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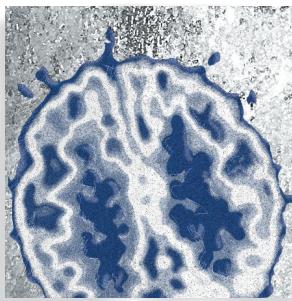
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Sex-biased cellular signaling: molecular basis for sex differences in neuropsychiatric diseases

Rita J. Valentino, PhD; Debra A. Bangasser, PhD



Introduction

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hat there are disparities in disease prevalence, severity, and onset determined by sex has long been recognized; however, the underlying mechanisms have not been well delineated. Historically, the conventional thinking has been that circulating sex hormones alter physiological systems in a way that determines these factors. This extended to the concept that the actions of hormones specifically occurring during critical windows of development organize morphology and neural circuitry in a sex-specific manner.¹ Sex hormones can regulate gene expression patterns in sexually specific and regionally selective ways that then become expressed as sex-specific behaviors.² More recent but less well-studied mechanisms for sex biases involve genetic differences that result from differential encoding of genes on sex chromosomes. Through studies that use the four-core-gene mouse model to distinguish the roles of chromosomal and gonadal sex, evidence has been provided for a genetic basis contributing to sex differences in

The recognition that there are fundamental biological sex differences that extend beyond those that define sexual behavior and reproductive function has inspired the drive toward inclusion of both sexes in research design. This is supported by an underlying clinical rationale that studying both sexes is necessary to elucidate pathophysiology and develop treatments for the entire population. However, at a more basic level, sex differences, like genetic differences, can be exploited to better understand biology. Here, we discuss how sex differences at the molecular level of cell signaling and protein trafficking are amplified to create a state of vulnerability that under the right conditions can result in symptoms of neuropsychiatric disease. Although this dialogue focuses on the specific example of corticotropin-releasing factor, the potential for analogous sex differences in signaling and/or trafficking of receptors for other neuromodulators has broad biological and therapeutic implications.

Keywords: amygdala; arousal; β -arrestin; corticotropin-releasing factor; G_s-GTP binding protein; locus coeruleus; norepinephrine; stress

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Basic research

Selected abbreviations and acronyms

CRF	corticotropin-releasing factor
CRF-OE	CRF-overexpressing mice
CRF₁	CRF subtype 1 receptor
G_s	stimulatory G-protein
LC	locus coeruleus

certain social behaviors, habit formation, nociception, and sensitivity to morphine.³ This model has also suggested that sex differences in the prevalence of certain autoimmune diseases are genetically based.³ A recently identified chromosomal mechanism for conferring sex differences is bias in parent-of-origin selection.⁴ For example, cortical glutamatergic neurons of female mice preferentially inherit the maternal X chromosome. These examples underscore the diversity and complexity in the mechanisms through which sex differences in disease vulnerability can arise.

Sex disparities in the major psychiatric diseases have been well documented. The disorders that are nearly twice as prevalent in females than in males include posttraumatic stress disorder (PTSD), affective disorders, and anxiety disorders.⁵⁻⁸ Notably, these have all been associated with stress. Stress precipitates or worsens symptoms. Likewise, patients with these diseases often present with end points of hypothalamic-pituitary-adrenal (HPA)-axis dysfunction, such as dysregulated plasma cortisol rhythms and adrenal hypertrophy.⁹ Substance abuse, and particularly the phenomenon of relapse, has also been linked to stress.¹⁰ Although rates of substance abuse disorders are generally higher in males, females may be more vulnerable because they start abusing substances at lower doses, show steeper rates of escalation, and are more prone to relapse.¹¹ Another salient feature of stress-related psychiatric disorders is altered arousal seen as sleep disturbances, inability to concentrate, and inappropriate responses to stimuli. Given that stress and altered arousal are two common threads through the psychiatric disorders that prevail in females, the sex disparity in pathophysiology should lie at the intersection between neural circuits that convey information about stress and those that underlie arousal. A major point of intersection between stress and arousal circuits is at the synapses between axon terminals containing the stress neuropeptide corticotropin-releasing factor (CRF) and dendrites of norepinephrine-containing locus coeruleus (LC) neurons.¹²

Communication here is the means by which arousal is heightened and attention and cognitive processes are altered to optimally respond to a life-threatening challenge. In this review, we describe how at this node, a convergence of three sex differences in the cellular and molecular substrates of this communication can be amplified and translated to sex differences in behavior and psychopathology.

