Estrogen Receptors and the Regulation of Neural Stress Responses

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Abstract

It is now well established that estrogens can influence a panoply of physiological and behavioral functions. In many instances, the effects of estrogens are mediated by the 'classical' actions of two different estrogen receptors (ERs), ER α or ER β . ER α and ER β appear to have opposing actions in the control of stress responses and modulate different neurotransmitter or neuropeptide systems. Studies elucidating the molecular mechanisms for such regulatory processes are currently in progress. Furthermore, the use of ER α and ER β knockout mouse lines has allowed the exploration of the importance of these receptors in behavioral responses such as anxiety-like and depressive-like behaviors. This review examines some of the recent advances in our knowledge of hormonal control of neuroendocrine and behavioral responses to stress and underscore the importance of these receptors as future therapeutic targets for control of stress-related signaling pathways.

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Introduction

Estrogens influence both reproductive- and nonreproductive-related neurobiological functions. Given that the brains of both males and females are exposed to varying amounts of estrogens, as a result of either gonadal secretions or local synthesis from androgenic precursors, deciphering the role of estrogens in brain function is important in understanding normal physiology as well as pathological responses. Recent studies indicate novel roles for estrogens in the regulation of neuroendocrine, autonomic and behavioral responses to stress. In this regard, estrogens have also been shown to modulate related functions such as inflammatory processes, pain, anxiety, depressive-like behaviors and cognitive function [1–4]. In this review, we focus on some of the roles for estrogens and estrogen receptors (ERs) in the control of stress and stress-related behaviors and address a few of the molecular mechanisms that might drive these regulatory processes.

ERs in the Brain

The actions of estrogens are mediated by at least two different ERs. Currently, the two major types of ERs described are ER α (also known as ESR1 or NR3A1) and

ER β (also known as ESR2 or NR3A2). Of these two receptors, ER α was the original ER to be identified and cloned [5], whereas ER β was not described until the subsequent decade [6]. Both receptors share considerable homology within the ligand-binding and DNA-binding domains and, in similar fashion to all members of the nuclear receptor superfamily of proteins, act as ligand-activated transcription factors. These receptors are characterized by their ability to alter transcriptional activity by binding to estrogen response elements (EREs) in the DNA sequence of gene promoters, thereby providing a direct link between steroid signals and transcriptional responses (for review, see [7]).

It has now become apparent that nonclassical, or 'rapid', estrogen actions also occur in the brain and are mediated by extranuclear estrogen receptors [8, 9, for review see 10, 11]. ER α and ER β have been found in the plasma membrane and cytoplasm where they can regulate intracellular signaling pathways through phosphorylation events [12, 13]. Other receptor proteins, such as GPR30 [14], a G protein-coupled receptor that binds estradiol with high affinity, or a yet to be described G protein-coupled receptor for the diphenylacrylamide, STX [15], have also been suggested as functional receptors that transmit rapid actions of estrogens through second messenger pathways. However, these nonclassical actions of steroid hormones can also result in gene expression changes [16], underscoring the complex relationship between the rapid and genomic actions of ER α and ER β .

In the mammalian nervous system, ER α and ER β are expressed differentially throughout the brain and spinal cord where they have unique but overlapping expression patterns. Of importance for this review, brain regions such as the preoptic area, bed nucleus of the stria terminalis and medial amygdala, express both ER types. ER α is the predominant receptor found in the ventromedial nucleus of the hypothalamus, whereas ER β is the predominant form found in the suprachiasmatic, supraoptic and paraventricular hypothalamic nuclei [17–19]. The results of these anatomical studies emphasize the fact that ER α and ER β can affect numerous complex physiological and behavioral functions of animals.

An interesting and perhaps confounding aspect of ERs is the fact that they are variably spliced under normal and pathological conditions. Several splice variants of ER α have been identified, although most of these have been found in carcinomas and peripheral reproductive tissues [20, 21]. Prior to the discovery of ER β , an ER variant (likely ER α) lacking the 4th exon was described in rodent brain [22]. A 52-kDa ER α variant of unknown etiology

has been found in plasma membrane of neurons [23], and several ER α splice variants have been described in human brain [24].

