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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 (ER), alpha or beta. Estrogen receptor alpha and beta 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 ERalpha and ERbeta 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.

Keywords

estradiol; hypothalamus; pituitary; adrenal; behavior; anxiety

I. Introduction

Estrogens influence both reproductive and non-reproductive 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.

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II. Estrogen Receptors in brain

The actions of estrogens are mediated by at least two different estrogen receptors (ERs). Currently, the two major types of ERs described are ERalpha (also known as ESR1 or NR3A1) and ERbeta (also known as ESR2 or NR3A2). Of these two receptors, ERalpha was the original ER to be identified and cloned (5) whereas it was over a decade later before ERbeta was described (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, they act as ligand-activated transcription factors. These receptors are characterized by their ability to alter transcriptional activity by binding to estrogen response elements 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 non-classical, or "rapid", estrogen actions also occur in the brain that are mediated by extranuclear estrogen receptors (8, 9). ERalpha and ERbeta have been found in the plasma membrane and cytoplasm where they can regulate intracellular signaling pathways through phosphorylation events (10, 11). Other receptor proteins, such as GPR30 (12), 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 (13), have also been suggested as functional receptors that transmit rapid actions of estrogens through second messenger pathways. However, these non-classical actions of steroid hormones can also result in gene expression changes (14), underscoring the complex relationship between the rapid and genomic actions of ERalpha and ERbeta.

In the mammalian nervous system, ERalpha and ERbeta 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 (BST) and medial amygdala, express both ER types. ERalpha is the predominant receptor found in the ventromedial nucleus of the hypothalamus whereas ERbeta is the predominant form found in the suprachiasmatic n., supraoptic n, and paraventricular hypothalamic nuclei (15–19)). The results of these anatomical studies emphasize the fact that ERalpha and ERbeta 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 ERalpha have been identified, although most these have been found in carcinomas and peripheral reproductive tissues (20, 21). Prior to the discovery of ERbeta. an ER variant (likely ERalpha) lacking the 4^{th} exon was described in rodent brain (22). A 52kDa ERalpha variant of unknown etiology has been found in plasma membrane of neurons (23), and several ERalpha splice variants have been described in human brain (24).

For ERbeta, 6 splice variant mRNAs have been shown in rodents, including the originally described wild-type form (designated ERbeta1; 25, 26). Alternative splicing of the 8 exons encoding ERbeta result in transcripts that have an additional 18 amino acids in the ligand binding domain (ERbeta2) or a deletion of Exon 3, encoding the 39 amino acids in the carboxy terminus of the DNA binding domain (ERbeta – delta3), or Exon 4 (ERbeta – delta4). Combinations of the delta variants with ERbeta1 and ERbeta2 result in beta1/delta3, beta2/delta3, beta1/delta4 and putatively beta2/delta4. Each of these splice variants alters the binding affinity, association kinetics, cellular trafficking and transcriptional efficacy of the receptor, thus providing functional diversity to ERbeta 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 ERbeta1 type (25–27).

Unfortunately, the ability to detect protein levels of the specific ERbeta variants has been hampered by the inability to generate specific antibodies against the exon-deletion variants. Anti-peptide antibodies have been generated against the unique sequence in the ligand-binding domain of ERbeta2 (29,30). Mapping studies in brain have shown that ERbeta2 has a distribution that largely matches ERbeta1 with high amounts in the cortex, raphe and supraoptic nucleus (30).

III. Estrogen Receptors 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 of the hypothalamus (PVN), 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 adrenocorticotropic hormone (ACTH) from the anterior pituitary. CRH is the most potent ACTH secretogogue 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 responsivity 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 ERalpha can amplify HPA reactivity to stress, whereas selective activation of ERbeta 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 ERalpha and ERbeta with near equivalent affinities can selectively increase or decrease HPA reactivity under physiological conditions remains to be determined.

Estrogen Receptor alpha and the HPA axis

ERalpha 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 et al (47) have shown that ERalpha 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 trans-synaptic 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 ERalpha 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 ERalpha regulation of HPA function. Accordingly, it has been suggested that ERalpha enhancement of HPA axis function may

work through a reduction in inhibitory tone normally provided by GABAergic neurons that surround the PVN (47).

Estrogen Receptor beta and the HPA axis

In contrast to ERalpha, ERbeta is expressed at high levels by neurons within the PVN (44) suggesting that by binding to ERbeta, estradiol could directly alter the function of PVN neuropeptide neurons. A large number of PVN ERbeta cells are oxytocin (OT) and vasopressin (AVP) immunoreactive (44, 50–52). CRH neurons of the PVN also express ERbeta although to 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 ERbeta in the PVN suggest that estradiol could directly alter the function of PVN neuropeptide 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 ERbeta 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 ERbeta, such as diarylpropionitrile (DPN), WAY-200070 or 3 beta 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).

IV. Molecular mechanisms of ER signaling: controlling *crh* through alternate pathways

In vivo, CRH neurons can be considered a prototypical 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 estrogen response elements (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 kushner) or one that involves a “tethering” response element (60 - Lefstin). For the ER, such regulation has been demonstrated for multiple transcription factors, two of the best described being AP-1 and Sp1 (59, 61 Kusher, Safe). 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 co-transfections of non-neuronal CV-1 cells with ER

(alpha) and *crh*:reporter constructs (62) and showed that treatment with estradiol led to an approximate 2-fold increase in *crh* promoter activation.