The corticotropin-releasing factor–locus coeruleus synapse

Although stress is generally considered in terms of the pathophysiology with which it is associated, the stress response is adaptive and critical to survival. CRF orchestrates the stress response through a dual role as a neurohormone within the HPA axis and a neurotransmitter in neural circuits outside of this axis.¹³ The presentation of an acute life-threatening challenge initiates coordinated CRF release in parallel circuits to integrate endocrine, behavioral, and autonomic responses. A component of the CRF-mediated stress response is its engagement of the forebrain-projecting norepinephrine system through its actions on the pontine nucleus, LC.¹² The LC-norepinephrine system is a substrate by which salient stimuli, regardless of valence, initiate arousal and guide attention.¹⁴ CRF axon terminals synapse with LC dendrites; through its actions on the CRF subtype 1 receptor (CRF₁), CRF shifts the mode of LC discharge from a phasic state, characterized by moderate tonic activity and robust responses to discrete sensory stimuli, to a high tonic state in which cells discharge at a relatively high frequency and are not selectively responsive to discrete stimuli.¹⁵⁻¹⁷ Whereas phasic LC discharge is associated with focused attention and maintenance of task performance, the high tonic state is associated with hyperarousal, labile attention, going off-task, and behavioral flexibility.¹⁸ In the context of a dynamic environment with life-threatening challenges, these consequences of high tonic LC activity are optimal for survival. However, if they are initiated outside of the context of acute stress or if they are maintained long after stress termination, these same responses would become expressions of the arousal-related pathology that characterizes PTSD, depression, and anxiety. Consistent with this is evidence for excessive CRF and hyperactivity of the brain norepinephrine system in PTSD and depression.^{19,20}

Sex differences in LC dendritic morphology—structural basis for emotional arousal

Communication between CRF and LC neurons is topographically organized such that functionally distinct populations of CRF neurons terminate either within the compact nuclear region or in the peri-LC, where LC dendrites extend for hundreds of microns away from the nucleus.²¹ The LC nuclear core is relatively sparsely innervated by CRF axon terminals, and these arise from autonomic-related CRF neurons. One source of CRF terminals in the nuclear LC is Barrington's nucleus, a nucleus known for its regulation of parasympathetic innervation of the pelvic viscera.²² The other source of CRF that terminates in the LC nucleus is the nucleus paragigantocellularis (PGi) in the ventrolateral medulla.²³ The PGi regulates sympathetic preganglionic neurons that control blood pressure. Projections of Barrington's nucleus and PGi CRF neurons to the LC provide a mechanism whereby central noradrenergic activity can be coordinated with autonomic activity in response to an acute stress. CRF neurons in the paraventricular hypothalamic nucleus (PVN) project to the LC and terminate primarily in the medial dendritic zone.²⁴ These neurons are distinct from those that project to the median eminence to initiate adrenocorticotropin release, suggesting that the endocrine and arousal limbs of the stress response can be initiated independently. However, the function of these LC-projecting CRF neurons of the PVN and the stimuli that engage them are currently unknown. In contrast to the sparse CRF innervation of the LC nucleus, a dense CRF terminal field exists in the dorsolateral peri-LC where LC dendrites extend.²⁵ Here, CRF terminals are found in synaptic contact with LC dendrites, indicating proof of direct communication. The CRF terminals that synapse with LC dendrites here derive primarily from the central nucleus of the amygdala (CNA), a nucleus that is central to the generation of emotions.²⁵ As engagement of the LC-norepinephrine system initiates arousal, synapses between CNA axon terminals and LC dendrites can be considered a structural basis for emotional arousal. Notably, unlike the cell-rich compact LC nucleus, the peri-LC is less dense and more heterogeneous, containing neurochemically diverse neurons.²⁶ This provides a potential for complex modulation of this structure through the integration of multiple signals.