For ER β , 6 splice variant mRNAs have been shown in rodents, including the originally described wild-type (WT) form (designated ER β 1; [25, 26]). Alternative splicing of the 8 exons encoding ER β result in transcripts that have an additional 18 amino acids in the ligand-binding domain (ER β 2) or a deletion of exon 3, encoding the 39 amino acids in the carboxy terminus of the DNA-binding domain (ER β - δ 3), or exon 4 (ER β - δ 4). Combinations of the delta variants with ER β 1 and ER β 2 result in β 1/ δ 3, β 2/ δ 3, β 1/ δ 4 and putatively β 2/ δ 4. Each of these splice variants alters the binding affinity, association kinetics, cellular trafficking and transcriptional efficacy of the receptor, thus providing functional diversity to ER β signaling [25–28]. To date, the mRNAs of these splice variants have been shown in multiple tissue, including brain, with levels of some splice variants sometimes exceeding that of the native ER β 1 type [25–27]. Unfortunately, the ability to detect protein levels of the specific ER β variants has been hampered by the inability to generate specific antibodies against the exon-deletion variants. Antipeptide antibodies have been generated against the unique sequence in the ligand-binding domain of ER β 2 [29, 30]. Mapping studies in brain have shown that ER β 2 has a distribution that largely matches ER β 1 with high amounts in the cortex, raphe and supraoptic nucleus [30].

ERs and the Hypothalamo-Pituitary-Adrenal Axis

The hypothalamo-pituitary-adrenal (HPA) axis consists of a cascade of neural and humoral signals driven by both the circadian pacemaker as well as the environment. Changing environmental conditions or perceived threats to homeostasis activate the HPA by funneling information through neurons located in the paraventricular nucleus (PVN) of the hypothalamus, a brain region that integrates multiple positive and negative inputs. Central to HPA axis regulation are neurons in the parvocellular part of the PVN that express corticotropin-releasing hormone (CRH). The release of CRH into the hypophyseal portal vasculature enhances the synthesis and release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. CRH is the most potent ACTH secretagogue and its actions at the anterior pituitary can be enhanced by other hypothalamic factors such as vasopressin and oxytocin [31, 32]. In turn, ACTH acts on the adrenal cortex to stimulate the synthesis and secretion of corticosterone

(CORT). Circulating CORT subsequently feeds back at the level of the pituitary, hypothalamus and higher brain areas to limit further hormone secretion [33, 34].

Over the years, multiple studies have demonstrated that estradiol, or the activation of ERs, can influence HPA responses to stress. Early studies, such as those by Gaskin and Kitay [35, 36], demonstrated that sex steroid hormones could modulate adrenal function. In many cases, ovariectomy reduced, whereas estradiol treatment of gonadectomized females enhanced, the HPA responsiveness to a stressor [36–39]. However, studies suggesting that estradiol treatment can inhibit HPA activity have also been reported [40, 41]. Whether such discrepancies are due to estradiol acting at different levels of the HPA axis, the amplitude or duration of hormone exposure, or the length of time following ovariectomy remains to be determined [39–41]. Importantly, recent studies have also demonstrated that selective activation of ER α can amplify HPA reactivity to stress, whereas selective activation of ER β can reduce the reactivity of the HPA axis to stressors [42, 43]. Curiously, the cellular mechanisms whereby a single hormone, such as estradiol, that binds ER α and ER β with near equivalent affinities can selectively increase or decrease HPA reactivity under physiological conditions remains to be determined.

ER α and the HPA Axis

ER α is found at low levels in rodent PVN neurons [44–46] and although some increases have been noted under conditions such as fasting [46], the distribution suggests the possibility of an indirect mode of estradiol action on PVN responses to stress. In support of this, Weiser and Handa [47] have shown that ER α is expressed by GAD67-ir neurons in the peri-PVN region of the hypothalamus, placing these neurons in a strategic position to modulate HPA axis function through transsynaptic mechanisms. GAD67 is a rate-limiting enzyme for the production of GABA, and GABA-a receptor blockade can increase CRH expression in the PVN [48]. Indeed, glucocorticoid inhibition of PVN activity is also mediated through increased GABA release onto parvocellular neurons of the PVN [49]. Further, the administration of estradiol and ER α agonists can impair glucocorticoid receptor-mediated negative feedback regulation of the HPA axis when implanted adjacent to the PVN [47], thereby implicating these neurons in ER α regulation of HPA function. Accordingly, it has been suggested that ER α enhancement of HPA axis function may work through a reduction in inhibitory tone normally provided by GABAergic neurons that surround the PVN [47].