After the discovery of ER-mediated alternate pathways (*e.g.59.63*), and the discovery of ERbeta and ERbeta 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). ERalpha activated the *crh* promoter weakly in the presence of either ligand. In contrast the ERbeta splice variants activated *crh* expression more robustly. Furthermore, each of the ERbeta splice variants exhibited a different profile of response. The ERbeta1/delta3 variant was most potent, increasing activation by about 12 fold in the presence of estradiol. Thus, it is clear that ERbeta 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; that was 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, IVBs, 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 ERalpha and beta as detected by immunocytochemistry (Gregg and Uht, unpublished) and functional ERs, as measured by *crh* reporter assays (67).

One seeming incongruity in the use of IVB cells is that they express both ERalpha and ERbeta where as *in vivo* data suggests that possibility that ERalpha effects on PVN function are indirect, and mediated through GABArgic interneurons [47]. This does not rule out direct effects of ERalpha 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 ERalpha is minimally expressed in PVN neurons, Estacio et al (46) have shown that following a 48-hour fast, the number of ERalpha containing neurons in the PVN and A2 region of the nucleus of the solitary tract (NTS) increased significantly. Unfortunately, the phenotype of these new ERalpha expressing neurons have never been determined which leaves open the possibility that, under certain circumstances, PVN CRH neurons may express ERalpha.

Kinetics of Chromatin Occupancy

Kinetic approaches for examining promoter occupancy have been performed for several transcription factors and coregulators including ERalpha (72–74). In 2003, Metivier et al published a comprehensive kinetic report of cyclic occupancy of the pS2 promoter region by

ERalpha and identified proteins with which it might form a regulatory complex (75). 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 one minute ERalpha co-occupies the promoter with SRC-1. By 3 mins. the putative ERalpha/SRC-1 complex no longer predominates. Rather, ERbeta 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 ERalpha and of histone 4 in the case of ERbeta.

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.

V. ER beta and stress-related behaviors

In addition to effects on stress responsive hormone secretion, estrogens modulate several non-reproductive functions including mood, fear, anxiety, depression, cognition and memory in both humans and laboratory animals (77–79). Depending upon 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 ERβ mediates estradiol's anxiolytic functions in the central nervous system (83–86). In ovariectomized female rats, pharmacological administration of the ERβ-specific agonist, DPN, but not ERα-specific agonist propyl-pyrazole-triol (PPT), 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 depressive-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 paraventricular nucleus (PVN) of the hypothalamus, a brain region containing some of the highest levels of ERβ expression. Administration of non-selective ER antagonist, tamoxifen, blocks the inhibitory actions of DPN, confirming the role of ERβ in the modulation of stress-reactivity (43).

Using gonad-intact female mice with a disruption of the gene for either ERα (ERαKO) or ERβ (βERKO), Krezel et al (90) demonstrated increased anxiogenic behaviors by βERKO mice compared to their wild type (WT) littermates or ERαKO 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 βERKO mice, suggesting a role for ERβ in anxiety responses (91). Hughes et al (92) have also demonstrated a role for ERβ in modulation of anxiety and depression using another ERβ selective agonist, WAY-200070

(Wyeth, Princeton, NJ) in gonadectomized male βERKO and WT littermates. Interestingly, ERβ modulation of anxiety also appears to involve serotonin and dopamine neurotransmission (92 – 94)

Although, ERβ-specific agonists and βERKO mice have provided substantial proof of involvement of ERβ in anxiety behaviors, the mechanism(s) by which the ERβ 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 βERKO mice, we recently examined ERβ action on anxiety-related behavioral measures, the corresponding stress hormonal response to HPA axis reactivity, but also the neuroanatomical targets of ERβ action. Peripheral administration of S-DPN decreased anxiety-like behaviors in open field activity, light/dark exploration and elevated plus maze in ovariectomized, WT female mice, but not βERKO female mice. S-DPN treatment also resulted in a significant decrease in plasma CORT and ACTH levels in WT, but not in βERKO mice. Such decreases in CORT and ACTH secretion in wild type animals have also been reported upon administration of ERβ selective agonists, racemic DPN, WAY-200070, and S-DPN (43, 87, 92).

Increased *c-fos* mRNA expression was observed in anterior dorsal part of the medial amygdala (MEAad) and BST regions in S-DPN treated WT mice but not in βERKO 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 ERβ-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 ERβ agonist in WT mice, following EPM. Taken together, the available data suggest that ERβ 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 ERβ regulation in stress reactivity.

VI Summary and Conclusions

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, ERalpha and ERbeta. Furthermore, in most cases, these receptors influence neuroendocrine function and behavior by differing mechanisms of action. In regards to neuroendocrine responses, ERalpha and ERbeta 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 ERbeta. For behavioral responses, ERbeta 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 ERalpha and beta to regulate stress responses. Nonetheless, ERalpha and ERbeta appear to be logical therapeutic targets for treatment of dysfunctional HPA regulation and anxiety.

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