LC dendritic processes are more extensive and complex in female than male rats.²⁷ For example, LC dendrites of females have more nodes and ends. Their dendritic trees are longer, have more branches, and have longer branch lengths. A Sholl analysis indicated that the LC dendritic structure is of increased complexity in females versus males.²⁷ Consistent with this, LC dendrites in females make more synaptic contacts than LC dendrites in males, as indicated by an increased density of synaptophysin, a synaptic vesicular protein.²⁷

Because functionally distinct sources of CRF innervate the core and peri-LC dendritic zone, the sexual dimorphism of LC dendrites can bias the type of information that regulates LC activity (*Figure 1*). By having a dendritic system of higher complexity that extends further into the peri-LC and makes more synaptic contacts, the female LC has a greater probability of communication with CRF terminals from the CNA that are conveying emotion-related information. Thus, as a result of sexual dimorphic dendritic properties, the

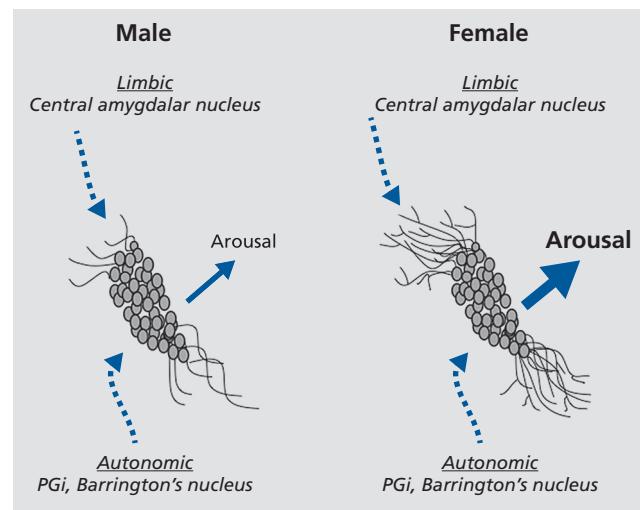


Figure 1. Schematic depicting how the topographical arrangement of locus coeruleus (LC) afferents interacts with sex differences in LC dendritic morphology to determine the magnitude of emotional arousal. LC neurons of female rats have longer and more complex dendrites than neurons of males. As a result, the probability that LC dendrites will contact corticotropin-releasing factor (CRF)-containing amygdalar afferents that convey emotion-related information and terminate in the peri-LC rather than the core is greater in females than in males. This would be predicted to result in a greater magnitude of arousal in response to emotion-related stimuli. PGi, paragigantocellularis

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structure for emotional arousal through which CRF transmits information is potentially greater in females than in males.

Sex differences in CRF receptor-G_s protein coupling

In the absence of stress, CRF is not tonically released into the LC, and LC neuronal activity is comparable in males and females.²⁸ Spontaneous discharge rates are similar, and they respond to sensory stimuli by a similar magnitude. However, female LC neurons are more sensitive to CRF and are activated by concentrations of CRF that have little effect on male LC neurons.²⁸ As expected, increased sensitivity of female LC neurons to CRF translates to a greater magnitude of activation elicited by stressors that release CRF into the LC.²⁸ Interestingly, this sex difference is unrelated to adult circulating sex hormone levels, suggesting that it has a basis either in an early organizational effect of sex hormones or in sex chromosomes. CRF excites LC neurons by binding to CRF₁ on the plasma membrane. The CRF₁ is a 7 transmembrane G-protein-coupled receptor that is primarily coupled to the stimulatory G-protein (G_s) in brain and signals within the cell through activation of adenylyl cyclase and formation of cyclic adenosine monophosphate (cAMP).^{29,30} The degree of CRF-G_s coupling determines the magnitude of the neuronal response, and receptor immunoprecipitation studies in which the amount of G_s pulled down with CRF₁ was quantified indicated greater CRF₁-G_s coupling in females.³¹ Like the neuronal response to CRF, this sex difference is independent of circulating hormone levels, occurring both in ovariectomized and intact females. This molecular sex difference can account for the functional sex difference expressed as increased neuronal sensitivity to CRF and stressors of female rats. At the time of discovery, this example of a sex difference in coupling of a receptor to its G-protein was unique. However, recent studies using guanosine 5'-O-[*thio*]triphosphate (GTP γ S) binding as an indicator of receptor G-protein coupling demonstrate similar sex differences with other receptors, including opioid-receptor subtypes.^{32,33} Given the predominance of G-protein-coupled receptors, their diverse functions, and their ability to serve as therapeutic targets, the potential for sex differences in coupling of some of these receptors to their G-proteins has broad clinical implications.