ER β and the HPA Axis

In contrast to ER α , ER β is expressed at high levels by neurons within the PVN [44]. A large number of PVN ER β cells are oxytocin (OT)- and vasopressin (AVP)-immunoreactive [44, 50–52]. CRH neurons of the PVN also express ER β , although to a much lesser extent than do AVP and OT neurons [44, 53, 54]. Further, both OT and AVP can be colocalized with CRH in some parvocellular neurons of the PVN [55–57]. Stimulation of parvocellular neurons also causes the dendritic release of OT and AVP within the PVN, which works to inhibit CRH secretion [58]. The high levels of ER β in the PVN suggest that estradiol could directly alter the function of PVN neuro-peptide neurons. Indeed, a central, PVN, site of action in the regulation of HPA reactivity has been demonstrated by the studies of Lund et al. [42] who placed ER β agonists in an area adjacent to the PVN and demonstrated a reduction of stress-responsive CORT and ACTH secretion in ovariectomized female rats. Furthermore, compounds with selective affinity for ER β , such as diarylpropionitrile (DPN), WAY-200070 or 3 β -diol, whether delivered centrally or peripherally, appear to inhibit HPA reactivity to stressors [42] as well as reduce activation of neuropeptide neurons in the PVN, as demonstrated by altered expression of *c-fos* [43].

Molecular Mechanisms of ER Signaling: Controlling *crh* through Alternate Pathways

In vivo, CRH neurons can be considered a prototypic example of a systems integrator, incorporating large amounts of information from various sensory inputs to control a specific neuroendocrine or autonomic output. Elucidating the detailed molecular mechanisms whereby a single input, such as estradiol, can modulate *crh* expression is necessary for a complete understanding of the manner by which CRH-expressing neurons integrate information. In this case, salient information to CRH neurons concerning reproductive status is necessary given the close association between level of stress reactivity and reproductive success. Importantly, the analysis of integration at the molecular level initially requires simpler systems than in vivo models.

ER Regulation through Alternate Pathways

The prototypic pathway for an estrogen to exert its effect involves its binding to an ER with the resultant holo-receptor subsequently binding to specific sites in DNA. In the case of ERs, this ‘classic’ mode of action involves

binding to palindromic EREs that regulate genes in *cis*. In addition to this mode of regulation, ERs regulate expression by recruiting other transcription factors and/or shared coregulatory proteins. This *trans* mode of regulation, is generally referred to as regulation via an 'alternate pathway' [59] or one that involves a 'tethering' response element [60]. For the ER, such regulation has been demonstrated for multiple transcription factors, two of the best described being AP-1 and Sp1 [59, 61]. Given that the *crh* promoter does not have a full palindromic ERE, thereby precluding a 'classic' mechanism of action, an appreciation of alternate pathways is critical for understanding the regulatory mechanisms of *crh* expression. To this end a number of cell-based techniques have been used for examining CRH regulation by estradiol. The earliest of these were performed by Vamvakopoulos et al. [62]. Given the absence of an ERE in the *crh* promoter, the investigators focused on 3' ERE half-sites. These initial analyses of estrogen-regulated *crh* expression used transient cotransfections of nonneuronal CV-1 cells with ER (α) and *crh*:reporter constructs [62], and showed that treatment with estradiol led to an approximate twofold increase in *crh* promoter activation.

After the discovery of ER-mediated alternate pathways (e.g. [59, 63]), and the discovery of ER β and ER β splice variants, ER regulation of *crh* promoter expression was reassessed. Using reporter-based assays in HeLa cells, Miller et al. [54] focused on estrogen regulation of a more proximal region of *crh* promoter activity. Because the existence of alternate pathways had been well established by then, it was logical to examine the proximal promoter even in the absence of either a palindromic ERE or an ERE half-site. Experiments were carried out in the presence of estradiol or tamoxifen. The latter was used to assess whether an alternate pathway was in play [64]. ER α activated the *crh* promoter weakly in the presence of either ligand. In contrast, the ER β splice variants activated *crh* expression more robustly. Furthermore, each of the ER β splice variants exhibited a different profile of response. The ER β 1/ δ 3 variant was most potent, increasing activation by about 12-fold in the presence of estradiol. Thus, it is clear that ER β is able to play a significant role in *crh* promoter activity and can do so in the absence of palindromic EREs.

