other brain regions suggest that E_2-induced effects via membrane 'ERs' are likely to be involved in neural circuit formation through regulating morphological and physiological properties in neuritogenesis, synaptogensis and synaptic transmission.

Conclusions

The preceding studies document a high likelihood that aromatized estrogen induces physiological and morphological gender-dependent differences in the hypothalamus during the critical developmental period and that the effects of estrogen are exerted through binding to ER, the gender-specific expression of which is also patterned by perinatal hormone exposure. Recent studies have revealed new roles for ERs, ERα and ERß, in addition to their direct binding to DNA and regulation of transcription. ERs can rapidly affect channel activities as well as many cellular states including modifying transcriptional and translational activities via signaling cascades. Moreover, the existence of membrane 'ERs' and their commitment to regulation of signaling cascades and channel activities have also been shown. The cross-talk between ERs and intracellular signaling cascades in the brain, which can modify the transcriptional activity of estrogen, is beginning to be appreciated (Vasudevan et al., 2005). Complex interactions between ER and signaling cascades and/or channels in different cellular locations and at varied times affords the opportunity for exquisite control of estrogen function. During the critical period for sexual differentiation in the hypothalamus, physiologically distinct neurons therein possibly receive different signals from estrogen because of modified signaling cascades and/or channels. Therefore, to fully elucidate the mechanism underlying sexual differentiation in the hypothalamus, effects of estrogen not only on transcriptional activities but also on signaling pathways and channel activities in physiologically identified neurons must be examined in future studies.

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