Sex differences in CRF₁-β-arrestin 2 association and CRF₁ trafficking

Like other G-protein-coupled receptors, CRF₁ becomes internalized into early endosomes after agonist binding.^{29,34} This process is initiated by phosphorylation of CRF₁ at its carboxyl tail and recruitment of β-arrestin 2, which promotes CRF₁ internalization into early endosomes. From here, the receptor can either be recycled back to the plasma membrane or recruited to multivesicular bodies, where it is degraded with the consequence of downregulation at the plasma membrane. Receptor internalization and subsequent downregulation is an adaptive process that protects cells against overstimulation by agonists. Both agonist- and stress-induced CRF₁ internalization have been documented in male LC neurons.^{35,36} For example, at both 1 hour and 24 hours after swim stress, the percentage of total CRF₁ labeling that is present in the cytoplasm of LC neurons shifts from 50% in the unstressed state to 80% by 1 hour after swim stress, and this is maintained at 24 hours after stress.³⁶ As time increases after the stress, increasingly more CRF₁ is found in multivesicular bodies, consistent with receptor degradation and downregulation. This cellular response has functional consequences that are expressed as a decreased magnitude of LC activation elicited by subsequent CRF exposures (Figure 2).^{37,38} In contrast to male LC neurons, female LC neurons do not internalize CRF₁ after swim stress.³¹ Notably, following swim stress, a significantly greater amount of receptor labeling is present on the plasma membrane of female LC neurons, which could reflect either a unique recruitment to the plasma membrane or decreased synthesis. However, the overall CRF₁ protein level is not altered. The inability of female LC neurons to internalize CRF₁ can account for the observation that unlike male LC neurons, the response of female LC neurons to CRF is not diminished after swim stress.²⁸ Thus, the cellular mechanism for adapting to overstimulation of CRF₁ is compromised in female LC neurons. Taken with evidence for a lack of agonist-induced CRF₁ internalization in female LC neurons described below, this implies that the molecular mechanisms underlying this important adaptive response to CRF₁ stimulation are impaired in females.

Using the same receptor immunoprecipitation approaches described above that provided evidence for increased CRF₁-G_s in females, it was discovered that

stress-induced association of CRF₁ with β-arrestin 2 was greatly diminished in females compared with males regardless of circulating hormonal status.³¹ In the absence of stress, CRF₁-β-arrestin 2 association is minimal in both males and females. However, shortly after stress, β-arrestin 2 becomes associated with CRF₁ selectively in males. This molecular sex difference is not surprising given the spatial competition between G_s and β-arrestin in their association with G-protein-coupled receptors.^{39,40} Because of this competition, a receptor bias toward G_s, as seen in females, is a bias away from β-arrestin 2. Importantly, the impairment in this molecular step probably contributes to the inability of female LC neurons to internalize CRF₁, rendering them more susceptible to hyperstimulation.

Convergence of sex differences at the CRF-LC synapse

The three sex differences discussed above converge on the CRF-LC synapse to make the LC-norepinephrine system of females more sensitive to stress. At the pre-synaptic level, female LC dendrites are differentially structured so as to receive more CRF-amygadalar input that conveys information about emotion than do male LC dendrites. The neuronal response to this increased CRF influence will then be magnified in females because at the molecular level, CRF₁ is designed to be more responsive to agonist stimulation through its en-

hanced coupling to G_s. Finally, following its activation, CRF₁ in female neurons is less able to adapt because of an impairment in β-arrestin 2 association, which compromises internalization. These three sex differences create a stronger structure for emotional arousal in females. However, in spite of these differences, LC neurons of males and females are quite comparable in the unstressed state, underscoring that the expression of sex differences in this system is only apparent under the specific condition of CRF release. Additionally, the differences should be magnified when CRF is present in excess, as has been proposed to occur in stress-related psychiatric disorders such as depression and PTSD. The condition of excessive CRF can be modeled by mice genetically modified to overexpress CRF.