At the time, a critical component of cell-based experiments was missing, i.e. the availability of well-characterized neural cell lines that express CRH. Currently, there are a number of hypothalamic cell lines derived from rodents, which have been characterized with respect to their neuropeptidergic phenotype [65, 66]. One such line,

IVB, derived from rat embryo hypothalami, has many characteristics of paraventricular parvocellular neurons that express CRH and regulate the HPA axis [65]. These characteristics include the ability to synthesize and secrete CRH, express glucocorticoid receptors and reduce *crh* expression in response to glucocorticoid treatment. Thus, they provide a good model system for determining mechanisms of *crh* expression. The IVB line has proved suitable for analyzing estrogen regulation of *crh* expression, as well. The line expresses ER α and ER β as detected by immunocytochemistry (Gregg and Uht, unpubl.) and functional ERs as measured by *crh* reporter assays [67].

One seeming incongruity in the use of IVB cells is that they express both ER α and ER β , whereas in vivo data suggests the possibility that ER α effects on PVN function are indirect and mediated through GABAergic interneurons [47]. This does not rule out direct effects of ER α in other CRH neurons, such as those found in the central nucleus of the amygdala or elsewhere [68]. Moreover, it is important to consider that every cell has the potential to express a given protein and what is not apparent in a given in vivo state does not preclude its being expressed in another. For example, CRH and AVP are expressed in the PVN at rather low levels in the presence of endogenous glucocorticoids, but expression can be greatly increased following removal of glucocorticoids, such as after adrenalectomy [69, 70]. Moreover, following kainate-induced seizures, CRH expression is induced in areas such as the piriform and cingulate cortices, bed nucleus of the stria terminalis and globus pallidus at relatively high levels [71]. Similarly, although ER α is minimally expressed in PVN neurons, Estacio et al. [46] have shown that following a 48-hour fast, the number of ER α containing neurons in the PVN and A2 region of the nucleus of the solitary tract (NTS) increased significantly. Unfortunately, the phenotype of these new ER α -expressing neurons has never been determined, which leaves open the possibility that, under certain circumstances, PVN CRH neurons may express ER α .

Kinetics of Chromatin Occupancy

Kinetic approaches for examining promoter occupancy have been performed for several transcription factors and coregulators including ER α [72–74]. In 2003, Metivier et al. [75] published a comprehensive kinetic report of cyclic occupancy of the pS2 promoter region by ER α and identified proteins with which it might form a regulatory complex. The data permitted modeling of regulatory complex assembly and disassembly.

Such an approach has been reported for analysis of *crh* regulation in an amygdalar cell line that expresses CRH

[76]. These data permit predictions of ER complexes with coregulators. Specifically, at 1 min ER α co-occupies the promoter with SRC-1. By 3 min the putative ER α /SRC-1 complex no longer predominates. Rather, ER β co-occupies the promoter with CBP. The occupancy of the two complexes *together* corresponds to the kinetic profile of CRH mRNA levels. Individually, they do not. The importance here is that if taken alone, the mRNA data could suggest that there is one mechanism involved in the E₂ response. With an evaluation of occupancy, it is clear that that is not the case. That the two putative complexes are functionally distinct is suggested by an increased acetylation of histone 3 in the case of ER α and of histone 4 in the case of ER β .

In summary, molecular regulation through alternate pathways and phasic occupancy of the *crh* promoter may play significant roles for integrating incoming information, such as a change in reproductive state, to adjust stress reactivity and autonomic function. Certainly, changing the gain of stress responses across the reproductive cycle is likely a necessary component for controlling appropriate behaviors and physiological functions, thereby ensuring reproductive success.

ER β and Stress-Related Behaviors

In addition to effects on stress responsive hormone secretion, estrogens modulate several nonreproductive functions including mood, fear, anxiety, depression, cognition and memory in both humans and laboratory animals [77–79]. Depending on multiple factors, such as age and stage of the reproductive cycle, estrogens have been reported to exhibit either anxiogenic or anxiolytic properties in rodents. Elevated levels of estradiol during proestrus or estradiol replacement to ovariectomized females elicit anxiolytic actions [37, 80, 81]. Removal of endogenous estrogens by ovariectomy enhances anxiety-like behaviors, while estradiol replacement has been reported to increase or decrease behavioral measures of anxiety in rodents [82].