Modeling stress-related psychiatric diseases with CRF-overexpressing mice

That CRF is overexpressed or hypersecreted in PTSD and depression is evidenced by greater levels of CRF in the cerebrospinal fluid of patients, increased numbers of CRF-immunoreactive neurons in the paraventricular hypothalamic nucleus of depressed patients, and increased CRF messenger RNA, as determined by in situ hybridization.⁴¹⁻⁴⁶ Notably, CRF is also elevated in the LC of depressed patients.⁴⁷ Taken with evidence for normalization of CRF levels after antidepressant treatment, the findings imply a causal link between elevated CRF and psychopathology.⁴⁸⁻⁵¹ The presence of estrogen- and androgen-responsive elements in the promoter region of the CRF gene offers a mechanism for sex-specific regulation of CRF levels.^{52,53} However, there is no evidence for sex differences in CRF levels that can explain female vulnerability to stress-related diseases. CRF levels in patients with depression are comparable between males and females; in healthy human subjects,

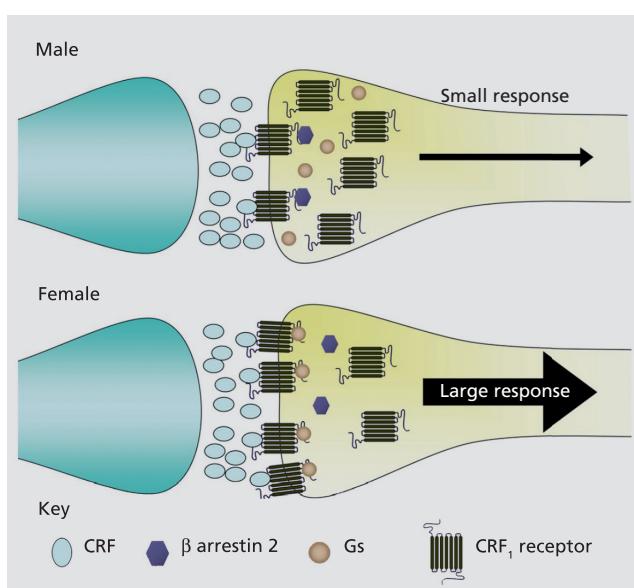


Figure 2. Schematic depicting sex differences in corticotropin-releasing factor subtype 1 receptor (CRF1) trafficking. In males, when corticotropin-releasing factor (CRF) released from pre-synaptic axon terminals (blue) binds to CRF1 on the plasma membrane of locus coeruleus dendrites (yellow), β-arrestin 2 associates with CRF1 and initiates receptor internalization. This results in an attenuated response to subsequent CRF. In females, stress-induced association of β-arrestin 2 with CRF1 is less than in males, perhaps because of the increased CRF1-Gs association. As a result, stress does not induce CRF1 internalization and the response of locus coeruleus neurons to CRF is relatively larger in females. Gs, stimulatory G-protein

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males actually have more CRF neurons in the paraventricular hypothalamic nucleus than females.^{42,46,54} These findings implicate postsynaptic mechanisms as a basis for sex differences in stress vulnerability.

The putative link between CRF hypersecretion and psychiatric disorders inspired the development of animal models of CRF hypersecretion to delineate how this condition alters neurons to lead to pathology. The most commonly used models are mice that are genetically altered to overexpress CRF.⁵⁵⁻⁵⁸ These include conditional models in which CRF-overexpression is confined to certain brain regions and/or can temporally be controlled by doxycycline administration. Sex differences have been studied in a transgenic mouse in which CRF expression is under control of the metallothionein (mMT1) promoter.⁵⁹ In this model, CRF is elevated primarily in neurons that normally express CRF, as well as in certain peripheral tissues, although it is not present in the circulation.⁵⁷ This may better model the human condition of CRF overexpression than some of the conditional models in which CRF is expressed in neurons and glia and in some regions that do not typically express CRF.

In both male and female CRF-overexpressing mice (CRF-OE), CRF overexpression is apparent in the dense innervation of the LC, compared with wild-type littermates, and there is no sex difference in the density of CRF innervation.⁵⁹ Although this would be predicted to translate to higher LC firing rates in CRF-OE mice than in wild-type littermates regardless of sex, this is not the case.⁵⁹ As expected, because wild-type mice should be analogous to the unstressed condition in rats where there is no tonic release of CRF, LC neuronal discharge rates are comparable between males and females. In spite of the massive innervation of LC neurons by CRF in male CRF-OE mice, LC discharge rates are similar to wild-type mice, suggesting that male CRF-OE mice have an adaptation that confers protection from excess CRF. Importantly, this adaptation was not present in female CRF-OE mice, which had LC discharge rates that were nearly three times greater than those of wild-type mice or male CRF-OE mice. This sex difference in CRF-OE mice could be attributed to a different pattern of CRF₁ cellular localization.⁵⁹ Like unstressed male rats, CRF₁ is relatively equally distributed between the cytoplasm and plasma membrane in male wild-type mice. Analogous to the stressed male rat, CRF₁ is primarily internalized into the cytoplasm in male CRF-OE mice, thereby conferring protection

from excess synaptic CRF. Female wild-type and CRF-OE mice show an opposite pattern, with greater cytoplasmic localization of CRF₁ in wild-type mice and less internalization in CRF-OE mice. The lack of CRF₁ internalization in female CRF-OE mice would render their LC neurons vulnerable to the excess CRF in the CRF-LC synapse. Thus, under the specific condition of CRF overexpression, a condition that is thought to be present in stress-related psychiatric disorders, the female LC-norepinephrine system will be tonically hyperactive because of an inability to internalize CRF₁. Given that tonic LC hyperactivity is associated with the arousal-related symptoms that characterize anxiety disorders, depression, and PTSD, this could account for the increased prevalence of these disorders in females.

Sex-biased cellular signaling

By regulating CRF₁ trafficking and the number of receptors on the plasma membrane, the association of CRF₁ with β-arrestin 2 determines the magnitude of the response to CRF released during stress or under pathological conditions. More importantly, it determines the quality of the response by governing, along with G_s, the cellular signaling that transduces the binding of CRF to CRF₁. Because β-arrestins serve as a scaffold between receptors and G_s-independent signaling cascades, the degree to which receptors associate with β-arrestins versus G_s defines the cellular signaling that is engaged by ligand-receptor interaction.⁶⁰ Sex differences in CRF₁ coupling to G_s and β-arrestin 2 should translate to sex differences in cellular signaling initiated during stress by the binding of CRF to CRF₁. For females, CRF₁ signaling will be biased toward G_s-dependent pathways; for males, it will be relatively biased toward β-arrestin 2-related G_s-independent pathways (Figure 3). In this way, stressors can elicit a series of distinct cellular reactions in males and females that can translate to different physiological and behavioral responses and distinct stress-related pathology. Notably, these sex differences will be magnified when CRF is elevated as in depression and PTSD.

An important function of G_s and β-arrestin-initiated signaling is to regulate the dynamic process of protein phosphorylation that controls the activation and inactivation of cellular proteins. As a result, sex biases in CRF₁ signaling should give rise to sexually distinct profiles of phosphorylated proteins when CRF receptors

are activated, and the distinctions between male and female phosphoproteomes may reveal the molecular basis for sex differences in stress-related neuropsychiatric disorders. A global phosphoproteomic analysis comparing phosphopeptides in the cortex of male and female CRF-OE mice and their wild-type littermates identified these distinctions in Alzheimer disease-related pathways that may account for an increased vulnerability of females to Alzheimer's disease.⁶¹