A large body of evidence suggests that ER β mediates estradiol's anxiolytic functions in the central nervous system [83–86]. In ovariectomized female rats, pharmacological administration of the ER β -specific agonist DPN, but not the ER α -specific agonist propyl-pyrazoletriol, decreased anxiety-related behaviors measured in the open-field arena, elevated plus maze (EPM) and light-dark box [87]. In addition to decreased anxiety in the open field, DPN-administration also resulted in less de-

pressive-like behavior in the forced swim test [88, 89]. DPN administration not only attenuates anxiety-like behaviors in ovariectomized rats, but also causes a reduction in stress-induced reactivity of the HPA axis [43]. This reduction in stress reactivity is reflected by a decrease in plasma CORT and ACTH levels and immediate early gene activation of *c-fos* in the PVN of the hypothalamus, a brain region containing some of the highest levels of ER β expression. Administration of the nonselective ER antagonist tamoxifen blocks the inhibitory actions of DPN, confirming the role of ER β in the modulation of stress reactivity [43].

Using gonad-intact female mice with a disruption of the gene for either ER α (ER α KO) or ER β (β ERKO), Krezel et al. [90] demonstrated increased anxiogenic behaviors by β ERKO mice compared to their WT littermates or ER α KO mice. Further, DPN administration to ovariectomized females increased open-field entries and the time spent in open arms in plus maze in WT mice, but not the β ERKO mice, suggesting a role for ER β in anxiety responses [91]. Hughes et al. [92] have also demonstrated a role for ER β in modulation of anxiety and depression using another ER β selective agonist, WAY-200070 (Wyeth, Princeton, N.J., USA) in gonadectomized male β ERKO and WT littermates. Interestingly, ER β modulation of anxiety also appears to involve serotonin and dopamine neurotransmission [92–94].

Although ER β -specific agonists and β ERKO mice have provided substantial proof of involvement of ER β in anxiety behaviors, the mechanism(s) by which the ER β containing neurons in neuroendocrine stress circuitry function to modulate stress responses are still unclear. Using S-DPN, a more selective enantiomer of the commonly used racemic-DPN, as a pharmacological tool in conjunction with β ERKO mice, we recently examined ER β action on anxiety-related behavioral measures, the corresponding stress hormonal response to HPA axis reactivity and neuroanatomical targets of ER β action. Peripheral administration of S-DPN decreased anxiety-like behaviors in open-field activity, light/dark exploration and EPM in ovariectomized WT female mice, but not β ERKO female mice. S-DPN treatment also resulted in a significant decrease in plasma CORT and ACTH levels in WT, but not in β ERKO mice. Such decreases in CORT and ACTH secretion in WT animals have also been reported upon administration of ER β -selective agonists, racemic DPN, WAY-200070 and S-DPN [43, 87, 92].

Increased *c-fos* mRNA expression was observed in the anterior dorsal part of the medial amygdala and bed nu-

cleus of the stria terminalis regions in S-DPN-treated WT mice, but not in β ERKO mice exposed to the EPM [95]. Similar activation of *c-fos* expression in the amygdala has also been reported in rats exposed to the EPM [96]. The absence of changes in *c-fos* expression in the PVN region by S-DPN in WT mice suggests the possibility that ER β -mediated effects on parvocellular neurons of the PVN may not be direct, but indirect. This latter possibility is supported by the reduced HPA axis reactivity by the ER β agonist in WT mice, following EPM. Taken together, the available data suggest that ER β activates an inhibitory circuit within the amygdala and extended amygdala that can then elicit reduced activation of outputs regulating anxiogenic responses. Further studies are in progress to determine the complexity of ER β regulation in stress reactivity.

Conclusion

It has become increasingly evident that estrogens can profoundly affect neuroendocrine and behavioral responses to stress. The effects of estrogens are mediated by

classical estrogen receptors, ER α and ER β . Furthermore, in most cases, these receptors influence neuroendocrine function and behavior by differing mechanisms of action. In regards to neuroendocrine responses, ER α and ER β work in opposition through different populations of neurons within or near the PVN. Increasing evidence supports the direct regulation of neuropeptide promoters, such as *crh*, by ER β . For behavioral responses, ER β appears to work through brain circuitry involving the amygdala. Further work is still required, however, to tease out the exact cellular and molecular mechanisms that are used by ER α and ER β to regulate stress responses. Nonetheless, ER α and ER β appear to be logical therapeutic targets for treatment of dysfunctional HPA regulation and anxiety.

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