In addition to psychiatric disorders, Alzheimer disease is an example of a disease that has been associated with stress and that is more prevalent in females.⁶²⁻⁶⁴ In animal models, both stress and CRF overexpression accelerate the formation of plaques and cognitive deficits.⁶⁵⁻⁶⁹ This has been attributed in part to activation of protein kinase A (PKA). For example, CRF elicits β -amyloid secretion—a process that contributes to the formation of plaques—in primary cultures of hippocampal neurons of mice that express a human form of amyloid precursor protein, and this effect requires PKA.⁶⁹ PKA is also involved in tau phosphorylation, a process that has been implicated in the formation of fibrillary tangles.⁷⁰ Given that PKA activation is a primary component of the G_s signaling cascade, the female bias toward CRF₁- G_s signaling would favor Alzheimer

disease pathology under conditions of stress or excess CRF. This can explain how stress and sex interact to account for an increased female prevalence of Alzheimer disease. Sex-biased CRF signaling may be a common link underlying the comorbidity between Alzheimer disease and depression.⁷¹

Future considerations

Identifying disease processes that arise from the sex bias in CRF₁ signaling is an important translational goal. The comparison of phosphoproteomes under the optimal condition of CRF overexpression described above is one approach toward this goal. Manipulating CRF₁-expressing neurons to exhibit a specific signaling bias and determining the consequences of this is another approach that addresses causality. Another important question to address relates to how sex differences in CRF₁ coupling to interacting proteins arise. The lack of evidence for a role of circulating hormones implicates an effect that is organized by hormones early in life or a genomic mechanism. Currently, there is no evidence for sex differences in the gene encoding CRF₁. Alternatively, the sex difference could be a posttranslational modification of the receptor that affects G_s and/or β -arrestin 2 binding. Consistent with this, preliminary findings from the global phosphoproteomic study show sex differences in phosphorylation of the ³⁹⁶S on the carboxyl tail adjacent to a putative binding site for β -arrestin 2.

Although this review has focused on sex-biased CRF₁ signaling, it would be surprising if this characteristic was unique to CRF₁. Evidence is emerging for sex differences in signaling of other receptors, including γ -aminobutyric acid (GABA), dopamine, and opioid receptors.^{32,33,72-74} Of these, opioid receptors are of interest because stress-induced release of endogenous opioids is thought to mitigate the effects of stress and oppose those of CRF.⁷⁵ Given the many biological processes mediated by G-protein-coupled receptors and that these are targets of a pharmacopoeia of drugs, identifying sex differences in their signaling has the potential to break new ground in our ability to understand mechanisms underlying disease vulnerability, as well as guide the development of better treatments for psychiatric diseases in men and women. □

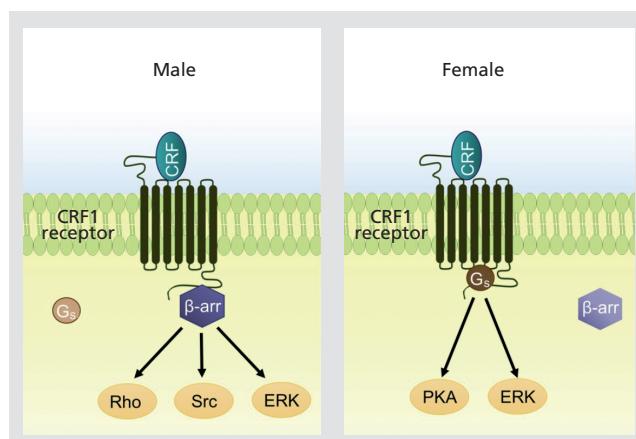


Figure 3. Schematic depicting sex-biased corticotropin-releasing factor subtype 1 receptor (CRF1) signaling. As a result of sex differences in CRF1 coupling to G_s (female bias) and β -arrestin 2 (male bias), corticotropin-releasing factor (CRF) released during stress can engage sexually distinct cellular signaling pathways. These different cellular reactions can translate to sexually distinct stress responses and pathology. β -arr, β -arrestin 2; ERK, extracellular signal-related kinase; G_s , stimulatory G-protein; PKA, protein kinase A; Rho, Rho family of GTPases; Src, proto-oncogene tyrosine protein kinase Src

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